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The effects of amniotic membrane on retinal pigment epithelial cell differentiation

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Purpose: To examine the characteristics of retinal pigment epithelial (RPE) cells cultured on amniotic membrane (AM). The present study examined how AM modulates RPE cell differentiation.
Methods: Human RPE cells were cultured on the basement membrane side of dispase treated AM. After one week of cellular confluence, cultures were terminated, conditioned medium was collected, and total RNA was extracted. The expression pattern of several genes considered to participate in the function of differentiated RPE was evaluated. Ultrastructural changes were evaluated by transmission electron microscopy.
Results: Morphologically, RPE cells cultured on AM exhibited ultrastructural epithelial features such as microvilli of the apical membrane and intercellular junctions. Gene expression of RPE65, CRALBP, bestrophin, and tyrosinase related protein (TRP)-2 was upregulated in RPE cells cultured on AM compared to cells cultured on plastic. In addition, protein production of vascular endothelial growth factor, thrombospondin-1, and pigment epithelium derived factor was markedly increased in cells cultivated on AM. Gene expression of cathepsin D, brain derived neurotrophic factor, and basic fibroblast growth factor, however, did not differ between RPE cells cultured on plastic or AM.
Conclusions: RPE cells cultured on AM demonstrated an epithelial phenotype morphologically and several growth factors important for maintaining retinal homeostasis were upregulated. AM might be a useful matrix substrate to retain the differentiated and epithelial phenotype of RPE for subretinal transplantation.

In the western world, age related macular degeneration (AMD) is the primary cause for visual impairment and blindness in individuals over 60 years of age. The most frequent form of AMD (dry AMD) is characterized by primary degeneration and atrophy of the retinal pigment epithelial (RPE) layer. Vision loss in patients with the exudative form of AMD (wet AMD) is caused by the formation of subretinal fibrovascular membranes.

RPE transplantation may be a new treatment strategy for both wet AMD, after removal of the submacular neovascular membrane, and dry AMD. There have been a number of experimental studies on human volunteers in which RPE was grafted into the eyes of patients with advanced AMD [1,2]. The fetal RPE grafts survived for 3 months after subretinal implantation, but there was no evidence of improved function in patients with AMD and subfoveal neovascular membranes [2]. Some of cells transplanted into the subretinal space are randomly organized in multilayers and do not appear to be differentiated [3].

The basement membrane promotes epithelial differentiation [4-6] and prevents epithelial cell apoptosis [7]. It may be that abnormalities of the basement membrane of Bruch's membrane in AMD or after surgical removal of the submacular neovascular membrane can impede the reattachment, survival, proliferation, and biologic function of the transplanted RPE graft. Some studies indicate that RPE does not repopulate as a monolayer over an experimentally damaged Bruch's membrane [8,9].

To preserve cell orientation, some researchers have tried to maintain cell monolayers and polarity using different biologic supports, such as Descemet's membrane [10], the lens capsule [11], Bruch's membrane, blood cryoprecipitates [12], or synthetic supports, such as collagen substrates [13] or biodegradable polymer films [14]. Most of these supports were either toxic to the cells or disintegrated on exposure to liquid media.

The amniotic membrane (AM), with its thick basement membrane and avascular stromal matrix, was recently used as a substrate for the transplantation of cultivated corneal epithelial cells [15]. It is a readily available tissue with many advantages for clinical application. In addition, AM seems to be an immune privileged tissue and contains some immunoregulatory factors, including HLA-G and Fas ligand [16]. We hypothesized that AM might be an ideal matrix substrate to induce and retain the differentiated epithelial phenotype of RPE cells for subretinal transplantation. Proper implantation and orientation of the grafted RPE cells are considered to be important factors for a successful graft because RPE cells are polar with distinct apical/basal characteristics. Implantation of RPE cells cultured on AM might provide a means

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of transplanting an organized sheet of cells for the restoration of normal RPE function over the long term.

As a first step to determine the usefulness of AM as a support matrix for RPE, the present study investigated whether RPE cells can be induced to adopt a differentiated phenotype when grown on AM.

METHODS

Reagents: We obtained Dulbecco's modified Eagle's media (DMEM), and TRIzol reagent from Invitrogen (Carlsbad, CA); plasticware from Falcon and Becton (Dickinson and Co. Franklin Lakes, NJ); fetal calf serum from Hyclone (Logan, UT); vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and brain derived neurotrophic factor (BDNF) enzyme linked immunosorbent assay (ELISA) kits, from Genzyme Techne (Minneapolis, MN); You-Prime First-Strand Beads, polymerase chain reaction (PCR) Beads, and Nylon membranes from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ); TSP-1 ELISA kit from Chemicon (Temecula, CA); and agarose from Takara (Ohtsu, Japan).

Culture of human RPE cells: Human RPE cells were kindly supplied by Peter A. Campochiaro, MD (Wilmer Eye Institute, Johns Hopkins University, Baltimore, MD). Human RPE cells were grown in DMEM supplemented with 10% fetal calf serum in a humidified incubator under 95% air and 5% CO_2. Cultures were demonstrated to be pure populations of RPE cells by immunocytochemical staining for cytokeratins (data not shown). Experiments were performed with third or fourth passage cells.

