Regulation of cell adhesion signaling by synthetic glycopolymer matrix in primary cultured hepatocyte

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Abstract Control of cell–matrix interactions is a central principle for the design of biomaterial in tissue engineering. In this study, we evaluated a synthetic glycopolymer, which is recognized by the asialoglycoprotein receptor (ASGPR) expressed on the surface of hepatocytes, as an artificial matrix to regulate integrin-mediated signaling. The phosphorylation of focal adhesion kinase was restricted in hepatocytes cultured on the glycopolymer compared with fibronectin. In addition, there was no reorganization of cytoskeleton-related proteins such as actin filaments, microtubules, and vinculin in hepatocytes cultured on the glycopolymer. DNA synthesis and cyclin D1 expression were suppressed in hepatocytes grown on the glycopolymer as compared with those grown on fibronectin and collagen. The data suggest that the glycopolymer will be a good artificial matrix to regulate integrin-mediated signaling and cell growth through the unique ASGPR–carbohydrate interaction.

Key words: Extracellular matrix; Cell adhesion; Integrin; Asialoglycoprotein receptor; Glycopolymer; Hepatocyte

1. Introduction

Extracellular matrix (ECM) is an insoluble macromolecular complex that forms an environmental substrate outside the cell. Cell–ECM contact plays a fundamental role in regulating such biological processes as wound healing, organogenesis, and metastasis. It is well known that some ECM components are cell adhesion molecules that facilitate attachment, whereas others inhibit cell adhesion. Integrins are a family of cell adhesion receptors linking the ECM to intracellular signaling molecules and the cytoskeleton network. Integrin signaling pathways that take place through the cell–matrix interaction are directly involved in cell survival, proliferation, differentiation, migration, and morphogenesis [1–3].

Control of cell–matrix interactions is indispensable for the design of biomaterial in tissue engineering. The introduction of biomolecules to polymer surfaces has become a relevant tool for controlling the cell–biomaterial interaction. Presently, most investigators are utilizing the knowledge of integrin–ligand recognition and using naturally derived or modified ECM components for the design of artificial matrices. Specifically, the Arg-Gly-Asp (RGD) peptide, many other oligopeptides that bind to integrins, has been incorporated into biomaterials in an attempt to understand and control the behavior of cells [4–6].

Knowledge of carbohydrate-mediated cell recognition has been applied to enhance selective interactions between materials and cells in tissue engineering. In our strategy, we have focused on the ability of multivalent galactose to bind to the asialoglycoprotein receptor (ASGPR) expressed on hepatocytes. ASGPRs are lectins for receptor-mediated endocytosis found at the hepatocyte cell surface that bind galactose/*N*-acetylgalactosamine-terminated ligands in a calcium-dependent manner [7,8]. We developed a galactose-derivatized polystyrene, poly-[*N*-*p*-vinylbenzyl-4-*O*-β-D-galactopyranosyl-D-gluconamide] (PVLA), to mimic the ASGPR–carbohydrate interaction. PVLA has an amphiphilic structural unit composed of oligosaccharide side chains covalently bound to a polystyrene (PS) backbone. PVLA is stably adsorbed to hydrophobic surfaces such as PS dishes due to its amphiphilic property. Hepatocyte adhesion to PVLA-coated dishes is inhibited by asialofetuin, a natural ligand for ASGPR, and further, hepatocytes require Ca^{2+} to adhere to PVLA-coated dishes [9,10]. Although ASGPR is a non-adhesion cell surface receptor, PVLA is an artificial matrix to guide hepatocyte adhesion through the unique ASGPR–carbohydrate interaction. In this study, we hypothesized that ASGPR-mediated cell adhesion might control integrin-mediated cell signaling and cell response.

2. Materials and methods

2.1. Materials

PVLA was prepared according to the method described previously [11]. PS plates or dishes were obtained from Becton Dickinson (Franklin Lakes, NJ, USA). Collagenase and trypsin inhibitor were purchased from Wako Pure Chemicals (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Type I collagen and bovine fibronectin (FN) were obtained from Koken (Tokyo, Japan). Williams' medium E and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Epidermal growth factor (EGF) was a gift from Hitachi Chemicals (Ibaraki, Japan). Phospho-focal adhesion kinase (FAK) (Tyr-397) antibody was acquired from Transduc-

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Abbreviations: ASGPR, asialoglycoprotein receptor; PVLA, poly-[*N*-*p*-vinylbenzyl-4-*O*-β-D-galactopyranosyl-D-gluconamide]; PLL, poly-L-lysine; ECM, extracellular matrix; FN, fibronectin; FAK, focal adhesion kinase; QCM, quartz crystal microbalance; AFM, atomic force microscopy; PS, polystyrene

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tion Labs (Lexington, KY, USA). Anti-tubulin antibody was purchased from Neo Markers (Fremont, CA, USA). Alexa 488-conjugated phalloidin, and Alexa 488- and Alexa 547-conjugated anti-mouse IgG were purchased from Molecular Probes (Eugene, OR, USA). Peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Anti-β-actin and anti-vinculin antibodies were purchased from Sigma. ICR mice were obtained from Japan SLC (Shizuoka, Japan).

