발생과 생식 제 10권 제 1호, 63~73 (2006)
Dev. Reprod. Vol. 10, No. 1, 63~73 (2006)

In Vitro Differentiation of Human Amniotic Membrane-derived Stem Cells into Hepatocyte-like Cells

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양막 유래 줄기세포의 간세포로의 분화 유도

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ABSTRACT : This study aimed to find out suitable culture conditions for the differentiation of human amniotic membrane-derived stem cells(HAM) into hepatocyte-like cells. Almost homogenous population of fibroblast-like cells was successfully isolated from the amniotic membrane. In comparison to the non-coated plates and in the absence of insulin/transferrin/selenium(ITS), HAM cultured on the fibronectin-coated plates and in the presence of ITS showed the more intense immunocytochemical staining against the albumin. Addition of both fibroblast growth factor(FGF)-1 and -2 to the differentiation medium gave stronger staining compared to the treatment with FGF-1 or -2 alone. Periodic acid Schiff's base staining of glycogen and morphological turnover of fibroblast-like appearance of HAM into round shape matched the results of immunocytochemical studies. When the efficiency of two-step culture method was examined on the differentiation of HAM into hepatocyte-like cells, all of the results of immunocytochemical staining, periodic acid Schiff's staining and morphological change exhibited effective hepatic differentiation of HAM compared to the continuous culture method. Immunoblot analyses of HAM- conditioned media against the albumin showed that the culture of HAM in the presence of both ITS and fibronectin always gave a stronger staining intensity than those in the absence of them, and that the addition of ether mixture of FGF-4 and either FGF-2 or transforming growth factor(TGF)-α to the culture medium significantly enhanced the albumin secretion by HAM.

Based on these observations, it is suggested that HAM could differentiate into hepatocyte-like cells under a culture condition consisting of fibronectin and ITS, and addition of FGF-4 with either one of FGF-2 or TGF-α could enhance the hepatic differentiation of HAM.

Key words : Hepatocyte, Human amniotic membrane-derived mesenchymal stem cells, Differentiation.

요 약 : 간질환 환자의 대부분은 간 조직 손상으로 인해 간세포의 재생 능력이 감소한다. 간세포 이식은 이러한 간질환을 치료하는데 있어 혁신적인 방법으로 대두되고 있으나, 여전히 많은 의문과 문제점이 제기되고 있다. 사람의 양막으로부터 얻은 줄기세포를 이용하여 간세포 분화를 위한 최적의 조건을 알아 보고자 하였다. 세포내 알부민에 대한 면역 화학적 방법, 세포내 글리코겐의 특이 염색법, 세포의 형태적 변화 연구 방법 등을 이용하여 여러가지 배양 조건을 조사한 결과, 배양 접시를 fibronectin으로 coating하고 배양액내에 insulin/transferrin/selenium(ITS)을 첨가하는 것이 양막 줄기세포의 간세포로의 분화에 효과적이었다. 또한 배양액내에 fibroblast growth factor(FGF)-1과 FGF-2를 함께 첨가하는 것이 둘 중 하나만 첨가하거나 첨가하지 않는 것보다 효과적이었다. 한편 분화 배양은 한가지 배양액을 사용한 지속적인 배양법(continuous culture method)보다 배양 조건을 달리하여 두 단계로 배양하는 2단계 배양법(two-step culture method)가 훨씬 효과적이었다. 마지막으로, 기본 배양액에 FGF-2와 FGF-4를 첨가한 조건과 FGF-4와 TGF-α를 첨가한 조건이 다른 조건 보다 알부민 분비를 많이 하는 것으로 보아 FGF-4가 간세포 분화 과정에 중요한 역할을 하는 것으로 여겨지며 FGF-2 및 TGF-α 첨가는 더욱 효과적인 배양 조건으로 관찰되었다. 따라서, 양막에서 유래한 성체 줄기 세포는 적절한 배양 조건이 주어질 때, 간세포로 분화가 가능하며, 분화 과정에서 FGF-4가 주도적인 역할을 하며 FGF-2와 TGF-α는 상승 효과를 갖는 것으로 사료된다.

INTRODUCTION

Mesenchymal stem cells(MSC) are referred to as highly proliferate and adherent fibroblastic cells featuring a unique expression profile of cell surface molecules. They

This study was supported by the Bahrom Research Grant 2006 of the Seoul Women's University.

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reside mainly in bone marrow(BM) although they can be also isolated from a variety of other connective tissues and peripheral blood. In BM, there are certain populations of stem cell sources including hematopoietic stem cells(HSC), marrow stromal stem cells and multipotent adult progenitor cells. These are adult stem cells that are an attractive source of cells for tissue therapy, and are though to have developmental potentials or plasticity of multiple cell lineages(Raff, 2003).

The liver is the largest organ in mammals and it serves a variety of important function. Hepatocytes, the primary cells of the liver, perform various functions, including metabolizing diverse dietary molecules, detoxifying compounds and storing glycogen. Liver diseases are caused by infectious agents, autoimmune attack, malignant transformation, inborn genetic deficiencies or secondary defects. Most liver diseases lead to 'hepatocyte dysfunctions' with the possibility of eventual organ failure. Liver organ transplantation is the only effective therapy for many hepatic disorders. However, the procedure is invasive and not widely available to patients at a given time point because of limited availability of donor livers.

