

# Induced Expression of Insulin-like Growth Factor-1 by Amniotic Membrane-Conditioned Medium in Cultured Human Corneal Epithelial Cells

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**PURPOSE.** To determine the effect of amniotic membrane-conditioned medium (AMCM), via insulin-like growth factor (IGF)-1 induction, on human corneal epithelial cell (HCEC) proliferation.

**METHODS.** HCECs were cultured from corneal limbal tissue with supplemented hormonal epithelial medium (SHEM). After administration of AMCM, cell proliferation was evaluated with an MTT assay and DNA synthesis with methyl-[<sup>3</sup>H]-thymidine incorporation assay. RT-PCR and Western immunoblot analyses were performed, to determine potential inducible factors that may be associated with AMCM-induced cell proliferation. Neutralizing anti-IGF-1 antibody and small interfering (si)RNA were also used to clarify the role of IGF-1 in AMCM-induced HCEC proliferation.

**RESULTS.** HCEC proliferation increased after AMCM treatment. Of the cytokines known to be associated with HCEC proliferation, only IGF-1 expression was upregulated in response to AMCM in a dose- and time-dependent manner. The IGF-1 induction effect was found on both AMCM from live AM and from cryopreserved AM. HCEC proliferation was also increased by addition of exogenous IGF-1. AMCM-induced HCEC proliferation was inhibited in the presence of neutralizing anti-IGF-1 antibody and IGF-1 siRNA. Finally, Akt phosphorylation was increased in HCECs after AMCM treatment and was inhibited by IGF-1 siRNA.

**CONCLUSIONS.** IGF-1 is induced by AMCM during HCEC proliferation, and this induction may play an important role in the amniotic membrane during HCEC proliferation and migration in several intractable corneal epithelial defects. (*Invest Ophthalmol Vis Sci.* 2006;47:864–872) DOI:10.1167/iov.05-0596

The ocular surface serves as a key barrier that protects the internal structures of the eye from the external environment. The integrity of the ocular surface and the healing of corneal wounds depend on a delicate balance among cellular proliferation, differentiation, migration, and apoptosis.<sup>1</sup> By their regulation of these functions, a variety of growth factors

produced by the corneal epithelium, stroma, and endothelium play a vital role in the maintenance of normal corneal function.<sup>2,3</sup> As yet, the soluble factors and intracellular signaling pathways involved in this process are not fully understood. Several factors may have the potential to modulate cell migration and growth and may therefore be involved in wound healing, including insulin-like growth factor (IGF)-1, insulin, substance P (SP), epidermal growth factor (EGF), hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), and nerve growth factor (NGF).<sup>1,4–6</sup>

IGF-1 and its receptors have been identified in many tissues throughout the body, and they have been demonstrated to play an important role in cell proliferation and differentiation.<sup>7,8</sup> In rabbit and rat cornea, IGF-1 has been demonstrated to stimulate corneal epithelial migration in both organ culture and in vivo models.<sup>5,9</sup> However, few studies have examined the relationship between cell proliferation and IGF-1. Furthermore, the effects of IGF-1 on human corneal epithelial cell (HCEC) proliferation and migration have not yet been fully elucidated.

The amniotic membrane (AM) is the innermost layer of the fetal membranes and consists of a simple epithelium, a thick basement membrane, and an avascular stroma. When appropriately procured, processed, and preserved, AM has been successfully used as a substrate replacement for ocular surface reconstruction and is effective in the treatment of many severe and progressive epithelial defects, including neurotrophic corneal ulcers.<sup>10–14</sup> Recently, the underlying action mechanism explaining how AM transplantation works during ocular surface reconstruction has been reviewed.<sup>15</sup> Although one plausible mechanism explaining how AM may promote corneal epithelial growth is to release nerve growth factor,<sup>16</sup> few studies have demonstrated whether the AM stimulates cytokines, thereby facilitating wound healing.

In this study, we investigated the effects of AM on HCEC proliferation, by using AM-conditioned medium (AMCM), which contains substances and growth factors released from AM. We also assessed whether any cytokines play a role in AM-induced HCEC proliferation.

## MATERIALS AND METHODS

### Chemical Reagents and Cell Culture Media

Dulbecco's modified eagle medium (DMEM), F-12 nutrient mixture, fetal bovine serum (FBS), HEPES buffer, amphotericin B, and gentamicin were purchased from Invitrogen-Gibco (Grand Island, NY). Other reagents and chemicals, including mouse-derived EGF, cholera-toxin (subunit A), dimethyl sulfoxide (DMSO), hydrocortisone, transferrin, and human insulin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Dispase II was obtained from Roche Applied Science (Mannheim, Germany). Plastic cell culture dishes (35 and 60 mm), polyester membrane inserts (six-well, pore size 0.4  $\mu$ m; Transwell), a 96-well plate, and 15- and 50-mL sterile centrifuge conical tubes were purchased from Corning-Costar (Acton, MA). Affinity-purified goat polyclonal antibodies against human IGF-1, keratinocyte growth factor

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(KGF), EGF, and platelet-derived growth factor (PDGF) were purchased from R&D Systems, Inc. (Minneapolis, MN). The horseradish peroxidase (HRP)-conjugated secondary antibody was obtained from Dako (Glostrup, Denmark).

### Preparation of Human AM and AMCM

Human tissues were handled according to the tenets of the Declaration of Helsinki, with the approval of institutional review boards (IRBs). Human placenta was obtained from an elective Cesarean section performed on a seronegative (human immunodeficiency virus, human hepatitis type B and C, and syphilis) woman. Under a laminar flow hood, the placenta was cleansed of blood clots with sterile phosphate-buffered saline (PBS) solution containing 50  $\mu\text{g}/\text{mL}$  penicillin, 50  $\mu\text{g}/\text{mL}$  streptomycin, and 2.5  $\mu\text{g}/\text{mL}$  amphotericin B. After the membrane was cleansed three times with PBS, it was cut into several 100-mm<sup>2</sup> pieces. For cryopreservation, some membrane pieces were then attached to a nitrocellulose membrane (Immobilon-NC; Millipore, Billerica, MA), by placing the epithelial side up. The membrane was stored at  $-20^{\circ}\text{C}$  in DMEM containing glycerin (Invitrogen-Life Technology Inc., Gaithersburg, MD) in a ratio of 1:1 (vol/vol) for 2 weeks. Other fresh AM pieces were incubated in 10 mL DMEM without FBS in a 100-mm culture dish for 24 hours at  $37^{\circ}\text{C}$  in 95% humidity and 5%  $\text{CO}_2$ . The medium was then collected and preserved at  $-70^{\circ}\text{C}$  before use. AMCM was obtained from cryopreserved AM by thawing and washing the cryopreserved amniotic membrane, which was stored for 2 weeks at  $-20^{\circ}\text{C}$  three times with sterile PBS. The membrane was then cultured in the same medium, temperature, and humidity to obtain the AMCM from fresh AM for 24 hours. The collected medium was also preserved at  $-70^{\circ}\text{C}$ .

