

# Carboxymethylcellulose Binds to Human Corneal Epithelial Cells and Is a Modulator of Corneal Epithelial Wound Healing

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**PURPOSE.** In this study, the ability of carboxymethylcellulose (CMC), used in artificial tear formulations, to interact with corneal-epithelial-cells (HCECs) and facilitate corneal epithelial wound healing was investigated.

**METHODS.** HCECs were incubated with fluorescein-labeled CMC (F-CMC). CMC-epithelial binding was measured by spectrophotometry. The effect on F-CMC binding by hyaluronic acid (HA) or glucose was measured after preincubation in HA, mAb to CD44, or glucose, or mAb to GluT-1. F-CMC binding to fibronectin or collagen was measured by incubating proteins with F-CMC. The wound widths were measured 18 hours after confluent HCECs were scratch wounded. The ability of CMC to induce cell chemotaxis, proliferation, or migration was measured by quantitative assay. The efficacy of CMC in promoting epithelial wound healing was also tested in a rabbit epithelial scrape-wound model.

**RESULTS.** CMC remained bound to the HCECs for 2 hours. Preincubation of HCECs with glucose or mAb to GluT-1, but not with HA or mAb to CD44, reduced the binding of CMC to HCECs from 43.7% to 67.2% or 10.9% to 25.3%, respectively. CMC bound significantly to fibronectin (3.1-fold) or collagen (9.3-fold) compared with the control (BSA), and such binding enhanced cell adhesion. CMC stimulated re-epithelialization of HCECs scratched in vitro and in vivo rabbit cornea epithelial scrape wounds. CMC stimulated cell migration but not proliferation.

**CONCLUSIONS.** CMC probably binds to HCECs through interaction of its glucopyranose subunits with glucose transporters. CMC binding to the matrix proteins stimulated HCEC attachment, migration, and re-epithelialization of corneal wounds. (*Invest Ophthalmol Vis Sci.* 2007;48:1559-1567) DOI:10.1167/iov.06-0848

Carboxymethylcellulose (CMC), a high-molecular-weight polysaccharide, is one of the most common viscous polymers used in artificial tears to achieve their prolonged residence time on the ocular surface. It has been shown to be

efficacious in the treatment of aqueous tear-deficient dry eye symptoms and ocular surface staining,<sup>1,2</sup> and this effect has been found to be dose-dependent, with greater improvement observed with 1.0% CMC than with 0.5% CMC (Prather et al., *IOVS* 2002;43:ARVO E-Abstract 3152). It is generally understood that it is the physical properties of CMC, such as its viscous and mucoadhesive properties, that contributes to its prolonged retention time in the ocular surface. It is not known, however, whether there are any direct interactions of CMC with the ocular surface, in particular, with the epithelium.

CMC-based artificial tears have also been widely used after laser in situ keratomileusis (LASIK) to accelerate postoperative ocular surface recovery and to minimize dry eye symptoms.<sup>3</sup> CMC has been reported to be effective in reducing the incidence of epithelial defects during LASIK.<sup>4,5</sup> Also, it has cytoprotective properties on the ocular surface when used before contact lens insertion.<sup>6</sup> Contact lenses preconditioned with CMC before insertion produces less corneal staining and limbal and conjunctival redness and are more comfortable to wear than are nonconditioned lenses.<sup>7</sup> Although the precise role of CMC in the protective effect observed is not known, these findings suggest that CMC may be involved in the repair of the ocular surface.

This study was undertaken to investigate the interaction of CMC with corneal epithelial cells by synthesizing a fluorescently labeled form of the molecule. The time course of CMC-cell binding was determined, and competitive assays were performed to identify properties of the CMC binding sites. The potential of CMC to promote corneal epithelial wound healing has been explored in an in vitro wound healing model, and its efficacy has been tested in an animal model.

## METHODS

### Cell Culture

An immortalized human cornea-limbal epithelial (HCLE) cell line derived from primary cultures of HCLE cells (a kind gift from Ilene Gipson, Schepens Eye Research Institute, Boston, MA) was used in the study. HCLE cells were cultured as previously described.<sup>8</sup> Briefly, the cells were maintained on plastic at  $2 \times 10^4/\text{cm}^2$  in a keratinocyte serum-free medium (K-SFM; Invitrogen-Gibco, Grand Island, NY), supplemented with 25  $\mu\text{g}/\text{mL}$  bovine pituitary extract, 0.2 ng/mL epidermal growth factor (EGF; Invitrogen, Mount Waverley, VIC, Australia), and 0.4 mM  $\text{CaCl}_2$  and were grown at 37°C in a 5% carbon dioxide atmosphere. To enhance nutrient composition, the cultures were switched at approximately 50% confluence to a 1:1 mixture of K-SFM and low calcium DMEM/F12 (Invitrogen), to achieve confluence. All the experiments involving the culture of HCLE cells were performed at 37°C in a 5% carbon dioxide atmosphere unless otherwise indicated.

### Fluorescence Labeling of CMC

Pharmaceutical grades of sodium carboxymethylcellulose (Aqualon CMC) were obtained from Hercules (Wilmington, DE). For these studies, we used a mixture of high- and medium-viscosity types of CMC

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(approximate molecular weights 700,000 and 250,000, respectively) in a ratio of 35:65. This combination of CMC polymers has been found in certain commercial eye drop formulations, and recently patented.<sup>9</sup> CMC was labeled by modification of carboxyl groups of CMC with primary amine groups of a fluorescence label using EDC (1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide) and NHS (*N*-hydroxysulfadiazinimide). Briefly, 0.54 mL of 5-aminoacetamido fluorescein (fluoresceinyl glycine amide; Invitrogen-Molecular Probes, Inc. Eugene, OR) made in dimethylformamide (5 mg/mL), 0.5 mL CMC solution in water (2.5%), 0.13 mL EDC (100 mg/mL), and 0.1 mL NHS (100 mg/mL) were mixed, and pH was adjusted to 4.5 to 5.0. The reaction was performed for 1.5 hours at ambient temperature (AT) before adjustment of the pH to 8.0. After a further 48 hours at AT, unreacted fluorescence label, NHS or EDC, was removed with an ultra-centrifuge tube (molecular weight cutoff, 30,000; Amicon; Millipore, Bedford, MA). The fluorescein-labeled CMC (F-CMC) was freeze-dried and weighed. The degree of labeling of each batch of F-CMC was determined with a multiwell spectrophotometer (QLAB; Brisbane, QLD, Australia). The fluorescence at 485 and 535 nm (excitation and emission) was read, and the substitution was calculated by using a standard curve created by using fluorescence labeled solutions at concentrations of 1, 5, 10, and 20 ng/mL in water. The average substitution was 0.45% wt/wt. All the experiments involving measuring the level of F-CMC were determined by measuring the fluorescence at 485 and 535 nm.

