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ACIDIC FIBROBLAST GROWTH FACTOR PROMOTES HEPATIC DIFFERENTIATION OF MONKEY EMBRYONIC STEM CELLS

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SUMMARY

Embryonic stem (ES) cells can replicate indefinitely and differentiate into all cell types, including hepatocytes. Research using primate ES cells is considered to be important for studies of potential cell therapies. Recently, we established cynomolgus monkey ES cells designated as CMK6. The CMK6 cell line is a useful tool for investigating the mechanism of differentiation in primate ES cells and developing cell therapies, because of its biological similarity to human ES cells. To examine whether cynomolgus monkey ES cells differentiate into hepatocytes, CMK6 cells were cultured with or without acidic fibroblast growth factor (aFGF). Evaluation of the hepatic differentiation was performed by analysis of the mRNA expression in early hepatic marker genes using the reverse transcriptase–polymerase chain reaction (RT-PCR). The protein expression of albumin (ALB) was also studied by immunocytochemistry. RT-PCR analyses revealed mRNA expressions of alpha-fetoprotein, transthyretin, and ALB in the presence of aFGF at 3 wk of differentiation, whereas no mRNA expression of these genes was detected in cells without aFGF. The protein expression of ALB in the presence of aFGF at 3 wk of differentiation was also confirmed by immunocytochemistry. However, tyrosine aminotransferase, which is a mature hepatic marker, was not detected in the presence or absence of aFGF at any stage of differentiation. These results suggested that aFGF successfully promoted in vitro differentiation of cynomolgus monkey ES cells to an early hepatic lineage.

Key words: culture; primate; regenerative medicine; liver; hepatocytes.

INTRODUCTION

Liver transplantation is the only currently established treatment for fatal liver disease. However, most patients are unable to receive this treatment, because of limitations such as high cost, immune rejection, and donor shortage. Some studies have presented the potential use of cellular transplantation (Demetriou et al., 1991; Chowdhury et al., 1998). Source materials such as bone marrow cells, umbilical cord blood, immortalized cells, and embryonic stem (ES) cells have attracted attention for potential use (Petersen et al., 1999; Kobayashi et al., 2000; Kakinuma et al., 2003). Among these cells, ES cells are the most useful resource, because they can differentiate into all cell types found in the adult body and are able to proliferate extensively in culture. Initially, embryonic stem cells were isolated from the inner cell mass of mouse blastcysts in 1981 (Evans and Kaufman, 1981; Martin, 1981). Since then, it has been shown that mouse ES cells can differentiate into various cell types in vitro and in vivo, including neural cells, cardiac cells, skeletal muscle cells, hematopoietic cells, osteoclasts, and hepatocytes (Nakano et al., 1994; Klug et al., 1996; Okabe et al., 1996; Yamane et al., 1997; Hamazaki et al., 2001; Toumadje et al., 2003). These studies have also shown the use of several growth factors to be effective for differentiation into the desired cell types.

Recently, primate embryonic stem cell lines, including rhesus, marmoset, and cynomolgus monkeys, as well as humans, have also been established (Thomson et al., 1995, 1996, 1998; Suemori et al., 2001). Human ES cells, as with mouse ES cells, exhibit pluripotency, and can be induced to embryoid bodies (EBs); EBs express specific markers for all three germ layers in vitro, and generate teratomas organized from multiple cell lineages in vivo (Itskovitz-Eldor et al., 2000; Shuldiner et al., 2000). The establishment of human ES cells has enabled a further potential usage for ES cells in the cell therapy of various human diseases. However, ethical considerations have limited the availability of human ES cells. In this regard, monkey ES cells are useful tools for the investigation of differentiation mechanisms and the development of preclinical models for cell therapy, because of their biological similarity to human cells. Recently we established a monkey ES cell line designated as CMK6 (Suemori et al., 2001; Takada et al., 2002; Asano et al., 2003). CMK6 was confirmed to express alkaline phosphatase (ALP), octamer-binding transcription factor-4 (Oct-4), and stage-specific embryonic antigen-4 (SSEA-4), which are the markers of undifferentiation in primate ES cells.

In the case of ES cells from mice, many studies have reported mechanisms of liver cell development and differentiation of mouse ES cells into mature hepatocytes producing albumin (ALB)(Hamazaki et al., 2001; Jung et al., 1999; Chinzei et al., 2002). During the embryonic development of mice, the initial event of liver ontogeny occurs at embryonic d 9. At this stage, fibroblast

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growth factors, which are secreted from the adjacent cardiac mesoderm, commit the foregut endoderm to form the liver bud (Jung et al., 1999). Hamazaki et al. (2001) demonstrated that acidic fibroblast growth factor (aFGF) could promote mouse ES cells to differentiate into the early hepatic lineage in vitro. In the case of a human, the liver bud is also derived from the foregut endoderm adjoining the cardiac mesoderm. Therefore, aFGF was used to induce hepatic lineage in monkey ES cells.

In the present study, the mRNA expression of hepatic marker genes and the protein expression of ALB were analyzed by reverse transcriptase–polymerase chain reaction (RT-PCR) and by immunocytochemistry, using the monkey ES cell line CMK6 in the presence or the absence of aFGF.

Material and Methods

Cell culture. The CMK6 ES cell line was maintained as undifferentiated with mitomycin C-treated (5 μg/ml, 37° C for 2 h; Kyowa Hakko, Tokyo, Japan) feeder cells, on gelatin-coated tissue culture dishes (Iwaki Glass, Chiba, Japan). The cell line at passage numbers 20–30 was used. The ES cell culture medium consisted of 75% Dulbecco's modified Eagle's medium nutrient mixture F-12 ham (DMEM) (Sigma, St. Louis, MO) supplemented with 25% Knockout Serum Replacement (Gibco BRL, Grand Island, NY), 2 mM L-glutamine (Nacalai Tesque, Kyoto, Japan), 1 mM sodium pyruvate (Sigma) and 1% MEM nonessential amino acids solution (Sigma). The medium was changed daily, and ES cell colonies were split every 2–3 d. After incubation in 0.1% collagenase IV (1 mg/ml, 37° C for 5 min; Invitrogen Corp, Carlsbad, CA) and mild centrifugation, dissociated cells were replated onto dishes with new feeder cells. To induce differentiation, EBs were prepared using dissociated ES clumps by the hanging drop method (Hamazaki et al., 2001). The cell density of one drop was approximately 3000 cells per 30 μl of DMEM containing 16% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS), 2 mM L-glutamine (Nacalai Tesque), 1 mM sodium pyruvate (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin (Nacalai Tesque), and 0.1% sodium bicarbonate solution (Sigma). After 7 d in a hanging drop culture using the differentiation medium, the resulting EBs were plated onto plastic 35-mm dishes coated with collagen type I (Vitrogen, JRH Biosciences, Lenexa, KS), and allowed to attach and spread for 3 wk in the presence or absence of aFGF (Sigma). In this report, 20 ng/ml of aFGF was used according to previous reports that described successful differentiation of hepatocytes using this concentration with murine ES cells. (Chinzei et al., 2002).

RNA RT-PCR analysis. To assess the differentiation of CMK6 cells into a hepatic lineage, the gene expression of the hepatocyte markers was examined by RT-PCR analysis. The expressions of alpha-fetoprotein (AFP), alpha-1 antitrypsin (AAT), transthyretin (TTR), hepatocyte nuclear factor 3β (HNF-3β), tyrosine aminotransferase (TAT), ALB, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in the culture, differentiated either in the presence of or absence of aFGF, were investigated.

Total RNA was extracted from undifferentiated and differentiated CMK6 cells every week, using an RNeasy Mini Kit (Qiagen, Stanford, CA). Hepatocytes of an adult cynomolgus monkey were used as a positive control. The extracted total RNA was treated by deoxyribonuclease (DNase) with DNA-free (Ambion, Austin, TX). One microgram of DNase-treated total RNA was used for the first strand synthesis of the complementary DNA. This reaction was performed using Super ScriptII first-strand synthesis system with oligo (dT) primer (Gibco BRL) according to the manufacturer's instructions. The resulting complementary DNA was amplified by the use of Platinum PCR Super Mix (Invitrogen, Carlsbad, CA) and Gene Amp PCR 9700 (Perkin-Elmer Corporation, Norwalk, CT) with the following sets of primers: for AFP, 5′-TGCCAACTCAGTGAGGACAA, 5′-TCCAACAGGCCTGAGAAATC; for AAT, 5′-CTGTCCAGCTGGGTGCTGCTGATG, 5′-GATGGTCAGCACAGCC TTATGCAC; for TTR, 5′-CCTCTGATGGTCAAAGT, 5′-GCCGGAGTCGTT GGCTGTGAA; for HNF-3β, 5′-CCCATGGCGTACGCGCCGTC, 5′-CAGCG TCCAGTAGGAGCCCTT; for TAT, 5′-GGGCTGGTGAAGCTGAGTCAGCG, 5′-TGGGAGGCAGTGGACAGACTGCTC; for ALB, 5′-TTGGAAAAATCCC ACTGCCT, 5′-CTCCAAGCTGCTCAAAAAG.; and for GAPDH, 5′-GTCTTC ACCACCATGGAGAAGGC, 5′-CATGCCAGTGAGCTTCCCGTTCA.

The cycling parameters were as follows: denaturation at 94° C for 30 s; annealing at 55–60° C for 40 s (depending on the primer); and elongation at 72° C for 1 min. The amplified products were separated by 1.5% agarose gels and stained with ethidium bromide.

Immunochemistry. To determine whether differentiated cells produce ALB, immunocytochemical detection was attempted using anti-rabbit polyclonal antibody against monkey ALB, which does not cross react with mouse ALB. Cells that had differentiated for 2 and 3 wk, with or without aFGF, were immunostained. The adult liver tissue of a cynomolgus monkey was used as a positive control.