2.2. Coating of PS dishes

PVLA was dissolved in ultrapure water, FN (10 μg/ml) was dissolved in phosphate-buffered saline (PBS), and type I collagen (10 μg/ml) was dissolved in acetic acid (pH 3) before coating the PS plates or dishes. After 4 h at 37°C, non-adsorbed polymer solution was removed from the dishes that were then washed with ultrapure water three times. The dishes were treated for 2 h at 37°C with PBS/0.5% BSA to prevent non-specific cell adhesion, and then washed with ultrapure water three times.

2.3. Hepatocyte isolation and culture

Hepatocytes were isolated from livers of male ICR mice (5–7 weeks old) using a modification of the in situ collagenase perfusion method that includes perfusion with EGTA before collagenase treatment, as described previously [12]. Isolated hepatocytes were allowed to adhere onto the coated PS surface in Williams' medium E, pH 7.4 (containing 10 mM HEPES, 10 mM $NaHCO_3$, 50 μg/ml penicillin and streptomycin, and 100 μg/ml neomycin) (WE base) in a humidified atmosphere of 5% CO_2 and 95% air for 3 h at 37°C. The medium was replaced by fresh WE base to remove unattached cells, after which the adhered cells were cultured for a specific time period. The morphology of adherent hepatocytes was observed using a phase contrast microscope (IX70; Olympus, Tokyo, Japan) and photographed using digital Hi-Vision microscope equipment (VQ-7000; Keyence, Osaka, Japan).

2.4. Immunofluorescence assay

For actin, phospho-FAK (pFAK), and vinculin staining, hepatocytes cultured for 24 h on PVLA or collagen surfaces were fixed for 10 min in 4% paraformaldehyde and permeabilized for 10 min with 0.2% Triton X-100. For detection of F-actin, the cells were incubated with Alexa 488–phalloidin for 30 min. For the detection of pFAK and vinculin, the cells were incubated for 1 h with anti-pFAK and anti-vinculin and subsequently incubated for 30 min with Alexa 488- and Alexa 547-conjugated anti-mouse IgG, respectively. For microtubule staining, the cultured hepatocytes were fixed for 10 min in 1% glutaraldehyde and permeabilized for 10 min with 0.2% Triton X-100. The cells were incubated with anti-tubulin antibody for 1 h and subsequently incubated for 30 min with Alexa 488-conjugated anti-mouse IgG. Stained cells were observed using a fluorescence microscope (IX70; Olympus, Tokyo, Japan).

2.5. Western blot analysis

Cultured hepatocytes were solubilized in lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 2 μg/ml aprotinin) for 1 h at 4°C. Lysates then were clarified by centrifugation at 15 000×*g* for 30 min at 4°C, diluted in Laemmli sample buffer containing 2% SDS and 5% (v/v) 2-mercaptoethanol, and heated for 5 min at 90°C. Proteins were separated by SDS–polyacrylamide gel electrophoresis using 8% or 12% resolving gels followed by transfer to polyvinylidene difluoride membranes (Millipore). The membranes were incubated with primary antibody for 2 h at room temperature. For detection, peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG and the ECL method (Amersham Pharmacia Biotech) were used as described by the manufacturer. Films were scanned with a photoscanner (Epson, Tokyo, Japan), using Adobe Photoshop software to create images for figures. Bands were quantified using the software NIH Image 1.62.

2.6. Atomic force microscopy (AFM)

AFM images were taken via scanning probe microscopy (SPM; NanoScope IIIa, Digital Instruments, USA). SPM was performed in air with an etched 125 μm silicon cantilever operating in Tapping™ mode with a scan size of 200 nm. The spring constant was from 20 to 100 N/m, and its frequency was approximately 0.5–0.7 Hz.