Cultures of RPE on AM: Preserved human AM was purchased from Bio-Tissue (Miami, FL). AM was preserved according to the method described by Lee and Tseng [17]. Briefly, AM derived from cesarean section derived placentas was rinsed in phosphate buffered saline (PBS) containing 100 U/ml penicillin and 0.2 mg/ml streptomycin and stored in a solution of 50% DMEM and 50% glycerol at -80 °C for at least 3 months. After thawing at room temperature, AM was treated with 1.2 U/ml sterile dispase-I solution (Godo-Shusei, Tokyo, Japan) for 30 min and then gently scrubbed with an epithelial scrubber (cell scraper; Costar, Corning, NY), to remove the amni-

Table 1. Primer sequences used for semi-quantitative PCR

Gene	Primer sequence (5'-3') position in mRNA	Annealing temp (°C)	Product size (bp)	Reference
RPE65	F: CCTTTCTTCATGGAGTCTTTG R: ATTGCAGTGGCAGTTGTATTG	52	390	[57]
CRALBP	F: ATGTCAGAAGGGGTGGG R: TCAGAAGGCTGTGTTCTCA	60	953	[57]
TRP2	F: GGAGAAAAGTACGACAGAGACAAGG R: AGAAAAGCCAACAGCACAAAAAGAC	60	1542	[25]
tyrosinase	F: GCATCATTCTTCTCCTCTTGG R: ATAGGATCGTTGGCAGATCC	60	359	[25]
cathepsin D	F: GTGCTTCACAGTCGTCTTC R: GAGCCATAGTGGATGTCAAAC	55	172	[58]
bFGF	F: GCCTTCCCGCCCGGCCACTTCAAGG R: GCACACACTCCTTTGATAGACACAA	55	179	[57]
BDNF	F: ATGACCATCCTTTTCCTTACTATGGT R: TCTTCCCCTTTTAATGGTCAATGTAC	52	741	[57]
PEDF	F: GGACGCTGGATTAGAAGGCAG R: TTGTATGCATTGAAACCTTACAGG	64	675	[39]
FGFR	F: GGGTCCATCAATCACACGTAC R: GGCGTGTTGTTATCCTCACCAG	60	643	[21]
bestrophin	F: GGCCAGATCTATGTACTGGAATAAGCCCGAGC R: GGCCCTCGAGTTAGGAATGTGCTTCATCCCTG	65	773	[36]
β-actin	F: GAGCACAGAGCCTCGCCTTTGC R: GGATCTTCATGAGGTAGTCAGTCAGG	65	620	[57]

This table describes the primer sequences used in RT-PCR. The results of RT-PCR are shown in Figure 4.

otic epithelium without breaking the underlying basement membrane. The AM was sutured onto a culture insert (Millicell-CM, Millipore, Bedford, MA) with a non-absorbable suture with the basement membrane facing up and placed in a 6 well tissue culture plastic plate, as described by Meller and Tseng [18]. RPE cells were seeded at a density of $1.0x10^5$ cells/culture insert on the AM. The cultures were incubated at 37 °C in 5% CO_2 and 95% air, and the medium was changed every 2 days.

In some experiments, RPE cells were cultured on uncoated plastic in DMEM with 10% FBS and used as controls. Each condition was prepared in triplicate, and experiments were performed at least three times with reproducible results.

F-actin immunostaining of RPE cells on AM: RPE monolayers were washed three times with PBS, fixed with 1.0% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 for 5 min in PBS, washed in PBS, stained with FITC conjugated phalloidin for 40 min then washed two times in PBS. The distribution of F-actin was visualized and de-

Table 2. Primer sequences used for real-time quantitative PCR

Gene	Primer sequence (5'-3') position in mRNA	Annealing temp (°C)	Product size (bp)	Accession number
RPE65	F: CCTTTCTTCATGGAGTCTTTG R: ATTGCAGTGGCAGTTGTATTG	50	122	BC075035
CRALBP	F: GCTGCTGGAGAATGAGGAAACT R: TGAACCGGGCTGGGAAGGAATC	61	149	BC004199
TRP2	F: GGTTCCTTTCTTTCCAGT R: GAAGAAAAGCCAACAGCACAA	60	185	S69231
bestrophin	F: ATGGGGCCTTGATGGSAGCAC R: GGCGAAGCATCCCCATTAGG	58	227	BC015220
GAPDH	F: TGAACGGAAGCTCACTGG R: TCCACCACCCTGTTGCTGTA	65	307	M33197

Some of the primers listed in Table 1 were used for the detection of tyrosinase, cathepsin D, bFGF, BDNF, PEDF, and FGFR.