Cells differentiated for 3 wk (1.0×10^5 cells) were also injected into the spleen of a 50% hepatectomized female BALB/cA nu/nu mice (CLEA, Tokyo, Japan) and 4 wk later, immunohistochemical analysis of liver sections was performed using the same antibody.

Cultured cells and sections were fixed with pure methanol for 30 min at −20° C. The fixed sample was incubated with a primary antibody (1:500) (IgG fraction of rabbit polyclonal antibody against monkey ALB, Nordic Immunological Laboratories, Tilburg, Netherlands) for 1 h at room temperature, and then with a secondary antibody (1:100) (Texas Red conjugated goat anti-rabbit IgM + IgG, Southern Biotechnology Associates, Birmingham, AL) for 30 min. The treated samples were examined using a fluorescence microscope (IX71, Olympus, Tokyo, Japan) equipped with a CCD camera (Roper Scientific, Tucson, AZ).

Results

Monkey ES cells and differentiation. CMK6 cells formed flat colonies with individual cells displaying small cytoplasmic/nuclear ratios and clearly distinguishable nucleoli (Fig. 1*a*).

Differentiation was initiated by culturing the cells in hanging drops of ES medium containing FBS. After 2 or 3 d of culture in hanging drops, most of the ES cell clumps formed simple EBs. Extended culture resulted in the formation of large EBs and cystic EBs. EBs at d 7 are presented in Fig. 1*b* and *c*. Upon further culture of EBs on the dishes, distinctive cells with varied morphologies appeared. Epithelial-like cells, fibroblast-like cells and larger cells containing multiple nuclei can be observed at 3 wk (Fig 1*d*). There was no obvious morphological and proliferative difference observed between the culture in the presence, or the absence of, aFGF at any stage of further culture.

Expression of hepatic marker genes. Undifferentiated ES cells did not express any endodermal or hepatic markers (Fig. 2*a*, Lane 1). Both HNF-3β and AAT were expressed at 2 and 3 wk of differentiation, whereas there was no mRNA expression of AFP, TTR, ALB, or TAT observed in the cells differentiated without aFGF for 3 wk (Fig. 2*a*, Lanes 3, 4).

On the other hand, mRNA expression of AFP, TTR, and ALB were observed in the presence of aFGF at 3 wk of differentiation (Fig. 2*b*, Lane 2). The expression of HNF-3β and AAT was also detected in the presence of aFGF at 2 and 3 wk of differentiation. However, the expression of TAT, which is a marker of mature hepatocytes, was not detected at any stage.

Immunocytochemistry for ALB. ALB was detected only in those cells differentiated with aFGF for 3 wk, and the ALB was localized in the cytoplasm. ALB-positive cells had large nuclei and abundant cytoplasm, which is characteristic of hepatocytes (Fig. 3*a*, *b*). A similar expression pattern was observed in the liver section prepared from an adult monkey (Fig. 3*c*, *d*). ALB-positive cells and total cells in the dish were counted under microscope, which were 182 ± 33 ($n = 4$) and about 2.0×10^4 respectively. Therefore, we calculated the yield of ALB-positive cells was approximately 0.9%.

In a cell transplantation experiment using 3-wk differentiated ES cells, ALB-positive cell clusters were also found in the liver 4 wk posttransplantation (Fig. 4).

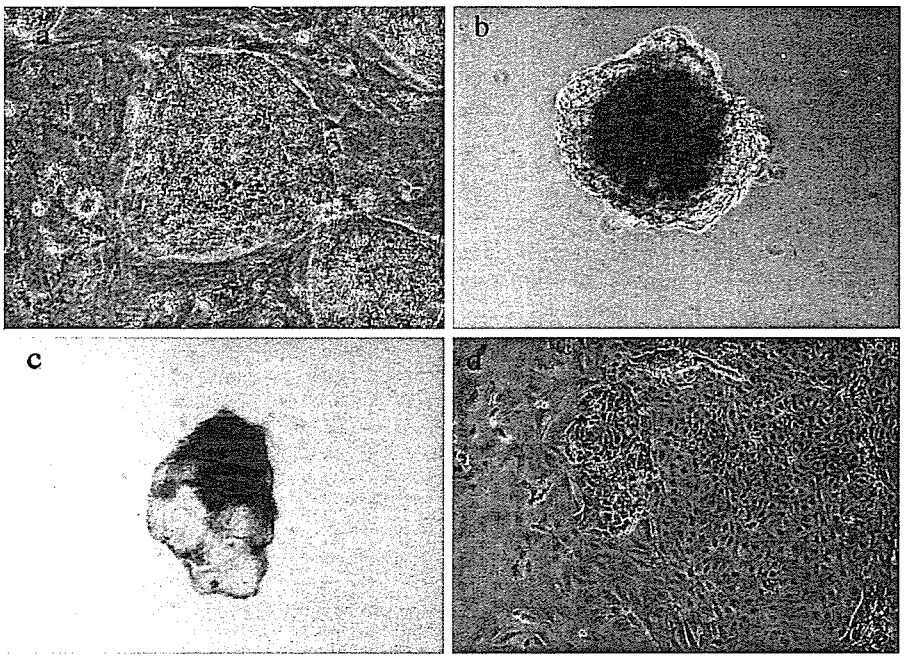

FIG. 1. Micrographs showing various stages of cynomolgus monkey ES cells. (*a*) Colonies of undifferentiated ES cells cultured for 3 d (×100), (*b*) EB with a dark central core cultured for 7 d (×40), (*c*) EB with cysts (×40), (*d*) morphologies of ES cells differentiated for 3 wk (×40).

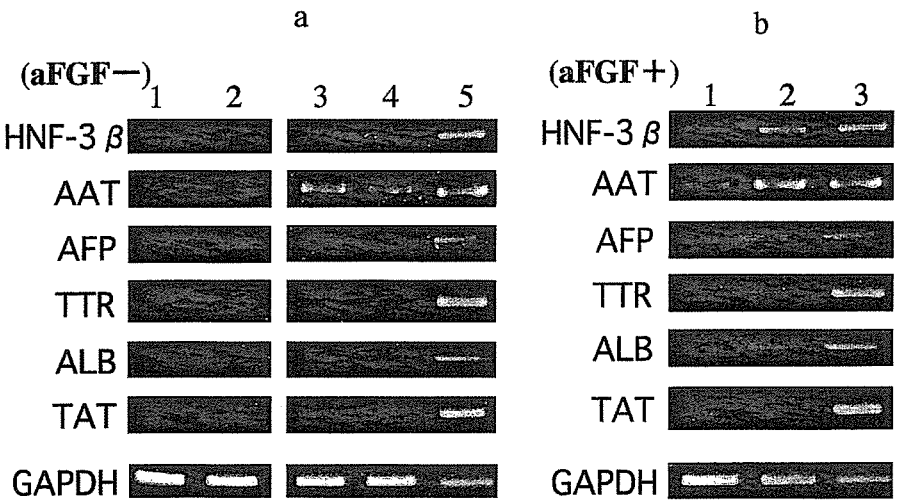

FIG. 2. Effects of aFGF on the hepatic differentiation of CMK6 shown by RT-PCR analysis. (*a*) Differentiation in the absence of aFGF. Lane 1: undifferentiated ES cells; Lane 2: ES cells differentiated for 1 wk (EBs); Lane 3, for 2 wk; Lane 4, for 3 wk; Lane 5, adult hepatocytes as a positive control. (*b*) Differentiation in the presence of aFGF. Lane 1: for 2 wk; Lane 2: for 3 wk; Lane 3: adult hepatocytes as a positive control.

DISCUSSION

Cell based therapy has potential use in the treatment of fatal liver disease in humans. Using animal models, cell transplantation has been reported as an effective treatment for hepatic failure and metabolic liver disease (Demetriou et al., 1991). However, for clinical application of cell therapy, it is difficult to proliferate sufficient numbers of primary hepatocytes. Therefore, it is important to produce sufficient hepatocytes in vitro. Recently, some studies have reported the generation of multiple-lineage cell types from tissue-specific stem cells (such as bone marrow, umbilical cord cells, and adipose tissue) and ES cells (Goodwin et al., 2001; Zuk et al., 2001; Jiang et al., 2002; Zuk et al., 2002; Blanpain et al., 2004). In regard to hepatocytes, it was demonstrated that pluripotent progenitor cells derived from human bone marrow and umbilical cord blood cells could be differentiated into functional hepatocytes (Schwartz et al., 2002; Kakinuma et al., 2003). These studies indicated the possibility of adopting adult stem cells for cell therapy. However, the use of these cell types have limitations, in that they are obtained by extraction from bone marrow or umbilical cord blood, require complex procedures for purification, and are difficult to proliferate extensively in culture. As an alternate source, ES cells have the ability to differentiate into specific cell types and proliferate extensively, and therefore hold immense potential for cell and gene therapy. In 1998, human ES cells, which have the ability to differentiate into all cell types, were established by Thomson et al. (1998). The potential of human ES cells has attracted strong attention for use in

