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e-Gnosis, núm. 4, 2006, p. 0

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Available in: http://www.redalyc.org/articulo.oa?id=73000410
ATTACHMENT EVALUATION OF EMBRYONIC STEM CELLS ON A PAU-COATED NON-WOVEN FABRIC: A POTENTIAL SOURCE FOR BIOARTIFICIAL ASSIST DEVICES

EVALUACIÓN DE LA FIJACIÓN DE CÉLULAS MADRE EMBRIONARIAS EN UN PARCHE PTFE DE PAU: UNA FUENTE POTENCIAL PARA INSTRUMENTAL BIO-ARTIFICIAL DE SOPORTE

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Recibido: abril 6, 2006 / Aceptado: junio 19, 2006 / Publicado: junio 26, 2006

ABSTRACT. Embryonic stem (ES) cells have been recently studied as an attractive source of bioartificial organs. The proliferation and differentiation of ES cells on such device should be considered. We have currently developed a hollow fiber Poly-urethane-coated unwoven PTFE fabric-typed (PAU-coated PTFE) bioartificial liver module. Here, we have evaluated the attachment capacity of ES cells, embryonic bodies and the functional capacity of hepatocyte-like cells derived from mouse embryonic stem cells on nonwoven PTFE fabric. To efficiently produce a large number of hepatocyte like cells from mouse ES cells, we formed embryonic bodies and plated on matrigel in the presence of fibroblast growth factor-2, and the deleted form of hepatocyte growth factor as well of dimetilsulfoxide and dexametason. Cellular adherence and morphology on PAU-coated unwoven PTFE fabric was regularly assessed by a scanning electron microscope (SEM). For hepatocyte differentiation, gene expression of albumin was examined. To test the function of these hepatocytes in vitro we evaluated the metabolizing capacity using ammonia and lidocaine and found that the cells cultured on PTFE expressed and secreted better quantities of albumin and significantly better metabolizing capacities. In the present work we have demonstrated the successful attachment of mouse ES cells on PAU-coated unwoven PTFE fabric and subsequent hepatic differentiation. Thus, culture and subsequent differentiation of ES cells on PAU-coated unwoven PTFE fabric could be a useful new scaffolding biomaterial towards to develop bio-artificial liver and a new model for drug screening.

KEYWORDS: embryonic stem cells, hepatocytes-like cells, attachment, Scanning Electron Microscope, differentiation, fiber-and PAU-coated unwoven clothes (PTFE), bio-artificial support.

RESUMEN. Las células embrionarias han sido estudiadas recientemente como un recurso atractivo para la construcción de órganos artificiales. La proliferación y subsiguiente diferenciación de las células embrionarias dentro de los mismos dispositivos artificiales deben ser consideradas. Recientemente, hemos desarrollado un hígado bioartificial usando una tela sintética con fibras huecas recubiertas con un poliaminouretano, el cual facilita la atracción celular dentro del dispositivo hepático. En esta ocasión hemos evaluado la capacidad de atracción celular de las células embrionarias y de cuerpos embrionarios así como la funcionalidad de hepatocitos derivados de células embrionarias de ratón en la fibra sintética. Para diferenciar un gran número de hepatocitos derivados de las células embrionarias, nosotros formamos cuerpos embrionarios y los expusimos al factor 2 de crecimiento de los fibroblastos y a la forma sintética del factor de crecimiento de los hepatocitos así como dimetilsulfoxido y dexametasona. La adherencia celular y morfología sobre la fibra sintética fue evaluada regularmente usando microscopio electrónico (SEM). Para evaluar la diferenciación celular examinamos la expresión del gen de la albúmina. Para probar la función de los hepatocitos derivados In Vitro, nosotros evaluamos la capacidad de metabolizar amonio y lidocaina, encontramos que las células cultivadas sobre la fibra sintética expresan y secretan mayores cantidades de albúmina y metabolizan significativamente mejor que los controles. En este trabajo de investigación hemos demostrado que existe una adecuada atracción de las células embrionarias de ratón cultivadas sobre la fibra sintética y que también son capaces de diferenciarse en hepatocitos funcionales. Por esta razón, el cultivo y subsiguiente diferenciación de las células embrionarias sobre la fibra sintética podría ser útil en el desarrollo de
Biosoportes para la construcción de hígados bio artificiales y un nuevo modelo de cultivo para el estudio in vitro de nuevos medicamentos.