BM-derived cells of mice were shown to differentiate into hepatocytes both *in vivo* and *in vitro*(Shi *et al.*, 2005). Embryonic stem cells of mice were also shown to differentiate into hepatocytes *in vitro*(Teratani *et al.*, 2005). In humans, embryonic stem cells, BM-derived cells(Schwartz *et al.*, 2002; Lee *et al.*, 2004) and cord blood cells(Hong *et al.*, 2005; Wang *et al.*, 2005) have been shown to be possible candidate cell sources for hepatocyte transplantation. However, for the use of embryonic stem cells, it is necessary to determine how to avoid tumor formation and immune rejection, to induce differentiation into hepatocytes in an efficient fashion, and to resolve the ethical issues surrounding use of material from embryos.

Recent reports indicate that amnion tissue could be a novel source of multipotent stem cells that can be used in pharmacological studies and clinic. The amnion consists of a single layer of epithelial cells on a thicker basement membrane and collagen spongy layer containing MSC(Meinert *et al.*, 2001). The amnion has been applied clinically, e.g., for the treatment of burn lesions, to cover surgical wounds to avoid collusion, and in ocular surface reconstitution, since human amniotic epithelial cells(HAE) seem to be relatively resistant to rejection even after allotransplantation. They have been shown not to express immunosuppressive factors, such as CD-59(Rooney & Morgan, 1992) and soluble HLA-G(Rebmann *et al.*, 1999). Under the appropriate culture conditions, HAE have been observed to differentiate into a variety of somatic cells including neural cells, pancreatic beta cells and hepatocyte-like cells(Takashima *et al.*, 2004). In contrast, human amniotic membrane-derived stem cells(HAM) express phenotypes of neuroglial progenitor cells(Sakuragawa *et al.*, 2004) and exhibit some characteristics of cardiomyocytes(Zhao *et al.*, 2005). Because the amniotic tissue is disposed after parturition, it is easy to obtain and overcomes the ethical issues associated with use of fetal tissues.

The present study was performed to examine whether the HAM could differentiate into hepatocyte-like cells and what type of culture conditions could be good ones to produce high outcome of albumin-producing cells.

MATERIALS AND METHODS

1. Chemicals

Acrylamide, bisacrylamide and *N,N,N',N'*-tetramethylethylenediamine were purchased from Bio-Rad(Hercules, CA, USA). Mouse monoclonal antibody against human albumin was purchased from Zymed(San Francisco, CA, USA). Fibroblast growth factor(FGF)-1, FGF-2, FGF-4, transforming growth factor(TGF)-α and hepatocyte growth factor(HGF) were purchased from Peprotech(Princeton, NJ, USA). Auroprobe^{TM}BL PLUS goat anti-mouse immunoglobulin G(IgG) and reagent A and B were purchased from Amersham(Buckinghamshire, England). Collagenase A and DNase were purchased from Roche(Rotkreuz, Switzerland). Dulbecco's Modified Eagle Medium(DMEM- LG), trypsin, and ITS premix containing 1.0g/L insulin, 0.55g/L transferrin and 0.67 mg/L selenium was supplied from the Gibco(Grand Island, NY, USA). Unless specified elsewhere, all other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO, USA).

2. Isolation and Culture of HAM

Placenta was obtained at cesarean section from the volunteers, with the informed consent. The amnion was peeled off mechanically from the chorion and washed in phosphate-buffered saline(PBS) to remove blood and cellular debris. The human amniotic epithelial cells, which were layered the surface of the amnion that bathed by amniotic fluid *in vivo*, were removed as follows. The amnion tissue was minced and placed in DMEM-LG containing 0.25% trypsin. The flask was maintained at 37℃, with stirring for 30 min. After centrifugation, the first digestion supernatant, which consisted primarily of red blood cells, was discarded. The residual tissue was minced and incubated again at 37 ℃ with 0.25% trypsin for 30 min with stirring. Liberated cells, mostly epithelial cells, were removed by centrifugation and the procedure was repeated again. The remaining tissue pieces were minced and placed in PBS containing 2mg/mL collagenase A and 0.05mg/mL DNase. The mixture was incubated at 37℃ for 2 h with stirring. The dispersed mesenchymal cells were collected by centrifugation at 800×g for 10 min. After several washes with DMEM-LG, the cells were plated in 75mL-size culture flask(Nunc, Rochester, MN, USA) containing DMEM supplemented with 100U/mL penicillin, 0.1mg/mL streptomycin, 3.7mg/mL sodium bicarbonate, and 10% fetal bovine serum(FBS, Gibco). The medium was changed twice a week after 7 days from the primary culture. When achieving confluence, cells were treated with 0.125% trypsin and 1mM ethylenediamine tetraacetic acid(EDTA) for 3 min. Released cells were collected and subcultivated again. Morphologically homogeneous population of fibroblast-like cells, namely HAM, was obtained after 1 or 2 subcultures.

Human hepatoblastoma cell line(HepG2; HB8065) were obtained from ATCC and used after subculture using the maintenance medium.