### In Vitro Binding of F-CMC to HCLE Cells

HCLE cells were seeded in 96-well plates (black, flatbottomed) at  $1 \times 10^4$  cells/well and cultured in K-SFM until the cells were 85% confluent. The medium was replaced with fresh K-SFM media containing F-CMC (0.5%), CMC (0.5%), or the fluorescence label (fluoresceinyl glycine amide; 0.05 mg/mL) used for the labeling of CMC. This concentration of CMC or controls was selected because it is the same as is found in commercial artificial tear preparations. The cells were cultured in test media for 1 hour. At the end of culture, the cells were washed with culture medium extensively, to remove the unbound F-CMC, CMC, or fluorescence labels before measuring the fluorescence. Wells without cells were used as background control samples. For imaging F-CMC binding to HCLE cells, the cells were seeded on eight-well chamber slides (Nalge Nunc International, Naperville, IL) at 5000 cells/well. The cells were cultured and treated with the test medium under the same conditions as described earlier. After the cells were fixed and stained (Diff-Quik; Merck, Darmstadt, Germany), binding of F-CMC to the cells was observed by fluorescence microscope, and images were obtained (Polaroid DMC le Low Light System software, ver. V1.5; Electron Microscopy Sciences, Hatfield, PA).

### Binding of F-CMC to HCECs Collected by Corneal Irrigation

HCECs were collected from healthy subjects by using corneal irrigation. The subject was asked to place the forehead in a horizontal headrest of the irrigation chamber. The irrigation cylinder, with a fine blunt needle fitted in the center, was brought close to the center of the cornea (~2 mm away). The subject's cornea was irrigated for 10 seconds with 10 mL of warm, sterile saline to collect loosely adherent epithelial cells. The collected irrigating fluid pooled from five subjects was immediately centrifuged at 1500 rpm. The cells were resuspended in 1 mL of K-SFM culture medium in the presence or absence of F-CMC (0.5%) and incubated for 1 hour with shaking at 37°C in a standard incubator with 5% CO<sub>2</sub> with 95% air. The cell suspension was then passed through a filter unit containing a 5- $\mu$ m polycarbonate filter disc, and the cells collected on a filter disc were washed with phosphate-buffered saline (PBS), fixed, and stained (Diff-Quik; Merck). The binding of F-CMC to the corneal epithelial cells was visualized by fluorescence microscopy, and the images were obtained. The protocol adhered to the guidelines in the Declaration of Helsinki, and institutional ethics committee approval for the use of human subjects for corneal irrigation was granted.

### In Vitro Retention Time of F-CMC to HCLE Cells

Cultured monolayers of HCLE cells in black 96-well plates were preincubated with F-CMC in culture medium for 1 hour. The excess F-CMC was washed off with 200  $\mu$ L of culture medium before the initial fluorescence measurement. This represented a retention time of 0 hours. The cells were continually cultured in culture medium for a further 0.5, 1, 1.5, 2.5, 3.5, 5.5, 8.5, and 24 hours. At each time point, the cells were washed with culture medium, and the fluorescence of the remaining F-CMC bound to the cells was measured.

### Competitive Binding between F-CMC and Hyaluronic Acid or Glucose to HCLE Cells

To investigate whether CMC shares the same binding site as hyaluronic acid (HA) or glucose on HCLE cells, HCLE cells were incubated at 85% confluence for 2 hours with concentrations of HA (0.1, 0.5, 1.0, and 2.0 mg/mL; Fluka, St. Gallen, Switzerland) or the mAb to CD44, the receptor for HA on corneal epithelial cells (1.0, 10, and 50  $\mu$ g/mL; Clone A3D8, MFC00164629; Sigma-Aldrich, St. Louis, MO), or glucose (3.125, 25, and 100 mM) or the antibody to glucose transporter 1 (GluT-1, G3900-05D; US Biological, Swampscott, MA), the binding site for glucose on corneal epithelial cells (1.0, 10, and 50  $\mu$ g/mL),<sup>10</sup> respectively. The cells were washed with culture medium to remove unbound HA, glucose, or mAbs before further incubation with F-CMC (0.05 mg/mL) for 2 hours. The cells were then washed with culture medium to remove unbound F-CMC and the fluorescence of F-CMC bound to HCLE cells was measured. HCLE cells without HA or glucose or mAb treatment were used as control cultures.

### In Vitro Scratch-Wound Closure in Response to CMC Treatment

A scratch-wound assay with HCLE cells was used to determine whether CMC could promote wound closure. HCLE cells were cultured to a confluent monolayer on eight-well chamber slides coated with collagen I (10  $\mu$ g/cm<sup>2</sup>; Auspep, Parkville, VIC, Australia) before being wounded by scratching with a 100- $\mu$ L pipette tip. The scratch-wounded HCLE cells were washed with fresh medium to remove detached cells before incubation in the medium in the absence or presence of CMC (2 mg/mL) for 18 hours. To ensure that the wounds with the same wound area were compared, multiple positioning marks were made at the center of the denuded surface with a small needle, and the mean distance between the wound edge ( $n = 10$ ) was measured. Eighteen hours after wounding, the monolayers were fixed and stained, and the wound areas in a marked field of view were imaged. The mean distance between the migrated cell edge ( $n = 10$ , measurements in three separate samples) was determined using an image analysis system (Image J 1.33o; available by ftp at [zippy.nimh.nih.gov/](http://zippy.nimh.nih.gov/) or at <http://rsb.info.nih.gov/nih-image/>); developed by Wayne Rasband, National Institutes of Health, Bethesda, MD) and the percentage of wound closure by HCLE cells in response to CMC was compared with that of the control medium with no added CMC.