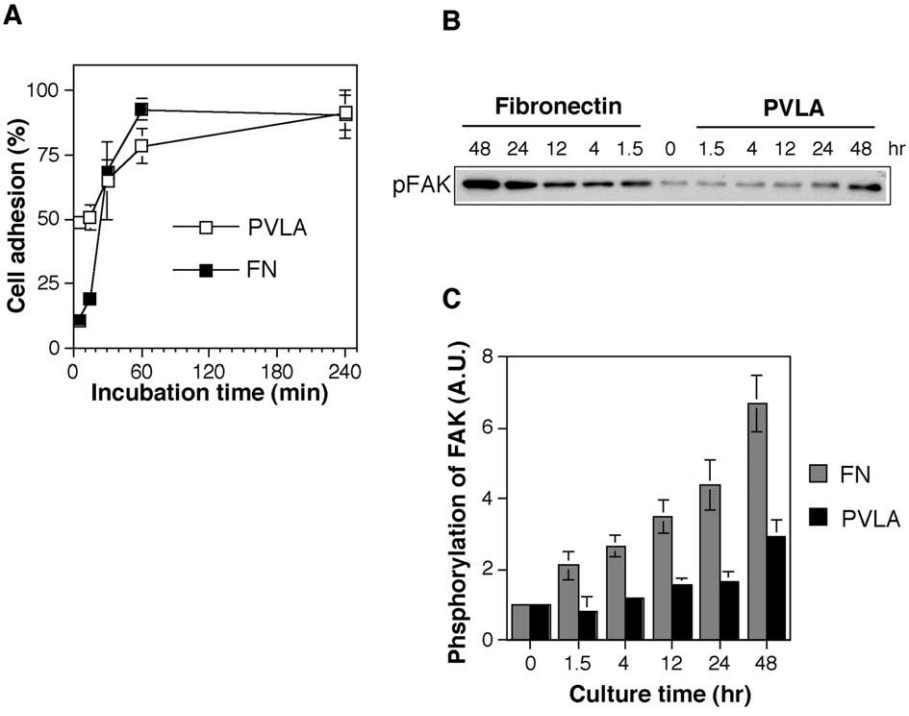

Fig. 1. FAK is not phosphorylated in hepatocytes adhered onto a PVLA matrix. A: Kinetics of hepatocyte adhesion: hepatocytes were allowed to adhere for an indicated time to PS dishes coated with 100 μg/l PVLA or 10 μg/ml FN. The percent of adhered cells was measured by protein assay. All values were normalized to the percentage of maximum adhesion, which was 100%. B: Western blot analysis of FAK phosphorylation: hepatocytes were allowed to adhere for an indicated time. C: The mean intensity of the band was quantified as described in Section 2; the values were determined from experiments performed separately at least three times. All values were normalized to the ratio of phosphorylated FAK to total FAK, which was 1.

2.7. Quartz crystal microbalance (QCM)

A slightly modified QCM technique was used to examine the adsorption behavior of PVLA onto the PS surface. Briefly, the experimental apparatus consisted of a 4.9 mm^2 27 MHz AT-cut crystal (Showa Crystals, Chiba, Japan) and an oscillator that drives the crystal at its resonant frequency. Gold electrodes on the crystal were treated with H_2SO_4/H_2O_2 (3/1, v/v) solution for 10 min three times followed by 5 mM *n*-octadecyl mercaptan for 15 min. Four μl of 0.1 mg/ml PS dissolved in chloroform was cast onto the gold electrodes on the crystal and the frequency of the crystal, the initial frequency (F_0), was measured under dry conditions. The cast crystal was immersed in PVLA solution for 2 h at 25°C, dried after being rinsed thoroughly, and the frequency of the crystal (F) was measured. All aqueous solutions were filtered with membrane filters of pore size 0.2 μm (Millipore).

3. Results and discussion

It has been reported that FAK is a key molecule involved in the formation of focal contact that regulates cellular processes such as cell growth, survival, spreading, and migration, mediated through integrin signaling [13–15]. Integrin-mediated adhesion of cells to the ECM triggers autophosphorylation at the Tyr-397 residue of FAK [16]. To elucidate whether integrin-mediated signaling occurs in hepatocytes cultured on PVLA, we evaluated the phosphorylation of FAK in hepatocytes cultured on PVLA compared with those cultured on FN by Western blot analysis with anti-pFAK (Tyr-397) antibody. Phosphorylated FAK was detected in hepatocytes seeded on FN-coated dishes by 1.5 h after seeding. The phosphorylation level of FAK was sustained during culture. In contrast, phosphorylated FAK was not detected in hepatocytes cultured on PVLA, even 12 h after seeding (Fig. 1B,C). Fig. 1A shows the kinetics of hepatocyte adhesion to PVLA and FN. Most hepatocytes adhered to PVLA within 15 min after seeding. Hepatocytes adhered more rapidly to PVLA than FN. The hepatocyte adhesion rate was almost the same for both matrices at 1 h post seeding. These results indicate that the down-regulation of FAK phosphorylation in hepatocytes cultured on PVLA is not due to cell adhesion kinetics.