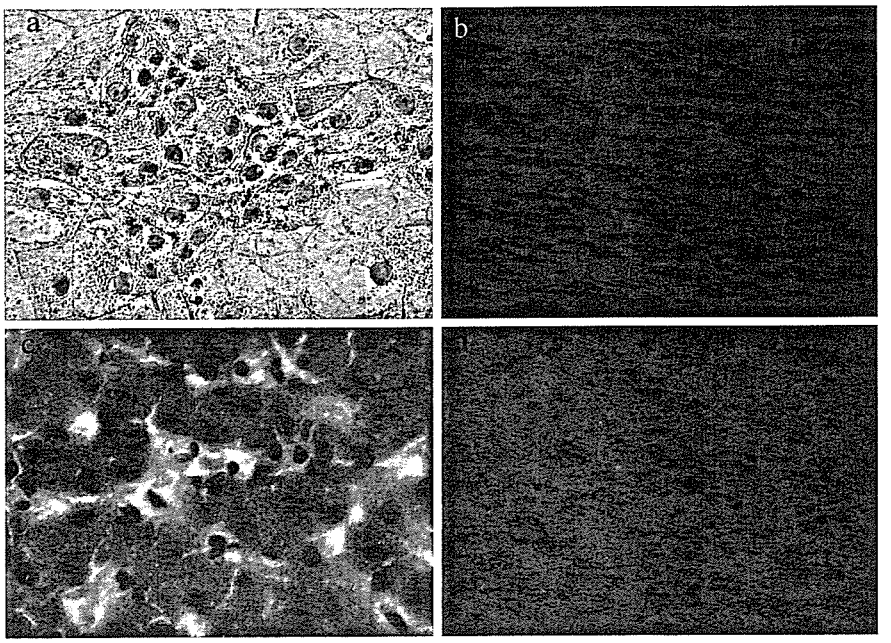

FIG. 3. Immunocytochemical staining for ALB. (*a*) CMK6 differentiated with aFGF for 3 wk are shown under a phase contrast microscope (×200). (*b*) The fluorescent image of the same field as (*a*) is shown. Texas Red-positive signals represent ALB-producing cells (×200). (*c*) Hematoxilin-eosin (HE)-stained liver section from an adult monkey (×200). (*d*) Fluorescent image of the liver section from an adult monkey stained with ALB antibody (×200).

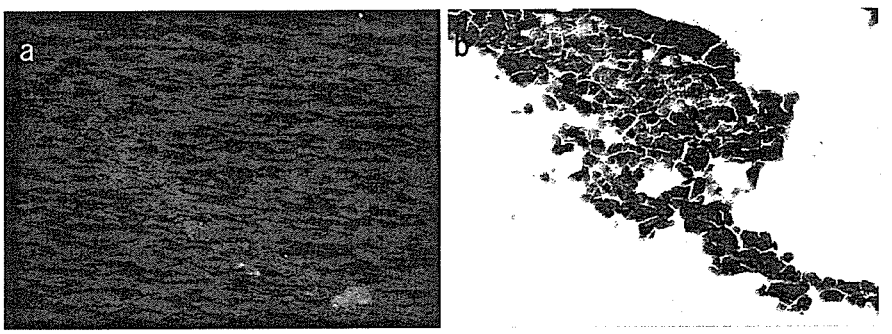

FIG. 4. ALB-synthesizing cells in the liver of monkey ES cell-transplanted mouse. (*a*) Fluorescent image of the liver section from a cell-transplanted mouse stained with ALB antibody (×100). (*b*) Hematoxilin-eosin (HE) staining of the serial section.

cell transplantation therapy for human organ dysfunction. However, ethical and practical problems have limited their application. Therefore, monkey ES cells provide a useful model for examining the practical application of cell therapy in humans.

Recently, it has been shown that monkey ES cells can be differentiated into various cell types—including neurons, hematopoietic cells, and pancreatic cells—using growth factors (Kuo et al., 2003; Honig et al., 2004; Lester et al., 2004). Although several reports described endodermal differentiation using mouse and human ES cells (Shuldiner et al., 2000; Hamazaki et al., 2001; Chinzei et al., 2002; Lavon et al., 2004), there have been no reports showing the differentiation of monkey ES cells into hepatocytes. In the case of mouse ES cells, Hamazaki et al. (2001) tested the effect of aFGF, hepatocyte growth factor (HGF), oncostatin M, dexamethasone, and ITS (insulin, transferrin, selenium) (Gibco BRL) to induce hepatocytes. These growth factors induced expressions of ALB and TAT. However, Chinzei et al. (2002) reported conflicting results, with no observation of TAT expression using these growth factors. In the case of human ES cells, Lavon et al. (2004) tested the effect of aFGF, basic fibroblast growth factor (bFGF), bone morphogenetic protein 4 (BMP-4), and HGF in hepatic differentiation. The report showed that only aFGF was effective to induce early hepatocytes that express ALB. Schuldiner et al. (2000) examined the differentiation potential of bFGF, transforming growth factor β, activin-A, BMP-4, HGF, epidermal growth factor, nerve growth factor (NGF), and retinoic acid in human ES cells. This study reported that only NGF and HGF allowed differentiation into endodermal lineage. These studies were carried out using different differentiation protocols. Therefore, the timing and period for addition of these factors seems to be important, affecting subsequent differentiation

In this study, to reveal whether monkey ES cells (CMK6) could be induced to hepatic lineage, the expressions of liver-specific markers were examined in the presence or the absence of aFGF. Subsequently, it has been shown that the addition of aFGF could

induce early hepatocytes. Accordingly, aFGF was effective in the promotion of monkey ES cells into early hepatocytes. However, hepatic differentiation was inefficient (0.9%) and this level of differentiation would make it difficult to perform functional studies in vitro. Therefore, an attempt was made to transplant 3-wk differentiated ES cells into the spleen of a 50% hepatectomized mouse. Although some ALB-positive cell clusters, which were derived from the ES cells, were found in the liver 4 wk posttransplantation, expression of TAT was not observed in these clusters using RT-PCR.

The gestation periods of mice, cynomolgus monkeys and humans are 20, 165, and 280 d, respectively. According to the gestation periods, it was expected that a longer culture period would be required to induce mature hepatocytes in monkey ES cells than in those of their mouse counterparts. Several studies of murine ES cells reported that the early hepatic markers, such as AFP, ALB, and AAT, are detected after 8 d (Hamazaki et al., 2001; Chinzei et al., 2002). This study revealed that early hepatic markers are detected in 3 wk for monkey ES cells, but mature hepatocyte markers were not detected. The effect of aFGF on hepatocyte differentiation is considered to be time-dependent; therefore, a differentiation period of more than 3 wk may be required to induce mature hepatocytes. However, it was difficult to maintain viability of the differentiated cells for 4 wk in vitro. Therefore, a culture system that maintains differentiated cells for more than 3 wk must be considered. It has been reported that a simple two-dimensional (2D) culture decreases the activity of hepatocytes, and that 2D sandwich cultures or three-dimensional (3D) cultures improve the longevity of hepatocytes (Richert et al., 2002). To further analyze the effects of aFGF, culture systems that allow longer culture in vitro, such as 3D cultures, will be required. In addition, the effect of other growth factors used to induce mature hepatocytes, such as HGF, should be investigated.

In conclusion, the present study suggests that cynomolgus monkey ES cells (CMK6) could be differentiated into early hepatocytes using aFGF in vitro. However, hepatic differentiation was inefficient under these culture conditions. The results have also suggested that large improvements would have to be made before hepatic differentiation of primate ES cells in vitro could have any therapeutic applications. Therefore, other culture systems and growth factors should be tested to induce mature and functional hepatocytes with high efficiency from primate ES cells in vitro.

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In Vitro expression of natriuretic peptides in cardiomyocytes differentiated from monkey embryonic stem cells

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Abstract

Functional characterization of ES cell-derived cardiomyocytes is important for differentiation control and application to the cell therapy. One of the crucial functions of cardiomyocytes is a production of atrial and brain natriuretic peptides (ANP and BNP, respectively), which have important endocrine, autocrine, and paracrine functions. In this study, we focused on the functional aspect of the cardiomyocytes differentiated from monkey ES cells in vitro and investigated the expression of ANP and BNP. Spontaneously contracting cells showed nodal-like action potentials, and expression of ANP and BNP by RT-PCR and immunocytochemistry. Interestingly, ANP and BNP expressions were detected as immunoreactive granules in the perinuclear area and these signals appeared to co-localize with trans-Golgi network. These findings suggest that monkey ES cells were able to differentiate into cardiomyocytes with functional characteristics in vitro and therefore can be used as a useful model to study mechanisms and functions in early cardiogenesis.

Keywords: Cardiomyocytes; Monkey ES cells; Expression; ANP; BNP; In vitro

Pluripotent ES cells differentiate into a variety of cell lineages in vitro after aggregation into 3-dimensional structures termed embryoid bodies (EBs) [1]. EBs originate a variety of specialized cell types, including cardiomyocytes [2]. Because the early development of cardiomyocytes cannot be easily observed in vivo, ES cell-derived cardiomyocytes will provide a useful model to study molecular mechanisms involved in the early stages of cardiogenesis [3]. In addition, cardiomyocytes differentiated from ES cells might be suitable as a renewable source for grafting therapies [4].

The heart is the first functioning organ in the embryo and any impairment of its function leads to early lethality [5]. Cardiomyocytes do not regenerate after birth, and they respond to mitotic signals by increasing in the size (hypertrophy) rather than by cell division (hyperplasia) [6]. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are cardiac hormones, predominantly secreted into circulation by the atria and ventricles, respectively [7]. They regulate blood pressure and fluid homeostasis by causing natriuresis, diuresis, vasorelaxation, and inhibition of the renin–angiotensin–aldosterone system [8]. In addition, cell-based studies have shown that ANP and BNP exhibit important autocrine and paracrine functions such as modulating myocyte growth, apoptosis, and proliferation in smooth muscle cells [9] and cardiac myocytes [10], and suppress cardiac fibroblast proliferation [11] and extracellular matrix secretion [12,13]. Moreover, it has been reported that ANP inhibits the norepinephrine-induced growth of cardiac myocytes [14].