### HCLE Cell Proliferation

HCLE cell proliferation, in the presence of CMC, was determined with a cell proliferation assay (CyQuant kit; Invitrogen-Molecular Probes) incorporating a fluorescent dye (CyQuant GR; Invitrogen-Molecular Probes), which exhibits strong fluorescence enhancement when bound to DNA (excitation wavelength at 485 nm and emission at 535 nm).<sup>11</sup> Briefly, HCLE cells were seeded into 96-well tissue culture plates at a density of  $1 \times 10^4$  per well in K-SFM medium and cultured for 24 hours. K-SFM was removed, and the cells were washed once with PBS before addition of 1:1 SFM-low-calcium DMEM/F12 (Invitrogen) containing concentrations of CMC (0, 0.02, 0.2, and 2 mg/mL) and incubated for a further 0, 8, 24, and 48 hours. After the medium was removed, 200  $\mu$ L of the CyQuant GR dye-cell lysis buffer was added to each well and incubated for 2 to 5 minutes at room temper-

ature. The fluorescence intensity, related to the number of viable cells, was measured at 485/535 nm.

### HCLE Cell Migration: An In Vitro Dispersion Assay

To investigate the ability of CMC to stimulate HCLE cell migration, the dispersion assay described by Pilcher et al.<sup>12</sup> was used, with modifications. HCLE cells were seeded and cultured to confluence with supplemented K-SFM in siliconized cloning cylinders (Sigma-Aldrich, Sydney, NSW, Australia) on collagen I-coated 6-well plates (10  $\mu\text{g}/\text{cm}^2$ ; Sigma-Aldrich). The cells were cultured for a further 24 hours in the presence of 100  $\mu\text{M}$  hydroxyurea (Sigma-Aldrich) to induce growth arrest.<sup>13</sup> After the rings were removed, the cells were thoroughly washed with K-SFM and treated with CMC (2 mg/mL) or left untreated and allowed to migrate for 24 hours. The cells were fixed and stained, and images of the dispersion areas were taken.

### Chemotactic Migration of HCLE Cells

To determine the chemotactic migration response of HCLE cells to CMC, an in vitro assay (QCM Chemotaxis 96-well Cell Migration Assay Kit; Chemicon, Temecula, CA) was performed according to the manufacturer's instructions.<sup>14</sup> Briefly, HCLE cells were seeded into the upper migration chamber ( $5 \times 10^4$  in 100  $\mu\text{L}$ ), which was then placed into the lower chamber containing 150  $\mu\text{L}$  of culture medium in the presence or absence of CMC at 0.5, 2, and 5 mg/mL or 2 mg/mL HA in culture medium, and the cells were incubated overnight. After the cells on the top side of the insert were gently removed, migratory cells on the bottom of the insert membrane (8- $\mu\text{m}$  pore size) were dissociated from the membrane with cell-detachment buffer. These cells were subsequently lysed and stained with fluorescence dye that exhibits strong fluorescence enhancement when bound to cellular nucleic acid. Cell migration was determined by measuring the fluorescence of the cell solutions at 485/535 nm.

### Effect of CMC on Attachment of HCLE Cells to Fibronectin and Collagen

To assess whether CMC binds to extracellular matrix (ECM) proteins, black 96-well-culture plates were precoated with matrix proteins, fibronectin (human plasma; Sigma-Aldrich) and collagen type I at 5 and 10  $\mu\text{g}/\text{cm}^2$ , respectively. Coating with bovine serum albumin (1% BSA; Sigma-Aldrich) was used as a control. After uncoated matrix proteins were washed off and nonspecific binding sites blocked with BSA, the plates were further incubated for 2 hours in culture medium with or without F-CMC (2 mg/mL) or in culture medium containing the fluorescent label only (2  $\mu\text{g}/\text{mL}$ ). The fluorescence of F-CMC or fluorescent label bound to the matrix proteins was measured after unbound F-CMC or fluorescence labels were washed off with PBS.

To assess whether the binding of CMC to matrix proteins further enhances cell adhesion, HCLE cells were seeded in 96-well culture plates precoated with fibronectin, collagen or BSA (as just described), and further incubated with CMC (2 mg/mL) as described earlier. Cells were incubated for 4 hours. Nonadherent cells were removed by washing with fresh culture medium. The amount of adherent viable cells was determined by using the cell proliferation reagent WST-1 incorporating water-soluble tetrazolium (Roche, Sydney, NSW, Australia). Briefly, 10  $\mu\text{L}$  WST-1 was added to each well containing fresh culture medium, and the plate was incubated for 2 hours. The absorbance at 450 nm, related to the number of adherent cells converting the reagent to colored formazan crystals, was measured.

### Animal Studies

All procedures were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

NZ white rabbits weighing 2 to 3 kg (all 1-year-old females) were used in the study. Nictitating membranes in all rabbits were removed from both eyes at least 2 weeks before surgery. Animals were anesthe-

tized with an intramuscular injection of ketamine 35 mg/kg and xylazine 5 mg/kg to a depth of stage 3, plane 2. The central corneal epithelium was removed with a 6-mm trephine and a corneal gill knife, leaving the basement membrane intact, resulting in an epithelial scrape wound with a diameter of 6 mm. The wound size was determined by staining the surface of the eye with fluorescein and photographing the cornea with a slit lamp camera equipped with a cobalt excitation filter. The area of the corneal scrape wound was quantitated from the photographs by using a computer image-analysis system. Two groups of six rabbits were used in the study, with each group receiving either PBS vehicle or 1% CMC. Each dose (50  $\mu\text{L}$ ) was topically administered to both wounded and unwounded eyes, with the first dose being administered immediately after the initial measurement of wound size followed by administration four times a day. The wounded eye was photographed immediately after the first dose (0 hour) and at 24 hours after initiation of treatment, and the wound area was quantitated at each time point.

### Statistical Analysis

All results are expressed as the mean  $\pm$  SD of results for six samples, unless otherwise indicated; experiments were repeated two to three times. One-way analysis of variance (ANOVA) was performed with commercial computer software (SPSS; SPSS Inc., Chicago, IL), to analyze statistical significance. Post hoc multiple comparisons were analyzed incorporating the Bonferroni correction. Statistical significance was set at  $P < 0.01$ , to make sure that the inferences from the study would have the lowest level of type 1 error.