3. Hepatogenic Differentiation

To induce the differentiation of HAM into hepatocyte-like cells, 6-well or 24-well culture plates(Nunc) were coated with 10 μg/mL human fibronectin solution diluted in PBS. The plates were left overnight at 37℃, and then were washed with PBS three times. HAM were seeded on non- or fibronectin-coated plates.

In the Experiment 1, cells were either cultured on non-coated or fibronectin-coated plates for 3 weeks in the basic medium(BM) consisted of DMEM-LG, 10% FBS, 20ng/mL HGF, 20ng/mL oncostatin M(OSM) and 1 μM dexamethasone(Dex) to which either 20ng/mL FGF-1, 20ng/mL FGF-2, ITS premix alone or their combinations were added. Medium was changed twice a week. After culture, cells were analyzed for the hepatocyte-related functions by immunocytochemistry, immunoblotting and periodic acid-Schiff's (PAS) staining.

In the Experiment 2, cells were cultured in BM supplemented with either 20ng/mL FGF-1, 20ng/mL FGF-4, 20ng/mL TGF-α, ITS premix alone or their combinations. Medium was changed twice a week. After 3 weeks, the cells were analyzed for the presence of albumin by immunoblotting.

In the Experiment 3, cells were cultured in BM supplemented with ITS premix and 0.1mM *L*-ascorbic acid 2-phosphate, to which one of combinations consisting of 20ng/mL FGF-1, 20ng/mL FGF-2 or 20ng/mL FGF-4 was added. Medium was changed twice a week. After 3 weeks, the cells were analyzed for the presence of albumin by immunoblotting.

In the Experiment 4, the efficiency of the two-step culture method was compared to the continuous culture method for the hepatic differentiation of HAM. In the first initiation step, 3×10^4 cells were initially cultured in 6-well dishes in a medium consisting of DMEM-LG, 10% FBS, 20ng/mL HGF, 20ng/mL FGF-1, 20ng/mL FGF-2 and ITS. After culture for 10 days, cells were divided into two groups for the second maturation step. One group of HAM as a control was cultured again for 10 days in the same medium as in the initiation step and the other group was cultured for 10 days in the same medium wherein FGF-1 and -2 were eliminated but 20ng/mL OSM and 1 μM Dex were added to. Half of each group was also examined for the effect of fibronectin by using non-coated plates or fibronectin-coated plates.

4. Immunocytochemical Analysis

For the immunocytochemical analyses, HAM harvested from the above experimental groups after termination of

the culture were seeded onto 8-well chamber slides(Nunc). After culture, they were fixed with 4% paraformaldehyde in PBS for 1 h at 4℃, and then rinsed with PBS. They were permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature. After several washing, cells were incubated in 3% hydrogen peroxide for 15 min to quench the endogenous peroxidase activities. They were rinsed with Tris buffer consisting of 0.15M NaCl, 0.05M Tris-HCl(pH 7.6) and 0.05% Tween 20 and incubated in blocking solution containing 2% bovine serum albumin(BSA) for 1 h at room temperature. Cells were then incubated with 1 μg/mL mouse monoclonal antibodies against human albumin, which were diluted in 0.05M Tris-HCl with 0.1% Tween 20 and 0.015M sodium azide. After incubation with the primary antibodies for 17 h at 4℃, cells were washed in Tris buffer. And cells were incubated with biotinylated goat anti-mouse IgG and anti-rabbit IgG for 20 min at room temperature. The cells were rinsed three times, and then incubated with horseradish peroxidase-conjugated streptavidin (DAKO, CA, USA) for 20 min at room temperature. Immunoreactivity against the albumin was visualized utilizing the 3,3'-diaminobenzidine tetrahydrochloride. The color reaction was stopped with water. Finally they were counterstained with Mayer's Haematoxylin and then observed under a light microscope(LSM410; Carl Zeiss, Oberkochen, Germany).

5. PAS Stain for the Glycogen Granules

Cells cultivated on fibronectin-coated 8-well chamber slides were fixed in 4% paraformaldehyde for 30 min at room temperature and rinsed with PBS. They were permeabilized with PBS consisting of 0.1% Triton X-100 for 10 min. After several washing, the cells were oxidized in 1% periodic acid for 5 min at room temperature and rinsed three times in deionized H_2O(dH_2O). After treatment with Schiff's reagent for 15 min at room temperature, they were rinsed in dH_2O for 5～10 min. Cells were counterstained with Haematoxylin for 3 min, rinsed in dH_2O and observed for the staining using a light microscope.

6. Immunoblotting Analysis for the Albumin

Cells were cultured on fibronectin-coated plates of 8-well chamber slides in BM containing one of cytokine combinations consisting of FGF-1, FGF-2, FGF-4 or TGF-α, in the absence or presence of ITS and *L*-ascorbic acid 2-phosphate. After 3 weeks of culture, cells were transferred to a serum-free DMEM-LG and then cultured again for 12 h. These starvation media were harvested at the end of culture and followed by concentration(1:20) using Centricon (Millipore, Bedford, MA, USA). Then they were mixed with an equal amount of 2× sample buffer consisting of 0.125M Tris-HCl(pH 6.8), 4% sodium dodecyl sulfate(SDS), 10% 2-mercaptoethanol, 20% glycerol and 0.004% bromophenol blue, and then boiled at 95℃ for 5 min.