Both ANP and BNP exert their biological actions by binding to the natriuretic peptide receptor A (NPR-A), resulting in generation of the second messenger cGMP

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[15]. Furthermore, all natriuretic peptides bind to natriuretic peptide receptor C, a clearance receptor which mainly serves for cellular internalization and degradation of natriuretic peptides [16].

Gene expression of ANP has been used as cardiac marker for cardiomyocytes differentiated from ES cells [17,18]. However, it has been reported that proliferative activity of cardiomyocytes during embryonic cardiogenesis correlates with elevated levels of ANP. Shortly after birth as the proliferation of cardiomyocytes stops, the expression of ANP rapidly declines [19,20]. Occurrence of natriuretic peptides immunoreactivity has been previously observed in the cardiac myocytes predominantly related to the perinuclear Golgi region in immature and mature endocrine secretory granules [21,22]. As the expression pattern of natriuretic peptides during in vivo development was different among species [23], it would be interesting to investigate the expression in cadiomyocytes derived from monkey ES cells.

In addition, physiological functions of ES cell-derived cardiomyocytes are important for transplantation therapy and several questions need to be addressed before ES cell-derived cardiomyocytes can find their way into cardiac cell therapy in human. One of these questions is their abilities to function as normal cardiac cells, which is not investigated in detail. Therefore, in the present study, we investigated the functional properties especially expression of ANP and BNP in the cardiomyocytes differentiated from monkey ES cells in vitro.

Materials and methods

ES cell culture and differentiation. Monkey undifferentiated ES cells of CMS-A-2-G1 and CMS-A-2 lines were cultured on the mitotically inactivated embryonic fibroblast feeder layer using mitomycin C. The culture medium consisted of Dulbecco's modified Eagle's medium/nutrient mixture F-12 ham (DMEM/F-12; Sigma, St. Louis, MO, USA) supplemented with 20% knockout serum replacement (Gibco-BRL, Grand Island, NY, USA), 2 mM L-glutamine (Nacalai Tesque, Kyoto, Japan), 1 mM sodium pyruvate (Sigma), 1% MEM non-essential amino acid solution (Sigma), and 0.1 mM 2-mercaptoethanol (Sigma). The cells were dissociated into small clumps using 0.1% collagenase (Wako, Osaka, Japan) and differentiation was induced by culturing dissociated ES cells in hanging drops for 10 days using differentiation medium consisted of DMEM/F-12 (Sigma) supplemented with 15% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA), 1 mM Na pyruvate, 2 mM L-glutamine (Nacalai), 0.1% sodium bicarbonate (Sigma), 0.1 mM 2-mercaptoethanol (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin (Nacalai). After 10 days in drop culture, EBs were brought in a suspension culture in 35 mm Petri dishes for more 7 days. Then EBs were plated into 35 mm gelatin coated dishes (2–4 EBs/dish). Beating cell clusters were mechanically isolated, dissociated into small cell clumps using 0.05% trypsin, and replated onto the new gelatin-coated dishes. These beating cells were cultivated with differentiation medium.

Electrophysiology. Membrane potentials were recorded with an EPC-8 patch–clamp amplifier (HEKA, Lambrecht, Germany). A glass coverslip (5 mm × 3 mm) with adherent ES cardiomyocytes was placed on the glass bottom of a recording chamber (0.5 ml in volume) mounted on the stage of a Nikon TMD-300 inverted microscope (Tokyo, Japan). The chamber was maintained at 35 ± 1 °C and was continuously superfused with normal Tyrode solution containing 140 mM NaCl, 0.33 mM NaH_2PO_4, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.5 mM glucose, and 5 mM Hepes (pH adjusted to 7.4 with NaOH). Electrodes were made from glass capillaries (outside diameter 1.5 mm; Narishige Scientific Instrument Laboratory, Tokyo, Japan) using a Sutter P-97 microelectrode puller (Novato, CA, USA), and the tips were then fire-polished with a microforge. Electrode resistance ranged 2.5–4.0 MΩ when filled with an internal solution containing 70 mM potassium aspartate, 50 mM KCl, 10 mM KH_2PO_4, 1 mM $MgSO_4$, 3 mM Na_2-ATP, 0.1 mM Li_2-GTP, 5 mM EGTA, and 5 mM Hepes (pH adjusted to 7.2 with KOH). Voltage signals were sampled at 1.0 kHz through an LIH-1600 AD interface (HEKA) controlled by Patchmaster software (HEKA).

Reverse transcription-polymerase chain reaction (RT-PCR). RNA was prepared on day 5 after initiation of beating. Five beating cell clusters were mechanically isolated and total RNA was extracted using RNeasy Mini kit (Qiagen, Stanford, CA) according to manufacturer's instructions. The total RNA was then reverse-transcribed into cDNA using Superscript III first-strand cDNA synthesis Kit (Invitrogen). Genomic DNA was digested with DNase I (Qiagen). One microliter of cDNA reactions was used for a 50 μl PCR mixture using Blend *Taq* polymerase (Toyobo, Japan). The absence of genomic DNA contamination in RNA samples was confirmed with RT-PCR without superscript III. GATA4 and GAPDH primers were designed based on the human sequence. ANP and BNP primers were designed based on monkey sequences. The primer sequences and the expected sizes of RT-PCR products are given in Table 1.

The amplification profile consisted of initial step of denaturation at 95 °C for 2 min, 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, followed by a final extension at 72 °C for 2 min. The PCR products were electrophoresed onto 1.5% agarose gel and stained with ethidium bromide.

Immunocytochemistry. The cells were rinsed briefly with phosphate-buffered saline (PBS) and fixed for 20 min in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The cells were permeabilized with 0.1% Triton X-100 with 0.1 M phosphate-buffered saline, pH 7.4 and then rinsed two times with Tris-buffered saline with 0.05% Tween 20, pH 7.4 (TBST). The cells were blocked with 4% normal goat serum in PBS or 2% bovine serum albumin (BSA) for 30 min at room temperature.

Then they were incubated at 4 °C overnight with the primary antibody. The primary antibodies used were: monoclonal anti-bovine cardiac troponin I (cTnI) (1:1000, Chemicon), monoclonal anti-human cardiac actin (Nichirei, Japan), goat polyclonal anti-GATA-4 IgG (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-human ANP (1:500, Osaka Institute, Japan), polyclonal anti-human BNP (1:250, IBL, Japan), and monoclonal anti-human p230 trans Golgi (1:500, BD Bioscience). After three washes with TBST, cells were incubated with the appropriate secondary antibodies for 45 min at room temperature: Cy3-labelled goat anti-mouse IgG (1:500, Amersham Biosciences, UK), Rhodamine-conjugated donkey ant-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA), Alexa Fluor 488-labelled anti-mouse IgG (1:500, Molecular Probes) or Cy3-labelled goat anti-rabbit IgG (1:500, Chemicon). Cells were rinsed

Table 1
PCR primers and their products

Gene	Sense	Antisense	Product size (bp)
GATA4	GGCCTCTACATGAAGCTCCA	GGCTGTTCCAAGAGTCCTGC	403
ANP	GAACCAGAGGGGAGAGACAGAG	CCCTCAGCTTGCTTTTTAGGAG	406
BNP	AGCCTCCGCAGTCCCTCCAGAGAC	AGGTGTCTGCAGCCAGGACTTCCT	454
GAPDH	ATGGGGAAGGTGAAGGTCGG	GGAGTGGGTGTCGCTGTTGAA	876

three times in TBST and then covered with PBS. We counterstained nuclei with Hoechst33342 (1 μg/ml) and then examined under the fluorescence microscope (IX71, Olympus). Images were captured using a color CCD camera (coolSNAP cf, Roper Scientific).

Results

Spontaneously contracting cardiomyocytes derived from monkey ES cells

Spontaneously contracting cells appeared as clusters in the differentiated EB outgrowth at differentiation day 20 to 26 (3–9 days after plating). We observed some beating clusters were stopped to beat due to the overgrowth of other differentiating cells which caused structural hindrance on the beating cells. These cells were started to beat again after mechanical separation and enzymatic dissociation to new gelatin-coated dishes. We isolated most of the beating areas from EB outgrowth after few days from the beginning of beating and transferred to new dishes to increase the number of beating cells. Most of the transferred cells restarted to beat after 1 day and showed cell division. Some cells formed synchronized beating clusters again, other cells continued to beat as single cells. The beating cardiomyocytes were characterized by large euchromatic nucleus to cytoplasmic ratio. The shape of beating cells was rounded to oval before isolation from EBs, it became elongated and with various shapes after isolation and dissociation. The beating cells continued to beat in culture for a period of observation of up to 86 days.

Electrophysiology

Fig. 1 demonstrates a representative example of action potentials (upper panel) and the first derivative of membrane potential (dV/dt, lower panel) recorded from beating cells differentiated from monkey ES cells. Spontaneous and repetitive action potentials were triggered by a characteristic slow diastolic depolarization, which appears to be similar to the automaticity observed in sino-atrial (SA) node cells. The following parameters of action potentials were measured from beating cells (Table 2), namely, spontaneous rate, action potential duration measured at 50% and 90% repolarization (APD_{50} and APD_{90}, respectively), maximum upstroke velocity of action potential (dV/dt_{max}), action potential amplitude (APA), and maximum diastolic potential (MDP). Based on the presence of slow diastolic depolarization leading to spontaneous action potentials, slow dV/dt_{max}, and small APA, these action potentials recorded from beating cells can be classified as nodal-like [24,25].

