**KGF Prevents Oxygen-Mediated Damage in ARPE-19 Cells**

**R. Christopher Geiger,** ¹,² **Christopher M. Waters,** ³ **David W. Kamp,** ² and **Matthew R. Glucksberg**¹

**PURPOSE.** Oxidative stress has been implicated in a variety of diseases of the eye. In several other tissues, keratinocyte growth factor (KGF) has been shown to prevent negative cellular changes associated with oxidative insult, such as permeability increases and nuclear DNA damage. In this study, we looked at whether KGF provided these same protective effects to cultured human retinal pigment epithelial (RPE) cells (ARPE-19).

**METHODS.** Reverse transcriptase–polymerase chain reaction (RT-PCR) using a published primer pair sequence followed by restriction endonuclease digestion with Avai and HincII was used to look for the KGF receptor message in ARPE-19 cells. Cellular response to KGF was verified through proliferation assays and Western blot analysis for mitogen-activated protein kinase (MAPK). Single-cell gel electrophoresis was used to assess DNA damage. Western blot analysis was used to assay actin cytoskeletal changes, and electrical resistance and tracer experiments with Transwell tissue plates were used to assess permeability changes. Immunostaining was used to verify the existence of the tight junction protein occludin.

**RESULTS.** It was verified through RT-PCR that the ARPE-19 cell line exhibited the message for FGFR2-IIb, otherwise known as KGFR. KGF was also shown to increase cellular proliferation and activated the MAPK p44/p42 cascade. KGF ameliorated nuclear DNA damage and cytoskeletal rearrangement caused by oxidative stress through the addition of exogenous hydrogen peroxide but was unable to prevent permeability changes.

**CONCLUSIONS.** KGF was shown to significantly reduce DNA damage and cytoskeletal rearrangement caused by oxidative stress in cultured ARPE-19 cells. This result may be useful in targeting future therapies to combat a multitude of diseases of the eye that result from increases in reactive oxygen species.


From the ¹Department of Biomedical Engineering, Northwestern University, Evanston, Illinois; the ²Division of Pulmonary and Critical Care Medicine, Northwestern University Medical School, Chicago, Illinois; and the ³Department of Physiology, University of Tennessee Memphis, Memphis, Tennessee.

Supported by National Institutes of Health Grants EY13002 and EY13015. Supported in part by a National Eye Institute Multidisciplinary Visual Sciences Training Grant T32 EY07128–05, National Institutes of Health Grant EY09714, National Heart, Lung and Blood Institute Grant HL6981, and the Fight for Sight research division of Prevent Blindness America.

Submitted for publication December 17, 2004; revised March 27 and May 19, 2005; accepted July 22, 2005.

Disclosure: R.C. Geiger, None; C.M. Waters, None; D.W. Kamp, None; M.R. Glucksberg, None.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Matthew R. Glucksberg, Biomedical Engineering Department, Northwestern University, 2145 Sheridan Rd., Evanston, IL 60208-3107; m-glucksberg@northwestern.edu.

The eye is a unique structure in that light passes through an optically clear medium to be focused on a delicate, metabolically active tissue (the retina). It contains high concentrations of polyunsaturated fatty acids and is positioned next to highly vascularized tissue (the choriocapillaris). This combination of focused light, oxygenated blood, and metabolically active tissue offers multiple pathways for reactive oxygen species (ROS) formation and may explain both the prevalence and the variety of antioxidants present in the retina. Because elevated ROS formation is implicated in the pathogenesis of many diseases, including neurodegenerative diseases, diabetes, and cancer, as well as “normal” aging, the discovery of biochemical mechanisms that either protect cells or promote cellular recovery from damage is important.

Many diseases of the retina are characterized by permeability increases in the blood-retinal barrier (BRB). Through the formation of tight junctions, the retinal endothelial cells and the retinal pigment epithelium (RPE) form an almost impermeable boundary that can prevent molecules as small as ions from crossing this barrier. Increases in barrier permeability develop during the progression of diseases such as diabetic retinopathy, cystoid macular edema, proliferative vitreoretinopathy, age-related macular degeneration (AMD), and radiation retinopathy, or they result in part from permeability changes. Although the exact mechanism for the breakdown of the BRB is still widely debated, it is apparent that preventing permeability increases is a first step in battling these debilitating ocular diseases.