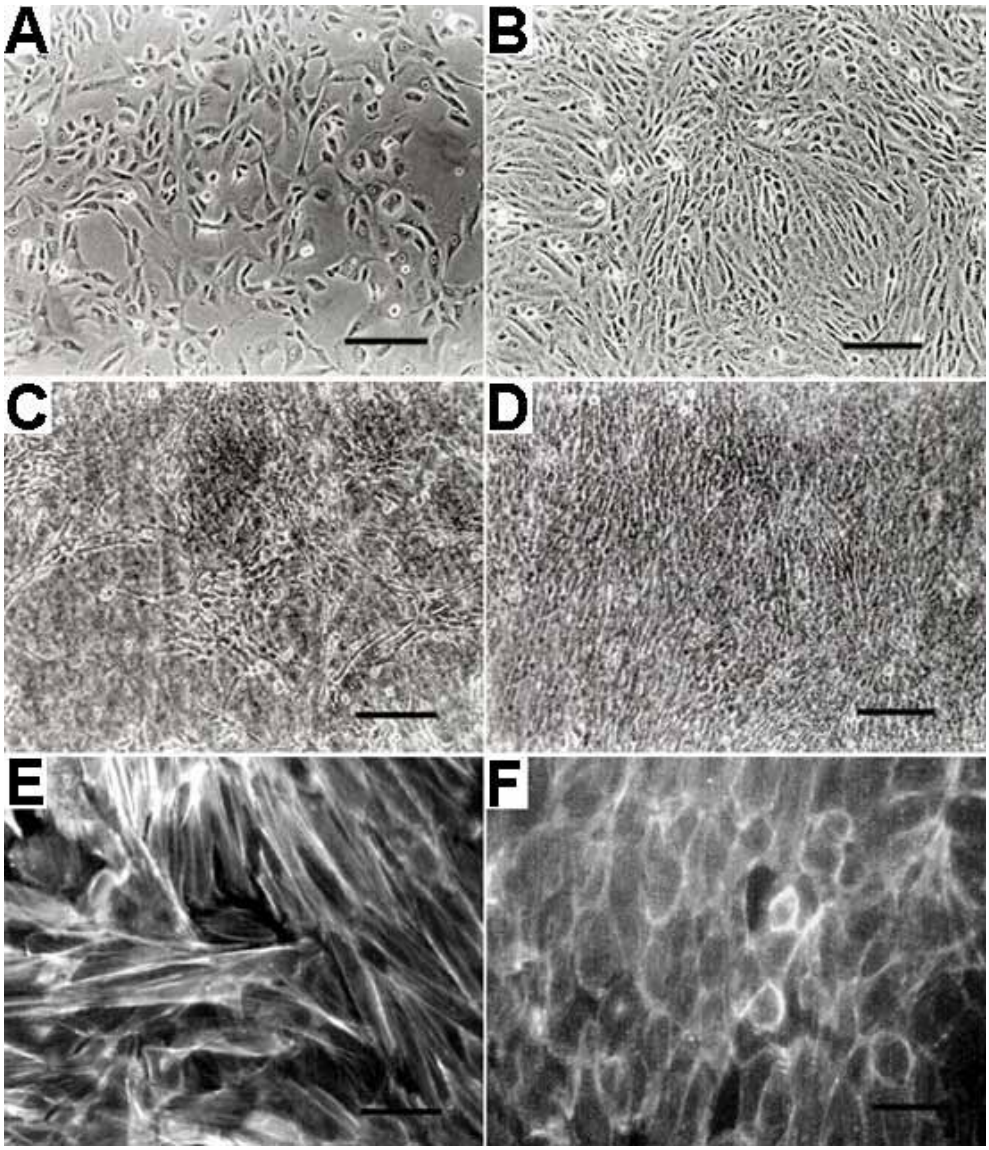

Figure 1. Phase contrast micrographs and F-actin immunostaining. Human RPE cells cultured on plastic one day after seeding (**A**) and at confluence (**B**). Human RPE cells cultured on amniotic membrane (AM) one day after seeding (**C**) and at confluence (**D**). F-actin immunostaining in confluent RPE cells on plastic (**E**) and on AM (**F**). Scale bars in **A**-**D** represent 100 μm; scale bars in **E** and **F** represent 15 μm.

tected under a fluorescence microscope.

Electron microscopy: RPE cell cultures on denuded AM were examined by transmission electron microscopy. On day 14, cultures on denuded AM were fixed in 2.5% glutaraldehyde in 0.1 M PBS for 2 h. The cultures were washed overnight at 4 °C in the same buffer and post-fixed with aqueous osmium tetroxide with 0.1 M PBS for 2 h. The pellets were dehydrated through a graded ethanol series, and embedded in Epon 812. Semi-thin (1 μm) sections for light microscopy were collected on glass slides and stained for 30 s with toluidine blue; ultrathin (70 nm) sections were collected on copper grids and double stained with uranyl acetate and lead citrate and then examined with an H-7100 transmission electron microscope (Hitachi Ltd., Hitachinaka, Japan).

Semiquantitative reverse transcription-PCR: Total RNA was extracted from RPE cells using TRIzol reagent. cDNA was synthesized from 2 μg of total RNA using You-Prime First-Strand Beads according to the manufacturer's protocol, and reaction product was submitted to PCR amplification using GeneAmp PCR System (Perkin-Elmer Cetus Corp., Norwalk, CT). The expression of mRNA for the discriminative cellular markers RPE 65, cellular retinaldehyde binding protein (CRALBP), and bestrophin was examined. The growth factors and trophic factors investigated were pigment epithelium derived factor (PEDF), BDNF, and bFGF. Also, the enzymes involved in melanin synthesis, tyrosinase and tyrosinase related protein (TRP)-2, were examined. A special lysosomal enzyme in RPE cells, cathepsin D, was also examined [19]. The expression of FGFR2, an isoform of the FGF receptor that is upregulated as a function of differentiation in RPE cells [20], was also examined. Oligonucleotide primers complimentary to the 5' and 3' ends were used in the RT-PCR studies (Table 1). PCR products (8 μl) were electrophoresed with a 2% agarose gel that contained 0.5% ethidium bromide, and specific DNA bands were examined under an ultraviolet transilluminator.

