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Hepatocytes maintain their function on basement membrane formed by epithelial cells

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Abstract

To establish liver tissue engineering, the effective substratum for hepatocytes culture should be developed. Up to now, it is believed that Matrigel, which contains several basement membrane proteins produced by sarcoma cells, is the most effective substratum. Matrigel does not contain extracellular matrix molecules derived from epithelial cells although the space of Disse contains the molecules such as laminin-511/521 (laminin-10/11). Therefore, the basement membrane formed by epithelial cells can be more effective substratum than Matrigel. In this study, we evaluated hepatocytes behavior on basement membrane (rBM) formed by alveolar epithelial cells. The viability of hepatocytes on rBM is higher than that of Matrigel within 5 days. Also, the expression of *Cyp1a2* induced by β-naphthoflavone can be observed in hepatocytes on rBM but not in Matrigel. These results indicate that rBM is a more effective substratum for hepatocyte culture than Matrigel.

Keywords: Basement membrane; Matrigel; Laminin; Tissue engineering; Hepatocytes

To form tissue architecture, epithelial and endothelial cells adhere to basement membrane which has a highly integrated sheet structure composed of extracellular matrix (ECM) molecules, including type IV collagen, laminin, entactin (nidogen), and perlecan, a heparin sulfate proteoglycan [1]. Depending on the biological activity of each component, the basement membrane can regulate various kinds of cellular functions such as adhesion, migration, proliferation, and differentiation [2,3]. For the expression of tissue-specific functions, the components of basement membrane are strictly decided in each tissue. For instance, laminin-111 (laminin-1) is expressed in fetal tissue but not adult tissue [4]. These ECM molecules in basement membrane of epithelial tissue are produced by both epithelial cells and stromal cells [1,5]. The interaction between epithelial cells and stromal cells is necessary for the formation of basement membrane.

In the liver, the basement membrane structure cannot be observed. However, the ECM molecules are distributed in the space of Disse [6]. ECM pattern in the space of Disse alters depending on the physiological condition of the liver such as regenerating one [7,8]. Also, hepatocyte behaviors are influenced by the types of ECM *in vitro*, indicating that ECM plays an important role in regulating hepatocyte behavior [2,9,10]. For this reason, ECM has been focused to establish the culture system of hepatocytes for liver tissue engineering. To maintain the liver-specific functions and the viability of hepatocytes *in vitro*, Matrigel which contains several basement membrane proteins has been used. The expression of liver-specific functions in the hepatocytes cultured on Matrigel was maintained for longer time than those on stromal ECMs such as type I collagen and fibronectin [2,11,12], suggesting that basement membrane proteins are effective in keeping the liver-specific

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functions. Matrigel is secreted from Engelbreth–Holm–Swarm (EHS) sarcoma cell. Therefore, ECM molecules produced by EHS sarcoma cell do not include ECM molecules derived from epithelial cells. Indeed, Matrigel contains laminin-111 (laminin-1) but not laminin-511/521 (laminin-10/11) which exists in the space of Disse [13,14]. It is expected that basement membrane formed by the epithelial cells can be effective to establish the culture system of hepatocytes.

Previously, it was reported that immortalized alveolar type II epithelial cells (SV40-T2 cells) cultured on fibroblast-embedded collagen gel could form a continuous lamina densa of basement membrane and were able to assemble basement membrane components on the lamina densa [5]. Instead of using a fibroblast-embedded collagen matrix, the basement membrane could be also formed by the SV40-T2 cells on fibrillar collagen substratum with the supplement of Matrigel [15]. The Matrigel served as an exogenous source of basement membrane components. In addition to the components of Matrigel, ECM molecules produced by SV40-T2 cells were assembled to this basement membrane. The alveolar type II epithelial cells *in vitro* expressed abundant laminin α5 chain and some laminin α3 chain which are observed in adult epithelial tissue [16]. Therefore, it is expected that hepatocytes cultured on this basement membrane can maintain the liver-specific functions for longer time than those on Matrigel.