PALABRAS CLAVE: células madre embrionarias, células tipo hepatocitos, vinculación o apego, microscopio electrónico de barrido, diferenciación, parches de PTFE (Poli tetra fluoro etileno, soporte bio-artificial).

Introduction

Embryonic stem cells can be differentiated into representative derivatives of all three embryonic germ layers (endoderm, ectoderm, and mesoderm) both in vivo and in vitro [1]. Embryonic stem (ES) cells have drawn much attention in the field of regenerative medicine, cell therapy and tissue engineering [2]. Especially in the field of liver diseases tissue engineering methods are being examined as a means to replace damaged hepatic tissue or provide short-term support until the damaged liver spontaneously recovers or a donor liver becomes available for transplantation. However, the shortage of donor livers, high costs, and life-long requirement for immunosuppression limit the use of orthotopic liver transplantation and primary hepatocyte transplantation [3,4]. Therefore, effective therapies, such as bioartificial livers (BALs) have been explored [5,6]. Thus, researchers have been exploring alternative sources of human hepatocytes, such as immortalized human hepatocytes [7] and embryonic stem (ES) cell-derived hepatocytes [8-11]. One of the unique advantages of ES cells over immortalized hepatocytes is that ES cells can be grown without any genetic manipulation and when provided an appropriate environment of differentiation, the cells can differentiate into any types of mature functional cells [1]. Currently, efficient conversion of ES cells into hepatocyte like-cells have been achieved by a combination of various growth factors [12-14]. Such cells are a potential cell source for BAL. Thus, selecting the material for such a scaffold is extremely important to provide a three dimensional structure with the goal of creating a functional environment for the success of such devices [15]. Previously our group has developed a Poly-amino-urethan-coated (PAU) non-woven PTFE fabric for the development of a newly bioartificial liver and pancreas (BAL) [15].

In the present work, we have investigated the feasibility of the mES cells to attach, grow and function on a novel three-dimensional PTFE fabric coated with a Poly-amino-Urethan that provided cell attachment in its different stages of differentiation. In addition this PTFE created by our laboratory for bioartificial support was evaluated to allow mES cells and demonstrate that are a promising substrate for future bioartificial support technology. The results from this study suggest that the differentiation stage of the mES cells plays a role on the effective attachment to the PTFE probably legated to the expression of certain adhesion molecules in the cell membrane like the integrin family. In this work we demonstrated the effective attachment of the mES cells according to the subsequent differentiation into hepatocytes-like cells. We consider that this is valuable information for the future experiments and the progress of embryonic stem (ES) cells-based therapies in regenerative medicine.

Materials and methods

Culture of mouse ES (mES) cells

Mouse ES cells derived from Balb/c × 129sv embryos were expanded on a feeder layer of mitomycin-treated mouse embryonic fibroblasts [16], seeded on 0.1% gelatin-coated plates (R-ES-006B, Dainippon Pharmaceutical Co., Osaka, Japan), and cultured in Dulbecco’s Modified Eagle Medium containing 15% fetal bovine serum, 1% nonessential amino acid, 1% nucleosides, 2-mercaptoethanol (110 μM), 1% penicillin/streptomycin, 1% glutamic acid, and 500 U/mL leukemia inhibitory factor (LIF) (Dainippon Pharmaceutical Co., Osaka, Japan).
Embryonic body (EB) formation of mES cells

ES cells (2.5x10^5 cells / ml) in suspension were cultured by suspension method for 5 days with Dulbecco’s Eagle’s medium Nutrient Mixture F-12 (DMEM/F12) (Gibco BRL), containing 4.5 mg/ml glucose, 2 micro M L-Glutamine, 25 micro M Hepes (Gibco BRL) 100 microgram/ml penicillin, 100 microgram/ml streptomycin (Sigma), 3% albumin from bovine serum (Sigma) without LIF and fetal bovine serum and 10% fetal bovine serum (CCT, Canada).