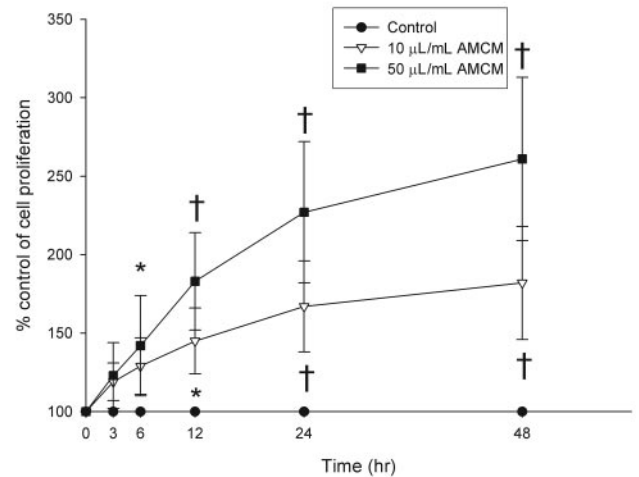
### Human Limbal Explant Culture

Human corneal limbal tissue was harvested from the donor corneal button after keratoplasty. The corneal limbal tissue was washed three times with DMEM containing 50  $\mu\text{g}/\text{mL}$  gentamicin and 1.25  $\mu\text{g}/\text{mL}$  amphotericin B. After removal of excessive conjunctiva, sclera, and iris tissue, the remaining tissue was placed in a culture dish and exposed for 1 hour to 1.2 U/mL dispase II in  $\text{Mg}^{2+}$ - and  $\text{Ca}^{2+}$ -free solution at  $37^{\circ}\text{C}$  in 95% humidity and 5%  $\text{CO}_2$ . The epithelial tissue was separated from the rest of the tissue and cut into 2.0-mm<sup>2</sup> tissue sections with a no. 15 blade and scissors. The segmented epithelial tissue was placed on the upper chamber of a cell-migration culture dish (Transwell; Corning-Costar), which has a lower chamber containing 3T3 fibroblasts, and was cultured in supplemental hormonal epithelial medium (SHEM) made of an equal volume of HEPES-buffered DMEM containing bicarbonate and Ham's F12 supplemented with 10% FBS, 0.5% DMSO, 50  $\mu\text{g}/\text{mL}$  gentamicin, 1.25  $\mu\text{g}/\text{mL}$  amphotericin B, 2 ng/mL mouse EGF, 5  $\mu\text{g}/\text{mL}$  insulin, 5  $\mu\text{g}/\text{mL}$  transferrin, 0.5 mg/mL hydrocortisone, and 30 ng/mL cholera toxin. Human corneal limbal epithelial cells were maintained at  $37^{\circ}\text{C}$  under 95% humidity and 5%  $\text{CO}_2$ . The medium was changed every other day, and cell outgrowth was monitored daily for 3 weeks with inverted phase microscopy. When the cultured corneal epithelium was 80% to 90% confluent, the cells were subcultured with 0.25% trypsin and 5.0 mM EDTA with a 1:3 split. In all experiments using cultured human corneal epithelium, we used the second- and third-passage cells.

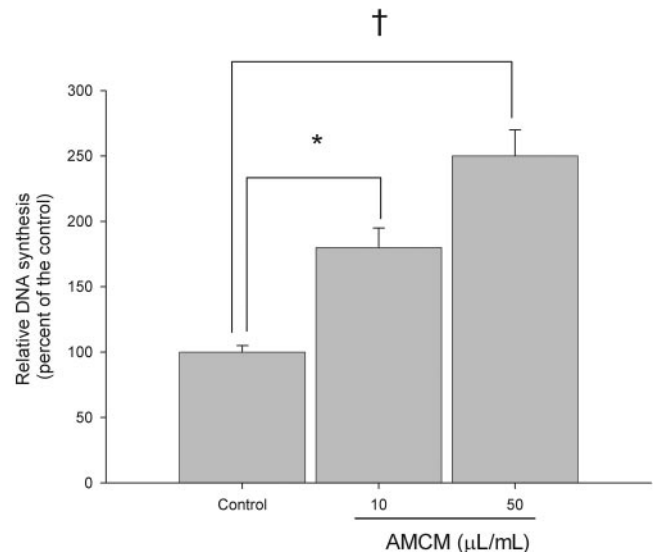
### MTT HCEC Proliferation Assay

Confluent cultures of HCECs were trypsinized and plated into 96-well plates at a density of  $2 \times 10^5$  cells/mL and allowed to attach for 48 hours at  $37^{\circ}\text{C}$ . Then, a cell-proliferation assay was performed. After the medium was removed, and cells were washed twice in PBS, HCECs were serum-fasted for 12 hours. The cells were then incubated with AMCM (10, 50  $\mu\text{L}/\text{mL}$ ) or IGF-1 (10 or 50 ng/mL) in serum-free DMEM for 12, 24, and 48 hours. The cell proliferation assay was performed by using the MTT colorimetric assay system, which measures the reduction of the tetrazolin component (i.e., MTT) into an insoluble formazan product by the mitochondria of viable cells. One hour before cell