Cell adhesion to the ECM induces reorganization of cytoplasmic cytoskeleton networks and influences cell morphology. The morphology of hepatocytes adhered to PVLA was compared with hepatocytes adhered to collagen type I and poly-L-lysine (PLL) (Fig. 2). The adherent hepatocytes were much more spread out on PS surfaces coated with collagen type I (Fig. 2A) than on surfaces coated with PVLA or PLL (Fig. 2B–D). Interestingly, the morphology of adherent hepatocytes appeared to be less spread out on PS surfaces coated with 100 μg/ml PVLA (Fig. 2D) than with 0.5 μg/ml PVLA (Fig. 2C) or 5 mg/ml PLL (Fig. 2B) at 16 h after hepatocyte seeding. In addition, hepatocytes were spread out on dishes coated with 0.05–1 μg/ml PVLA but not with more than 10 μg/ml PVLA (data not shown). These morphological data show that hepatocytes are more spread out on PVLA surfaces with lower coat density and suggest that ASGPR-mediated adhesion to PVLA is a negative modulator of hepatocyte spreading. F-actin, microtubule and vinculin are major cytoskeletal proteins involved in integrin-mediated signaling and cell spreading [17,18]. Hepatocytes cultured on collagen or PVLA were stained with the F-actin-specific probe Alexa 488–phalloidin, anti-microtubule antibody, and anti-vinculin antibody (Fig. 3). Hepatocytes do form extensive actin filaments and microtubules on collagen (Fig. 3B,C). Vinculin and phosphorylated FAK were localized at the points of focal adhesion (Fig. 3A,D). However, there was no reorganization

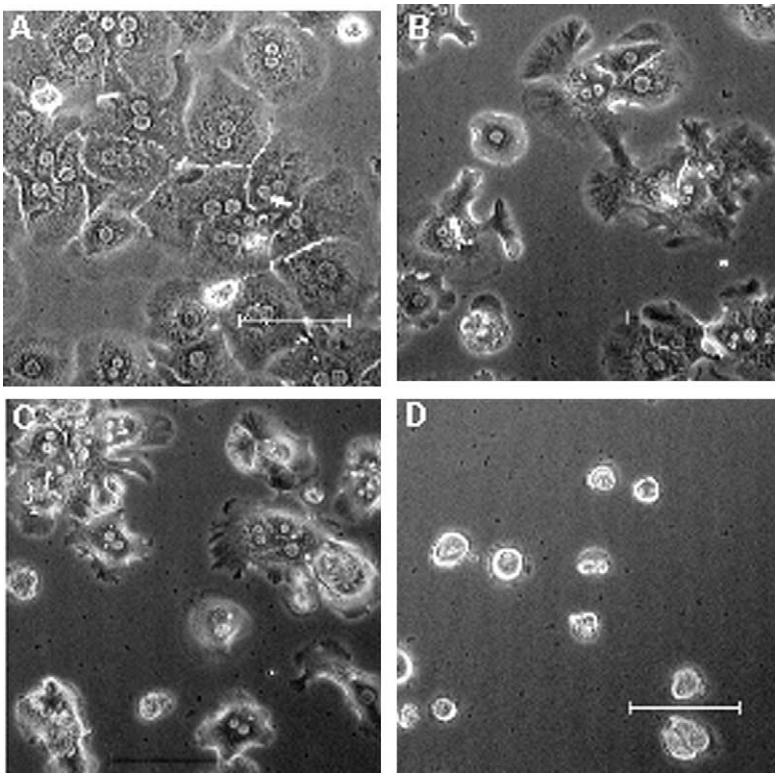

Fig. 2. Hepatocyte morphology depending on adhesion matrix. Hepatocytes were cultured for 16 h in WE base on PS dishes coated with (A) 10 μg/ml collagen type I, (B) 5 mg/ml PLL, (C) 0.5 μg/ml PVLA, or (D) 100 μg/ml PVLA; scale bar = 100 μm.