In this study, we evaluated the hepatocyte behaviors on the basement membrane formed by SV40-T2 cells and compared the hepatocyte behaviors among Matrigel, fibrillar collagen substratum, and poly (*N*-*p*-vinylbenzyl-4-*O*-β-D-galactopyranosyl-D-gluconamide): PVLA substratum. PVLA substratum is a synthetic substratum to which hepatocytes can specifically adhere and maintain the liver functions such as bile acid production and cytochrome P450 expression for longer time than those on type I collagen substratum [17–19]. Adhesion rate of hepatocytes, the viability, the responsibility of hepatocyte to the drug, and the morphology of hepatocytes were compared among the substrata above.

Materials and methods

Materials. Matrigel were obtained from Beckton–Dickinson Biosciences (San Jose, CA). Neutralized type I collagen (acid-extracted type I collagen from bovine dermis) was purchased from KOKEN (Tokyo, Japan). PVLA, a synthetic sugar-carrying polymer, was prepared according to the method described previously [20]. Non-treated 6-well polystyrene plates and cell culture insert with a polyethylene terephthalate membrane was obtained from Beckton–Dickinson Biosciences. Williams' medium E was obtained from Sigma (St. Louis, MO). β-Naphthoflavone was purchased from Wako (Osaka, Japan). Seven weeks old ICR mice were obtained from Japan SLC, Inc. (Shizuoka, Japan).

Preparation of cell culture substrata. PVLA coating was carried out as previously described [21]. Matrigel (50 μl/cm^2) was added to a cell culture insert and gelated in a CO_2 incubator for 45 min. To naturalize the Matrigel-coated cell culture insert, the Matrigel-coated cell culture insert was incubated with Williams' medium E for 15 h.

Type I collagen was cast on a cell culture insert and allowed to gelate in a CO_2 incubator for 24 h. After that, the gel was air-dried and used as collagen fibrils. The reconstructed basement membrane substratum (rBM) was prepared as previously described [15,22]. To naturalize the collagen fibrils and rBM, these substrata were incubated with Williams' medium E for 15 h.

Isolation and culture of hepatocyte. Hepatocytes were isolated by *in situ* collagenase perfusion method [23] with slight modification [24]. The isolated hepatocytes were allowed to adhere onto the substrata at the indicated cell density in Williams' Medium E, pH 7.3, containing 10 mM $NaHCO_3$, 50 μg/ml penicillin and streptomycin, and 100 μg/ml neomycin (WE medium) in a CO_2 incubator for 3 h. The medium for cell attachment was replaced by fresh WE medium to remove non-adherent hepatocytes and the adherent cells were cultured for an indicated time.

Hepatocyte adhesion assay. Hepatocytes were harvested on the substrata at a density of about 65,000 cells/cm^2. After 3 h of incubation, non-adherent cells were removed by washing with PBS containing 0.5 mM $CaCl_2$ and 0.9 mM $MgCl_2$ three times. Adherent cells were quantified by colorimetric MTT assay. Results were expressed as percent of hepatocytes adhered onto Matrigel.

Lactate dehydrogenase (LDH) release assay. To determine the percent of cell death, LDH release was quantified in the supernatants of the samples with CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, Madison, WI) as instructed by the manufacturer. For the estimation of cell death, the cell membrane damage was expressed as percent of cell lysis.

Reverse transcription-polymerase chain reaction analysis. Total RNA was extracted from hepatocytes using TRIzol reagent according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). Total RNA (0.5 μg) was used as a first-strand reaction that included oligo(dT) primer and reverse transcriptase (Invitrogen). Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed using rTaq DNA polymerase (TOYOBO, Tokyo, Japan) with specific mouse primer sets; *Gapdh*, 5′-CTCTTGCTCAGTGTCCTTGC-3′ and 5′-CTCATGACCACAGTCCATGC-3′ [25]; *Cyp1a2*, 5′-TGTTCTGGATGGTCAGAGCC-3′ and 5′-CCTCATGGATCTTCCTCTGC-3′ [26]. All primers were obtained from Espec Oligo Service Corp (Ibaraki, Japan). For each experiment, *Gapdh* was amplified to normalize the expression of other gene in the sample. The PCR products were analyzed by 1% agarose gel electrophoresis.