## RESULTS

### The Binding of CMC to Corneal Epithelial Cells

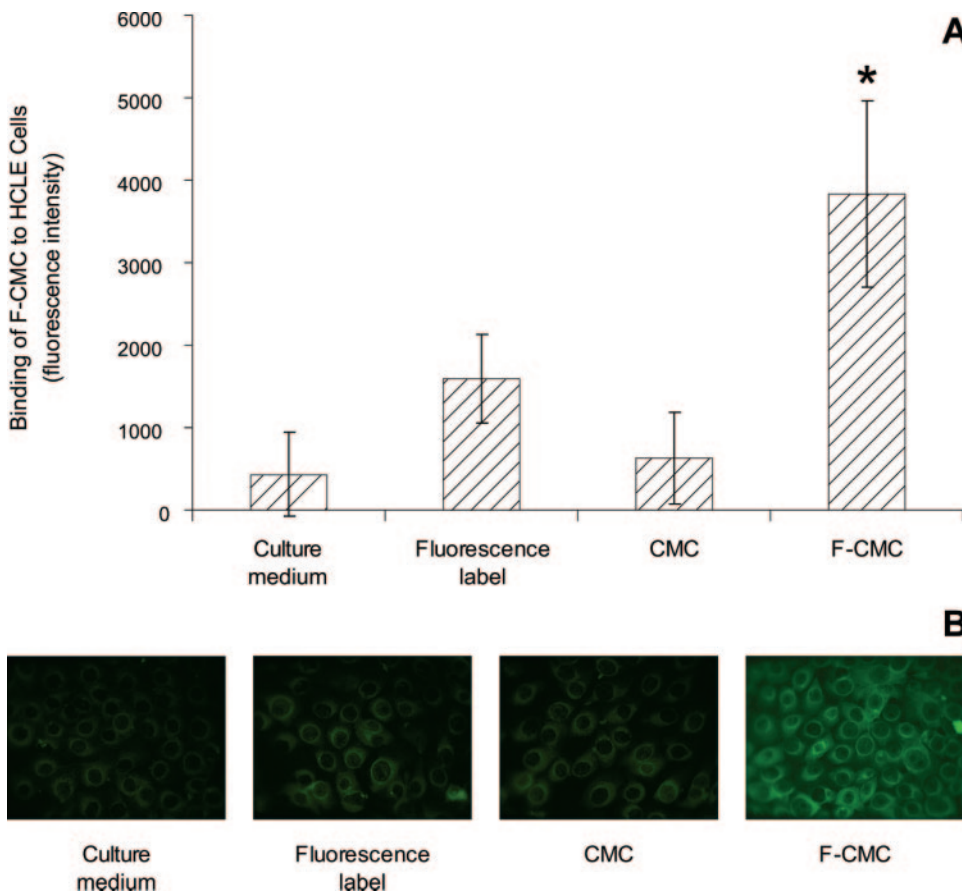
F-CMC bound to HCLE cells (Fig. 1A). The fluorescence intensity of the F-CMC-treated cells was significantly higher than that of the cells treated with control medium ( $P < 0.001$ ) or medium containing unlabeled CMC ( $P < 0.001$ ). Although the fluorescence intensity of the cells treated with the fluorescence label used for labeling of CMC was also higher than the control, it was significantly lower than the F-CMC treated cells ( $P < 0.001$ ), indicating that the observed binding of F-CMC to HCLE cells was due to the interaction of the cells with CMC but not with the fluorescence label. The binding of F-CMC to HCLE cells was also visualized by the use of fluorescence microscopy (Fig. 1B). F-CMC binding to corneal epithelial cells collected from healthy subjects by corneal irrigation was also observed (Fig. 2).

### Retention Time

A consistent level of F-CMC on the cells remained for 24 hours, indicating that there was some long-lasting binding between F-CMC and HCLE cells (Fig. 3). We assumed the remaining fluorescence at 24 hours to be long-term binding and subtracted this value from the rest of the data to find short-term binding. Exponential fit to the short-term binding indicated a half-life of approximately 2 hours, suggesting that F-CMC binds to corneal cells with a residence time of approximately 2 hours (Fig. 3).

### Binding Site of CMC to Corneal Epithelial Cells

CMC has many of the properties of HA, such as anionic charge, high microviscosity, and retention on the cornea for a prolonged period. The molecular structure of CMC subunits is also similar to glucose, and so we tested to determine whether CMC, like HA, binds to the CD44 receptor or like glucose bound to glucose transporters. Binding of F-CMC to HCLE was not significantly reduced in the presence of HA itself at concentrations of  $\geq 0.5$  mg/mL, or by a monoclonal antibody to



**FIGURE 1.** In vitro binding of F-CMC to HCLE cells. The affinity of CMC to the cultured HCLE cells is presented as the fluorescence intensity of F-CMC at 485/535 nm (A) and visualized by fluorescent images. Magnification,  $\times 50$ . (B) The HCLE cells were incubated in the culture medium (K-SFM), and the culture medium containing fluorescence labels (0.05 mg/mL), unlabeled CMC (0.5%), and F-CMC (0.5%) for 1 hour, followed by extensive washing with PBS before the fluorescence was read. Data are the mean  $\pm$  SD of results for six samples. \*Significant difference between the culture medium ( $P < 0.001$ ), CMC ( $P < 0.001$ ), and fluorescence label ( $P < 0.001$ ).

CD44 (Table 1). However, when HCLE cells were preincubated with glucose or the antibody to the glucose transporter-1 (GluT-1), the binding of F-CMC to the HCLE cells was decreased in a dose-dependent manner (Table 1).

**Effect of CMC on HCLE Wound Closure**

Eighteen hours after wounding the monolayers of HCLE, CMC-treated HCLE had closed the wound significantly more than the control medium-treated wounds ( $P = 0.00952$ ; Fig. 4), indicating that CMC promotes HCLE wound closure.