Cellular proteins were separated by electrophoresis on 10% SDS-polyacrylamide gel with a pre-stained protein molecular marker. Subsequently, gels were soaked in a transfer buffer composed of 25mM Tris(pH 8.4), 192mM glycine and 10% methanol for 15～30 min. To hydrate polyvinylidene difluoride(PVDF) membranes(Immobilon-P; Millipore), they were soaked in absolute methanol for 15 sec, soaked in distilled water for 2 min and then equilibrated in transfer buffer for 5 min. Proteins on the gel were electrotransferred onto a PVDF membrane for 60 min at 4℃ at 100V. To saturate non-specific binding sites, membranes were incubated at 37℃ for 1 h in a washing buffer containing 0.8% NaCl, 0.02% KCl, 0.14% $Na_2HPO_4 \cdot 2H_2O$, 0.02% KH_2PO_4, 0.2% Tween 20, 10mM sodium azide and 5% BSA. Membranes were then incubated for 1 h in washing buffer containing 1% normal goat serum and 1 μg/mL mouse monoclonal antibody against human albumin. Following several washes with the washing buffer containing 0.1 % BSA, membranes were incubated for 1 h in washing buffer containing 1:100 diluted gold-labeled goat anti rabbit IgG. After reaction, the signal was visualized using an Inten SE BL kit according to the manufacturer's instructions.

RESULTS

1. HAM Cultivation

After two weeks from the beginning of primary culture of HAM, cells of a fibroblast-like colony surrounded by population of various types of cells appeared. However, during the subsequent 2～3 passages, most cells of other types disappeared but a homogenous population of fibro-

blast-like cells remained and showed active proliferation. During maintenance culture in DMEM-LG supplemented with 10% FBS, fibroblastic appearance of HAM remained unchanged until 10th passage(Fig. 1A, 1B & 1C). Human hepatoblastoma cell line HepG2 was polyhedral shape which also did not change during maintenance(Fig. 1D & 1E).

2. Immunocytochemical Analysis for Albumin

In the Experiment 1, HAM were either cultured on non-coated or fibronectin-coated plates using BM. When they were immunocytochemically analyzed after culture, the intensity of albumin staining was considerably stronger in the HAM cultured in the fibronectin-coated plates than those in the non-coated plates(Fig. 2A & 2B). However, regardless whether HAM were cultured in non-coated or fibronectin-coated plates, HAM cultured in the presence of ITS showed stronger staining than those in the absence of ITS (Fig. 2A & 2B). Among the hepatogenic media containing FGF-1 and/or FGF-2, BM containing both FGF-1 and -2 showed the strongest staining intensity against the albumin. BM containing none of them exhibited the weakest staining.

In the Experiment 4, the efficiency of the two-step culture method was compared to the continuous method and the effect of fibronectin was also examined on these methods. All experimental groups of HAM showed discernable albumin staining. However, the staining intensity of HAM cultured by the two-step method was much stronger than that of HAM cultured by the continuous method as seen in Fig. 2C. Regardless of the culture method, fibronectin-coated plates consistently gave a stronger staining than non-coated plates.

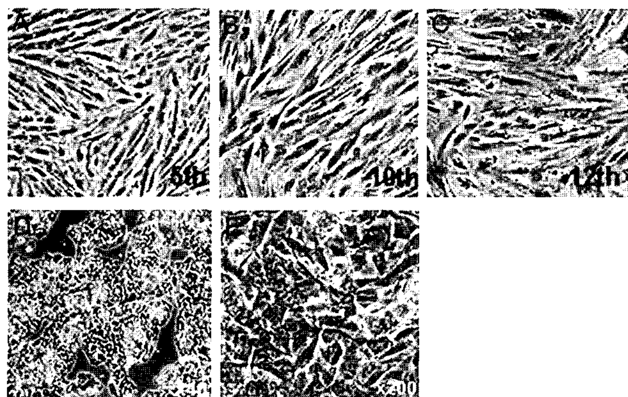

Fig. 1. Morphology of the HAM and HepG2 cell line cultured *in vitro*. HAM at 5th(A), 10th(B) and 12th(C) passage(× 100), and HepG2 cells(D, E).

The negative control in which cultivated HAM were incubated with anti-mouse IgG antibody did not show specific staining, while the positive control in which HepG2 cell line was incubated with anti-human albumin antibody gave