Hepatic differentiation of mES cells in culture

The resulting EBs were transferred onto nonwoven PTFE fabric coated with poly-amino-urethan (PAU) which has cellular adhesive property, treated for 3 days with 100 ng/ml basic fibroblast growth factor (FGF2) (PreproTech EC, UK), then with 100 ng/ml dHGF (a deleted variant of hepatocyte growth factor) and 1% dimethyl sulfoxide (DMSO) for 8 days, and eventually with 10^-7 M dexamethasone (DEX)(Sigma) for 3 days.

Morphological Assessment

ES-Heps 8 hours after were plated onto PAU-coated PTFE were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Electron microscopic examinations were performed as previously reported[17]. Ten different areas were randomly chosen and examined. Then, the cells inoculated onto unwoven PTFE fabric coated with PAU were subjected to micro-structural analysis using scanning electron microscope (SEM) (Hitachi S-2300, Hitachi Co. Ltd., Tokyo, Japan).

Measurement of cellular activity of the cells attached on PAU-coated non-woven PTFE

An MTT assay was performed to analyze the number of viable cells attached onto PTFE fabric where 1x10^6 of mES, EBs and mES derived-hepatocytes were inoculated on PTFE (1cm^2) by using 0.5 mg/ml of 3-(4,5-dimethyl- thiazole- 2- yl)- 2.5- diphenyltetrazolium bromide (MTT reagent) (Sigma), as previously reported [18]. First, we inoculated the cells into 12-well plates, cultured for 8 hours and washed two times with PBS and transfer to a new plate, finally the cellular activity was analyzed by viable cell number attached on PAU-coated non-woven PTFE by an MTT assay. The cell number was confirmed every time using trypan blue dye exclusion test.

Gene expression analysis

Total RNA from 1 × 10^6 undifferentiated mouse ES cells, EBs, mES derived-hepatocytes ES-Heps), mouse primary hepatocytes, and mouse embryonic fibroblasts (MEF) were extracted using RNA Trizol (Invitrogen) according to the manufacturer’s protocol. Reverse transcription (RT) was performed at 22°C for 10 min and then 42°C for 20 minutes using 1.0 μg of RNA per reaction to ensure that the amount of amplified DNAs was proportional to those of specific mRNAs in the original samples, as previously reported[17] Polymerase chain reaction (PCR) was performed with specific primers in volumes of 50 μl containing 2.0 μg reverse-transcriptase products, according to the manufacturer’s protocol (PCR kit; Perkin-Elmer/Celtus, Norwalk, CT). The amplification reaction involved denaturation at 95°C for 1 minute and 72 °C for 1 minute using a thermal cycler (Perkin/Elmer, Foster City, CA). The PCR products were resolved on 1% agarose gels and visualized by ethidium bromide staining. Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control for the efficiency of mRNA isolation and cDNA
synthesis. PCR primers used and experimental conditions were as follows: ALB (5’-GCTAGGCACACAGTGCTTG, 5’-CAGGATTGCAGACAGATAGTC); the beta 1 integrin sequences were taken from Tominaga et al.[19]: (5`-TGCCTACAACTCTTTCTTC, 5`-CACCACATTCACAAATACC); GAPDH (5`- TGAAGGTCGGTGTGAACGGATTTGGC, 5`-TGTGGGGGCCGAGTTGGGATA).

**Evaluation of metabolic capacities of ES cells, EBs and hepatocyte-like cells**

At the end of hepatic differentiation on matrigel (BD, USA), mouse ES cells cultured were transferred to PAU-coated PTFE and cultured for 24 hours and subjected to metabolic and synthetic tests. Ammonium sulfate (0.56 mM) and lidocaine (1 mg/ml) were added to individual wells of 12-well plates and the amount of each substrate remaining in the media after culture for 4 hours was measured. The ammonia concentration was determined using a Fuji Dri-Chem slide (Fuji Co., Tokyo, Japan) and concentration of lidocaine was measured by SRL (Tokyo, Japan) as previously reported [20]. These functional parameters were compared per microgram cell.