### A

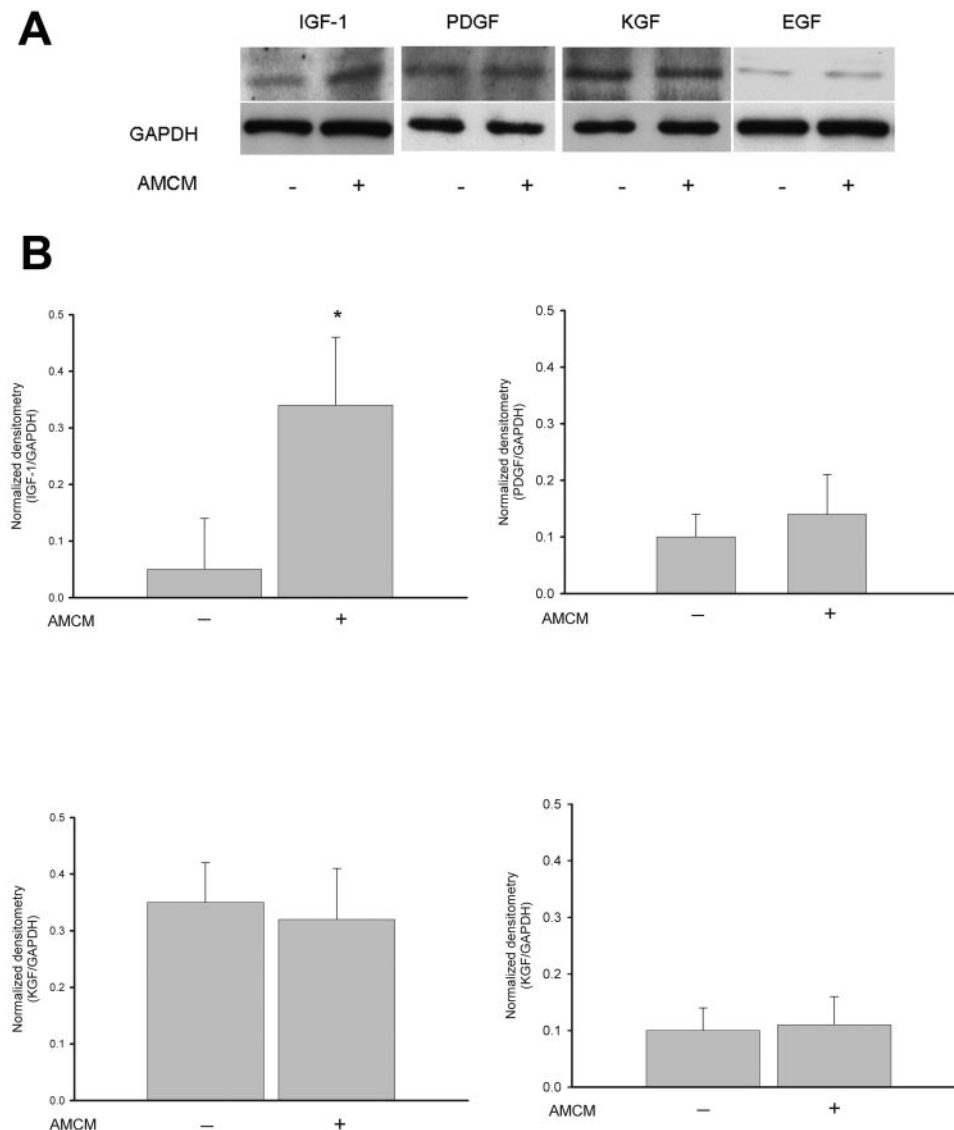


### B



**FIGURE 1.** The effect of AMCM on HCEC proliferation. (A) HCECs were cultured with serum- and growth factor-free medium for 12 hours and then treated with AMCM (10 or 50  $\mu\text{L}/\text{mL}$ ) or left untreated. Cell proliferation was measured with the MTT assay for 48 hours. (B) AMCM induced DNA synthesis. For the methyl- $^3\text{H}$ -thymidine incorporated assays, the HCECs were incubated with SHEM for 24 hours, with or without AMCM (10 or 50  $\mu\text{L}/\text{mL}$ ). Before stimulation with AMCM or no stimulation, cells were starved of serum and growth factors for 12 hours. After 22 hours with or without AMCM, the cells were labeled with 5  $\mu\text{Ci}$  methyl- $^3\text{H}$ -thymidine for 2 hours ( $^*P < 0.05$ ,  $^\dagger P < 0.01$ ).

harvest, the culture medium was replaced with serum-free medium, and 100  $\mu\text{L}$  MTT solution (5 mg/mL in PBS) was added to each well. After a 1-hour incubation, the medium was replaced by 0.4 mL acidic isopropanol (0.04 M HCl in absolute isopropanol) to solubilize the colored crystals. The samples were read using an ELISA plate reader (Labsystems Multiskan MCC/340; Fisher Scientific, Pittsburgh, PA) at a wavelength of 570 nm with background subtraction at 650 nm. The



**FIGURE 2.** Western immunoblot analysis of IGF-1, PDGF, KGF, and EGF expression in HCECs after 10  $\mu$ L/mL AMCM treatment. Twelve hours after treatment with AMCM or no treatment, expression of these growth factors in HCECs was evaluated with Western immunoblot analysis on cell lysates. IGF-1 expression was only significantly increased after AMCM treatment (first panel and densitometry; \* $P < 0.05$ , compared with control). However, expression of PDGF, KGF, and EGF did not significantly differ between control and AMCM-treated cells.

amount of color produced that normalized with the background is directly proportional to the number of viable cells and is represented as the proliferation index. For proliferation inhibition assay, 1 hour before treatment of AMCM, 10 ng/mL of neutralizing anti-IGF-1 antibody was inoculated. Then, the proliferation assay was as just described. All the assays were repeated three times with triplicate samples.

### Determination of DNA Synthesis with Methyl- $^3$ H-Thymidine

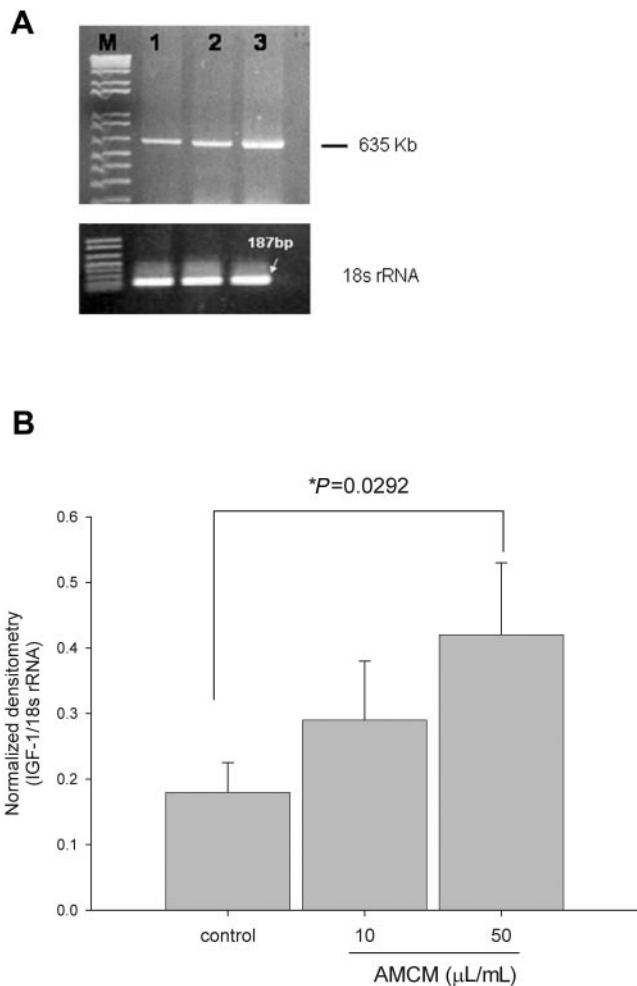
Cells were incubated in serum- and growth factor-free DMEM, with or without AMCM (10 or 50  $\mu$ L/mL) and IGF-1 (10 or 50 ng/mL), up to 24 hours. One hour before harvesting of the cells, the medium was then replaced with serum-free DMEM with 5  $\mu$ Ci methyl- $^3$ H-thymidine (90 Ci/mmol; GE Healthcare, Buckinghamshire, UK). Labeling was terminated by washing the cells with ice-cold PBS three times, followed by the addition of 0.5 mL of dissolution buffer (25 mM HEPES [pH 7.5] and 0.1% Triton X-100) and 5  $\mu$ L of 2% sodium deoxycholate. After cells were lysed, the lysate was collected into a 1.5-mL microfuge tube, precipitated with trichloroacetic acid to a final concentration of 10%, and centrifuged at 14,000 rpm for 10 minutes at 4°C. The pellet was then washed with 400  $\mu$ L of 10% trichloroacetic acid, dissolved in 200  $\mu$ L of 0.2 M NaOH and counted with a  $\beta$  counter (Beckman Instruments, Montreal, Quebec, Canada).