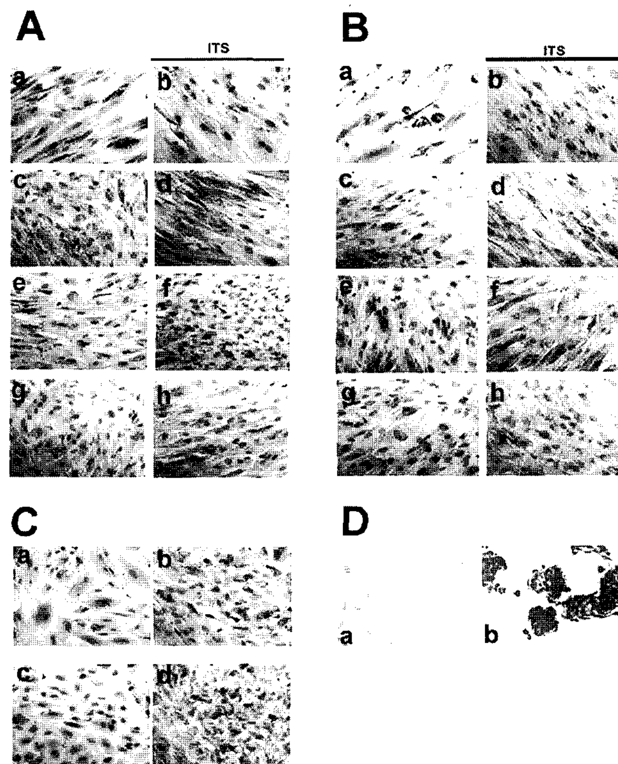

Fig. 2. Immunocytochemical analysis of the HAM after culture on non-coated or fibronectin-coated plates in hepatogenic medium. Immunoreactivity of antibody against the anti-human albumin of the HAM is colored brown. Nuclei stained with Haematoxyline are blue. A, HAM cultured on non-coated plates in the Experiment 1; B, HAM cultured on fibronectin-coated plates in the Experiment 1. a and b, DMEM-LG+10% FBS; c and d, BM(DMEM-LG+10% FBS+HGF + OSM+Dex) + FGF-1; e and f, BM+FGF-2; g and h, BM+FGF-1 + FGF-2; a, b, c and d, without ITS; b, d, f, and h, with ITS. C, a and b, HAM cultured on non-coated plates in the Experiment 4; c and d, HAM cultured on fibronectin-coated plates in the Experiment 4. a and c, HAM cultured by the continuous method; b and d, HAM cultured by the two step method. D, controls; a, negative control in which cultivated HAM were incubated with anti-mouse IgG antibody alone; b, positive control in which HepG2 cell line was incubated with anti-human albumin antibody. ×200.

very intense staining(Fig. 2D).

These observations demonstrate that fibronectin and ITS, respectively, could enhance the albumin synthesis by HAMunder the hepatogenic culture condition and addition of FGF-1 or -2 to these culture further increase the synthesis. The results also demonstrate that the two-step culture method consisting of the first step wherein FGF-1 and -2 are included and the second step wherein OSM and Dex replace FGF-1 and -2 might be better than the continuous culture method.

3. PAS Staining for Glycogen Storage

HAM prepared as in Experiments 1 and 4 were examined for the glycogen storage by using PAS staining. HAM cultured on the fibronectin-coated plates in the Experiment 1 gave a stronger PAS staining of glycogen storage than those on the non-coated plates(Fig. 3). Addition of ITS to the culture medium resulted in the stronger staining compared to the control group regardless of the presence or absence of fibronectin. The medium containing both FGF-1 and -2 showed the stronger staining intensity compared to the medium containing FGF-1 or -2 alone.

In the Experiment 4 in which the effects of two-step culture method and fibronectin coating were examined, the PAS staining intensity was stronger in HAM cultured by the two-step method compared to those cultured by the continuous step(Fig. 3C). Fibronectin-coated plates gave a stronger staining than non-coated plates. As a positive control, HepG2 cell line exhibited very intense staining(Fig. 3D).

These results demonstrate that glycogen synthesis by HAM during the continuous culture would be enhanced by fibronectin or ITS, and that addition of both FGF-1 and -2 could increase the synthesis. Again it is demonstrated that two-step culture method is better than the continuous culture method for the glycogen synthesis by HAM.

4. Morphological Appearance

Morphological change of HAM was examined in Experiments 1 and 4. In the Experiment 1, effects of fibronectin coating, ITS and various combinations of cytokines on the morphological change of HAM into hepatic round shape were examined. As seen in Fig. 2 and 3, neither fibronectin coating nor ITS affected the morphological change of HAM under all culture conditions examined. However, when HAM were cultured in BM supplemented with FGF- 2 alone or mixture of FGF-1 and -2, turnover of discrete fibroblastic morphology into round shape was observed during culture. Morphological change began to appear as early as the third day of culture FGF-2-containing media. FGF-1 did not affect the morphological appearance of HAM.