Real-time PCR: To confirm mRNA expression and to evaluate the mRNA expression levels in the cultured cells, real-time PCR was applied using specific primers (Table 2) with a Light Cycler™ instrument (Roche Diagnostics, Basel, Switzerland). Some primers were redesigned to produce a product of size between 100 bp to 200 bp. Each 25 μl of PCR

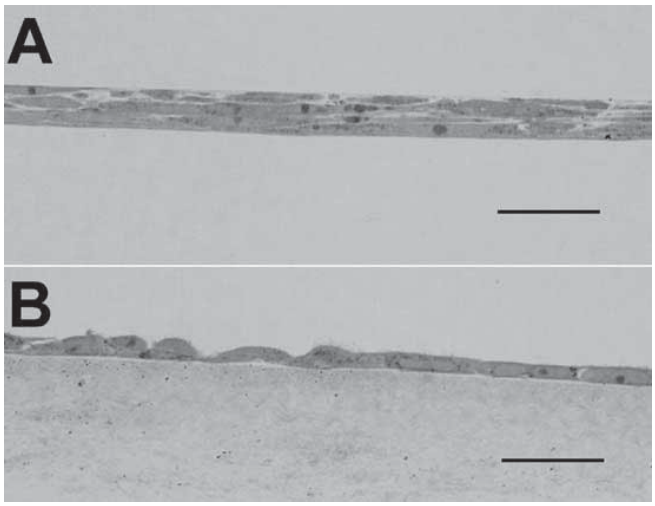

Figure 2. Different morphology of RPE cells cultured on plastic and on amniotic membrane. Toluidine blue stained sections of RPE cells on plastic (**A**) and RPE cells after 14 days cultivation on amniotic membrane (**B**). Scale bars represent 50 μm.

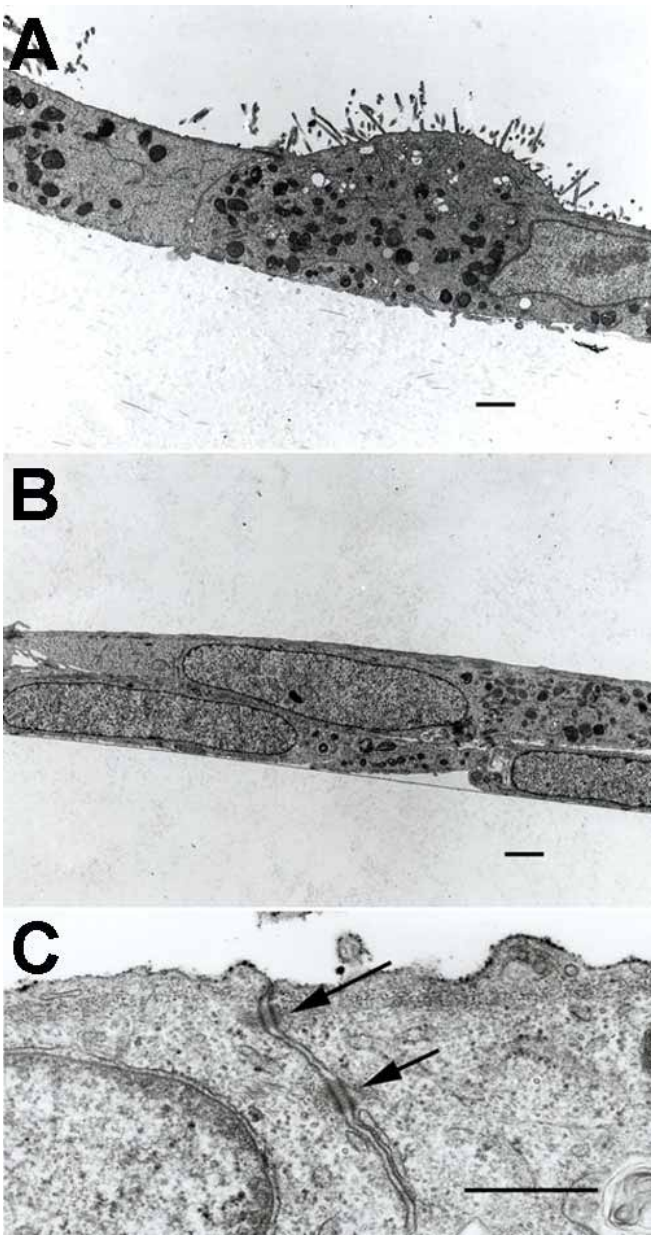

Figure 3. Transmission electron micrographs of cultivated RPE cells on denuded amniotic membrane for two weeks. **A**: Electron micrographs show a tight monolayer of cuboidal to spheroidal RPE cells growing over amoniotic membrane (AM). A heterogenous expression of apical microvilli was observed on the apical side. **B**: RPE cells cultured on plastic show elongated cell shape in multilayers. **C**: Electron micrographs reveal junctional specializations on the apical side between adjacent cells cultured on AM. Scale bars represent 1 μm.

mixture contained 0.3 μM of gene specific primers, 1 μl of sample cDNA solution, 12.5 μl of PCR mixture (Quantitect SYBR Green PCR Kit™ Qiagen Inc., CA) and 6.5 μl of supplied water. PCR was performed with the first cycle at 95 °C for 15 min to activate HotStar Taq polymerase in the premixture, this was followed by 45 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The signal intensity was measured at 72 °C during each cycle. The absence of bands other than the target band was verified by gel electrophoresis on 1.5% agarose stained with ethidium bromide under UV light after amplifications. The original PCR product containing 25 ng/μl of target DNA was diluted into concentrations of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} with distilled water, and these dilutions were simultaneously subjected to real-time PCR to establish a standard curve. To adjust the difference of concentration of mRNA reverse transcribed, GAPDH was used as an internal control. Relative quantity was evaluated by ratio of mRNA expression of the target gene and GAPDH.