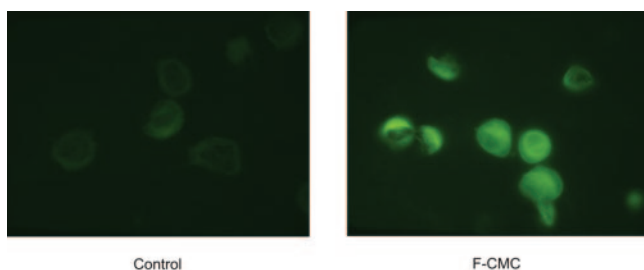
**Effect of CMC on HCLE Cell Proliferation**

CMC at low concentrations ( $\leq 0.2$  mg/mL) did not show any statistically significant effect on HCLE cell proliferation compared with the treatment with control culture medium without CMC (Fig. 5,  $P > 0.01$ ). However, at a concentration of 2

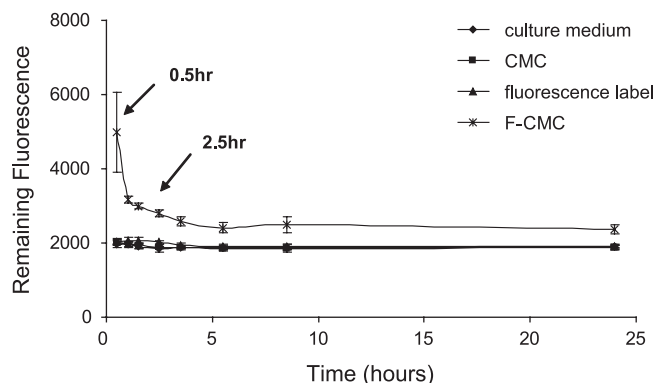
mg/mL, CMC showed a significant inhibitory effect on HCLE cell growth at 24 and 48 hours compared with the effect of the control culture medium (Fig. 5;  $P = 0.008$  and  $0.004$ , respectively). Phase contrast microscopy showed that the morphology of the CMC-treated cells, even at 2 mg/mL, remained the same as the control and we had observed no cytotoxic effects of CMC (data not shown).

**Effect of CMC on HCLE Cell Migration**

CMC-induced migration was assessed by the ability of the cells to move into an acellular area after growth arrest. CMC promoted cell migration, whereas no dispersion was seen in response to the control culture medium with no added CMC (Fig. 6).



**FIGURE 2.** F-CMC binding to HCECs collected by corneal irrigation. The binding of F-CMC to corneal epithelial cells was visualized by fluorescence microscopy after epithelial cells collected from healthy human subjects were incubated for 1 hour in K-SFM with added F-CMC (0.5%) and without F-CMC (control), respectively. Magnification,  $\times 100$ .



**FIGURE 3.** Fluorescence intensities of F-CMC remaining on HCLE cells after washing with PBS to remove unbound F-CMC. Cells were then washed 0.5, 1, 1.5, 2.5, 3.5, 5.5, 8.5, and 24 hours after initial adsorption of F-CMC (2 mg/mL). Error bars, SD of six samples.

TABLE 1. Competitive Binding of F-CMC to HCLE Cells

Treatments	Concentrations	Fluorescence Intensity		P*	P†	P‡	P§
		Mean	SD				
HA (mg/mL)	0	2243	208	—	—	—	—
	0.1	3487	904	0.005	—	—	—
	0.5	2332	418	0.827	0.008	—	—
	1	2784	616	0.191	0.093	0.272	—
	2	3124	1007	0.038	0.374	0.06	0.407
Anti-CD44 (µg/mL)	0	1912	515	—	—	—	—
	1	2658	1025	0.171	—	—	—
	10	2449	854	0.319	0.694	—	—
	50	2568	1125	0.226	0.866	0.823	—
Glucose (mM)	0	1614	248	—	—	—	—
	3.125	1438	191	0.132	—	—	—
	25	908	214	<0.001	<0.001	—	—
	100	529	76	<0.001	<0.001	0.003	—
Anti-GluT1 (µg/mL)	0	1730	171	—	—	—	—
	1	1708	127	0.741	—	—	—
	10	1541	61	0.009	0.009	—	—
	50	1293	49	<0.001	<0.001	0.001	—

Data were collected in the presence or absence of HA or the monoclonal antibody to CD44 (the HA receptor) and glucose or antibody to GluT-1 (the glucose-specific binding site). The binding of F-CMC is presented as fluorescence intensity of F-CMC bound to the HCLE cells after the cells were preincubated with concentrations of HA or antibody to CD44 or glucose or antibody to GluT-1, for 1 hour followed by extensive washing with culture medium and further incubation with F-CMC for 2 hours. Data are expressed as the mean ± SD of six samples.

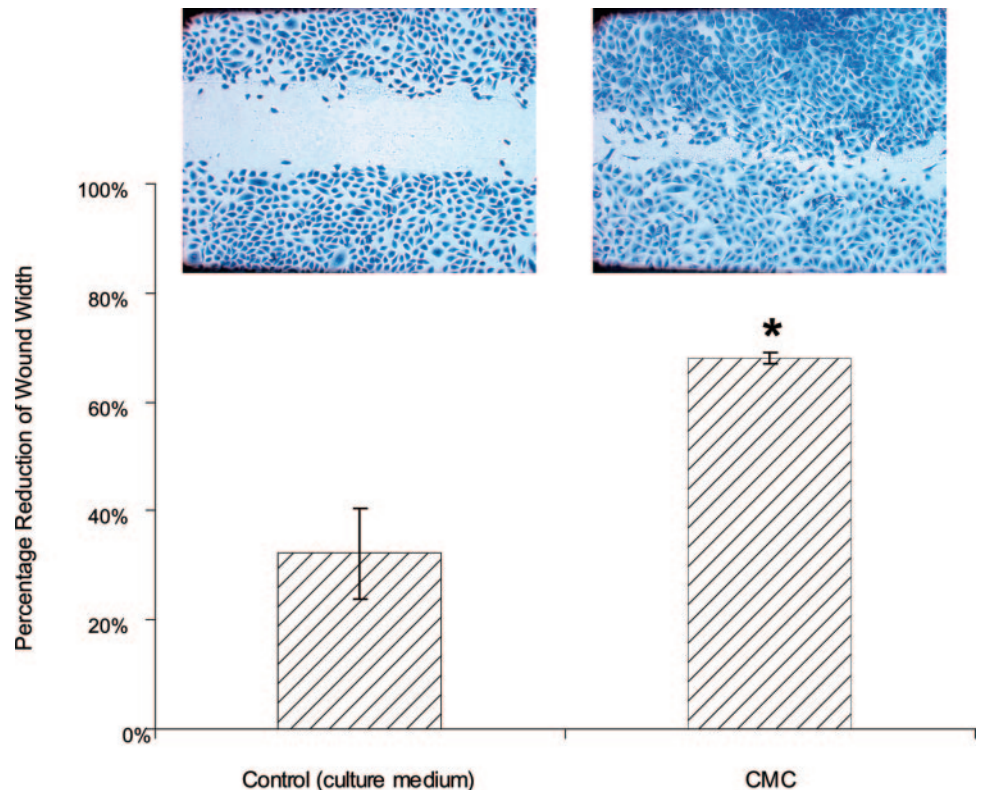
- \* Significant when compared with the first concentration.
- † Significant when compared with the second concentration.
- ‡ Significant when compared with the third concentration.
- § Significant when compared with the fourth concentration.