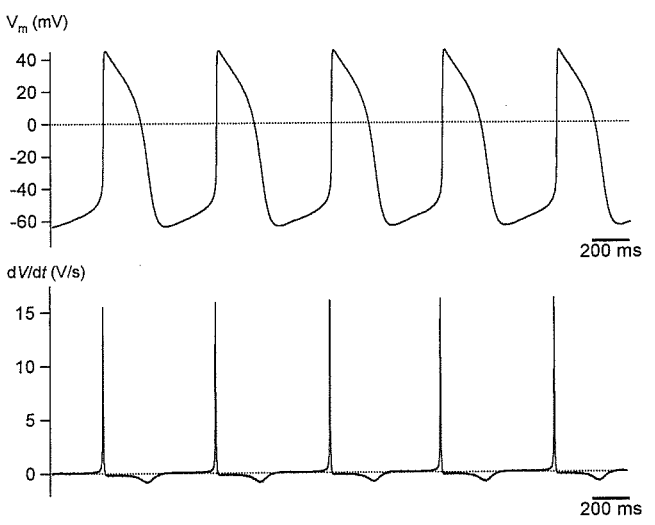

Fig. 1. Action potentials recorded from spontaneously contracting cardiomyocytes derived from monkey embryonic stem cells (upper panel) and dV/dt (lower panel). The *dotted line* indicates zero-potential level.

RT-PCR and immunocytochemistry

RT-PCR showed that monkey ES cell-derived beating cells expressed cardiac natriuretic peptides (ANP and BNP), as well as GATA-4 (cardiac transcription factor) (Fig. 2).

The immunofluorescent staining of beating cells showed the presence of cardiac specific proteins; the dispersed beating cells were stained positive for cardiac actin (Fig. 3A), and the actin fibers appeared more condensed underneath the cell membrane. Immunostaining of cardiac troponin I (cTnI) showed well-organized parallel myofilament and some cells showed a typical cross-striation which is specific for striated muscles and it was mainly observed in contracted cells (Fig. 3B). Also, cells showed positive staining for GATA-4 and it was localized in the nucleus of contracted cells (Fig. 3D). For ANP and BNP immunostaining, we used antibodies against human ANP and BNP according to our previous results obtained from molecular cloning of ANP and BNP from monkey (unpublished data) which showed high identity with human sequence particularly in the mature region of hormones. The beating cells showed ANP (Figs. 4A and B) and BNP (Figs. 4E and F) immunoreactivity which was observed as granules around the nuclei (Figs. 4A and E). The localization of these peptides was similar. To further analyze the localization, p230 trans-Golgi antibody was used as a marker of trans-Golgi network [26]. Double immunostaining of either ANP or BNP and p230 trans-Golgi antibodies (Figs. 4B, C, F, and G) showed that ANP and BNP signals appeared to co-localize with p230 trans-Golgi around the nucleus of beating cells (Figs. 4D and H).

Discussion

Gene expression, electrophysiological, and immunocytochemical data obtained in this study indicate that monkey ES cell-derived cardiomyocytes have some characteristics similar to those of differentiated in vivo. The generation of functional cardiomyocytes from ES cells has potential applications including myocardial repair through cell transplantation. Our data demonstrates that

Table 2
Action potential parameters detected in monkey ES cell-derived cardiomyocytes

Spontaneous rate (min^{-1})	APD50 (ms)	APD90 (ms)	dV/dt_{max} (V/s)	APA (mV)	MDP (mV)
87.1 ± 9.3	195.3 ± 25.9	272.7 ± 22.0	12.6 ± 1.8	98.3 ± 7.0	−61.2 ± 2.7

All data represent means ± SE ($n = 3$).

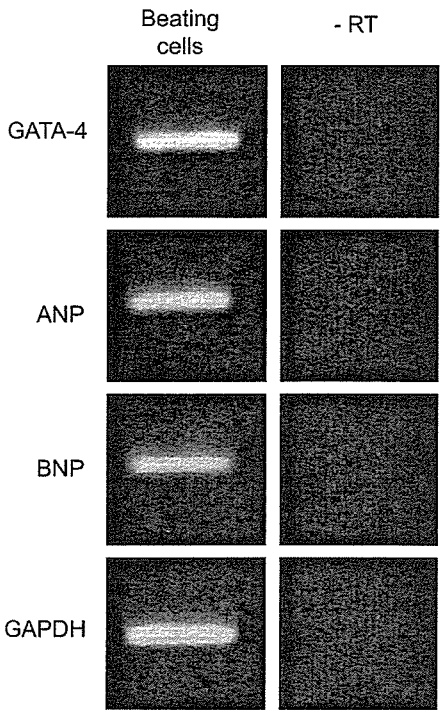

Fig. 2. Expression of natriuretic peptides genes. Reverse transcription-polymerase chain reaction (RT-PCR) analysis for GATA-4, ANP, BNP, and GAPDH expression in monkey ES cell-derived cardiomyocytes. (− RT) indicates PCR carried out using samples without reverse transcriptase.

monkey ES cells can be differentiated into functional cardiomoycytes which can produce natriuretic peptides.

The RT-PCR methods and immunocytochemistry in this study demonstrated that ANP and BNP were expressed in the cardiomyocytes differentiated from monkey ES cells. The immunoreactive granules of both peptides were observed in the perinuclear zone. The double immunostaining suggested that the ANP and BNP granules were co-localized with trans-Golgi network. Therefore, both peptides observed in this work are under processing in the Golgi apparatus and the most immunoreactive natriuretic peptides were considered to be immature. This phenomenon agrees with the previous findings in the embryonic [22] and adult [21] heart of human. Also, perinuclear localizations of ANP and BNP were observed in mouse ES cell-derived cardiomyocytes after ectopic transplantation [27]. Interestingly, immunoreactive granules of ANP and BNP were mainly detected in the contracted cardiomyocytes. These findings may support the concept that increased contraction of cardiac cells may affect the synthesis of RNA [28] and protein [29].

Our gene expression and immunocytochemical data confirmed the presence of GATA-4 in the contracted cells. In vertebrates, GATA-4 plays an important role in heart formation [30] and it was found to be a potent trans-activator of several cardiac specific promoters. Within the heart, GATA-4 transcripts are localized in ANP and BNP-expressing myocytes of both atria and ventricles, and they act as a potent activator of ANP and BNP hormones encoding genes [31]. It was found that certain regions of 5′ flanking sequences of the ANP and BNP genes confer cardiac-specific expression that may be regulated by transcription factors [31–33]. These findings suggest a role of GATA-4 as activator of natriuretic peptides to be expressed in ES-derived cardiomyocytes.

The actual role of natriuretic peptides in this study remains unclear. However, some possibilities are raised due to gene expression and perinuclear localization of both hormones and their co-localization with Golgi apparatus. Previous experiments which examined the effect of ANP

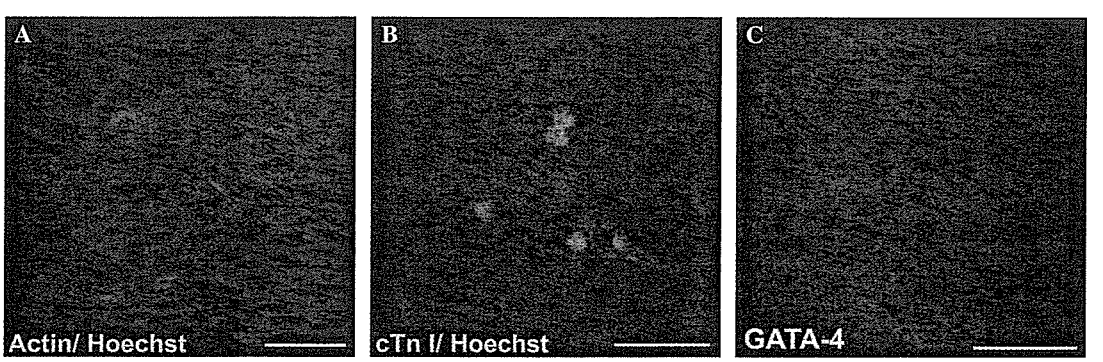

Fig. 3. Immunostaining of monkey ES cell-derived cardiomyocytes with cardiac marker antibodies. (A) Positive staining with anti-cardiac α-actin antibody. (B) Positive staining with anti-cardiac troponin I (cTnI) antibody. Note the cross-striation pattern in troponin positive cells. (C) Positive staining with anti-GATA-4 antibody. Scale bar = 50 μm.