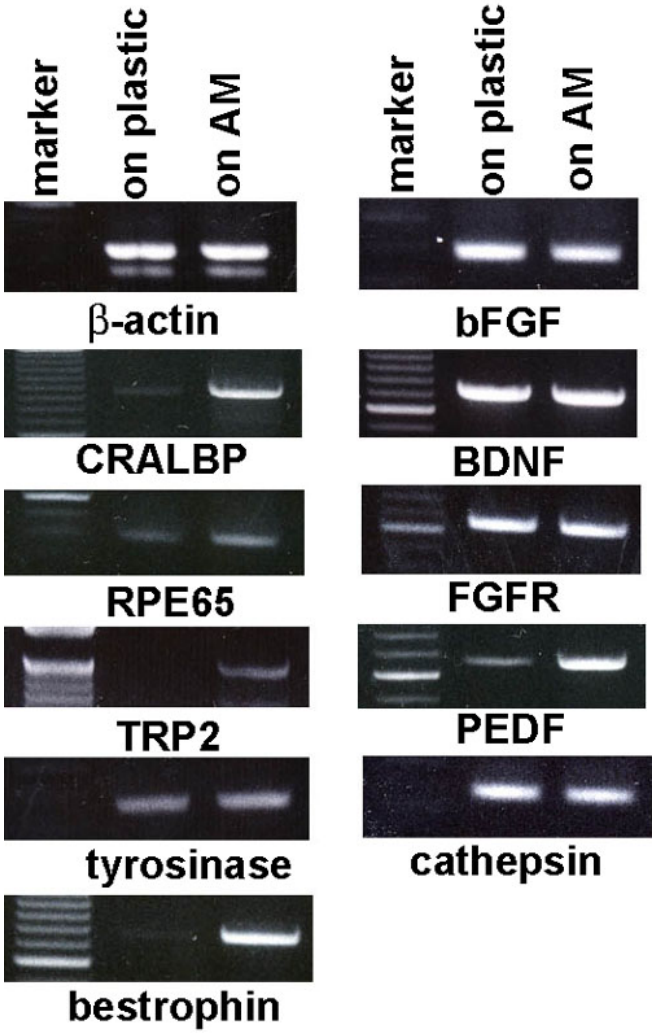

Figure 4. RT-PCR analysis of discriminatory molecules and growth and trophic factors in RPE cells. Total RNA was extracted from confluent RPE cells cultured on either plastic or amniotic membrane (AM). RT-PCR was performed on 2 μg RNA. For each gel shown, the left lane (labeled "marker") is a commercially supplied size marker, the middle lane (labeled "on plastic") is product from a reaction using RNA from RPE cells cultured on plastic, and the right lane (labeled "on AM") is a product from a reaction using RNA from RPE cells cultured on AM. cDNA product (2 μl) from RT-PCRs that used β-actin primers was used for all PCR reactions and β-actin was used as a positive control throughout.

ELISA of growth factors: Cells were incubated in serum free medium for 48 h before harvest for protein concentration. The amount of secreted VEGF, BDNF, and bFGF in RPE conditioned culture medium was determined using commercial ELISA kits (Genzyme Techne), according to the manufacturer's instructions. Also, the amount of secreted thrombospondin (TSP)-1 was determined using a commercial ELISA kit (Chemicon, Temecula, CA). The lowest detection limit of each molecule by ELISA assay was as follows: VEGF, 5 pg/ml; BDNF, 20 pg/ml; bFGF, 7 pg/ml; and TSP-1, 3.91 ng/ml. After the conditioned medium was collected, cells were washed with PBS, detached from the dish by treatment with 0.2% trypsin-EDTA, and suspended in 1 ml PBS. The number of viable cells was determined using a Coulter Counter Channelyzer (Coulter Electronics, Krefeld, Germany).

Western blot analysis of PEDF: Cells were allowed to condition serum free medium for 48 h before harvest for protein concentration. Final protein concentrations were determined using the BCA assay (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. Equal amounts of secreted protein (8 μg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transblotted onto nylon membranes. Nylon membranes containing transblotted proteins were pretreated with 1.0% nonfat dried milk in 50 mM Tris buffer (pH 8.0), followed by incubation overnight with a monoclonal antibody against human PEDF (dilution 1:4000, Transgenic Co., Kumamoto, Japan). PEDF immunoreactivity was visualized by exposing X-ray film to blots incubated with the ECL reagent.

Table 3. Results of real-time PCR

Gene	AM to plastic ratio
RPE	12.80
CRALBP	5.00
TRP2	12.30
tyrosinase	0.89
cathepsin D	0.78
bFGF	0.52
BDNF	1.02
PEDF	8.22
FGFR	1.11
bestrophin	13.50

The fold changes in mRNA levels of the selected genes were determined by real-time RT-PCR. Values represent fold changes of mRNA levels of RPE cells on amniotic membrane compared to RPE cells on plastic (AM to plastic ratio) after normalization for GAPDH content. Data are means of 3 to 4 samples per group.

RESULTS

Morphology of RPE cell cultures on AM: The human RPE cells cultured over dispase treated AM fragments were attached to the epithelial basement membrane side within 24 h of seeding. After settling down, RPE cells displayed many connected colonies comprised of small, polygonal, and uniform epithelial cells (Figure 1C). Three days after seeding, RPE cells cultured on plastic or AM reached confluence (Figure 1B,D). F-actin staining using FITC-phalloidin demonstrated the formation of actin stress fibers and altered cell shape to fusiform pattern in RPE cells cultured on plastic (Figure 1E). In contrast, the actin staining in RPE cells cultivated on AM revealed actin fibers arranged in a radial and circumferential pattern (Figure 1F). When RPE cells were seeded over AM, semithin sections stained with toluidine blue demonstrated that the cells were organized in a tight monolayer of round cells (Figure 2B). In contrast, RPE cells cultivated on plastic showed fusiform and elongated cells in multilayers (Figure 2A). Electron micrographs showed a tight monolayer of spheroidal RPE cells growing over AM (Figure 3A), different from elongated RPE cells in multilayers when cultured on plastic (Figure 3B). Ultrastructurally, RPE cells cultured on AM contained well developed cell organelles, including mitochondria, endoplasmic reticula, and Golgi apparatuses, and large quantities of free ribosomes. Electron micrographs revealed junctional specializations on the apical side between adjacent cells (Figure 3C) and showed a heterogenous expression of apical microvilli (Figure 3A).