In addition to permeability changes, many of these diseases also give rise to elevated ROS levels. For example, in patients with diabetes, age and elevated glucose levels have been implicated in ROS production and elevated levels of oxidative stress. Cholesterol esters and oxidized lipids accumulate because of the nondegradable end products resulting from phagocytosis of photoreceptor cell outer segments early in the development of AMD. Ninefold increases in hydrogen peroxide production have been measured in vivo and are attributed to this phagocytosis. Implied elevating ROS production is at least present in the early pathogenesis of AMD. In addition to disrupting barrier integrity, oxidative insults target many proteins and nucleic acids within the cell. Although the cells of the BRB have a robust antioxidant defense system, increased ROS formation in disease can overcome cellular antioxidant capacity, whereas in the aged, reduced capacity can increase susceptibility to ROS produced by normal cellular function. Many investigators believe that both nuclear and mitochondrial DNA damage are factors in cellular changes caused by oxidative stress. Previous studies have examined oxidative stress-induced DNA damage in the RPE in vitro under chronic and acute conditions. These types of stress can be used to model diseases of the eye such as AMD and complications resulting from radiation treatment, each of which has been shown to display ROS formation. These models may also be useful in describing the effects of aging.

In recent years, keratinocyte growth factor (KGF) has been shown to prevent permeability increases in endothelial and
epithelial cell barriers caused by insults such as radiation,22 hydrogen peroxide,23 hyperoxia,24 and acid instillation.25 KGF has also been shown to prevent DNA damage, enhance DNA repair,26–28 and prevent cytoskeletal rearrangement resulting from oxidative insult.30 Our purpose in this study was to examine whether KGF may help ameliorate damage caused by oxidative stress in the cells of the BRB, specifically the RPE as represented by ARPE-19 cell culture, using DNA strand break analysis and actin depolymerization as markers of oxidant injury. We also examined the changes in permeability as one functional manifestation of oxidative injury.

METHODS

Cell Culturing

Human adult RPE cells were obtained through ATCC (ARPE-19; Manassas, VA) and were grown in a 1:1 mixture of Dulbecco MEM (DMEM) and Ham F12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (all products from Gibco, Rockville, MD). On confluence, the cells were maintained in DMEM/F12 supplemented with 1% FBS and 1% penicillin-streptomycin. Cells were grown on tissue culture–treated flasks (Nalge Nunc International, Rochester, NY), and the medium was replaced every 3 to 4 days. On passaging with 0.05% trypsin-EDTA (Gibco), cells were replated at a 1:7 to 1:10 ratio. All experiments, except where noted, were conducted on confluent monolayers 10 to 14 days after plating and 4 to 7 days after confluence.

The SUM52-PE cell line is a human breast cancer cell line31 obtained through the University of Michigan Human Breast Cell/Tissue Bank. The cells were grown in serum-free medium32 on 60-mm collagen-coated Petri dishes (Fisher), and the medium was replaced every second or third day. Once the cells reached a confluent monolayer, they were passaged and replated at a 1:6 ratio. For the first 24 hours after plating, the growth medium was supplemented with 2% FBS to ensure cellular attachment to the substrate.

Treatment Protocols

Except where noted, all cells treated with KGF were pretreated at a concentration of 50 ng/mL in DMEM/F12 supplemented with 5% FBS for 24 hours before the experiment. Oxidative insult was achieved by way of a bolus addition of 0.5 mM H2O2. On the day of the experiment, a 10 mM stock solution of hydrogen peroxide (Acros Organics, Morris Plains, NJ) was made in Hanks balanced salt solution (HBSS; Gibco) supplemented with 0.5% BSA and 25 mM HEPES and titrated to a final pH of 7.4 (complete HBSS) and then further diluted to a final concentration of 0.5 mM.