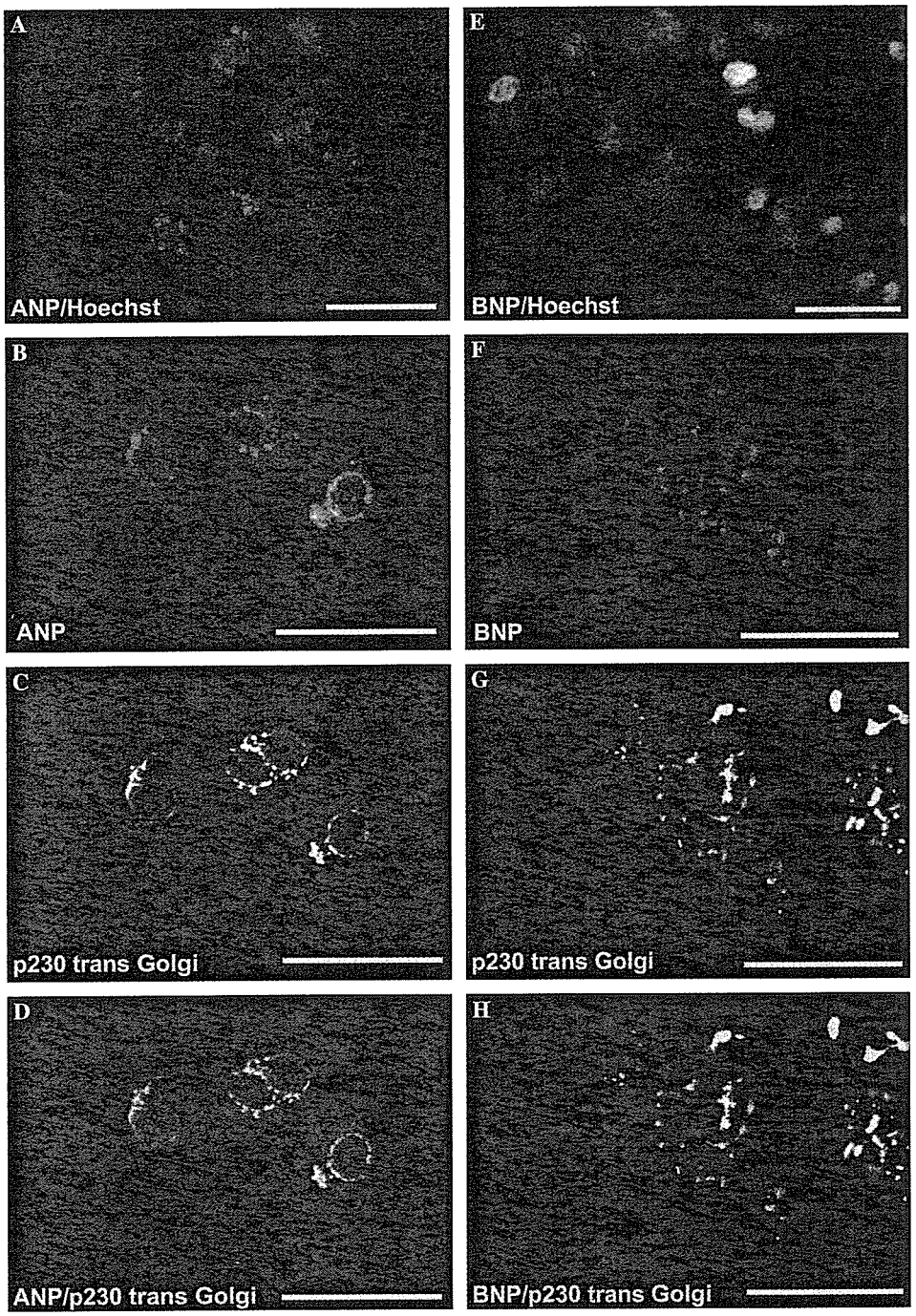

Fig. 4. Immunostaining of monkey ES cell-derived cardiomyocytes with natriuretic peptides antibodies. (A) Double image of positive staining with anti-ANP antibody and Hoechst nuclear stain. (B) ANP positive staining. (C) Perinuclear staining with p230 trans-Golgi antibody. (D) Double immunostaining of ANP and p230 trans-Golgi antibodies. (E) Double image of positive staining with anti-BNP antibody and Hoechst nuclear stain. (F) BNP positive staining. (G) positive staining with p230 trans-Golgi antibody. (H) Double immunostaining of BNP and p230 trans-Golgi antibodies. Note the co-localization of ANP and BNP with p230 trans-Golgi. Scale bar = 50 μm.

on the development of chick cardiomyocytes in vitro indicate that ANP increases the proliferative activity, expression of contractile proteins, and DNA synthesis through receptor-mediated pathway [34]. Furthermore, other studies showed inhibitory effect of endogenous natriuretic peptides on cardiomyocytes hypertrophy [10]. More recent data suggested that ANP might antagonize cardiomyocyte hypertrophy-promoting effects of vasoactive peptides and/or growth promoting factors [35]. However, mice with targeted deletion of BNP exhibit a different phenotype from that of the ANP-deficient mice. Mice without BNP do not have hypertension or cardiac hypertrophy; instead, they show focal ventricular fibrotic lesions with a remarkable increase in factors which was implicated in the generation and progression of ventricular fibrosis [36]. Therefore, the BNP may have a role as a local, paracrine

antifibrotic factor within the heart. These reports suggest complementary roles of ANP and BNP in the regulation of myocardial structure.

Receptor analysis has shown that various types of receptors for natriuretic peptides are found in the heart itself [16]. Thus, the in vitro expression of natriuretic peptides observed in the ES cell-derived cardiomyocytes, together with their elevated levels during normal heart formation in vivo [19], suggests a paracrine and/or autocrine function of natriuretic peptides. These observations suggest that cardiac natriuretic peptides (ANP and BNP) may play a role in the regulation of cardiomyocyte development in vivo and in vitro.

We could obtain large number of beating cardiomyocytes by mechanical isolation of beating cardiomyocytes and dissociation into small clusters or single cells. These findings are in agreement with that of cardiomyocytes derived from human ES cells [37]. This system should be a potent tool for increasing the number of beating cardiomyocytes to be used for cellular transplantation for cardiac diseases. Although several different types of cardiomyocyte preparations were successfully engrafted in animals [38], it is difficult to obtain a sufficient number of donor cells in human. ES cells may serve as alternative renewable source of donor myocytes.

In conclusion, the spontaneously produced cardiomyocytes from monkey ES cells can be enriched and they have the ability to produce natriuretic peptides in vitro. These results suggest a possible role of cardiac natriuretic peptides (ANP and BNP) in the regulation of cardiomyocyte development in vitro through paracrine and/or autocrine functions. In addition, these findings suggest that ES cell-derived cardiomyocytes are functional cardiac cells and can be used as a model to study mechanisms and functions in early stages of cardiogenesis.

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Brief Communication

Monkey Embryonic Stem Cells Differentiate into Adipocytes *In Vitro*

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ABSTRACT

Production of functional adipocytes is important in adipocyte research and regenerative medicine. In this paper, we describe the differentiation of monkey embryonic stem (ES) cells into insulin-responsive adipocytes. Treatment of embryoid body (EB) outgrowth with adipogenic hormones induced the expression of adipocyte-specific genes, such as PPARγ, C/EBPα, aP2, insulin receptor, and GLUT4. Expression of adipocytokines, leptin and adiponectin, was also detected. Furthermore, translocation of GLUT4 was observed by insulin stimulation in differentiated adipocytes. These results suggested that monkey ES cells can be a useful tool for studying adipogenesis in primate.

INTRODUCTION

EMBRYONIC STEM (ES) CELLS display self-renewal and pluripotency (Evans and Kaufman, 1981; Martin, 1981). They have shown to differentiate into various cell lineages, such as neurons (Bain et al., 1995), cardiomyocytes (Maltsev et al., 1993), osteoblasts (Buttery et al., 2001; Sottile et al., 2003; Yamashita et al., 2005), and adipocytes (Dani et al., 1997) *in vitro*. Adipose tissue is now recognized as a highly active metabolic and endocrine organ. It plays an important role not only for energy storage, but also for secreting a variety of bioactive peptides, known as adipocytekines (Arner, 2003). However, very little is known about the ontogeny of adipose tissue because of the lack of specific markers. Therefore, ES cells are considered to be useful tool to investigate molecular mechanism of adipogenesis. Differentiation into adipocytes was demonstrated only in mouse ES cells (Dani et al., 1997), and little is known about the function of adipocytes derived from ES cells. It has been reported that mouse preadipocyte cell line, 3T3-L1, displayed several metabolic and signal transduction differences compared with primate cells (Ryden et al., 2002). Therefore, it is worthy to investigate whether monkey ES cells can be differentiated into adipocytes.

In this study, we attempted to differentiate cynomolgus monkey ES cells into an adipocytic lineage and examined translocation of GLUT4 by insulin stimulation, as glucose uptake is a key function of mature adipocytes.

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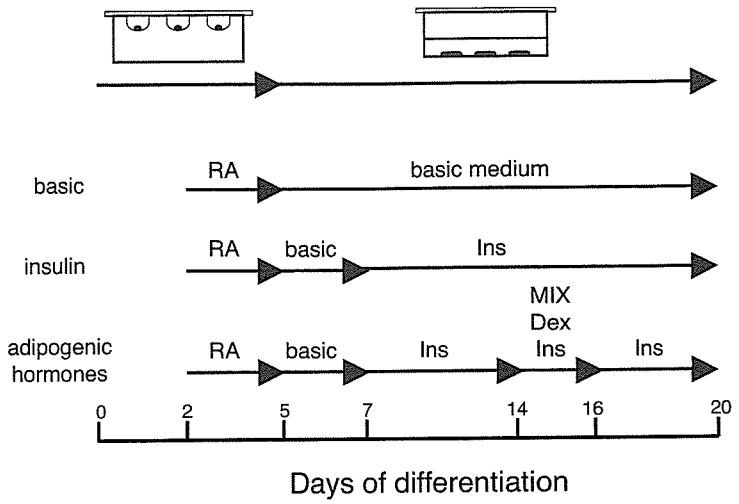

FIG. 1. Schematic presentation of the protocol to differentiate monkey embryonic stem (ES) cells into adipocytes. For adipocyte differentiation of ES cells, embryoid bodies (EBs) were prepared by hanging drop culture for 5 days and allowed to adhere onto gelatinized dish. EBs were treated with retinoic acid (RA; days 2–5) and subsequently cultured in the basic, insulin, or adipogenic medium.