### Determination of IGF-1, EGF, PDGF, and KGF Induction and Akt Phosphorylation in HCECs

The cultured HCECs were 80% to 90% confluent in all six-well migration chambers (Transwell; Corning-Costar). The lower chamber containing 3T3 fibroblasts was discarded, and the HCECs were washed with PBS. The lower chamber was then exchanged with a new one without a fibroblast feeder layer and was incubated for various times in serum- and growth factor-free DMEM, with or without AMCM (10 or 50  $\mu$ L/mL) from live AM or cryopreserved AM. Cells were then washed with ice-cold PBS and lysed with cell lysis buffer (20 mM HEPES [pH 7.2], 10% glycerol, 10 mM  $\text{Na}_3\text{VO}_4$ , 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.1 mM dithiothreitol, 1  $\mu$ g/mL leupeptin, 1  $\mu$ g/mL pepstatin, and 1% Triton X-100) on ice for 30 minutes. The lysate was subjected to sonication, and the cell homogenates were then centrifuged at 15,000g for 10 minutes. Protein concentration of the resultant supernatant was assessed with a Bradford reagent.

### Western Immunoblot Analysis

Twenty micrograms of protein were electrophoresed. After cell sample preparation (as described earlier), Laemmli sample buffer was added, and the samples were boiled for 5 minutes. Proteins were separated by SDS-PAGE (8% gels) and transferred to a polyvinylidene fluoride (PVDF; Millipore) transfer membrane. Antibodies against IGF-1 (1:1000), EGF



**FIGURE 3.** (A) Expression of IGF-1 mRNA in HCECs, as determined by RT-PCR after treatment with AMCM or no treatment (*lane M*: marker; *lane 1*: serum-free medium; *lane 2*: 10  $\mu\text{L/mL}$  AMCM; *lane 3*: 50  $\mu\text{L/mL}$  AMCM). (B) Densitometry showed that expression of IGF-1 mRNA (635 kb) was increased after treatment with 50  $\mu\text{L/mL}$  AMCM compared with the control.

(1:500), PDGF (1:1000), and KGF (1:500) were used at various dilutions. Phosphorylation of Akt, a major cellular substrate of phosphoinositide 3-kinase (PI3-K), was determined with a phospho-Akt-specific antibody. Secondary antibodies were conjugated to HRP. Immunoreactive bands were visualized using the enhanced chemiluminescence method (ECL kit; GE Healthcare).

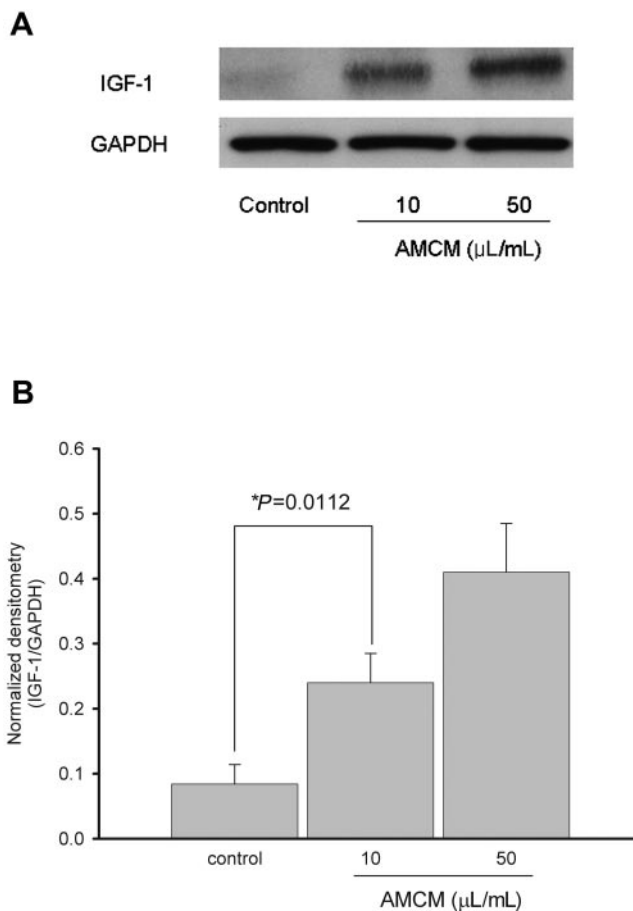
#### RT-PCR for IGF-1 Expression in HCECs after AMCM Treatment

Twelve hours after the addition of either 10 or 50  $\mu\text{L/mL}$  AMCM to cultured HCECs in serum- and growth factor-free medium, total RNA was isolated (SV Total RNA Isolation System; Promega, Madison, WI), according to the manufacturer's instructions. The concentration and purity of RNA was determined by spectrophotometric measurement (Gene Quant II; Pharmacia Biotech, Cambridge, UK). Total RNA was converted into cDNA by a first-strand synthesis system (Superscript; Invitrogen-Gibco) under the following conditions: 0.4  $\mu\text{M}$  each primer, 0.2 mM deoxynucleoside triphosphate mixture (Applied Biosystems, Inc. [ABI], Foster City, CA), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM  $\text{MgCl}_2$ , and 1 unit *Taq* polymerase (ABI). The reaction mixtures were incubated in a thermal controller (Model TPC-100; MJ Research, Watertown, MA) for 35 cycles of denaturation for 45 seconds at 95°C, annealing for 1 minute at 58°C, and extension for 45 seconds at 72°C.