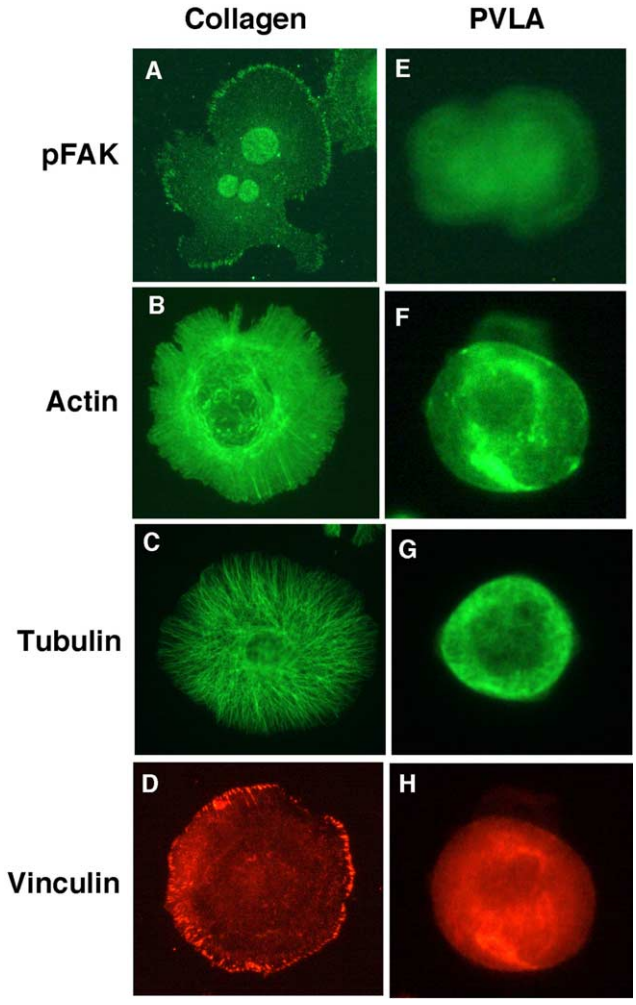

Fig. 3. Focal adhesion and cytoskeleton proteins are not reorganized in hepatocytes adhered to the PVLA matrix. Hepatocytes were cultured for 24 h on cover glasses coated with 10 μg/ml type I collagen (A–D) or 100 μg/ml PVLA (E–H). The hepatocytes were stained for the detection of pFAK (A,E), F-actin (B,F), tubulin (C,G), and vinculin (D,H).

of actin filaments, microtubules, and vinculin in hepatocytes adhered to PVLA (Fig. 3E–H). These results indicate that integrin-mediated signaling is not activated in hepatocytes cultured on PVLA.

Furthermore, to confirm that PVLA is directly related to the modulation of integrin-mediated signaling, we examined the effect of PVLA coating density on FAK phosphorylation in hepatocytes adhered to PVLA surfaces. Quantitative analysis of the coating density was performed by QCM. The dry weight of PVLA adsorbed onto a crystal cast with PS was determined from the frequency change (ΔF) using the Sauerbrey equation [19]. A 1 Hz decrease in resonant frequency corresponds to a mass increase of 0.612 ng/cm^2 on the PS surface. Fig. 4A shows the amount of PVLA adsorbed onto the PS surface versus the concentration of PVLA. The amount of adsorbed PVLA was increased with an increase in the concentration of PVLA solution as a Langmuir-type hyperbolic curve. The reciprocal plots of the adsorption isotherm presented a straight line (data not shown) indicating that PVLA is adsorbed onto the PS surface in a monolayer. AFM was used to characterize the topology of surfaces coated with PVLA. When a 100 μg/ml PVLA solution was used to coat the PS surface, the surface was densely covered by PVLA (Fig. 4Bb). In contrast, when a 0.5 μg/ml PVLA solution was used to coat the PS surface, the PVLA molecules were separated by areas of unoccupied space (Fig. 4Ba). These results indicate that PVLA is adsorbed uniformly to the PS surface in a dose-dependent manner. Subsequently, phosphorylation of FAK in hepatocytes cultured on low or high density PVLA-coated PS surfaces was compared. FAK phosphorylation was increased in hepatocytes cultured on 0.5 μg/ml PVLA compared with 100 μg/ml PVLA (Fig. 4C). Taken together, these results suggest that integrin-mediated signaling is regulated depending on the coating density of PVLA.

Cell growth is a major phenomenon regulated by integrin-mediated signaling. We examined the effect of the PVLA matrix on DNA synthesis and G1 cyclin expression in primary cultured hepatocytes. Nuclei were purified from freshly isolated hepatocytes or cultured hepatocytes and DNA content was measured by flow cytometric analysis of propidium iodide (PI)-stained nuclei. The majority of hepatocytes cultured on PVLA in the presence of EGF demonstrated 2N DNA content, compared with a distribution between 2 and 4N DNA content seen in hepatocytes cultured on FN (Fig. 5A). Cyclin D1 is a cell cycle protein that binds Cdk4/6 and is involved in the progression to S phase in proliferating cells [20]. Cyclin D1 was highly expressed in hepatocytes cultured on FN (Fig. 5C). However, cyclin D1 was rarely expressed in hepatocytes cultured on PVLA at high coat density (Fig. 5C). In hepatocytes cultured on PVLA at the lower coating density, DNA content and the expression of cyclin D1 increased (Fig. 5B,C). Cell viability was almost the same in hepatocytes cultured on PVLA-coated dishes as those cultured on type I collagen-coated dishes (data not shown), indicating that the down-regulation of DNA content and cyclin D1 expression was not due to cell viability of hepatocytes cultured on PVLA at high coating density. Taken together, these results indicate that hepatocytes cultured on PVLA are arrested in the G_0/G_1 phase of the cell cycle. Thus, the coating density of PVLA regulates hepatocyte cell growth.