Single-Cell Gel Electrophoresis

DNA damage was detected using the single-cell gel electrophoresis assay (CometAssay; Trevigen, Inc., Gaithersburg, MD). This assay has the ability to sensitively measure the single-stranded DNA strand breaks (DNA-SB) accumulated in the cell populations when exposed to oxidative insult. For the comet assay, cells were grown to confluence in 6-well plates. The day before the experiment, the cells were pretreated with KGF at the concentrations described in the permeability assay. The assay was performed per the manufacturer’s instructions and as outlined in Upadhyay et al.33 An alkaline solution was used to unwind the DNA, allowing the comet assay to measure single-strand breaks and alkali-labile damage. The next day the cells were viewed using an inverted fluorescence microscope (Nikon Eclipse TE 200; Nikon Instruments, Inc., Melville, NY). All images were acquired with a cooled 3-shot interline progressive scan charge-coupled device (Spot RT Slider; Diagnostic Instruments, Inc., Sterling Heights, MI) using a 20× objective and imaging software (Spot v3.1; Diagnostic Instruments, Inc.).

Reverse Transcriptase–Polymerase Chain Reaction Analysis of Exon IIIc/β Expression

Messenger ribonucleic acid (mRNA) was isolated from each of the cell populations through an isolation technique (TRIZOL; Invitrogen, Rockville, MD) that uses a premixed solution of guanidinium thiocyanate and phenol to separate the various cellular components. Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification was conducted using a synthesis system (SuperScript First-Strand Synthesis System; Invitrogen) for RT-PCR with the addition of 5 U/μL polymerase (Platinum Taq DNA Polymerase; Invitrogen). RT was carried out at 42°C for 50 minutes, followed by termination at 70°C for 15 minutes. The PCR reaction consisted of 40 cycles of DNA denaturing at 94°C, followed by 2 minutes of primer pair annealing to the DNA target sequence at 55°C, followed by 2 minutes of primer pair extension at 72°C. These cycles were preceded by an initial 2-minute 94°C incubation and concluded with a 5-minute cycle at 72°C. Primer constructs used for this were specific for exon III of FGR234,5 upstream and downstream of both the IIIb and the IIIC exons; hence, the RT-PCR product can potentially contain both receptor isoforms.35 To differentiate between the two splice variants of which only the IIb variant binds with high affinity to KGF, the RT-PCR product was subjected to digestion by the restriction endonucleases Aral and HincII (Promega, Madison, WI) overnight at 37°C to ensure maximal digestion of our samples per the manufacturer’s instructions. The IIb exon contains a single Aral restriction site, whereas the IIIC exon contains two HincII sites, allowing for the determination of which exon(s) were present in the cell lines.36 After digestion, the product was resolved on a 1% agarose gel at 50 V for 35 minutes, stained with ethidium bromide, and viewed under ultraviolet light. All primer pairs were sequenced at the Northwestern University Biotechnology Laboratory (NUBL; Chicago, IL).

Mitogen-Activated Protein Kinase Activation

ARPE-19 cells were plated onto tissue culture–treated 6-well plates (Costar, Cambridge, MA) at a seeding density of 200,000 cells/well and were allowed to grow to confluence; the medium was changed once a week. On confluence and before measuring MAPK activation, the samples were serum starved overnight in growth medium containing 0.1% BSA without FBS. On the day of the experiment, cells were initially incubated in fresh serum-free medium for 2 hours to reduce basal phosphorylation caused by secreted cellular factors. After this initial 2-hour incubation, fresh, serum-free growth medium containing 10% FBS, 0.1% BSA, or 10, 50, or 100 ng/mL KGF was added to the cells. The cells were then incubated for 10 minutes at 37°C to allow for MAPK activation.

Immediately after incubation, the cells were washed in PBS and then lysed through the addition of 100 μL 1× lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/mL leupeptin; Cell Signaling Technology, Beverly, MA). Lysed cells were scraped off the plate and transferred at 0°C to a microcentrifuge tube. After centrifugation, protein content was measured using a protein assay (Bio-Rad DC; Bio-Rad Laboratories, Hercules, CA). Thirty five microliters of the sample was loaded with a 3× loading buffer (187.5 mM Tris-HCl [pH 6.8], 6% [wt/vol] SDS, 30% glycerol, 0.125 M dithiothreitol, and 0.03% [wt/vol] bromophenol blue; Cell Signaling Technology) into a 12.5% polyacrylamide Tris-HCl gel and was resolved, along with 10 μL prestained standards (Kaleidoscope; Bio-Rad Laboratories), for 45 to 60 minutes at 140 V. After running the gel, proteins were transferred electrophoretically to a polyvinylidene difluoride membrane (Immobilon-P Transfer Membrane; Millipore, Billerica, MA) using a wet transfer process. The membrane was labeled overnight at 4°C with a phospho-p44/p42 MAPK monoclonal mouse antibody (Cell Signaling Technology) that only binds to p44/p42 MAPK dually phosphorylated at the threonine 202 position and the tyrosine 204 position. The next day, the membrane was washed in TBS-T and was labeled with a rabbit anti-mouse IgG horseradish peroxidase (HRP)–linked secondary antibody (Bio-Rad Lab-
KGF Proliferation Assay