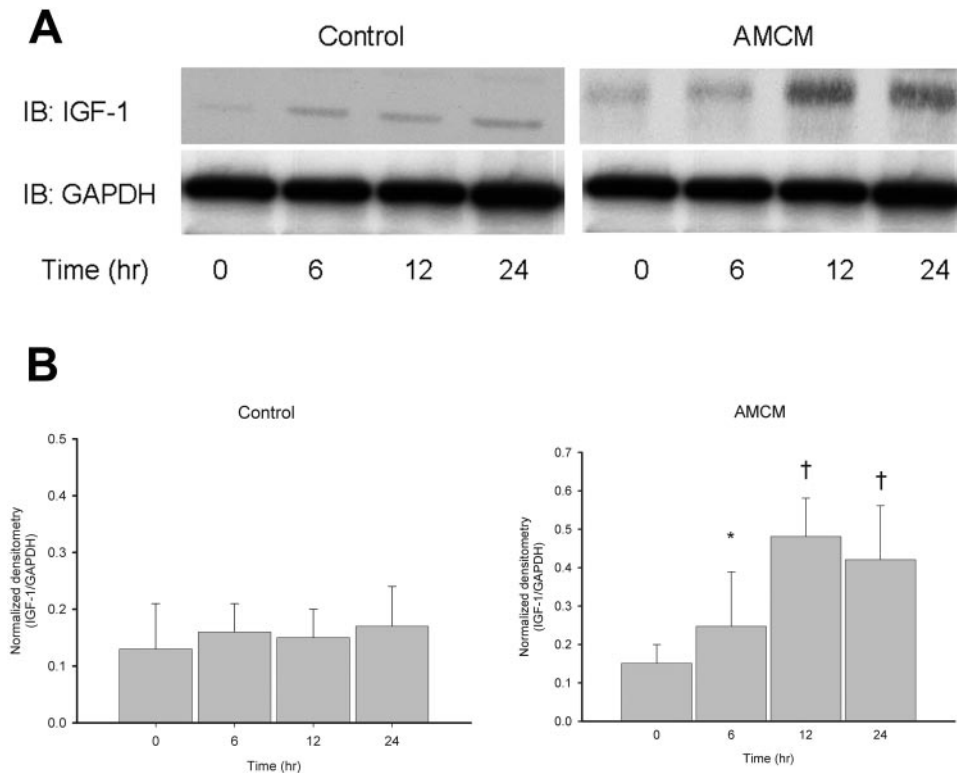
The quantity of the amplified products was analyzed with an image-documentation system (ImageMaster VDS; Pharmacia Biotech Inc., Uppsala, Sweden). The primer sequences specific to IGF-1 were 5'-GAG CCT GCG CAA TGG AAT AAA GTC-3' (forward) and 5'-CGG TGG CAT GTC ACT CTT CAC TC-3' (reverse). This primer sequence was already used to detect IGF-1 in a previous study.<sup>17</sup> The band area of IGF-1 was normalized with 18s RNA from each sample, and the IGF-1 mRNA level was then estimated.

#### IGF-1 Small Interfering RNA

IGF-1 small interfering (siRNA; Ambion, Austin, TX) transfection was performed according to the manufacturer's instructions. Briefly, cells were incubated until they reached 60% confluence, the transfection reagent (siPORT Amine; Ambion) was diluted into serum-free DMEM medium and incubated for 20 minutes at room temperature. The diluted transfection reagent was combined and mixed with the IGF-1 siRNA. The mixture was incubated for 20 minutes at room temperature to allow transfection complexes to form. The newly formed transfection complexes were then dispensed onto the cells for a final RNA concentration of 100 nM. Without swirling, the plate was gently rocked back and forth to distribute the complexes evenly. After a 16-hour incubation, the transfected cells were further incubated in serum-free DMEM for 6 hours. AMCM (200  $\mu\text{L/mL}$ ) was added to the transfected cells. For Western blot analysis, cells were harvested 1 hour after AMCM treatment. For the proliferation assay, cell growth was



**FIGURE 4.** Expression of IGF-1 in HCECs after AMCM treatment changed in a dose-dependent manner. Twenty-four hours after treatment with 10 or 50  $\mu\text{L/mL}$  AMCM or no treatment, the cell lysate was subjected to 8% SDS-PAGE (A) under reduced conditions and transferred to a PVDF membrane, followed by immunoblot analysis with IGF-1 antibody. IGF-1 expression increased in a dose-dependent manner (B).



**FIGURE 5.** Change of IGF-1 expression in HCECs after treatment with 50  $\mu$ L/mL AMCM or no treatment in a time-dependent manner. Twelve hours after serum and growth factor starvation, the medium was changed to serum-free medium with or without 50  $\mu$ L/mL of AMCM. The IGF-1 expression was evaluated with Western immunoblot analysis for 0, 6, 12, and 24 hours (A) and the expression was compared with densitometry (B). The protein preparation, electrophoresis, and PVDF membrane transfer conditions were the same as for the dose-dependent condition (Fig. 4). IGF-1 expression in AMCM-treated cells increased beginning at 6 hours after treatment and peaked at 12 hours. (\* $P < 0.05$ , † $P < 0.01$ , compared with the baseline).

measured 24 hours after AMCM treatment by the MTT proliferation assay (Roche Molecular Biochemicals).

### Statistical Analysis

Statistical analysis was performed on computer (SPSS ver. 11.0; SPSS Inc., Chicago, IL). Cell proliferation, DNA contents, and densitometry results were analyzed with the *t*-test or the Mann-Whitney test. Correlations between parameters were analyzed with the Pearson test. All tests were made assuming a level of statistical significance of  $P < 0.05$ .

## RESULTS

### Effects of AMCM on HCEC Proliferation and Migration

HCEC proliferation was determined by MTT assay and presented as a percent of the number of control cells at each AMCM concentration (Fig. 1A). HCEC proliferation was increased by AMCM in a dose- and time-dependent manner. No statistically significant difference in cell proliferation was observed between the control and AMCM-treated groups after 3 hours. However, at 6 hours, proliferation of HCECs treated with 50  $\mu$ L/mL AMCM was significantly increased compared with control cells but not with HCECs treated with 10  $\mu$ L/mL AMCM. At 12 hours, proliferation was also significantly increased in HCECs treated with 10  $\mu$ L/mL AMCM compared with control cells. Proliferation of HCECs grown in AMCM remained increased compared with the control at the 48-hour time-point. At 48 hours, the population of HCECs grown in the presence of 50  $\mu$ L/mL AMCM was twice as dense as the control population.

The total DNA content was assayed using methyl- $^3$ H-thymidine incorporation into DNA. Twenty-four hours after treatment of AMCM, total DNA content was increased by 2.4 times compared with serum- and growth factor-free control (Fig. 1B). It is possible that a potential artifact caused by radiation damage hampers the results when cell proliferation is determined

with  $^3$ H-thymidine.<sup>18</sup> Therefore, we evaluated the correlation of both methods—MTT assay and methyl- $^3$ H-thymidine incorporation DNA synthesis—and the correlation coefficient ( $r$ ) was 0.89 ( $P < 0.001$ , Pearson correlation coefficient).