Primary cultured hepatocytes are used widely for studies of liver-specific function. Hepatocytes are highly differentiated cells in the normal liver. However, hepatocytes quickly lose their differentiated phenotype after plating on culture dishes [21]. The growth activity and expression of a differentiated phenotype are known to be in a competitive relationship: as cells enter the proliferation state, differentiated functions of the cells decrease. Therefore, controlling growth potential in primary cultured hepatocytes is a prerequisite for cell differentiation. Numerous studies have shown that integrin-mediated signaling pathways initiated through cell–ECM contact were indispensable for cell proliferation. It was reported previously that DNA synthesis was affected by ECM components in primary cultured hepatocytes [22,23]. An ECM complex, such as Engelbreth–Holm–Swarm mouse sarcoma (EHS gel), was used as the culture substrate to improve differentiated function of primary cultured hepatocytes [24,25]. However, the interaction between the hepatocytes and the EHS gel was not fully understood.

We have developed artificial matrices, focusing on a ligand for a non-adhesion cell surface receptor to control cellular responses related to cell adhesion signaling. ASGPR, ex-

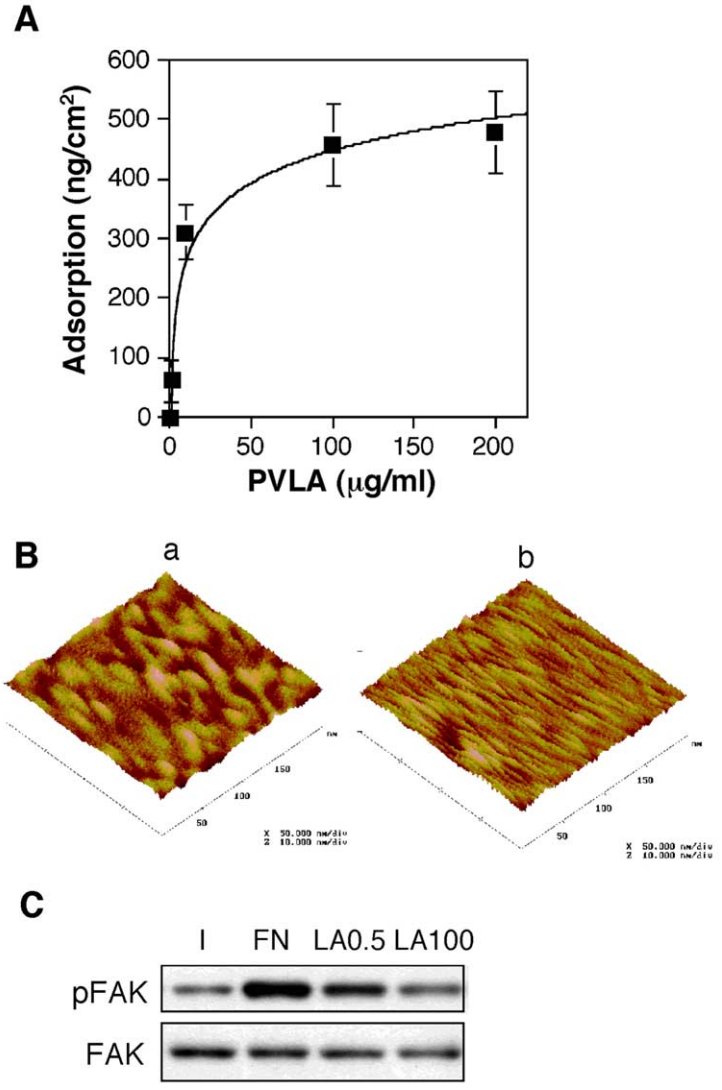

Fig. 4. Effect of the PVLA coating density on FAK phosphorylation. A: QCM analysis of PVLA adsorption to the PS surface. B: AFM image of a PS surface coated with (a) 0.5 or (b) 100 μg/ml PVLA. C: FAK phosphorylation in hepatocytes cultured on PS dishes coated with 10 μg/ml FN or 0.5 and 100 μg/ml PVLA.