The Western blot analyses of secreted albumin in the culture medium. The culture medium was collected everyday and used as samples for the determination of albumin secreted into the medium. The collected medium (20 μl) was separated on SDS–PAGE. After being transferred to a PVDF membrane (Immobilon, Millipore, Bedford, MA), proteins were reacted with antibodies and detected with ECL system (GE Healthcare Bioscience, Piscataway, NJ) following treatment with 5% of bovine serum albumin (BSA) (Sigma). The specific antibodies used for this experiment was anti-mouse albumin (MP Bio Japan).

Results and discussion

Hepatocytes can efficiently adhere to rBM

The affinity of murine primary hepatocytes to PVLA substratum, Matrigel, collagen fibrils, and rBM were compared. The affinity of hepatocyte to BSA-coated cell culture insert was also checked as a negative control of non-adhesive substratum. The amounts of attached hepatocytes were evaluated by MTT assay after 3 h of incubation. As shown in Fig. 1A, hepatocytes attached to rBM as well as Matrigel. Hepatocytes also attached on PVLA substratum and collagen fibrils although their affinities were lower, approximately 60% and 70% relative to Matrigel, respectively. Approximately 40% of hepatocytes attached to

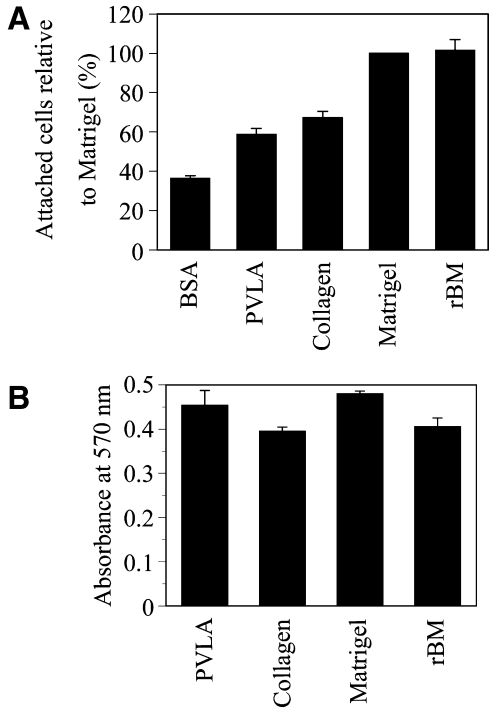

Fig. 1. Plating efficiency of hepatocytes on various types of substrata after 3 h of incubation. (A) Adhesion assay onto the substrata was performed after 3 h of incubation. Data shown are means ± SE ($n = 3$–5). (B) For the alignment of cell number attached to each substratum, the number of harvested hepatocytes is changed as shown in Table 1. Adhesion assay was performed after 3 h of incubation. Data shown are means ± SD ($n = 3$–4).

BSA-coated cell culture insert. This result indicated that the interaction between hepatocytes and basement membrane model such as Matrigel and rBM is stronger than that between hepatocytes and collagen fibrils as stromal ECM, and PVLA substratum. Moreover, comparing between Matrigel and rBM, laminin-511/521 (laminin-10/11) is included in rBM but not Matrigel (Mochitate, K., unpublished data). The interaction with laminin-511/521 (laminin-10/11) is stronger than the interaction with laminin-111 (laminin-1) [26,27]. It is expected that the number of hepatocyte attached to rBM is higher than that to Matrigel during the early time of culture.