### Identification of Possible Factors Responsible for the Proliferative Effects of AMCM in HCECs

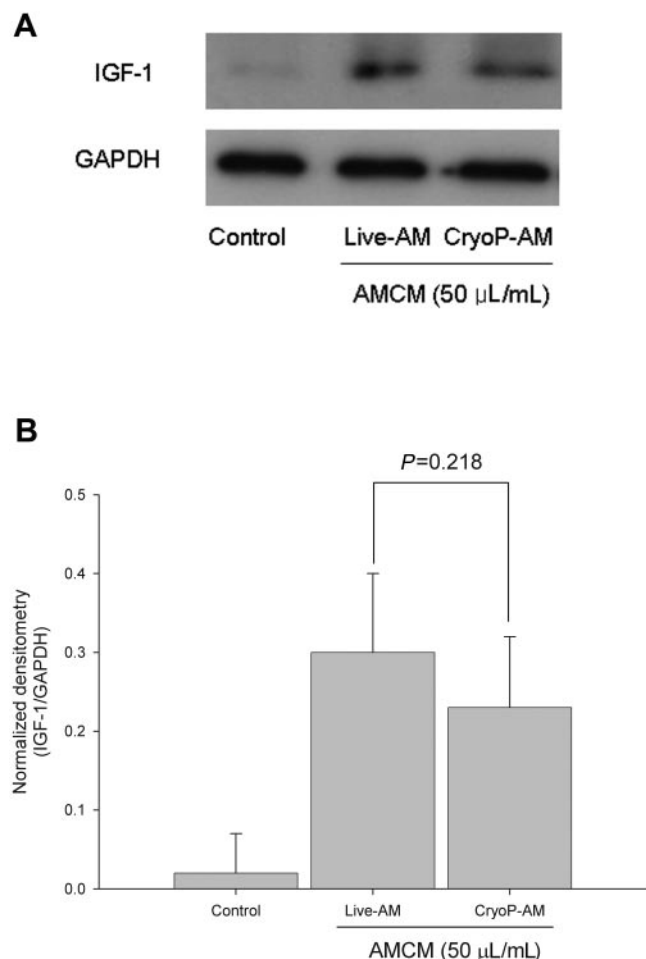
To determine whether cytokine expression in HCECs is induced by AMCM, we performed Western immunoblot analysis to detect the presence of IGF-1, PDGF, EGF, and KGF in HCEC lysates after treatment with 10  $\mu$ L/mL AMCM. Only IGF-1 expression was significantly increased compared with control cells (Fig. 2). The expression of KGF, EGF, and PDGF did not differ between the serum-free control and AMCM-treated cells.

### RT-PCR for Detection of IGF-1 mRNA in HCECs after AMCM Treatment

To determine whether the increased production of IGF-1 in AMCM-treated HCECs is regulated at the mRNA level, RT-PCR was performed. Using an IGF-1-specific primer, we found that IGF-1 mRNA expression was upregulated in AMCM-treated cells (Fig. 3, lanes 2, 3) compared with control cells (Fig. 3, lane 1). AMCM appeared to induce HCEC IGF-1 mRNA expression in a dose-dependent manner. These results suggest that AMCM-induced IGF-1 production in HCECs is, at least in part, regulated at the mRNA level.

### Western Immunoblot Analysis to Detect IGF-1 in HCEC Lysates after AMCM Treatment

To assess further the AMCM-induced expression of IGF-1 in HCECs, we next investigated IGF-1 protein expression at various AMCM concentrations and at various time points. IGF-1 expression was increased in a dose- and time-dependent manner. At 24 hours after AMCM treatment, IGF-1 expression



**FIGURE 6.** Comparison of IGF-1 expression in HCECs between AMCMs from cryopreserved and live AM. Twelve hours after serum and growth factor starvation, the medium was exchanged with the serum-free DMEM, 50  $\mu$ L/mL AMCM from live-AM, or 50  $\mu$ L/mL AMCM from cryopreserved AM (CryoP AM), followed by incubation for 12 hours. The cell lysate was processed by 8% SDS-PAGE and transferred to PVDF membrane, followed by immunoblot analysis with IGF-1 antibody.

increased in a dose-dependent manner until the AMCM concentration reached 50  $\mu$ L/mL (Fig. 4).

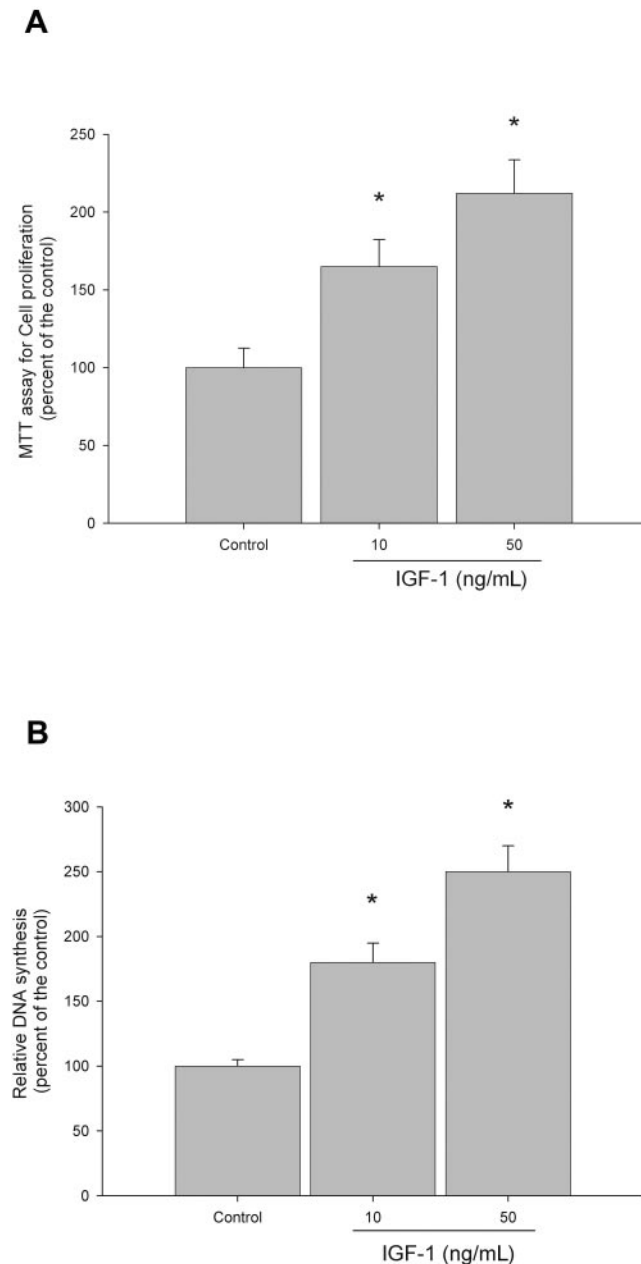
To investigate when the IGF-1 was induced by AMCM in HCECs, we determined the expression of IGF-1 in a time sequence. In the serum- and growth factor-free control, the IGF-1 expression was not significantly different from the baseline after 24 hours (Fig. 5A). In the AMCM-treated cells (50  $\mu$ L/mL), the expression of IGF-1 was not different from the baseline until 2 hours. At 6 hours, IGF-1 expression started to increase in AMCM-treated HCECs and was higher than baseline. This increased expression of IGF-1 was observed until 24 hours after AMCM treatment (Figs. 5A, 5B).