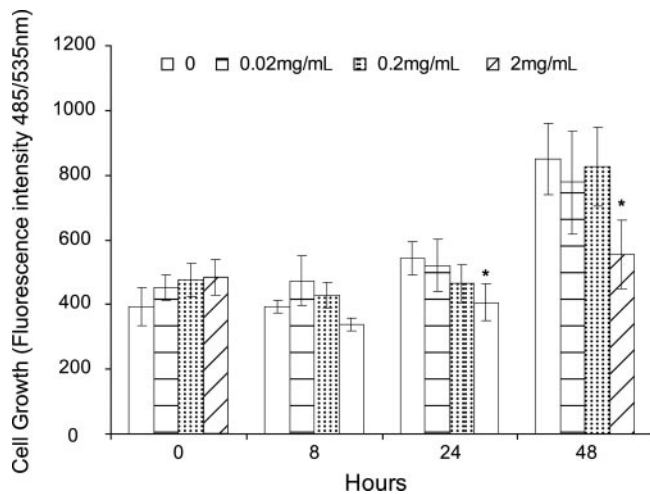
**Effect of CMC as a Chemoattractant for HCLE Migration**

HCLE cells exhibited a chemotactic response to CMC in a cell migration assay (Fig. 7; Transwell, Corning, Corning,

NY). Addition of CMC to the lower chamber caused an up to 1.5-fold concentration-dependent increase in the migration of HCLE cells toward CMC. As a positive control, migration of HCLE cells was induced by HA (2.0 mg/mL) in the lower

FIGURE 4. The closure of scratched HCLE wounds in response to CMC stimulation. The effect of CMC on wound closure was visualized and also represented as the percentage reduction of the average wound width 18 hours after confluent HCLE cells were scratch wounded and incubated in the culture medium in the absence or presence of CMC (2 mg/mL). Data represent the mean ± SD (n = 3) of the percentage reduction of the wound width which was measured as the average width of the 10 measurements along the wound edge. CMC stimulation was significant compared with the control (\*P = 0.00952).





**FIGURE 5.** Effect of CMC on cultured HCLE cell proliferation. Cells seeded at density of  $1 \times 10^5$  cells per well in 96-well microplate were cultured for 24 hours before treated with concentrations of CMC in K-SFM (0, 0.02, 0.2, and 2 mg/mL) for a further 8, 24, and 48 hours. Data represent the mean  $\pm$  SD ( $n = 6$ ) of the fluorescence intensity. At 2 mg/mL, CMC inhibition of cell proliferation was significant at 8, 24, and 48 hours compared with the control culture medium without CMC; \* $P = 0.008$  and  $0.004$ , respectively.

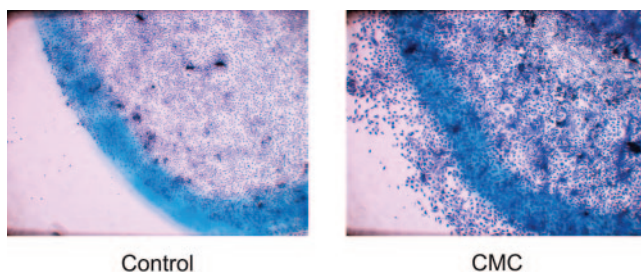
chamber, demonstrating the ability of the cell line to migrate.

### CMC Binding to Matrix Proteins and Results in Promotion of HCLE Cell Adhesion

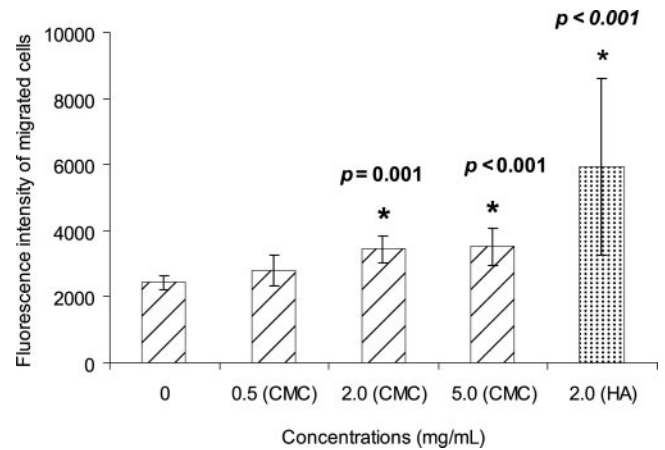
Epithelial cell migration requires alterations in actin cytoskeleton dynamics and cell adhesion which are modulated by ECM proteins. F-CMC bound to collagen or fibronectin but not to the control bovine serum albumin (BSA) coatings (Fig. 8A). F-CMC was also bound to collagen significantly more than to fibronectin (Fig. 8A). Preincubation of the matrix proteins with CMC significantly increased the number of cells attached to the fibronectin or collagen matrix, whereas no difference was observed in the cell attachment to the control BSA preincubated with CMC or culture medium (Fig. 8B).

### CMC Stimulation of Re-epithelialization of Rabbit Cornea Epithelial Scrape Wounds

In the rabbit corneal-reepithelialization model, the wound areas in the group that received the doses of CMC were smaller than those in the PBS-treated control group at 24 hours after wounding (Fig. 9). The mean percentage reduction of the



**FIGURE 6.** HCLE cell migration in response to CMC stimulation. Confluent HCLE cells in a colony ring were growth arrested by hydroxyurea. After removal of the culture ring, the cells were washed with PBS and then treated with (right) or without (left) CMC (2 mg/mL) for 24 hours. The curved blue line marks the edge of the culture ring. Magnification,  $\times 50$ .