pressed on the hepatocyte membrane, was selected as a non-cell adhesion receptor for controlling integrin signaling. We demonstrated that ASGPRs mediated the adhesion of hepatocytes to PVLA [9,10]. Our previous reports demonstrated that hepatocyte adhesion to PVLA was strikingly different from adhesion to ECM proteins [10,12]. For example, hepatocytes adhered to the PVLA surface but not to type I collagen and FN surfaces at 4°C. Hepatocytes required Ca^{2+} but not Mg^{2+} for adhesion to the PVLA surface, whereas hepatocytes required Mg^{2+} for adhesion to type I collagen and laminin, and Ca^{2+} or Mg^{2+} for FN. In this study, we demonstrated that intracellular signaling and the cellular response of hepatocytes cultured on PVLA were different from those in hepatocytes cultured on ECM proteins, indicating that ASGPR-mediated cell adhesion did not initiate integrin-mediated signaling. ASGPR accumulates in clathrin-coated pits for endocytosis [26]. Previous reports showed that hepatocyte adhesion to galactose-derivatized surfaces resulted in rapid accumulation of ASGPRs in a patch at the site of adhesion [27,28]. We also showed that ASGPR patches formed on membranes of hepatocytes that were adhered to the PVLA surface at high coating density. The patches were maintained during culture, demonstrating that the amount of ASGPRs bound to the PVLA surface increases with an increase in density of the coated PVLA [29]. It has been reported that isolated primary hepatocytes synthesize and secrete ECM proteins such as collagen and FN [30,31]. The possibility that ECM components secreted from hepatocytes affect cell behavior on PVLA surfaces during culture was investigated. The spreading of hepatocytes adhered to a PS surface coated with 0.5 μg/ml of PVLA was inhibited in the presence of RGD peptide (data not shown). PVLA-coated dishes were treated with BSA because ECM adsorption might be affected by surface hydrophilicity depending on the PVLA coating density. We showed that the hydrophilic property of PVLA-coated dishes was almost the same after BSA treatment, irrespective of the coating density of PVLA [32]. These results led to the following reasoning about the behavior of hepatocytes adhered to PVLA. At high PVLA density, ASGPRs on hepatocyte membranes are clustered within sites of focal adhesion

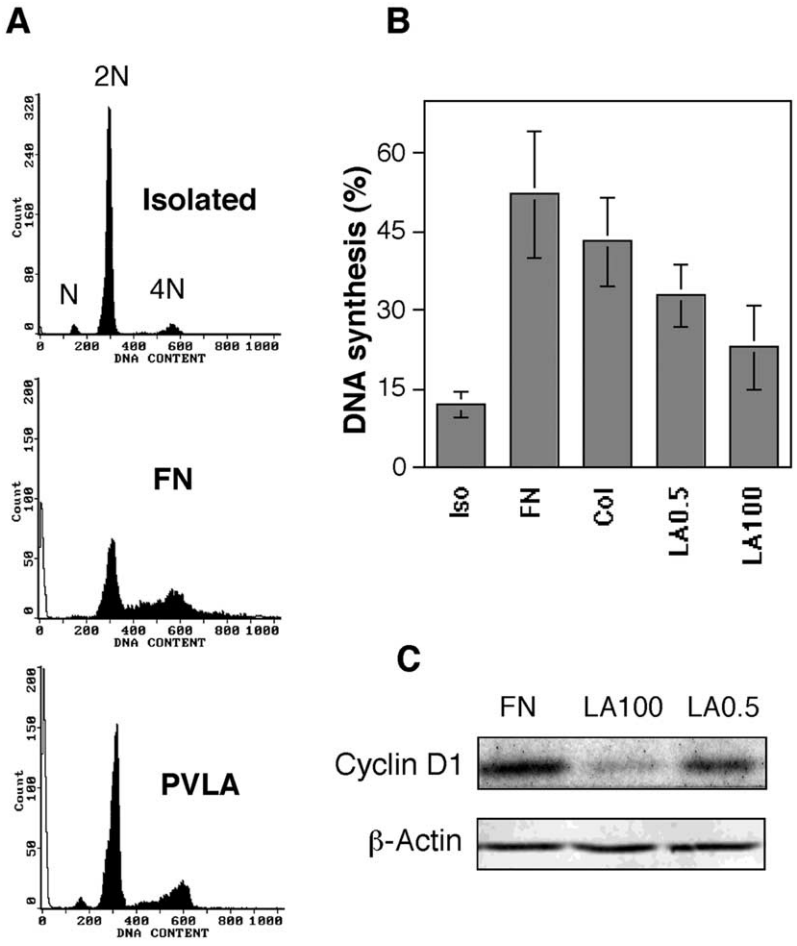

Fig. 5. Cell cycle is arrested in hepatocytes cultured on a PVLA matrix. A: DNA content in hepatocytes cultured on PVLA compared with those cultured on FN: a representative experiment is shown ($n = 3$). B: Effect of the PVLA coating density on DNA content. The nuclei were stained for DNA content with PI using the Cycle Test™ PLUS DNA Reagent kit (Becton Dickinson) and analyzed by flow cytometry. C: Effect of PVLA coating density on cyclin D1 expression; cyclin D1 expression was analyzed by Western blot.

as a large patch, and this clustering prevents integrin receptors from participating in adhesion processes during culture. At low PVLA density, hepatocytes allow integrin receptors to take part in adhesion processes within the space where ECM proteins are secreted during culture. Thus, the integrin receptors play a role in turning round hepatocyte shapes into spread shapes, and in FAK phosphorylation (Fig. 6).