To determine whether the cryopreserved AM could also induce the IGF-1 from HCECs, we compared the IGF-1 induction effect of AMCM between live and cryopreserved AM. Twelve hours after treatment with AMCM from either live or cryopreserved AM, IGF-1 was induced in the HCECs in both conditions (Fig. 6). Densitometry for IGF-1 did not show a significant difference.

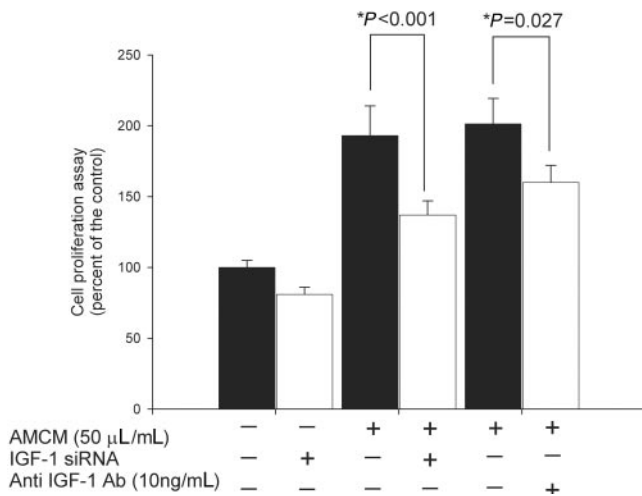
### IGF-1-Induced HCEC Proliferation

We next tested whether introduction of exogenous IGF-1 could enhance the cell proliferation and DNA content in

HCECs. At 24 hours, HCEC proliferation increased in response to IGF-1 in a dose-dependent manner. Treatment with 50 ng/mL increased cell proliferation twofold compared with the control (Fig. 7A). The DNA synthesis was also increased by treatment of IGF-1 (Fig. 7B). The cellular proliferative effect of IGF-1 in HCECs was well correlated with the increment of DNA synthesis ( $r = 0.92$ ,  $P < 0.0001$ ; Pearson correlation coefficient).



**FIGURE 7.** The effect of IGF-1 on HCEC proliferation and DNA synthesis. Before IGF-1 treatment, cells were serum starved for 12 hours. At 24 hours after treatment with 10 or 50 ng/mL IGF-1, cell proliferation was significantly increased in cells treated with 10 ng/mL IGF-1 and was two times higher than in the serum-free control cells treated with 50 ng/mL IGF-1 ( $*P < 0.05$ , compared with the control; A). The DNA synthesis was also increased by IGF-1 treatment in a dose-dependent manner (B). The data are representative of results in three experiments, each performed in triplicate.



**FIGURE 8.** Inhibitory effect of neutralizing anti-IGF-1 antibody and IGF-1 siRNA on AMCM-induced HCEC proliferation. One hour before treatment with 50  $\mu$ L/mL AMCM, 10 ng/mL neutralizing anti-IGF-1 antibody was added. For IGF-1 siRNA, the cells were incubated with 100 nM IGF-1 siRNA for 16 hours before AMCM treatment. Cell proliferation was evaluated with the MTT assay at 24 hours after AMCM treatment. AMCM-induced HCEC proliferation was significantly inhibited by 10 ng/mL anti IGF-1 antibody and IGF-1 siRNA.

### HCEC Proliferation Inhibition Assay Using Neutralizing Anti-IGF-1 Antibody and IGF-1 siRNA

To determine further the role of IGF-1 induction in AMCM-induced HCEC proliferation, we used neutralizing anti IGF-1 antibody and also designed IGF-1 siRNA to interfere with IGF-1 mRNA. Before AMCM treatment, HCECs were treated with the neutralizing antibody for 1 hour. For the IGF-1 siRNA assay, the cells were transfected with IGF-1 siRNA and then treated with AMCM for 24 hours. Consistent with the results described earlier, cell proliferation increased in HCECs treated with AMCM in the absence of either neutralizing anti IGF-1 antibody or IGF-1 siRNA. However, AMCM-induced HCEC proliferation was inhibited with neutralizing anti-IGF-1 antibody or in cells transfected with IGF-1 siRNA (Fig. 8). This result suggests that suppression of either IGF-1 by neutralizing antibody or gene expression by IGF-1 siRNA effectively inhibited AMCM-induced HCEC proliferation.

### Akt Phosphorylation after Treatment with AMCM and IGF-1 siRNA

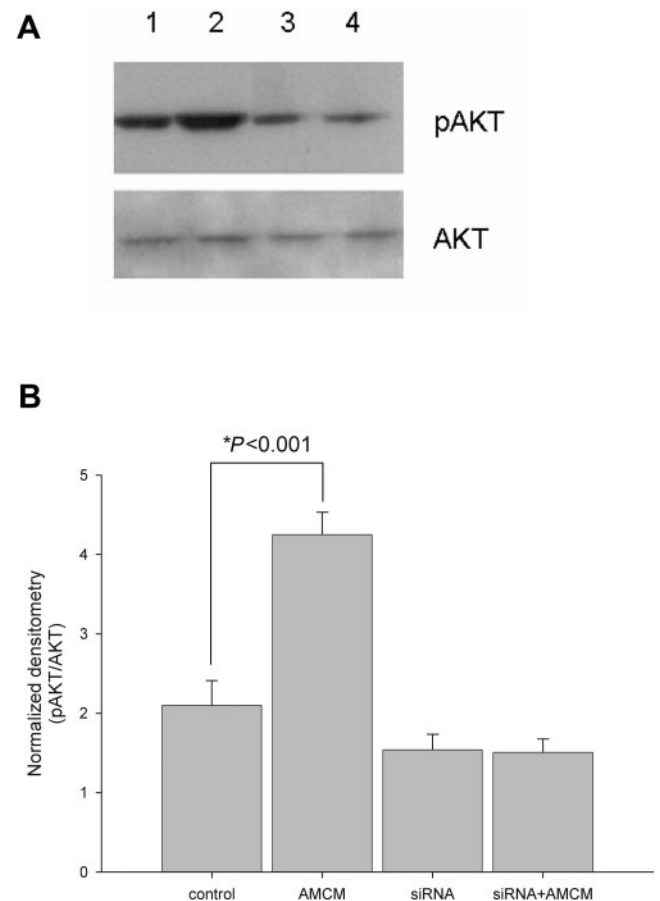
Because Akt is a known downstream effector molecule of the IGF-1 signaling pathway, we next investigated the effects of AMCM and IGF-1 siRNA on Akt phosphorylation in HCECs. Akt phosphorylation was increased in HCECs after AMCM treatment (Fig. 9). This phosphorylation peaked at 12 hours and then decreased in intensity over time. The total amount of Akt, however, did not change over time.