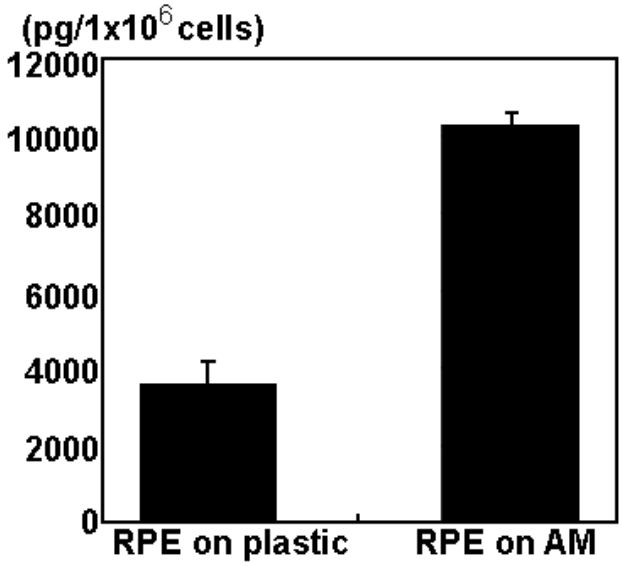

Figure 5. Increased secretion of VEGF in RPE cells cultured on amniotic membrane. Concentration of immunoreactive VEGF in supernatants of RPE cells cultured on either plastic or amniotic membrane. Cells were incubated in serum free medium for 48 h before harvest for protein concentration, and the amount of secreted VEGF in RPE conditioned culture medium was determined using commercial ELISA kits. The data show that VEGF secretion is increased in RPE cells cultured on amniotic membrane compared to cells on plastic ($p<0.01$).

Reverse transcription PCR: The discriminatory molecule RPE 65, an RPE specific molecule that is thought to have an important role in the RPE-photoreceptor vitamin A cycle, CRALBP, which is involved in the regeneration of visual pigment, and bestrophin, which is a late marker of RPE differentiation [21], were all detected by RT-PCR in RPE cells cultured on plastic and on AM (Figure 4). Expression levels of RPE 65, CRALBP, and bestrophin were significantly increased in RPE cells cultured on AM compared to cells cultured on plastic. According to the results of real-time PCR, the ratio of RPE 65/GAPDH, CRALBP/GAPDH, and bestrophin/GAPDH of cells cultured on AM compared to that of cells on plastic was 12.8, 5.0, and 13.5 times, respectively (Table 3). On the other hand, there was no apparent difference in mRNA expression of cathepsin D, a major photoreceptor outer segment digestive protease in the retina [22-24], between RPE cells cultured on either plastic or AM.

The expression of a panel of growth and trophic factors with the potential to affect both RPE and photoreceptor cell function and survival were also investigated by RT-PCR. Positive mRNA expression was observed for BDNF, bFGF, and PEDF (Figure 4). The expression of PEDF mRNA was markedly elevated in RPE cells cultured on AM compared to those cultured on plastic, while there was no apparent difference in the mRNA expression of bFGF and BDNF (Figure 4). According to the results of real-time PCR, the ratio of PEDF/GAPDH in cells on AM compared to that in cells on plastic was 8.2 times (Table 3). The expression and alternative splicing of FGF receptors is regulated by cellular differentiation in vitro [20]. mRNA expression of FGFR2 was detected both in RPE cells cultured on plastic those cultured on AM, but there was no apparent difference (Figure 4).

Tyrosinase and TRP-2 are the enzymes involved in melanin biosynthesis, and are expressed in melanocytes of neural crest origin and RPE cells [25,26]. There was no apparent difference in the expression of mRNA of tyrosinase, however, TRP-2 mRNA expression was markedly upregulated when cells were cultivated on AM (Figure 4). According to the results of real-time PCR, the ratio of TRP-2/GAPDH in cells on AM compared to that in cells on plastic was 12.3 times (Table 3).

Secretion of growth and trophic factors: ELISAs were performed on serial dilutions of RPE cell culture supernatants, conditioned for 2 days, from a minimum of three independent experiments. VEGF was positively detected in RPE cells cultured on plastic or AM. Secretion of VEGF in RPE cells cultured on AM was more than three fold higher than in RPE cells cultured on plastic ($p<0.01$, Figure 5). Also, the secretion of TSP-1 in RPE cells cultured on AM was approximately three fold higher than in RPE cells on plastic ($p<0.01$, Figure 6). On the other hand, BDNF and bFGF were not detected above the limit of the assays both in conditioned medium from RPE cells on plastic or on AM (data not shown).

Western blotting of PEDF: Western blot analysis using monoclonal antibody against purified PEDF protein (Transgenic, Inc., Kumamoto, Japan) was performed. PEDF antibody recognized a 50 kDa protein species in medium con-

ditioned by RPE cells cultured on AM (Figure 7). PEDF protein was not detected in the conditioned medium from RPE cells cultured on plastic or of AM only.

DISCUSSION

Although RPE cell transplantation remains a potentially useful approach for the treatment of AMD and other retinal diseases like retinitis pigmentosa, prolonged improvement of the vision of patients that received RPE cell suspension transplantations into the subretinal space has not been obtained. The main disadvantage of cell suspension transplantation is the random organization in multilayers of transplanted cells in the subretinal space of the host [3].