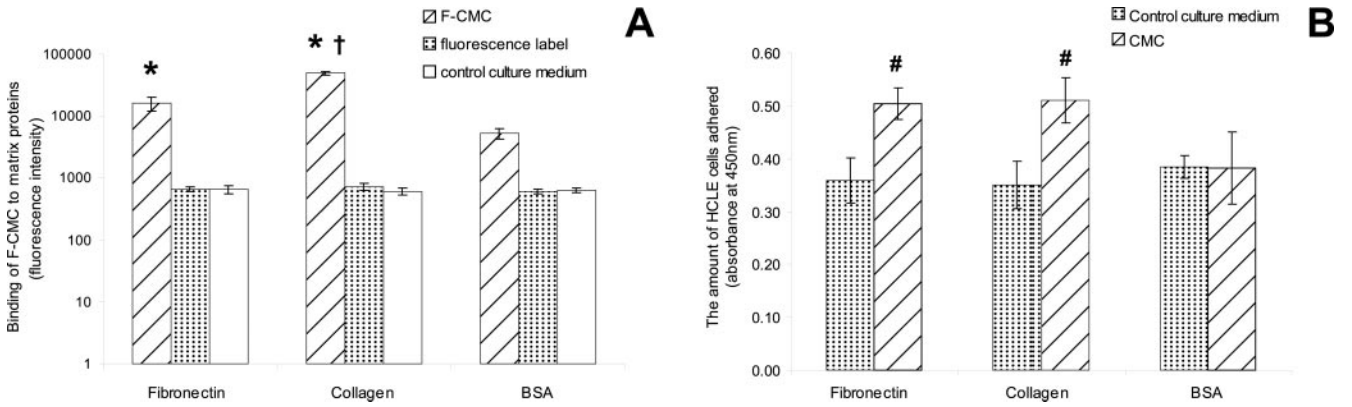
**FIGURE 7.** Chemotactic migration of HCLE cells in response to CMC. HCLE chemotaxis toward culture medium in the presence or absence of CMC at concentrations of 0.5, 2, 5 mg/mL and in the presence of HA (2 mg/mL). Error bars, SD in six samples. \*Significant difference from the control medium.

corneal defects for the CMC treatment group was greater than that for the PBS control group. Student's *t*-test showed statistically significant differences ( $P < 0.001$ ) between PBS control and the CMC group at 24 hours.

### DISCUSSION

Although CMC is widely used in artificial tears for treatment of dry eye,<sup>6,15,16</sup> the underlying mechanisms of how CMC interacts with corneal epithelial cells have not been reported. The present results demonstrate that CMC binds directly to corneal epithelial cells, and this binding utilizes, at least in part, the glucose receptor GluT-1. This finding is entirely plausible, as CMC is a polymer of glucopyranose subunits, which is also the predominant form of glucose in solution. Although CMC is similar to the naturally occurring mucopolysaccharide HA, the present results demonstrate that CMC does not bind to the HA receptor, the cell surface adhesion molecule CD44. Further work is needed to determine whether there are additional binding sites for CMC, and the relative affinity for CMC versus other glucose polymers to the glucose transporters.

Previous reports describing the residence time of CMC or other polymers on the ocular surface have used various methods, including mixtures of CMC solutions with radioactive<sup>16,17</sup> or fluorescent tracers,<sup>18,19</sup> retention of the fluid volume of an instilled drop (Jones LW et al. *IOVS* 2004;45:ARVO E-Abstract 144), or increased thickness of the tear film layer.<sup>20</sup> None of these methods has directly measured the presence of CMC itself, as in the current work. However, these other techniques have provided the advantage of actual on-eye measurements. In the on-eye situation, applied fluids are subject to mixture with ongoing tear secretions, physical action of the lids, effects of evaporation, and removal via the lacrimal drainage system. In the present experiments, *in vitro* cells were exposed to unaltered CMC solutions for 1 hour or more before measurement of binding, thus maximizing the opportunity for binding to occur. Attempts to model the ocular situation with shorter exposure times and/or dilution may be warranted, but such modeling is limited in describing the true dynamics of the on-eye tear film. In the present study, CMC bound to corneal cells at specific cell-surface sites, and, with repeated washing, the bound amount gradually declined over several hours to a residual level that showed no further decrement, at least for the 24-hour observation period. Thus, CMC binding appeared to have two