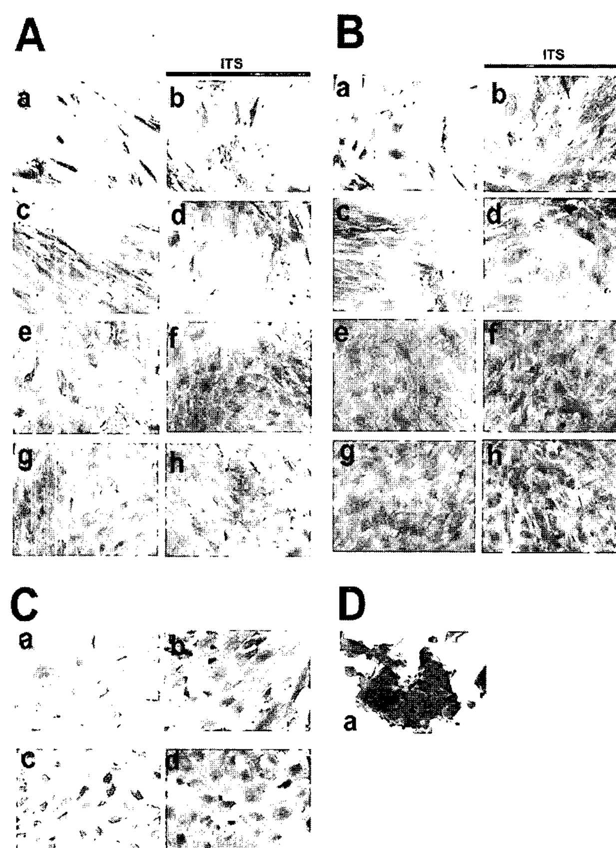

Fig. 3. PAS staining of the HAM after culture on non- or fibronectin-coated plates in hepatogenic medium. Glycogen storage of the HAM is stained purple. Nuclei stained with Haematoxyline are blue. A, HAM cultured on non-coated plates. B, HAM cultured on fibronectin-coated plates. a and b, DMEM-LG+10% FBS; c and d, DMEM-LG+10% FBS+HGF+OSM+Dex(BM-1)+FGF-1; e and f, BM-1+FGF-2; g and h, BM-1+FGF-1+FGF-2; a, b, c and d, without ITS; b, d, f and h with ITS. C, a and b, HAM cultured on non-coated plates; c and d, HAM cultured on fibronectin-coated plates. a and c, HAM cultured by the continuous method; b and d, HAM cultured by the two step method. D, HepG2 cell line. ×400 for HepG2 and ×200 for others.

In the Experiment 4 where the effect of two-step culture method was examined in comparison to the continuous culture method, there was an apparent morphological change of HAM after 10 days of the first initiation step. However, when cells were subsequently cultured for 10 days in the second maturation medium consisting of BM supplemented with ITS, they showed the remarkable transition from fibroblast-like morphology to round shape(Fig. 2C & 3C). In contrast, when cells were further cultured in the same initiation medium consisting of the maintenance medium supplemented with FGF-1, -2 and ITS, which was also used in the first initiation step, no further morphological change was observed.

These results demonstrate that for the morphological turnover of HAM, FGF-2 might be needed during the continuous culture, and that the two-step hepatogenic culture including OSM and Dex might be better than the continuous method.

5. Immunoblotting Analysis for Albumin

To examine the albumin secretion by HAM during hepatocyte differentiation, HAM were initially cultured in various culture conditions for 3 weeks as in Experiment 1 and then they were starved overnight in DMEM-LG without any supplement. When immunoblotting analyses were done against the albumin present in these starvation media, starvation medium obtained from HAM initially cultured in BM alone or BM containing ITS gave little Immunoreactivity (Fig. 4). In contrast, medium from HAM initially cultured in BM containing ITS and FGF-1 showed a discernable intensity and inclusion of FGF-2 instead of FGF-1 gave a stronger intensity. However, starvation medium from HAM initially cultured in the presence of both FGF-1 and -2 gave the strongest staining intensity among all media(Fig. 4).

In the Experiment 2, HAM were initially cultured in BM supplemented with various combinations of FGF-1, FGF-4 and TGF-α in the absence or presence of ITS for 3 weeks, and were starved in DMEM-LG without any supplement overnight. As seen in Fig. 5, compared to the non ITS-containing group, starvation media from HAM initially cultured in the presence of ITS gave a stronger reactivity. Among the ITS-containing media, the medium containing FGF-4 and TGF-α exhibited the strongest staining intensity. Addition of FGF-1 to this medium did not enhance staining intensity rather diminished the intensity. The intensity of the medium containing either combination of FGF-1 and TGF-α, or combination of FGF-1 and -4, was similar to each other but weaker than that containing both FGF-4 and TGF-α(Fig. 5).

In the Experiment 3, effects of various combinations of FGF-1, -2 and -4 were examined in the presence of ITS. As seen in Fig. 6, a combination of FGF-2 and FGF-4 gave the stronger intensity than combinations of FGF-1 + FGF-2 and of FGF-1 + FGF-4. Addition of FGF-1 to this mixture did not enhance the intensity(Fig. 6).

These results showed that ITS enhanced the albumin secretion by HAM during hepatic differentiation *in vitro*, and that addition of FGF-1 or -2 increased the amount of secretion. The results also showed that a combined treatment of FGF-1+FGF-2, FGF-1+FGF-4 or FGF-1+TGF-α could be better to induce the albumin secretion than single treatment with FGF-1 or -2 alone, and that particularly a combination of FGF-4 and either FGF-2 or TGF-α could further increase the secretion.

Fig. 4. Effect of ITS, FGF-1 and -2 on the secretion of albumin by HAM *in vitro*. A group of HAM was initially cultured in BM alone(BM). Another group of HAM was cultured in BM containing ITS in the presence of FGF-1(FGF 1), FGF-2(FGF 2), both (FGF 1 and FGF 2) or none. As a control, HAM were cultured in DMEM-LG containing 10% FBS alone(CON). After culture for 3 weeks, every group of HAM was starved overnight in DMEM-LG alone. Note that the amount of albumin is the greatest in starvation media obtained from HAM initially cultured in BM containing ITS, FGF-1 and FGF-2.