The human AM is a thin and elastic tissue that forms the inner layer of the amniotic sac. Human AM, with its thick basement membrane and avascular stromal matrix, has been successfully used for surface reconstruction in a variety of ocular surface disorders [27]. In the present study, we hypothesized that AM might be a feasible support substrate for transplanted RPE cells because AM can be obtained more easily than any other nonsynthetic carrier such as Descemet's membrane, and it has been used for cultured corneal epithelial cell transplantation with good clinical results [28,29]. AM was reported to induce non-goblet epithelial differentiation in cultured rabbit conjunctival epithelial cells [18]. Implantation of AM into the rabbit subretinal space is feasible and well tolerated by the rabbit retina [30].

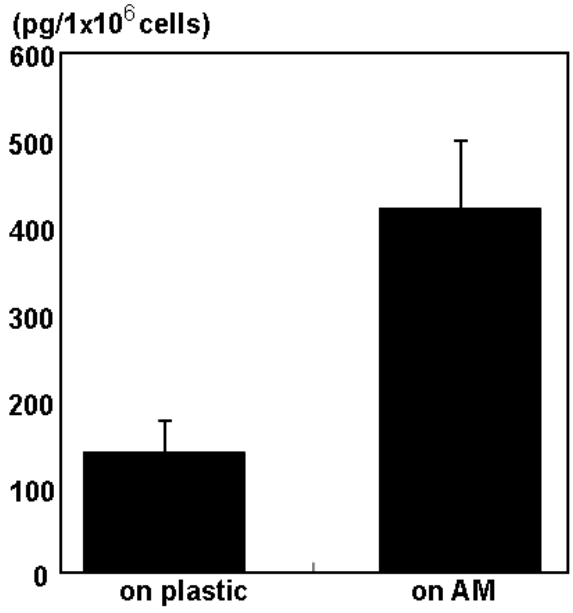

Figure 6. Increased secretion of thrombospondin-1 in RPE cells cultured on amniotic membrane. Concentration of immunoreactive thrombospondin-1 in supernatants of RPE cells cultured on either plastic or amniotic membrane. Cells were incubated in serum free medium for 48 h before harvest for protein concentration, and the amount of secreted thrombospondin-1 in RPE-conditioned culture medium was determined using commercial ELISA kits. The data show that thrombospondin-1 secretion is increased in RPE cells cultured on amniotic membrane compared to cells on plastic ($p<0.01$).

As a first step to evaluate the usefulness of AM as a support for transplanted RPE cells, we investigated the characteristics of RPE cells cultured on human AM. After seeding, human RPE cells spread easily and constituted tight colonies on AM. Using transmission electron microscopy, we identified a monolayer of RPE cells in which integration with the substrate and cell-cell contacts were detected, as reported by Capeans et al. [31]. We also observed that RPE cells cultured on AM exhibited ultrastructural epithelial features such as microvilli of the apical membrane and intercellular junctions, similar to RPE cells in situ.

We then investigated the expression pattern of several genes considered to participate in the function of differentiated RPE. Alge et al. [32] performed comparative proteome analysis of native differentiated and cultured dedifferentiated human RPE cells, and reported that RPE 65 and CRALBP were present only in native differentiated cells. Alizadeh et al. [33], using Northern blot analysis, also demonstrated that in ARPE-19 cells, a spontaneously differentiated RPE cell line, mRNA expression of CRALBP and RPE 65 was upregulated. Bestrophin is the protein altered in Best vitelliform macular dystrophy [34], an inherited retinal degenerative disease similar to AMD. Bestrophin is considered a very late marker of RPE differentiation during normal development [21], and bestrophin localizes to the basolateral plasma membrane of the RPE, which suggests that bestrophin contributes to the transepithelial potential of RPE [35]. In the present study, the mRNA expression of RPE 65, CRALBP, and bestrophin was clearly upregulated in RPE cells cultured on AM compared to cells cultured on plastic, which suggests that AM facilitates RPE expression of differentiated features [32,33]. On the other hand, we did not detect any difference in mRNA expression of cathepsin D between RPE cells cultured on plastic or on AM. Cathepsin D is a major digestive protease of photoreceptor outer segments in the retina [22]. Alge et al. [32] also reported that the expression pattern of proteins involved in phagocytosis and exocytosis, including cathepsin D, was not different between native RPE and dedifferentiated cultured RPE cells using comparative proteome analysis, which indicates that cathepsin D expression might not be influenced by the differentiation status of RPE cells.

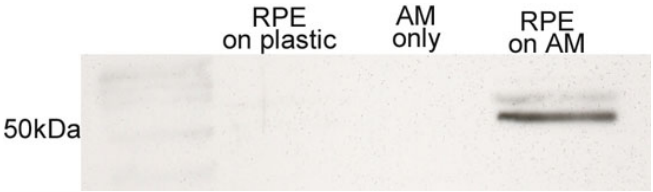

Figure 7. Representative western analysis of PEDF protein. The lanes from left to right are a commercially supplied size marker (unlabeled); proteins in medium conditioned for two days by RPE cells cultured on plastic (labeled "RPE on plastic"); proteins in medium conditioned for 2 days by amniotic membrane (AM) only, without seeding RPE cells on it (labeled "AM only"); and proteins in medium conditioned for two days by RPE cells on AM (labeled "RPE on AM").

The RPE is located between the neural retina and the vascular choroid and influences the structure and function of the cells in both, mediated mainly by secretion of various growth factors. In the present study, we focused on the expression of BDNF, bFGF, VEGF, PEDF, and TSP-1 from RPE cells among various factors secreted from RPE. Hackett et al. [36] showed that ARPE-19 has a high level of BDNF mRNA. Although mRNA expression of BDNF and bFGF was noted both in RPE cells cultured on plastic or AM in our study, we did not detect either protein in the RPE cell supernatant using ELISA. In addition, mRNA expression of FGFR-2, an FGF receptor that is upregulated by differentiation of ARPE-19 cells [20], was not different between cells cultured on plastic or on AM.