To align the number of attached hepatocytes after 3 h of incubation, the numbers of harvested hepatocytes was changed as shown in Table 1. To confirm whether the numbers of attached hepatocytes are aligned, the amounts of attached hepatocytes were evaluated by MTT assay after 3 h of incubation. As shown in Fig. 1B, the amounts of attached hepatocytes were similar among the substrata examined. To check the number of attached hepatocytes, the number of attached hepatocytes on PVLA substratum was calculated by the total activity of lactate dehydrogenase (LDH) in the adhered hepatocytes. The number of attached hepatocytes was approximately 180,000 cells *per* cell culture insert, indicated that almost all hepatocytes harvested on rBM and Matrigel were attached within 3 h.

Table 1
The number of harvested hepatocytes on the substrata

Substrata	PVLA	Collagen	Matrigel	rBM
Number of cells	312,900	273,000	183,500	181,000

Collagen, collagen fibrils. Unit, cells *per* cell culture insert.

The morphology of hepatocyte cultured on rBM

Hepatocyte functions are related to their morphology. Therefore, it is necessary to check the morphology of hepatocytes cultured on substrata (Fig. 2). After 3 h of culture, hepatocytes are round shape on the substrata examined (Figs. 2A–D). After 1 day of culture, hepatocytes cultured on PVLA substratum kept round shape (Fig. 2E), whereas hepatocytes cultured on collagen fibrils were spreading (Fig. 2F). Hepatocytes cultured on rBM were spreading after 1 day of culture (Fig. 2G) whereas hepatocytes cultured on Matrigel kept round shape (Fig. 2H).

Hepatocytes can survive on rBM for longer time than on Matrigel

To compare the viability of hepatocytes cultured on PVLA substratum, Matrigel, collagen fibrils, and rBM, the level of hepatic cell death was quantified as the activity level of LDH, which is released by cell death in the culture medium (Fig. 3). The number of hepatic cell death on PVLA substratum was higher than that on Matrigel, collagen fibrils, and rBM. The hepatic cell death on collagen fibrils was similar with that on Matrigel whereas the death on these matrices was higher than that on rBM until 5 days. After 7 days of culture, little difference of viability was observed between Matrigel and rBM. In this study, hepatocytes were cultured without the medium change. It seems that the viability of hepatocytes cultured was rapidly decreased because of the shortage of nutrients. This result suggests that rBM is more effective substratum for hepatocyte survival than Matrigel.

As shown in Fig. 3, the viability of hepatocytes on rBM is the highest among the substrata examined. It was reported that different laminin isoforms activated different intracellular signaling pathways [28,29]. In the hepatocytes, the activation of Akt enhances their survival in the adherent culture on natural ECM [30]. Also, laminin-511/521 (laminin-10/11) can activate Akt signaling pathway [31,32]. It seems that different laminin isoforms such as laminin-511/521 (laminin-10/11) activated Akt signaling pathway in the hepatocytes on rBM and prolonged the viability of hepatocytes compared with that on Matrigel.

Hepatocytes can maintain the responsibility to β-naphthoflavone on rBM and PVLA substratum

To investigate the maintenance of liver-specific function in the hepatocytes cultured on PVLA substratum, Matrigel, collagen fibrils, and rBM, the expression of *Cyp1a2*