One of the known effects of KGF on epithelial cells expressing the KGF receptor (KGFRI) is enhanced proliferation.21,36 We tested for the presence of this effect on the ARPE-19 cell line. Cells were seeded at a plating density of 10,000 cells per well on a tissue culture-treated 12-well plate (Costar) and were allowed to attach to the substrate for 24 hours. After this time period, the cells were serum starved for an additional 24 hours, at which time control cells were harvested to obtain a plating number. The remaining cells were divided into three test groups, and the media were replaced according to test group: regular medium (10% FBS), growth medium with 1% FBS, or growth medium with 1% FBS and 50 ng/mL KGF. Media were changed every other day for 6 days, at which time the remaining cells were counted in triplicate.

Measurement of Monomeric and Filamentous Actin

Monomeric actin (g-actin) and a small amount of filamentous actin (f-actin) not cross-linked to the cytoskeleton are soluble in NP-40. Once f-actin fibers are attached to the cytoskeleton, they are no longer NP-40 soluble. This difference in NP-40 solubility was used to quantify the portion of total cellular actin that contributes to the cytoskeletal structure of a cell using the technique of Forte et al.37 After application of various test conditions, cells on 12-well culture plates were washed twice with warmed (37°C) PBS and immediately scraped off the plates and pelleted at 400g for 1 minute. The pellet was resuspended in a 20× excess of extraction buffer (0.1% Nonidet P-40, 5 mM KH2PO4, 27 mM Na2HPO4 [pH 7.2], 2 mM MgSO4, 2 mM EGTA, 0.2 mM adenosine triphosphate [ATP], 0.5 mM dithiothreitol, 2 M glycerol, and 1 mM phenylmethylsulfonyl fluoride) and was agitated at room temperature for 15 minutes. The mixture was then pelleted at 400g for 1 minute, and the supernatant containing the non–cross-linked f-actin was collected and labeled g-actin. The cytoskeletal f-actin, and any other proteins associated with the cytoskeleton, remained in the pellet, and that fraction was resuspended in a 20× excess of the extraction buffer and labeled f-actin. The two samples were stored at ~80°C for Western blot analysis.

Cytoskeletal rearrangement was accomplished through the bolus addition of 0.5 mM H2O2 over a 2-hour period, as previously described in Boardman et al.39 Epithelial cells were used from passages 23 to 30 and were plated at a density of 200,000 cells/cm² onto tissue culture-treated 6-well plates. Cells were pretreated with KGF at a concentration of 50 ng/mL.

Permeability Experiment Protocol

All permeability experiments were conducted as described in Waters et al.23 Epithelial cells were used from passages 23 to 30 and were plated at a density of 200,000 cells/cm² onto collagen-coated, 0.4-µm, 12-well transwell inserts (Costar) pretreated with laminin at 10 µg/cm².39 Thirty minutes before the start of the experiment, cells were rinsed with complete HBSS, and 0.5 mM H2O2 was added to the cells apically and basolaterally for 30 minutes. At the start of the experiment, the complete HBSS in the apical chamber was removed and replaced with a sodium fluorescein tracer in complete HBSS, with or without 0.5 mM hydrogen peroxide. Permeability measurements were made at 15, 30, 60, 90, 165, and 245 minutes. Transepithelial resistance (TER) values were also measured at 30, 60, 165, and 245 minutes using a voltmeter (EVOM Voltmeter; World Precision Instruments, Fort Lauderdale, FL) coupled with a chamber (Endohm 12; World Precision Instruments).

Occludin Staining Protocol

Tight junction formation in our cellular monolayers was verified through staining for the tight junctional protein occludin by immunofluorescence staining. Cells were grown to confluence on sterile round microscope slide coverslips placed in a 12-well plate with an initial seeding density of 10,000 cells/well. Cells were fixed and stained according to the protocol outlined in Chapman et al.39 After fixation and immunostaining, the coverslips were mounted onto glass slides using mounting medium (Vectashield; Vector Laboratories, Burlingame, CA) with a counterstain (DAPI; Vector Laboratories) and were viewed under a 20× objective.