**Figure 1.** Schematic representation of the experimental strategy for evaluation and differentiation of mES cells to hepatocytes

Mouse embryonic stem cells (mES cells) were cultured, expanded and evaluated for cell attachment on mouse embryonic fibroblast (MEF) layer and after in suspension method for 5 days to form embryoid bodies. The resulting EBs were transferred onto non-woven polytetrafluoroethylene (PTFE) fabric coated with PAU and evaluated for cell attachment, the EBs were treated with fibroblast growth factor (FGF)-2
(100 ng/ml) for 3 days, then with the deleted variant of hepatocyte growth factor (dHGF; 100 ng/ml)) and 1% dimethyl sulfoxide (DMSO) for 8 days, and at the end stage with dexamethasone (DEX; 10^{-7} M) for 3 days for hepatic differentiation and evaluated for attachment and functional ability. As a control, mES cell-derived hepatocytes were cultured with Matrigel instead of PTFE fabric.

**Statistical analyses**

Mean values are presented with SDs. A Student's $t$ test was used to calculate the significance of difference in mean values. A $p$ value < 0.05 was considered statistically significant.

**Results**

EBs and ES-Heps attached favorably on PAU-coated non-woven PTFE fabric

We examined the grade of attachment of mouse ES cells by the quantity of cells observed by SEM and MTT after 8 hours of culture process of the mES cells onto the poly-amino-urethan-coated unwoven PFTE fabric previously developed by our laboratory for bioartificial systems. The net branch points and three-dimensional open spaces up to approximately 300 micrometers and were distributed throughout the structure and individual fibers generally exhibited a smooth surface as revealed by SEM micrographs (Fig. 2 A-C). These include undifferentiated ES cells (UnES) (Fig. 2 A), embryonic bodies (Fig. 2 B), and ES derived hepatocyte-like cells (Fig. 2 C). In the case of UnES the cells did not show a well attachment to PAU-coated PTFE fabric. mES cells were incubated by suspension culture method for 5 days and formed EBs. The resultant EBs attached favorably on the surface of non-woven PTFE fabric coated with PAU that has cellular adhesive properties. The EBs were uniformly distributed over the PTFE cloth (Fig. 2-B). At days 14 of hepatic differentiation process, mES-derived hepatocytes were uniformly distributed over the PTFE cloth (Fig. 2-C). Notably, these results were compatible with our data regarding cell activity after cell culture of the cells in their different differentiation stages showed by MTT (Fig. 2-D).

hES cell-derived hepatocytes showed albumin gene expression and beta-1 integrin expression according to their differentiation stage

The expression of albumin was detected in the time dependent manner of the process of hepatic differentiation of 14 days. No albumin expression was detected in either undifferentiated mES cells or MEF on day 5 (Fig. 3). Notably, in equivalent time of albumin expression, EBs and mES cell-derived hepatocyte showed more intense band for beta-1 integrin (Fig. 3). Such expression profile was also confirmed by a real-time PCR. The data were representatives from 3 independent experiments.
Figure 2. Morphology of hES cell-derived hepatocytes

(A) The mES cells did not show well attachment on the surface of non-woven PTFE fabric coated with PAU. (B) EBs formed by mES cells attached favorably to PAU-coated PTFE fabric. The EBs were uniformly distributed over the PTFE cloth. Bar= 100 micrometer. (C) At days 14 of hepatic differentiation process, mES cells were cultured 8 hrs and showed a uniform distribution and attachment over the PTFE fabric. Bar= 200 micrometer. (D) MTT assay of mES, EBs and mES cell-derived hepatocytes showed the cellular mitochondria activity of the cells that were attached to PAU-coated PTFE fabric. (O.D. Units; optical density units). * indicates p < 0.05.

mES-derived hepatocytes metabolized ammonia and lidocaine cultured onto PAU-coated PTFE

At days 14 of the hepatic differentiation process, mES cell-derived hepatocytes cultured on PAU-coated PTFE fabric after 24 hours metabolized 16 % of the loaded ammonia and 29.5 % of the loaded lidocaine, respectively (Fig. 4-A, B). Such metabolic activities were not observed in undifferentiated mES cells. The use of PAU-coated PTFE fabric significantly enhanced hepatic function of mES-driven hepatocytes in terms of ammonia (Fig. 4-A) and lidocaine metabolism (Fig. 4-B) compared to that of Matrigel. The values were compared per microgram cell. Data are means+ SD. The data were representatives from 3 independent experiments.
At 14 days during the process of differentiating mES cells into hepatocytes, the expression profile of albumin and beta-1 integrin was analyzed by RT-PCR. Normal mouse hepatocytes served a positive control, and undifferentiated mES cells and MEF served as a negative control. Such profile was confirmed by real-time RT-PCR analysis (Und, undifferentiated mES cells; EBs, embryonic bodies formed by mES cells on day 5; mES-Heps, mES-derived hepatocytes cultured on PAU-coated non-woven PTFE fabric on day 14 of hepatic differentiation).