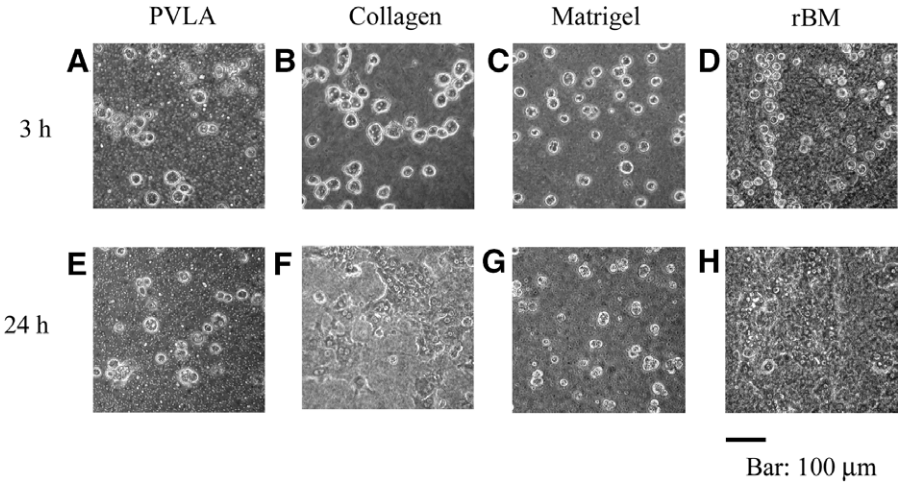

Fig. 2. The morphology of hepatocytes on various types of substrata. Hepatocyte morphology on the substrata was observed after 3 h (A–D) and 24 h (E–H) of culture. Hepatocytes were cultured on PVLA (A,E), collagen fibrils (B,F), Matrigel (C,G), and rBM (D,H). Scale bar: 100 μm.

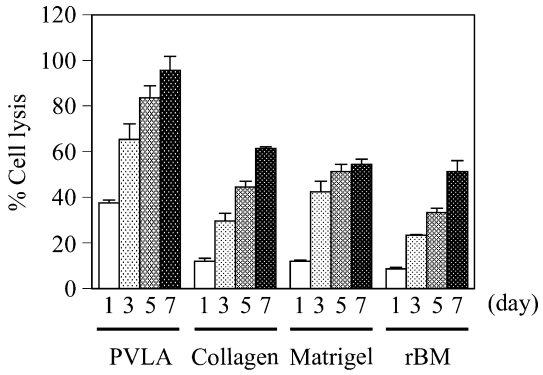

Fig. 3. The viability of hepatocytes cultured on various types of substrata. Percent of total cells releasing LDH activity was measured after 1, 3, 5, and 7 days of culture. Data shown are means ± SE ($n = 3$–4).

induced by β-naphthoflavone was checked as an indicator of liver-specific functions by RT-PCR (Fig. 4A). β-Naphthoflavone strongly induces the expression of *Cyp1a2* via the interaction between xenobiotic-responsive element (XRE) and activated aryllhydrocarbon receptor (AhR) [33]. After 1 day of culture, the expression of *Cyp1a2* was induced by β-naphthoflavone in the hepatocytes cultured on collagen fibrils. After 1 day of culture, the induction of *Cyp1a2* expression by β-naphthoflavone was similarly observed in the hepatocytes cultured on PVLA substratum, collagen fibrils, Matrigel, and rBM whereas after 3 days of culture, the induction of *Cyp1a2* was observed in the hepatocytes cultured on only PVLA substratum, collagen fibrils, and rBM but not Matrigel. After 5 days of culture, the expression of *Cyp1a2* was induced in the hepatocytes cultured on only PVLA substratum and rBM but not collagen fibrils and Matrigel. To further confirm whether hepatocytes on rBM can maintain the liver function for longer time than those on Matrigel, the amount of secreted albumin in the culture medium was measured by Western blot analysis (Fig. 4B). The level of albumin secretion in the culture medium on rBM was higher than that on Matrigel.