Statistical Analysis

All data were analyzed using an add-in (Excel; Microsoft, Redmond, WA) version X (XL-STAT; Addinsoft, Paris, France). Data were subjected to either a heteroscedastic or a homoscedastic two-tailed t-test, depending on the variance of the data collected. Data sets were analyzed using a two-tailed Wilcoxon Mann-Whitney nonparametric test. Significance was established for either test at P < 0.05 or better (95% confidence level) for each data set.

RESULTS

The purpose of our experiments was to test the hypothesis that KGF could ameliorate damage to the ARPE-19 caused by ROS, as measured by changes in monolayer permeability and DNA strand breaks. After characterizing the cellular response to hydrogen peroxide and to KGF and carefully examining monolayer formation, we conducted several sets of experiments to determine cytoskeletal rearrangement, nuclear DNA-SB formation, and monolayer permeability changes resulting from oxidative insult.

Fibroblast Growth Factor Receptor 2

The presence or absence of FGFR2 was evaluated in the ARPE-19 cell line using RT-PCR with primer sequences for FGFR2. Glycereraldehyde 3-phosphate dehydrogenase (GAPDH), a catalytic enzyme involved in glycolysis that is constitutively expressed at high levels in almost all tissues, was used as an internal control, whereas SUM52-PE cells were used as an internal control, whereas SUM52-PE cells were used as an FGFR2 control because they had previously been shown to express FGFR2.35 Figure 1 shows that the 367-bp product of FGFR2 was present in the ARPE-19 cell line.

Further studies were conducted on the ARPE-19 cell line to determine which receptor isoform was present. The restriction endonucleases AvaI and HincII were used to determine the presence of each isoform. Figure 2 shows that for our samples, AvaI digested both the SUM52-PE and the ARPE-19 samples, suggesting the only isoform present in these samples was...
FGFR2 IIIb. The result for the SUM52-PE cell line is consistent with prior findings.35

Mitogenic Response to KGF

KGF has previously been shown to be a potent mitogenic substance for epithelial cells.40,41 Furthermore, the activity of KGF is mediated at least in part through a MAPK-dependent process. Therefore, we chose to look at cellular proliferation and MAPK activation as two independent verifications of KGF activity. As can be seen in Figure 3, by day 6 there was a significant difference in the populations receiving 10% FBS or 1% FBS + KGF compared with those cells only receiving 1% FBS. From these data it is apparent that KGF has a proliferative effect on the ARPE cell line.

The second study targeted MAPK activity in response to increasing concentrations of exogenous KGF. As can be seen in Figure 4, increasing concentrations of exogenous KGF increased the amount of phosphorylated p42/44, as determined by densitometry. This result suggests KGF increases MAPK activity in a dose-dependent manner.

DNA Strand-Break Analysis

DNA strand breaks are one of the earliest cellular changes that occur in cells after oxidative stress. Wu et al.26 found that oxidative stress events as small as the bolus addition of 50 μM \( \text{H}_2\text{O}_2 \) caused DNA-SB formation in cultured A549 cells and rat alveolar type 2 cells. The principle of single-cell gel electrophoresis is that when denatured, cleaved DNA fragments are placed under an electric field, they migrate out of the cell faster than undamaged DNA. This causes damaged DNA to form a “tail” in the direction of the electric field as the smaller fragments migrate out of the cell “head” and as undamaged DNA remains within the confines of the nucleoid, causing cells with damaged DNA to look much like a comet, as can be seen in Figure 5.

As shown in Figure 6, the number of cells with measurable DNA-SBs was reduced significantly through the addition of 50 ng/mL KGF before the cells were subjected to bolus additions of \( \text{H}_2\text{O}_2 \), suggesting an increase in cells experiencing low (immeasurable) or no DNA-SB.

Cytoskeletal Rearrangement

Figure 7 shows that KGF prevented cytoskeletal rearrangement in the ARPE-19 caused by oxidative damage consisting of a bolus administration of 0.5 mM \( \text{H}_2\text{O}_2 \) for 2 hours. Through visual inspection and densitometry, a noticeable and significant decrease in the amount of filamentous actin could be seen when the cells were exposed to hydrogen peroxide. These changes were negated through pretreatment with 50 ng/mL KGF for 24 hours before the chemical insult.