Figure 3. Gene expression profile of albumin and beta-1 integrin of mES cells, EBs and mES cell-derived hepatocytes

Figure 4. Functional capacities of mES cell-derived hepatocytes

Application of PTFE fabric coated with PAU in hepatic differentiated cells significantly enhanced ammonia metabolism (A) and lidocaine metabolism (B), of mES cell-derived hepatocytes. * indicates $p < 0.05$. These
parameters were compared per microgram cell. Data are means ± SD. The data were representatives from 3 independent experiments.

Discussion

Normal human hepatocytes are an ideal source for BAL development, however, the shortage of donor livers severely limits their use of normal human hepatocytes in BAL. In addition, once human hepatocytes are available from discarded livers that are not suitable for organ transplantation, primary isolated hepatocytes do not proliferate in vitro. To overcome these problems, pluripotent stem cells have been investigated as a potential alternative to hepatocytes because they can unlimitedly replicate in the undifferentiated state and produce progenitors of various tissue specific cells. Although methods for hepatic differentiation of ES cells have been reported, none of them have described about drug-or ammonia-metabolizing activities [21-23]. In this study we have applied a poly-amino-urethane-coated non-woven polytetrafluoroethylene fabric (PAU-coated PTFE) to construct an engineered hepatic tissue that in vitro can maintain the function of mES cell-derived hepatocytes. This material previously developed for bioartificial pancreas by our group is able to provide a three-dimensional atmosphere and a well attachment circumstance to the cells. Such environment can maintain cell-cell contact and such interactions in hepatocyte culture coordinately modulates liver transcription factors and allows the cultured hepatocytes to perform sophisticated functions [24]. The extra cellular matrix environment has been known to be critical for hepatocyte differentiation, and gene inactivation studies have shown that b1-containing integrin receptors are essential for liver development [25]. The results of the present study are relevant to various hepatic cell types and physiological states. Because cell proliferation and differentiation are often mutually exclusive, the requirement for beta-1 integrin during hepatocyte differentiation may in principle reflect the ability of this kind of integrin to promote exit from the cell cycle and thereby favor differentiation [26]. In accordance with this hypothesis, our results show that, once plated on a PAU-coated PTFE substratum, the attachment ability of the cells vary according to the differentiation stage and apparently PAU-coated PTFE provide a cellular platform that assist liver functions.

Researchers have often used collagen or Matrigel for hepatic differentiation of hES cells as a scaffold, but we have to take it for granted that such a material is animal-derived. Considering the clinical application of hES-derived hepatocytes, we have to explore non-animal derived materials. PAU-coated non-woven PTFE fabric is free of animals and is chemically synthetic. We have already confirmed that the material was biocompatible when used as a component of an extracorporeal bioartificial pancreas in diabetic pigs. In fact, the use of PTFE fabric coated with PAU allowed the mES-derived cells to form 3-dimentional structure and cell-cell interactions. Thus, considering this material as a scaffold for cell differentiation is a important issue that can be perform in future experiments using human embryonic stem cells.

The present study has clearly demonstrated that cell attachment to the PAU-coated PTFE is according to the grade of differentiation and the gradual expression of the integrins super family. In vitro hepatic differentiation of mES cells is practically feasible. Further efforts will be required to generate more mature hepatocytes of which functions are compatible to normal hepatocytes. We believe that this work would be an important step toward the potential application of ES cells to treat the patients suffering from ALF with BAL.
Grant Support

This work was supported in part by the Ministry of Education, Science, and Culture, and the Ministry of Economy and Industry, and Life Science Project of 21st Century, Japan.

References

19. Tominaga S. Murine mRNA for the beta-subunit of integrin is increased in BALB/c-3T3 cells entering the G1 phase from the G0 state. FEBS Lett 1988; 238:315-319.