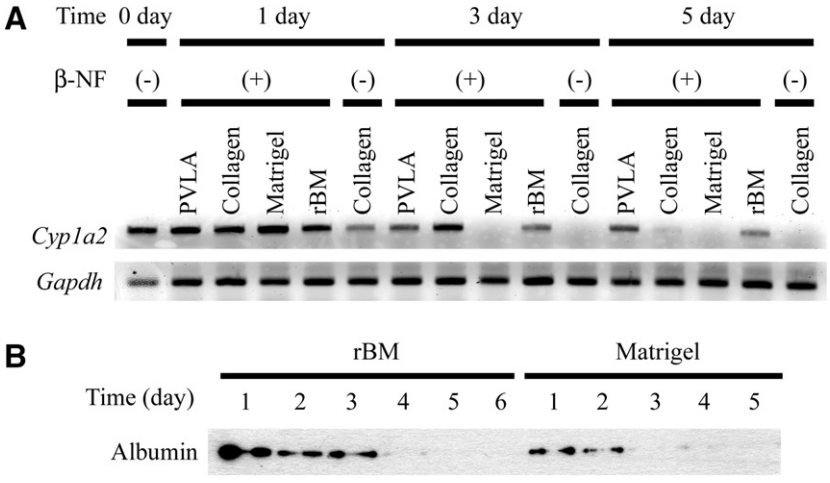

Fig. 4. The liver functions can be maintained in the hepatocytes on rBM. (A) Hepatocytes were treated with 50 μM of β-naphthoflavone after 3 h, 2 days, and 4 days of culture. After 24 h of exposure of β-naphthoflavone, semi-quantitative RT-PCR analysis of *Cyp1a2* was performed. β-NF is β-naphthoflavone. (B) Western blot analysis of secreted albumin in the culture medium.

These results indicated that rBM is more effective substratum for the maintenance of liver functions than Matrigel.

It is reported that laminin plays an important role in the expression of tissue-specific gene [34]. Laminin-511/521 (laminin-10/11) is contained in only rBM but not Matrigel. Cells attach to laminin-111 (laminin-1) substratum via integrin α6β1 and syndecans [35,36] although cells attach to laminin-511/521 (laminin-10/11) substratum via not only integrin α6β1 and syndecans but also integrin α3β1 [35,37]. Syndecans and integrin α3β1 play crucial roles in cell differentiation, especially hepatocytes [37,38]. It has been observed that hepatocytes cultured on rBM could maintain the liver-specific functions for longer time than those on Matrigel because of the binding to laminin-511/521 (laminin-10/11) via integrin α3β1 and syndecans. Not only laminin-511/521 (laminin-10/11) but also laminin α3 chain containing laminins, such as laminin-332 (laminin-5) can be contained in rBM because alveolar epithelial cells produce laminin α3 chain. These laminins also bind to integrin α3β1 and syndecan. In this study, we can not investigate the effect of these laminins on hepatocyte functions. This is an interest problem to be solved in the future.

Hepatocytes cultured on PVLA substratum also induced the expression of *Cyp1a2* by the β-naphthoflavone after 5 days of culture. It was shown that laminin-511/521 (laminin-10/11) were dominantly deposited beneath the hepatocytes cultured on PVLA substratum, suggesting that this deposited laminin-511/521 (laminin-10/11) maintain the liver-specific functions [25]. Therefore, it is seemed that at least laminin-511/521 (laminin-10/11) in rBM maintains the liver-specific functions in the hepatocytes cultured on rBM.

In this study, cell culture insert was used for hepatocyte culture. It is possible that endothelial cells attach to the opposite side of cell culture insert membrane to mimicking sinusoidal structure of the liver. When the liver is exposed by xenobiotics, such as drug, hepatocytes sometimes undergo cell death and liver will be damaged. The drugs can entry hepatocytes and nonparenchymal cell such as endothelial cells. Entering the drugs into endothelial cells, they secrete reactive oxygen intermediates, IL-1 and IL-6 [39,40]. These molecules can induce hepatic cell death to damage the liver. Therefore, to evaluate the drug toxicity, the mimicking sinusoidal structure of the liver will be important. Using cell culture insert, the culture system mimicking sinusoidal structure of the liver might be developed for drug evaluation system.

In conclusion, rBM, a basement membrane formed by epithelial cell, is an effective substratum for hepatocyte culture to develop bioartificial liver, bioreactor, and drug evaluation system using hepatocytes.

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