Epithelial Cell Permeability

Figure 8 shows that KGF was unable to prevent permeability changes in the ARPE-19 cells because of oxidative damage.
consisting of a bolus administration of 0.5 mM H_2O_2. This result was verified at the conclusion of our experiments with TER measurements. These results are summarized in Table 1. We chose to use 0.5 mM H_2O_2 in our experiments because at concentrations lower than this, permeability changes were negligible (data not shown). Previous studies from Garg and Chang have shown that this is a sublethal concentration of hydrogen peroxide in ARPE-19 cells resulting in no change in cell permeability, whereas Alizadeh et al. demonstrate that this level of exogenous H_2O_2 indeed increases intracellular reactive oxygen intermediates. In Figure 8 and Table 1, H_2O_2 cells are those that were administered H_2O_2 30 minutes before the experiment; H_2O_2 + KGF cells are cells that were pretreated for 24 hours with 50 ng/mL KGF before H_2O_2 insult 30 minutes before the start of the experiment; KGF cells are cells that were pretreated overnight with 50 ng/mL KGF; and control cells are cells that received neither KGF nor H_2O_2. Experiments were also conducted in cells that were cultured for 90 days, allowing for differentiation of the ARPE-19 cells, with no changes in the results (data not shown).

**DISCUSSION**

Cells from the central nervous system are protected from most substances normally found in the extracellular fluid. The retina, formed out of the embryonic brain, is no exception, hence, resulting in the formation of the blood-retinal barrier. Because the retina is nourished through two forms of circulation, two barriers exist—the inner and the outer BRB. Retinal vessels act as a boundary between the retinal circulation and the retina through the tight-junctioned endothelial cells lining the retinal vessels whereas the RPE separates the choroidal circulation and the retina.

### Tight Junction Formation

Occludin immunofluorescence staining was performed on the ARPE-19 cell line on day 9 after plating. As shown in Figure 9, fluorescence labeling showed regional variation for the presence of occludin. This result is consistent with findings in other studies of the ARPE-19 cell line.

### TABLE 1

Table Summary of Transepithelial (Transendothelial) Electrical Resistance (TER) Data

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Epithelial Resistance (Ω × cm^2)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>39.6 ± 3.44</td>
<td>12</td>
</tr>
<tr>
<td>KGF</td>
<td>40.6 ± 3.59</td>
<td>12</td>
</tr>
<tr>
<td>H_2O_2</td>
<td>12.5 ± 2.54</td>
<td>16</td>
</tr>
<tr>
<td>KGF + H_2O_2</td>
<td>8.75 ± 2.85</td>
<td>17</td>
</tr>
</tbody>
</table>

Resistance measurements were made at t = 30, 60, 165 and 245 minutes.
KGF is a heparin-binding mitogenic member of the fibroblast growth factor family that has recently been targeted in a number of studies as a substance that can protect cells from a variety of toxic insults, including oxygen-mediated damage.23–29 This protection occurs on a cellular and a subcellular level; KGF has been shown to protect barrier function and to aid in wound healing in many cell lines, and it has also been shown to prevent DNA-SB formation and to augment DNA repair. The purpose of our study was to examine the effects of KGF on cultured retinal pigmented epithelial cells. The cells were exposed to an oxidative-insult consisting of a bolus administration of H$_2$O$_2$ to determine whether KGF could attenuate cellular damage resulting from this exposure as measured by cytoskeletal rearrangement, DNA damage, and barrier function loss. Our studies showed that KGF activated MAPK at doses as low as 10 ng/mL and was effective in protecting cultured ARPE-19 cells from DNA-SB and cytoskeletal rearrangements at concentrations similar to those used in other studies involving KGF.25,26,30,39,45,46 It was unable to prevent a loss of barrier function in our culture system.