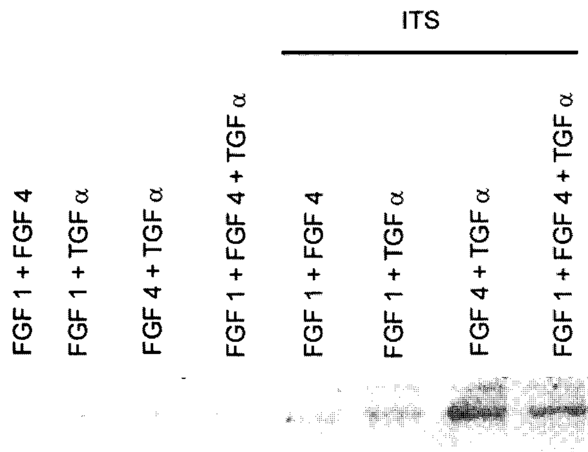

Fig. 5. Effect of ITS and various combinations of FGF-1, FGF-4 and TGF-α on the secretion of albumin by HAM *in vitro*. A group of HAM was cultured in BM without ITS and the other group was cultured in BM with ITS. Each group of HAM was further divided into 4 groups depending on the presence of cytokines; FGF-1 and FGF-4(FGF 1+FGF 4), FGF-1 and TGF-α(FGF 1+TGF-α), FGF-4 and TGF-α(FGF 4+TGF-α), and FGF-1, FGF-4 and TGF-α(FGF 1+FGF 4+TGF-α). Note that among these starvation media, albumin secretion is the greatest when HAM were initially cultured in BM containing ITS, FGF-4 and TGF-α.

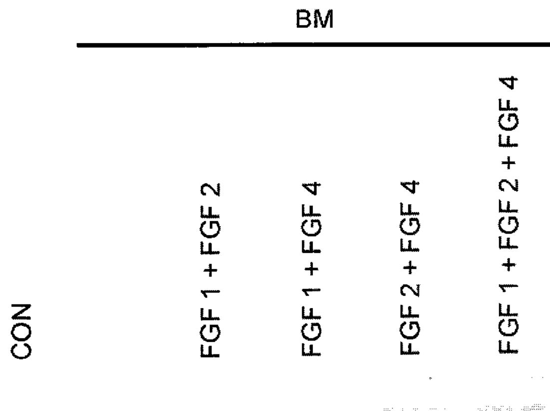

Fig. 6. Effect of various combinations of FGF-1, FGF-2 and FGF-4 on the secretion of albumin by HAM *in vitro*. HAM were initially cultured in BM supplemented with ITS and ascorbic acid in the presence of FGF-1 and FGF-2(FGF 1+FGF 2), FGF-1 and FGF-4(FGF 1+FGF 4), FGF-2 and FGF-4(FGF 2+FGF 4) or FGF-1, and FGF-2 and FGF-4(FGF 1+FGF 2+FGF 4) for 3 weeks on fibronectin-coated plates. A group of HAM was cultured in DMEM-LG containing 10% FBS alone(CON). Note that HAM initially cultured in the presence of both FGF-2 and -4 showed the greatest albumin secretion.

DISCUSSION

In the present study, we have observed for the first time that HAM could differentiate into hepatocyte-like cells *in vitro* and fibronectin, ITS and FGF family members might play important roles in the differentiation process.

HGF is well known to play an essential role in the development and regeneration of the liver(Miyazaki *et al.*, 2002). It induces differentiation of rat bone marrow cells into hepatocytes in culture as shown by the expression of albumin, cytokeratins 8 and 18, which are typically expressed in normal adult hepatocytes(Oh *et al.*, 2000). Dex, a synthetic glucocorticoid supplement in hepatocyte cultures, promotes stable levels of albumin secretion and tyrosine aminotransferase induction for up to 2 months (Tong *et al.*, 1990), and is essential trigger for hepatic maturation(Kinoshita & Miyajima, 2002; Zaret, 2002). During embryonic development in rodents, haematopoietic stem cells produce OSM, a member of the interleukin-6 cytokine family, has been reported to increase the cell size of hepatocytes, enhanced cell differentiation and formation of bile canaliculi, in combination with glucocorticoid as seen in Dex-induced maturation of hepatocytes(Kamiya *et al.*, 1999, 2001). Due by these properties, HGF, Dex and OSM are the most frequently used additives for the hepatocyte culture media.

However, despite the presence of these additives, the lack of terminal differentiation of primary hepatocytes in culture evidences that additional signals are necessary (Kinoshita & Miyajima, 2002). One of signals known to influence differentiation to hepatocytes is an extracellular matrix-derived signals. Previously it was shown using rat fibroblast that cell attachment was increased by extracellular matrix proteins of collagen I, fibronectin or entactin-collagen Ⅳ-laminin to a similar extent. However, cells cultured on fibronectin presented the maximal levels of expression for liver specific genes, such as albumin or alpha-fetoprotein coincidently with an increased expression of hepatocyte nuclear factor-4, compared with those cells cultured on others(Sanchez *et al.*, 2000). The present study also shows that coating of culture plates with fibronectin is more effective to induce the differentiation of HAM into

hepatocytes in comparison to the non-coating condition. Results of immunocytochemistry against the albumin and PAS staining of intracellular glycogen storage support the finding that fibronectin-coating of culture plates is superior to hepatic differentiation of HAM compared to the non-coating condition. These observations suggest that fibronectin could potentially modulate the local concentration of cytokines involved in the hepatocyte differentiation.