VEGF and PEDF expression was dramatically increased in RPE cells cultivated on AM. VEGF protein secretion was three times higher in the medium conditioned by RPE cells cultured on AM compared to that conditioned by RPE cells cultured on plastic. In addition, PEDF production was markedly increased when RPE cells were cultured on AM. The PEDF protein signal was completely absent in RPE cells cultured on plastic by western blotting; however, PEDF secretion dramatically increased in RPE cells cultured on AM. PEDF is a 50 kDa protein initially purified from the conditioned medium of human fetal RPE cells as a factor that induces neuronal differentiation of cultured Y79 retinoblastoma cells [37]. The gene is also called early population doubling level cDNA-1 [38]. PEDF is the most potent natural inhibitor of angiogenesis, and is a key factor associated with avascularity of the cornea, vitreous, and outer retinal layer of the eye [39]. Semokava et al. [40] also demonstrated that PEDF has a key role in retinal homeostasis by subretinal transplantation of genetically modified PEDF expressing iris pigment epithelial cells. We previously demonstrated a marked increase of VEGF and PEDF expression in differentiated RPE cells cultured on laminin, and suggested that a balance between high levels of both VEGF and PEDF might be important to maintain the homeostasis of the human retina [41]. Based on our previous studies [41,42], we speculated that in normal healthy conditions, the RPE has a positive survival effect on the maintenance of the highly vascularized, highly permeable fenestrated choriocapillaris on its outer basal aspect by secreting VEGF, while maintaining the complete avascularity of the photoreceptor layer internal to the RPE by secreting PEDF. Thus, expression of high levels of both VEGF and PEDF might be a property of the differentiated RPE.

TSP-1 protein secretion is also markedly increased in RPE cells cultured on AM. TSP-1 is a cell attachment factor with cell specific activity and the most prevalent TSP molecule found in vivo [43,44]. TSP-1 profoundly inhibits angiogenesis, both in vivo and in vitro [45-47]. Miyajima-Uchida et al. [47] reported that human RPE cells produce TSP-1, TSP-1 accumulates in the cytoplasm of RPE cells in vivo and in vitro, and suggested that TSP-1 is an important control factor in choroidal neovascularization. Considering these findings, increased production of TSP-1 by RPE cells cultured on AM might prevent the development of choroidal neovascularization and maintain the avascularity of the outer retina, together with PEDF.

Tyrosinase and TRP are enzymes involved in melanin synthesis. In particular, TRP-2 is an early differentiation marker for melanoblasts and RPE [48]. Although we did not detect any difference in mRNA expression of tyrosinase, TRP2 expression was markedly upregulated in RPE cells cultured on AM. Fang et al. [49] demonstrated that treatment of human cutaneous melanocytes with the differentiation inducer hexamethylene bisacetamide (HMBA) dramatically increased the expression of TRP-2 without changing the morphology, and suggested that the TRP-2 gene can be selectively modulated in melanocyte differentiation. TRP-2 might also have a role in the modulation of apoptotic pathways in melanoma cells [50]. Thus, accumulation of TRP-2 in RPE cells cultured on AM might protect transplanted cells from apoptosis.

Future studies are needed to determine which components of AM are responsible for controlling the phenotypic alterations of RPE. Growth factors contained in AM might have a role. Koizumi et al. [51] reported that AM preserved at -80 °C expressed the proteins of several growth factors (EGF, TGF-a, KGF, HGF, bFGF, TGF-b_1, TGF-b_2, TGF-b_3). AM without amniotic epithelium, however, had significantly lower levels of these growth factors, suggesting that the growth factors have an epithelial origin. In the present study, we removed the epithelium from AM before seeding the RPE. Neither BDNF nor bFGF protein, which induce differentiation in RPE cells [36,52,53], was detected in the supernatants of RPE cells cultured on AM. It is unlikely that these growth factors actively participate in phenotypic alteration of RPE cells cultured on AM.

Besides soluble factors, the extracellular matrix might have an important role in differentiation of epithelial cells. Immunohistochemical study revealed the presence of collagen (types 1, 3, 4, and 5) and fibronectin throughout the whole AM and the expression of collagen 7 and laminin-5 on the basement membrane side of AM [54]. A matrix component, laminin-5, has a crucial role in the proliferation, differentiation, and migration of corneal epithelial cells [55]. Laminin-5 might be one candidate for an important component in AM to induce phenotypic alteration in RPE cells.

In conclusion, this study demonstrated that AM induces phenotypic alterations in RPE cells. The epithelial phenotype is observed morphologically and upregulation of several growth factors important for maintaining retinal homeostasis occurs. This suggests that an RPE cell sheet cultivated on AM can be transplanted. By transplanting the RPE cell sheet maintaining the epithelial phenotype on AM, RPE cells on AM retain a monolayer and epithelial, differentiated phenotype in the subretinal space of the host for a long term. Simultaneously increased expression of a panel of genes necessary to maintain retinal homeostasis, as shown in the present study, might be needed for transplanted RPE cells. The use of AM as a basement membrane support might be expanded to other cell sources. Iris pigment epithelial cells, which have the same embryonic origin as RPE cells, are a possible candidate. More-

over, in regenerative medicine, there is a potential for embryonic stem cells [56] for use in clinical treatments. A combination of these cells with a basement membrane support like AM might be more advantageous for subretinal transplantation in the future.

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