METHODS

Monkey embryonic stem cell culture

The procedures for monkey ES cell culture were similar to those described by Thomson et al. (1995). Briefly, cynomolgus monkey ES cells (CMS-A-2) were grown on mitomycin C–treated (10 μg/mL, 37°C for 2 h) mouse embryonic fibroblast (MEF) feeder layers in gelatin-coated tissue culture dishes (Yamashita et al., 2005, 2006). The monkey ES culture medium consists of Dulbecco modified Eagle medium (DMEM/F-12 Ham 1:1; Sigma) supplemented with 20% Knockout Serum Replacement (KSR; Invitrogen), 1% nonessential amino acids (Sigma), 100 mM Na pyruvate (Sigma), 100 μM 2-mercaptoethanol (Sigma), and 200 mM L-glutamine (Sigma). Cells were kept at 37°C in a humid 5% CO_2 atmosphere, and the medium was changed every day. ES colonies were split every 3–7 days by incubation in 0.1% collagenase (37°C for 3–5 min; Wako) and replating collected cells after centrifugation onto dishes with new MEF feeder cells.

Differentiation of monkey embryonic stem cells

For differentiation of ES cells, entire ES cell colonies were loosely detached from MEF feeder cells by exposure to 0.1% collagenase (37°C for 3–5 min), and cultured in hanging drops (20 μL/drop) on the inner surface of 100-mm culture dish lids to make embryoid bodies (EBs) with basic differentiation medium, consists of DMEM/F-12 supplemented with 15% fetal bovine serum (FBS), 100 mM Na pyruvate, 200 mM L-gluta-

TABLE 1. SEQUENCES OF PCR PRIMERS

Genes	*Primer*		*Cycles*
PPARγ	For	5′-AAACTCTGGGAGATTCTCCT-3′	40
	Rev	5′-TCTTGTGAATGGAATGTCTT-3′	
C/EBPα	For	5′-TCGACATCAGCGCCTACATC-3′	40
	Rev	5′-CTTGTCCACCGACTTCTTGC-3′	
aP2	For	5′-ATGTGTGATGCCTTTGTGGGA-3′	40
	Rev	5′-TGCCCTTTCATAAACTCTTGT-3′	
Ins-R	For	5′-AACCAGAGTGAGTATGAGGAT-3′	30
	Rev	5′-CCGTTCCAGAGCGAAGTGCTT-3′	
GLUT4	For	5′-CCATTGTTATCGGCATTCTGATCG-3′	30
	Rev	5′-ATAGCCTCCGCAACATACTGGAAAC-3′	
leptin	For	5′-ATAGCATTGGGGAACCCTGTGCGG-3′	40
	Rev	5′-AGGTCCAGCTGCCACAGCATGTC-3′	
adiponectin	For	5′-AACATGCCCATTCGCTTTAC-3′	40
	Rev	5′-TCTCCTTCCCCATACACCTG-3′	
GAPDH	For	5′-ATGGGGAAGGTGAAGGTCGG-3′	25
	Rev	5′-GGAGTGGGTGTCGCTGTTGAA-3′	

mine, 7.5% sodium bicarbonate (Sigma), and 100 μM 2-mercaptoethanol. After 2 days of culture, 100 nM all-trans retinoic acid (RA; Sigma) was added in the medium and cultured for 3 more days. Then, EBs were transferred to tissue culture dish coated with 0.1% gelatin (Fig. 1).

To induce adipocyte differentiation, adipogenic hormones such as 3-isobutyl-L-methylxanthine (MIX), dexamethasone (Dex) and insulin were used. First, 100 nM insulin (Sigma) was added into the basic medium from day 7 to day 14. Then 0.5 mM MIX (Sigma), 0.25 M Dex (Sigma), and 100 nM insulin was added for 2 days, followed by the addition of insulin for another 4 days. To evaluate whether the addition of MIX and Dex is necessary for adipocyte differentiation, we also cultured cells only in the presence of insulin on days 7–20. These media were changed every 2 days. Their effects to differentiate ES cells into adipocytes were evaluated by reverse transcriptase–polymerase chain reaction (RT-PCR), Oil Red O staining, and GLUT4 immunocytochemistry.

RNA extraction and reverse transcriptase–polymerase chain reaction

Total RNA was isolated from cell culture at 20 days of differentiation using RNeasy Mini

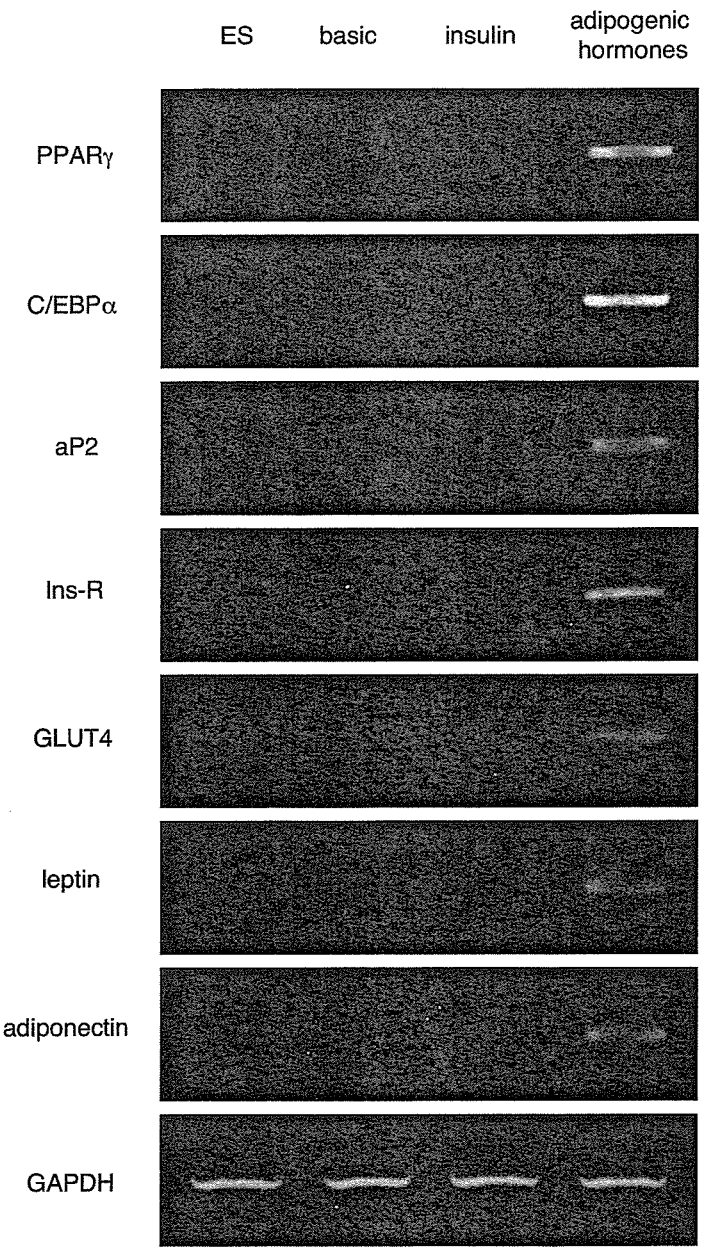

FIG. 2. Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of genes characteristic to adipocyte. Expression of peroxisome proliferator-activated receptor-γ (PPARγ), CCATT/enhancer binding protein-α (C/EBPα), adipocyte-specific fatty acid binding protein (aP2), Ins-R (insulin receptor), GLUT4, leptin, and adiponectin mRNAs were analyzed by RT-PCR at 20 days of differentiation. GAPDH was used as an internal control.

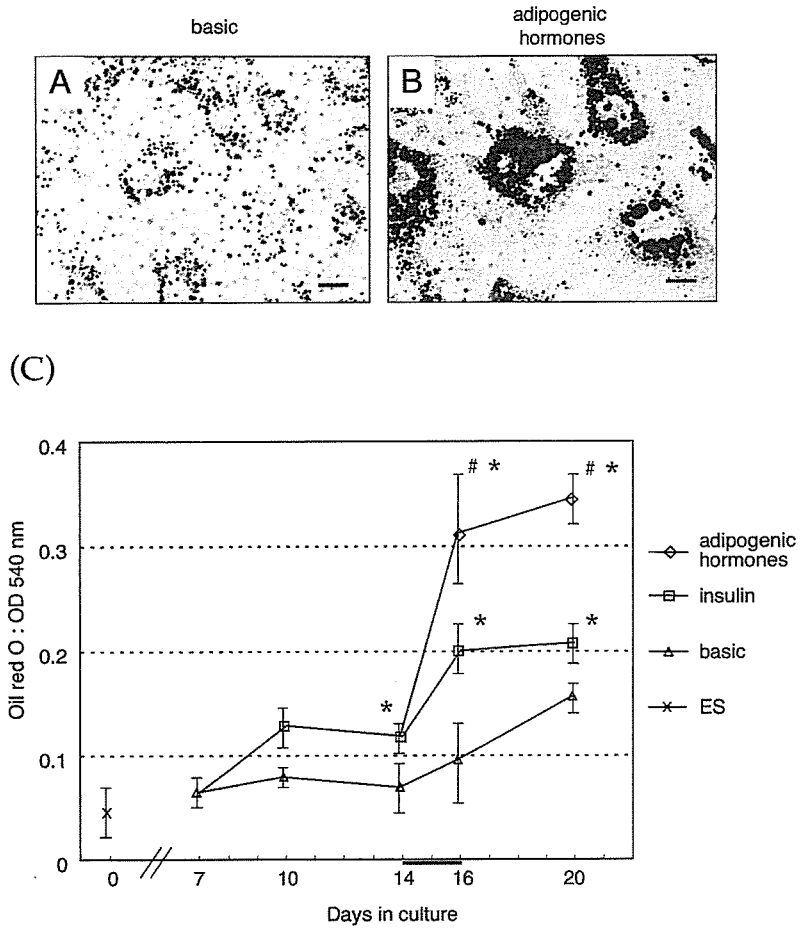

FIG. 3. Oil Red O staining. Lipid accumulation in adipocytes was visualized by Oil Red O staining at 20 days of differentiation. (A) Embryoid body (EB) outgrowth cultured in the basic medium (basic). (B) EB outgrowth cultured in the presence of adipogenic hormones (adipogenic hormones). Scale bar 10 μm. (C) Time course of lipid accumulation. Stained lipid was extracted from individual dish, and the absorbance at 540 nm was measured. Data were expressed as means ± SD ($n = 4$). *Significant difference from basic medium, $p < 0.05$ by Student's t-test. #Significant difference from insulin medium, $p < 0.05$ by Student's t-test.