Insulin has been found to stimulate the mitosis and albumin secretion of rat hepatocytes(Kaufmann *et al.*, 1999). In the present study, addition of ITS to the hepatogenic medium also remarkably increased albumin protein content and the amount of glycogen storage compared to the control. Addition of either FGF-1 or -2 to this ITS-containing medium enhanced the albumin content and the glycogen production by HAM. FGF-2 exerted better effect on the morphological change into round shape typical of hepatocyte than FGF-1. However, combined treatment of FGF-1 and -2 resulted in the more albumin content and glycogen storage than treatment of FGF-1 or -2 alone. Immunoblotting analyses also showed that the amount of albumin released into the medium was the greatest when HAM were treated with both FGF-1 and -2 in the presence of ITS. However, substitution of FGF-1 with FGF-4 resulted in more albumin production, suggesting that combined treatment of FGF-2 and-4 in the presence of ITS might be the best condition for the hepatocyte-like differentiation of HAM. TGF-α in combination with FGF-4 also produces similar effects as FGF-2 with FGF-4. From these observations, it is suggested that FGF-4 might play a more important role in the secretion of albumin by HAM. FGF-2 and TGF-α appear to play only supporting roles in the albumin release initiated by FGF-4.

FGF-1 and -2 induce the foregut endoderm to the hepatocyte lineage that is required to induce a hepatic fate(Jung *et al.*, 1999). However, previous study showed either cytokine failed to induce hepatic differentiation of human multipotent adult progenitor cells *in vitro*(Schwartz *et al.*, 2002), similar to our results. FGF-4 is important in the initial endoderm patterning and may play a role in endoderm specification(Shi *et al.*, 2005), and its expression in primitive streak-mesoderm can induce the differentiation of moue endoderm in a concentration-dependent manner(Wells & Melton, 2000). FGF-4 alone or in combination with HGF was shown to induce the hepatic differentiation of human multipotent adult progenitor cells *in vitro*(Schwartz *et al.*, 2002). TGF-α is an autocrine stimulator of hepatocyte proliferation that increases transiently the replication of hepatocyte both *in vivo* and *in vitro*(Fausto *et al.*, 2003).

Albumin is known to be the most abundant protein synthesized by hepatocytes. Its production starts in the early stage of liver development and reaches the maximum level in the adult liver(Kamiya *et al.*, 2001). Since hepatocytes consistently release albumin into the blood stream *in vivo*, it is critical to examine the presence of albumin in the culture medium to demonstrate the hepatic differentiation of targeted cells. In the present study, HAM cultured in the hepatic differentiation medium showed distinct albumin synthesis and secretion as revealed by both immunocytochemistry and immunoblotting. However, when immunoblotting analyses were done against the HAM-conditioned media, the signal was discernable only if the media was twenty-fold concentrated. Nevertheless most studies, so far, of hepatic cell differentiation have demonstrated the albumin synthesis using immunocytochemical analyses and little has proved the albumin secretion using immunoblotting analysis. In contrast, the present study demonstrates using immunoblotting assay that HAM could release albumin into the surrounding medium.

While a number of studies have tried to adopt stepwise culture method to accomplish efficient differentiation, only a few studies are known to use multi-step culture to achieve hepatic differentiation of human adult stem cells(Seo *et al.*, 2005) or to overcome de-differentiation, which occurs during continuous stimulation by means of growth factors (Auth *et al.*, 2005). In the present study, we observed that two-step method in which FGFs were treated first and then OSM and Dex replaced them was more efficient to induce differentiation of HAM into hepatocyte-like cells than the continuous culture method. To understand the mechanism of differentiation, however, further studies are needed to explore the expression profile of genes during the sequential treatment.

Stem cell-based therapy and transplantation are of po-

tential value in tissue and organ replacement and regeneration approaches. Many investigators have been intensively studying the efficient culture components and conditions for the hepatic differentiation of human umbilical cord blood-derived mononuclear cells(Kakinuma *et al.*, 2003), bone marrow cells(Schwartz *et al.*, 2002) and adipose tissue derived stem cells(Seo *et al.*, 2005). HAE was also shown to possess hepatocyte-like characteristics both *in vitro* and *in vivo*(Takashima *et al.*, 2004). They observed that HAE cells expressed a subset of hepatocyte-related genes and functions, and suggested the potential applicability of the HAE for the cell-based transplantation therapy. In this study, a type of cells of which morphology was similar to the BM-MSC was isolated from the human amniotic membrane at full term delivery. It was then examined whether the cells could differentiate into hepatocyte and what culture condition would be effective to induce the differentiation of HAM into hepatocyte *in vitro*. Taken together, these studies show that a variety of human adult stem cells could develop into hepatocyte-like cells under appropriate culture conditions.

In conclusion, HAM could readily differentiate into hepatocyte-like cells on fibronectin-coated plates in the presence of HGF, OSM, Dex, ITS, and combination of FGF-2 and FGF-4, or FGF-4 and TGF-α. The differentiation potential of HAM into hepatocyte-like cells might allow their therapeutic use for the hepatic diseases.

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