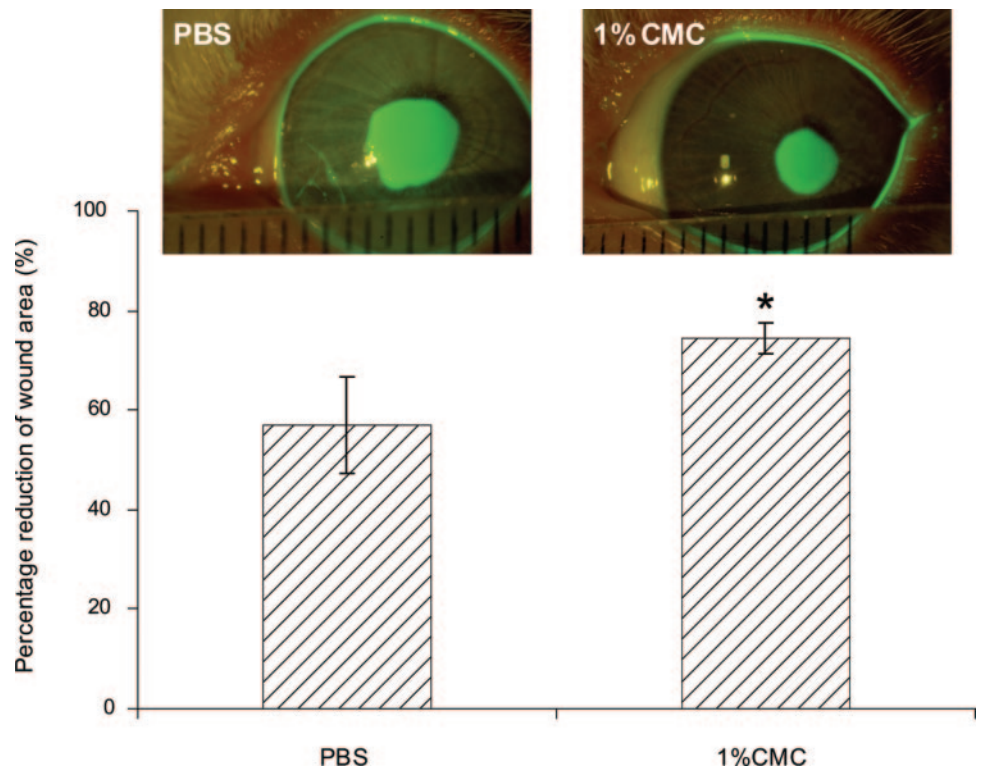


**FIGURE 8.** Binding of CMC to the matrix proteins fibronectin or collagen (A) and its effect on HCLE cell adhesion (B). The 96-well culture plates (black or clear) were precoated with fibronectin or collagen with BSA used as the control. Nonspecific binding sites were blocked with BSA before incubation for 2 hours in the culture medium in the absence or presence of F-CMC (2 mg/mL) or fluorescence labels (2 μg/mL) in a black plate or CMC (2 mg/mL) in a clear plate. The binding of F-CMC or fluorescence labels to the matrix proteins presented as the fluorescence intensity at 485/535 nm after the plates were washed with PBS to remove unbound F-CMC or fluorescence labels (A). For the cell adhesion assay, after unbound CMC was washed off, cells were seeded and incubated in the culture medium for 2 hours. Nonadherent cells were removed by washing with PBS before further incubation in the culture medium for 2 hours. The amount of adherent viable cells was determined with WST-1 and presented as the absorbance at 450 nm (B). Error bars, SD in six samples. F-CMC binding to fibronectin or collagen was significant compared with BSA (\* $P < 0.001$ ). A significant difference was also found between F-CMC binding to collagen and to fibronectin († $P < 0.001$ ). There was a significant difference between preincubation of CMC with fibronectin or collagen and the control without preincubation of CMC (# $P < 0.001$ ).

components, one somewhat labile, and the other fairly permanent. It is not clear whether these binding events represent more than one molecular process or a single process with variable kinetics.

Another goal of the present study was to determine whether CMC affects corneal epithelial wound healing and the mechanism of modulating the dynamics of tissue healing in vitro. CMC can accelerate post-LASIK ocular surface recovery and minimize dry eye symptoms.<sup>3</sup> CMC can also effectively reduce the incidence of epithelial defects during LASIK,<sup>4,5</sup> although the underlying mechanisms of how it works is not

clear. It was assumed that the protective effect of CMC observed in these past clinical studies was due to the strong mucoadhesive properties of the carboxyl groups of CMC serving as a lubricant.<sup>3,4</sup> In this study, we have demonstrated that CMC also had some biological functions. It stimulated the closure of in vitro HCLE wounds as well as in vivo re-epithelialization of rabbit corneal epithelial scrape wounds. Although it is not yet understood how CMC stimulates cell migration in vivo, our in vitro wound-healing model had its advantages in providing information on the basic principles involved and is widely used to assess exogenous agents that may modify the



**FIGURE 9.** The representative images of corneal defects and the percentage reduction of the cornea epithelial scrape wound area 24 hours after wounding in response to CMC (1%) or control PBS treatment. Error bars, SD in six samples. \*Significant difference from the control PBS treatment ( $P < 0.001$ ).

healing process.<sup>21-25</sup> In vitro, closure of wounds is a result of cell migration from the wound's edge as well as cell proliferation.<sup>21,26</sup> CMC demonstrated no stimulatory effect on cell proliferation at low concentrations (<2 mg/mL), and it appeared to inhibit cell proliferation at a concentration of 2 mg/mL. Further work is necessary to verify this inhibition and to explore possible mechanisms for the effect, if it occurs. However, CMC showed a potent stimulatory effect on HCLE cell migration. The cell migration in response to CMC in the present study was demonstrated by a direct interaction of the growth-arrested HCLE cells with substrate (collagen)-bound CMC in the dispersion colony assay, or was triggered by CMC as a chemoattracting stimulus in the cell migration assay.

Furthermore, cell migration in vivo is thought to result from the coordinated regulation of both cell-cell and cell-ECM interactions.<sup>27</sup> The initial step of epithelial wound healing employs provisional ECM matrix proteins such as fibronectin.<sup>28</sup> Our finding of CMC being able to bind to the matrix proteins fibronectin or collagen to form a CMC-fibronectin/collagen complex which facilitated the attachment of epithelial cells is of fundamental importance. There is evidence to suggest that extracellular fibronectin plays an essential role by providing a provisional matrix for wound healing onto which the migrating epithelial cells can adhere during the frequent cyclical processes of cleaving and attaching of the migrating epithelial cells.<sup>28,29</sup> Fibronectin appears on the wound surface after all types of insult, including penetrating or nonpenetrating incision of the cornea,<sup>30</sup> conventional keratectomy,<sup>31</sup> mechanical epithelial debridement,<sup>32</sup> and excimer laser keratectomy.<sup>33</sup> Fibronectin stimulates epithelial cells to produce plasminogen activator which, in turn, converts plasminogen to plasmin, which breaks down adhesions between cells and underlying subepithelial matrix. Like HA, which is known to facilitate corneal epithelial wound healing by its binding to a provisional fibronectin matrix,<sup>28</sup> CMC's potent effect in promoting wound closure may also lie in its binding to matrix proteins.

It should be noted that a glucose final concentration of 12.5 mM in the culture medium was used in our in vitro scratch-wound model and cell migration, adhesion, and proliferation assays. It has been reported that glucose at concentrations up to 17.5 mM has stimulatory effects on human corneal epithelial cell migration, adhesion, and proliferation, but concentrations above 31 mM have an inhibitory effect.<sup>34,35</sup> To eliminate a possible competitive effect of glucose present in the culture medium with the effect of CMC on the HCLE cell migration, cell adhesion, and proliferation and scratch-wound closure, we included a control group of culture medium containing 12.5 mM glucose but with no CMC in all our assays.

In conclusion, CMC, well-known for its mucoadhesive and viscous properties, binds to corneal epithelial cells and remains bound for at least several hours. Further, CMC stimulated epithelial cell migration through its binding to matrix proteins, and the enhancement of cell attachment to the matrix by CMC could be a major contributor to the observed closure of the scratched cell monolayer and re-epithelialization of rabbit cornea epithelial scrape wounds. The properties of this polymer may form the basis for the observed long-lasting benefits of clinical use of CMC.

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