Synthetic polymers such as PLL have been employed as adhesion matrices for cell biology studies related to cell adhesion-mediated signaling and cell morphology. However, the cell adhesion is mediated by non-specific physical interactions. There is little information about adhesion mechanisms that affect cell morphology and adhesion-related signal transduction. PVLA is a novel artificial matrix to control adhesion-mediated signaling through a specific receptor–ligand interaction. In addition, FAK phosphorylation and cell spreading were more suppressed in hepatocytes cultured on PVLA at high coating density than on PLL (data not shown, Fig. 2). It is argued here that adhesion mechanisms are key to understanding cell adhesion-mediated biology on artificial cell adhesion matrices. Moreover, elucidating the events that take place at surfaces or interfaces of biological systems is important for improving the design of highly functional biomaterials. ASGPR-mediated cell adhesion was a negative modulator of integrin signaling on PVLA surfaces, although adhesion processes that occur within the interface between PVLA surfaces and hepatocytes remain to be examined further. We suggest that the ASGPR–PVLA interaction may be a well-defined model for analysis and control of cellular responses related to integrin signaling in primary cultured hepatocytes.

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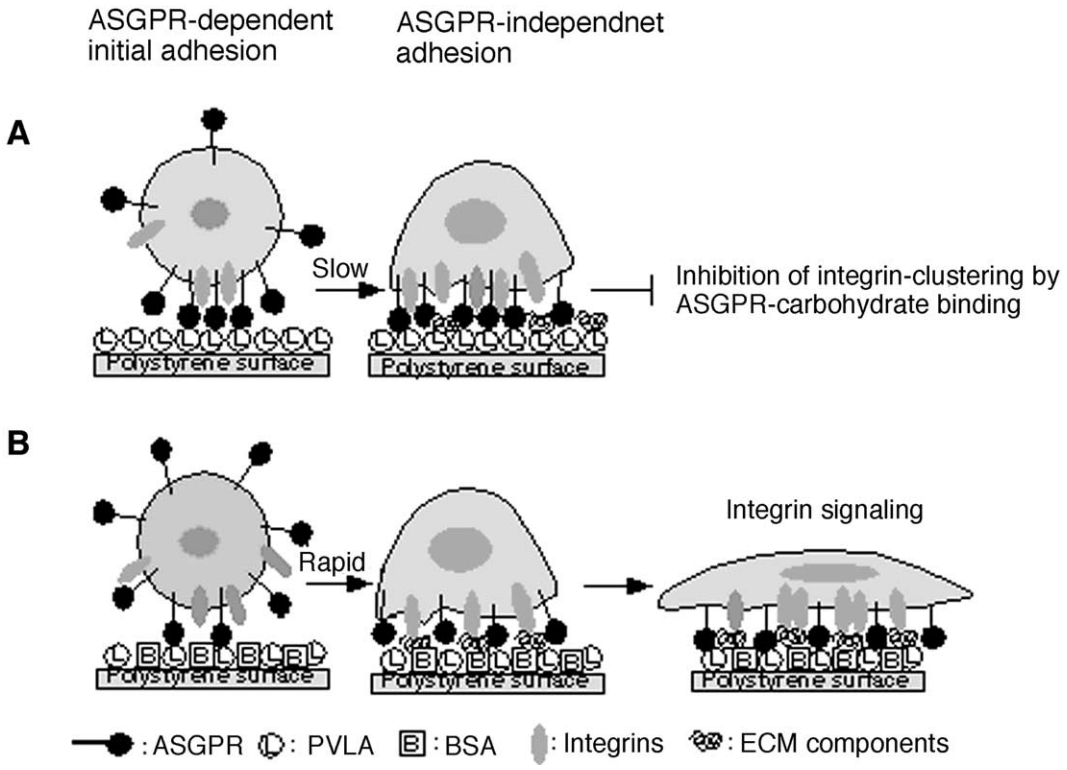

Fig. 6. Illustration of hepatocyte behavior on PVLA surfaces. ASGPR-dependent adhesion occurs initially within the interface between hepatocytes and PVLA surfaces both at high coat density (A) and at low coat density (B). However, ASGPR-independent adhesion takes place more rapidly at low coat density than at high coat density during culture [29]. It was expected that the ASGPR-independent adhesion would be induced by integrin receptors that have the opportunity to participate in cell adhesion mediated by ECMs secreted from hepatocytes following initial adhesion. Finally, the ASGPR-independent adhesion that occurs at low coat density causes hepatocytes to spread through integrin signaling. The concentrated ASGPR–ligand complexes at high coat density inhibit integrin signaling.

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