Kit (Qiagen) according to the manufacturer's instructions. After DNase treatment of the samples, reverse transcription was carried out using Super Script III First-Strand Synthesys System (Invitrogen). Expression of adipocyte-specific genes, peroxisome proliferator-activated receptor-γ (PPARγ), CCATT/enhancer binding protein-α (C/EBPα), an adipocyte-specific fatty acid binding protein (aP2), insulin receptor, GLUT4, leptin, and adiponectin was investigated. The PCR reactions (50 μL) were carried out using Blend Taq DNA Polymerase (Toyobo, Japan) according to the manufacturer's instructions. PCR conditions were 2 min at 94°C, 15-sec denaturation at 94°C, 30-sec annealing at 55°C, and 30-sec extension at 72°C. Primers were designed based on the human sequence and BLASTed for their specificity at the National Center for Biotechnology Information (NCBI). Primer sequences and cycle numbers were described in Table 1. PCR products were analyzed on a 1.5% agarose gel. Imaging and scanning were performed using an Epi-Light UV FA1100 system (Taitec, Japan).

Oil Red O staining and lipid accumulation assay

Accumulated lipid in adipocytes was visualized by staining with Oil Red O at 20 days of differentiation. Cells were washed three times with phosphate-buffered saline (PBS), followed by fixation with 4% paraformaldehyde for 15 min at room temperature. After fixation, the cells were stained with a filtered 0.3% Oil Red O solution for 1 h at room temperature. Cells were then washed twice with H_2O for 15 min. To evaluate lipid accumulation during differentiation, stained lipid was extracted by 100% isopropyl alcohol for 5 min, and the optical density of the solution at 540 nm was measured at days 7, 10, 14, 16, and 20.

GLUT4 immunocytochemistry and effect of insulin

EBs were attached on the coversilps pre-coated with 0.1% poly-L-lysine and differentiated into adipocytes using the same protocol as described above. Insulin was withdrawn from the medium

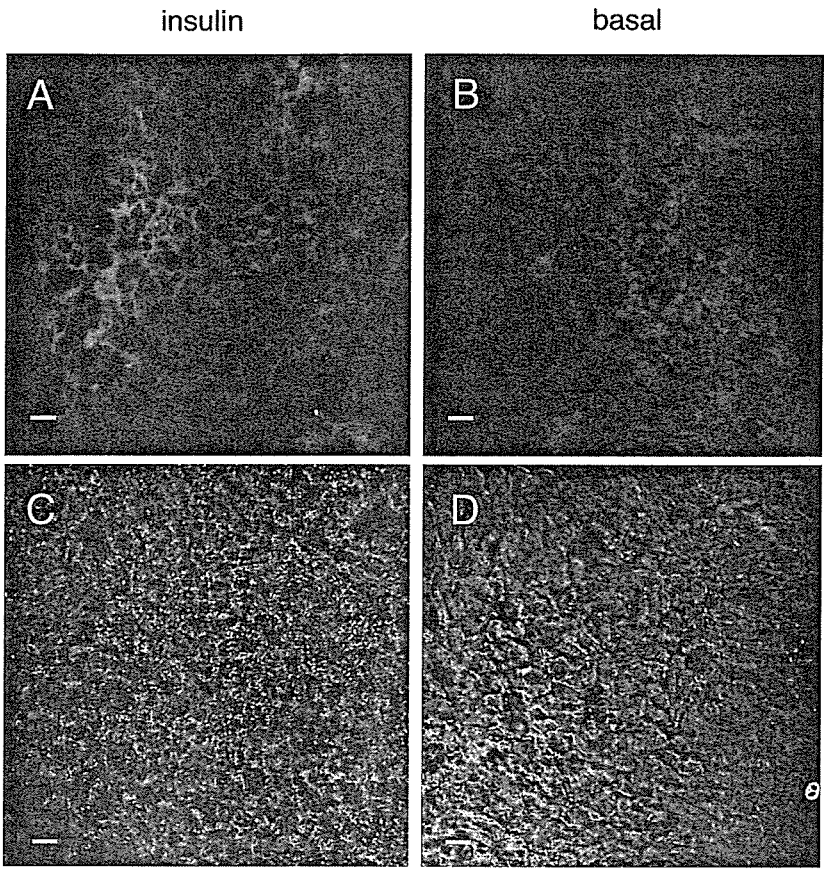

FIG. 4. Effect of insulin on the distribution of GLUT4 in adipocytes derived from monkey embryonic stem (ES) cells. The adipocytes derived from monkey ES cells were treated with insulin (insulin) (**A,C**) and vehicle (basal; **B,D**), as described in the text. The distribution of GLUT4 was analyzed using immunofluorescence confocal microscopy (A,B). Differential contrast image (C,D). Scale bar = 10 μm.

24 h before the experiments. At 21 days of culture, the medium was replaced with the fresh DMEM containing 4.5 g/L glucose and 10% FBS, and then the cells were incubated for 30 min at 37°C, in a humid 5% CO_2 atmosphere in the presence or the absence of 100 nM insulin. The cells were then rinsed twice with prewarmed PBS and were fixed with 3.7% formaldehyde in PBS for 1 h at room temperature. The fixed cells were washed three times with PBS for 10 min. To reduce non-specific background staining, cells were incubated with 0.2% Triton X-100 and 1% (w/v) BSA in PBS (blocking solution) for 30 min. The cells were then incubated with anti-GLUT4 antibody (1:500; kindly provided by Cushman, National Institutes of Health [NIH]) in the blocking solution for overnight at 5°C and washed three times with PBS for 10 min. The cells were incubated with Alexa fluor 546-conjugated goat anti-rabbit IgG (1:1000) in PBS for 3 h at room temperature. After washing three times with PBS for 10 min, each coverslip was mounted on a glass slip with the cell side down in a 1:1 solution of PBS/glycerol. Fluorescent signals were observed with a confocal laser-scanning microscope (Zweiss, LSM 510 META) mounted on a Zweiss Axiovert 200M inverted microscope with a 543-nm excitation filter and a 560-nm emission long-path filter.

RESULTS AND DISCUSSION

In this study, we have investigated whether monkey ES cells differentiate into adipocytes using adipogenic hormones (MIX, Dex, and insulin), which are frequently used for the differentiation into adipocytes (Gregoire et al., 1998).

The process of EB formation and differentiation was summarized in Figure 1. At 20 days of differentiation, RT-PCR was carried out to detect the expression of adipocyte specific genes. Differentiation from committed preadipocyte to mature adipocyte is characterized by the appearance of early transcription factors (PPARγ and C/EBPα) and late expression markers (aP2, insulin receptor, and GLUT4) (Gregoire et al., 1998). Expression of PPARγ, C/EBPα, aP2, insulin receptor, and GLUT4 was detected in cells treated with adipogenic hormones, whereas the expres-

sion of these genes was not detected in control cells cultured with basic and insulin medium (Fig. 2). These results suggest that monkey ES cells respond to adipogenic hormones by inducing adipocyte-specific genes, and the addition of MIX and Dex is necessary for their expression.

During the last decade, a growing number of adipocyte-derived hormones or adipocytokines have been identified, including leptin (Frayn et al., 2003) and adiponectin (Hu et al., 1996). These adipocytokines have been described to be involved in physiological regulations of fat store, development, and metabolism, and also play a role in obesity-associated disorders, including type II diabetes and hypertension (Kershaw and Flier, 2004). Therefore, we also investigated the expression of leptin and adiponectin in these cells. We found the expression of leptin and adiponectin in cells cultured in the presence of adipogenic hormones (Fig. 2). The expression of these adipocytokines, as well as late expression markers, suggest that these cells differentiated into mature adipocytes.

Next, lipid accumulation was visualized by Oil Red O staining. Cells treated with adipogenic hormones showed many lipid droplets in the cytoplasm (Fig. 3A,B). We also investigated the time course of lipid accumulation (Fig. 3C). The addition of MIX and Dex significantly increased the lipid accumulation, suggesting the importance of these adipogenic hormones for adipocyte differentiation in monkey ES cells. The increase of extracted lipid (OD at 540 nm) in cells treated with basic and insulin media may be mainly attributable to the increase of cell number by proliferation, rather than lipid accumulation, because these cells had some background staining similar to Figure 3, and did not show obvious increase of lipid droplets throughout the differentiation period.

Then, we investigated insulin responsiveness of GLUT4, an insulin-regulated glucose transporter. GLUT4 expression was confirmed by immunocytochemistry. In the absence of insulin, we obtained diffusive image of GLUT4 (Fig. 4B). However, localization of GLUT4 was altered probably to the plasma membrane by the addition of insulin (Fig. 4A). These data suggest that adipocytes derived from monkey ES cells possessed the same insulin-responsive GLUT4 as mature adipocytes found in the normal fat tissue (Ralston and Ploug, 1996; Slot et al., 1991, 1997; Bryant et al., 2002).

In summary, we have demonstrated the differentiation of monkey ES cells into an adipocytic lineage. Adipocytes derived from monkey ES cells expressed adipocytokines and insulin-responsive GLUT4 unique to mature adipocytes. This system will provide a useful model to elucidate the molecular mechanisms of adipogenesis in primate and to develop cell transplantation therapy.

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