The inhibitory effects of IGF-1 siRNA on Akt phosphorylation was confirmed by Western immunoblot after IGF-1 siRNA treatment. Akt phosphorylation decreased in HCECs transfected with siRNA-1, but not in control cells (Fig. 9). Specifically, transfection with siRNA-1 resulted in a  $\sim$ 70% reduction in Akt phosphorylation compared with the control. Based on these data, we believe that AMCM-induced HCEC proliferation is mediated by both IGF-1 production and Akt phosphorylation.

## DISCUSSION

In our study in HCECs, (1) addition of AMCM to serum-free medium significantly increased cell proliferation; (2) AMCM induced production of IGF-1; (3) neutralizing IGF-1 with anti IGF-1 antibody or IGF-1 siRNA inhibited the increase in cell proliferation; and (4) AMCM-induced IGF-1 expression stimulated phosphorylation of Akt. Taken together, these results suggest that AM-facilitated *in vitro* HCEC proliferation may at least in part be due to IGF-1 induction via unknown factors present in the AM.

Human AM is now widely used to reconstruct the ocular surface for the treatment of several conditions, including intractable epithelial defects, chemical burns, partial limbal cell deficiencies, ocular cicatricial pemphigoid, and Stevens-Johnson syndrome.<sup>19-22</sup> In addition, AM is used to enhance *in vitro* corneal epithelial cell culture systems.<sup>23</sup> However, precisely how AM promotes epithelialization, both *in vivo* and *in vitro*, has not yet been elucidated. Some hypotheses propose that AM facilitates epithelialization in the basement-membrane-like action and mechanical protection from lid movement and that it serves as a dressing material.<sup>19,24,25</sup> In addition to these functions, fresh or frozen AM has been shown to express many growth factors, including EGF, HGF, and KGF, primarily in the epithelial layer.<sup>15,26</sup> Moreover, Touhami et al.<sup>16</sup> reported that NGF is an important factor in AM-facilitated corneal epithelial



**FIGURE 9.** Changes in Akt phosphorylation in HCECs after AMCM and/or IGF-1 siRNA treatment (lane 1: control, serum-free medium; lane 2: 10  $\mu$ L/mL AMCM; lane 3: 100 nM IGF-1 siRNA only; lane 4: 100 nM siRNA + 10  $\mu$ L/mL AMCM). One hour after AMCM treatment, Akt phosphorylation was increased. However, this enhanced pAkt expression decreased after IGF-1 siRNA treatment.

cell migration. Their data helped explain why AM transplantation is effective in the healing of neurotrophic ulcer. However, few studies have investigated the role of cytokines, which may be released from the AM and may thereby affect cell proliferation and migration. In the present study, we used AMCM, which is a medium that contains the releasable factors from AMs. It may be possible to use AMCM to evaluate the effects of AM cytokines on cell proliferation and migration and to exclude physical effects of AM that may occur when it is applied to the in vivo cornea.

To clarify the existence of putative factors in AMCM that can induce IGF-1, we compared the effect of the AMCM from either live or cryopreserved AM. AMCM from either live AM or cryopreserved AM induced IGF-1. Also, densitometry for IGF-1 did not show a significant difference. Considering that no viable cells were found after the cryopreservation of AM,<sup>27</sup> the IGF-1-inducible factors from AMCM may already exist in AM itself, and may not be released by live cells in AM. However, the AMCM from the AM that had been cryopreserved for more than 3 months did not induce any IGF-1 (data not shown). Taken together, the IGF-1-inducible factors may exist in AM matrix and can be degraded when the cryopreservation of AM is prolonged.

We can speculate that Akt is involved in IGF-1 induction by AMCM. Activation of the IGF-1 receptor (IGF-1R) by IGF-1 is known to result in phosphorylation of PI3-K and Akt, which are known as cell proliferation and antiapoptotic effector molecules.<sup>28,29</sup> Accordingly, in the current study, AMCM-induced Akt activation was inhibited by IGF-1 siRNA. These results suggest that IGF-1-induced HCEC proliferation may be mediated by Akt phosphorylation. Also, because NGF signaling, which as reported by Touhami et al.,<sup>16</sup> is known to trigger the downstream of the Akt pathway, it remains unknown whether the NGF pathway is responsible for upregulating IGF-1 gene expression.

We have not been able to elucidate fully how AMCM application induces cell proliferation. Despite the fact that growth hormone (GH) is known to induce IGF-1 and that the GH-IGF axis is essential for cell proliferation, IGF-1 induction mechanisms have not yet been identified in most cell types.<sup>30,31</sup> Because of the central role of IGF-1 in cell migration and proliferation in the wound-healing process, further studies designed to elucidate how IGF-1 is induced in this process should be performed. Also, we used HCECs expanded on a cell-migration (Transwell; Corning-Costar) culture system with 3T3 fibroblasts as the feeder layer. The serum-free conditioned medium collected from 3T3 fibroblasts is well known to contain IGF-1.<sup>32</sup> Although we used only the fibroblast feeder during the cell-expansion period, to increase cell mass for preparing the experiments, and did not use the fibroblast feeder during the proliferation and immunoblot assays, it is possible that the IGF-1 from the fibroblast feeder already existed in the experimental condition. However, the Western blot analysis in the control group of our study showed a small amount IGF-1 expression in this situation. Therefore, we thought the effect of IGF-1 produced by 3T3 fibroblasts might be minimal, if any, in our experimental condition, even though such preconditioning of human corneal epithelial cells may have set the stage for them to be more dependent on IGF-1.

In conclusion, we have shown that AMCM can facilitate HCEC proliferation via induction of IGF-1. These results suggest that AMCM-induced IGF-1 induction may be an important modulator of corneal epithelial healing by AM. Further in vitro and in vivo studies are needed to investigate how AMCM induces IGF-1 in HCECs and what factors and intracellular signaling cascades are involved in the IGF-1 induction and cell proliferation processes in HCECs.

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