Hydrogen peroxide attacks a variety of cellular constituents; DNA is one of the most important targets. Lethality involves DNA damage by oxidants generated from iron-mediated Fenton reactions.30 Damage by Fenton oxidants may occur at the DNA bases or sugars, with the predominant consequence of sugar damage being strand breakage and eventual base release. Although mechanisms are in place to repair such damage, an accumulation of DNA damage can lead to cell death if the damage is not repaired properly.43 A study from Wu et al.55 suggests that the protective mechanism invoked by KGF involves prevention of DNA-SB and enhanced DNA repair. In our tests, not only was the severity of the DNA damage decreased by the pretreatment of KGF, as measured by comet tail length (data not shown), the number of cells experiencing some form of DNA damage was also drastically reduced. With 100 μM H$_2$O$_2$, only 16.6% of the total number of cells displayed no or immeasurable DNA damage (40 of 241 cells counted), and this number more than doubled (36.6%) when the cells were pretreated with KGF before H$_2$O$_2$ exposure (90 of 246 cells).

A second target of hydrogen peroxide is the cell cytoskeleton. It has been recognized that oxidative insult causes a disruption of cytoskeletal filaments.49 Furthermore, several studies have suggested that KGF protection of the epithelial cell barrier function is mediated through the cytoskeletal function.25,46,50 Two of these studies25,46 show that epithelial barrier function was retained through the pretreatment of KGF and that this barrier function corresponded to the retention of the perijunctional actin ring, which in insulted cells only occurred when those cells were pretreated with KGF. Liu and Sundqvist51,52 showed similar results in endothelial cells in that a dose-dependent increase in hydrogen peroxide led to an increase in permeability and an increase in actin rearrangement. In our studies, we saw a significant increase in the monomeric actin pool after 2-hour treatment with 0.5 mM H$_2$O$_2$ that was ameliorated through the pretreatment of the cell population with 50 ng/mL KGF. Based on the previous results suggesting that the retention of barrier function corresponds to the retention of filamentous actin, our results suggest that KGF may also be able to prevent permeability increases in the RPE monolayer caused by oxidative insult.

Although we were able to reduce the amount of oxygen-mediated DNA damage and cytoskeletal rearrangement, we were unable to reduce permeability increases associated with this damage. One concern with our results is the low measured TER values. Although in vivo RPE cells produce highly impermeable barriers, we have had little success in developing cultures with high TER values, which is a standard measurement for determining cell monolayer tightness. Our RPE TER measurements—though in line with values reported by Dunn et al.54 when they measured ARPE-19 monolayer permeability for cells grown in DMEM-F12 plus 1% FBS and close to the values reported by Chang et al.53 for freshly isolated rat RPE cells when grown in FBS—are still significantly lower than values expected from tight epithelium layers and are significantly lower than TER values we have measured in other epithelial cell lines. Chang et al.53 determined that serum is an inhibitor of tight junction formation in cultured RPE cells, though it does not completely inhibit junction assembly, nor does it affect cytoskeletal organization or cell-to-cell attachment. When Chang53 and Dunn54 grew their cells in a hormonally defined medium, an increase in TER was measured by both. This phenomenon of serum reducing epithelial cell monolayer permeability is not unique to RPE cells; several researchers have found serum factors that open tight junctions in a variety of epithelia.54–56 Even by switching to a hormonally defined medium, RPE TER measurements were still far lower than those of other epithelia.

In addition to serum inhibition of tight junctions, it has been shown that in vitro primary RPE cell cultures express large levels of phenotypic heterogeneity and that only certain phenotypes express prominent junctional distributions.57 As reported by Dunn et al.,58 even though ARPE-19 is a cell line, these cells were derived from primary cultures without any type of transfection. Using electron microscopy, they found that when the ARPE-19 cells were grown to post-confluence densities, they demonstrated junctional complexes, but the ultrastructure was not present in all the cells sampled. This was evident in our occludin studies, as shown in Figure 9, in which certain regions showed complete tight junction formation that was not present in other regions of the monolayer. Attempts made by Dunn et al.59 to subclone this cell line to enhance various cellular characteristics (such as increased TER) proved unsuccessful as the resultant sublines either rapidly senesced or did not result in line with improved properties. Other RPE cell lines, such as D407 cells50 and RPE-J cells, may be inappropriate because of their polysomy and transformation with SV40 virus, respectively.59 In our hands, D407 cells produced transepithelial resistances that were lower than those of ARPE-19 cells, and though Nabi et al.59 have reported high TER measurements in their RPE-J lines, these higher resistances are only obtained on differentiation requiring the use of permissive temperatures to achieve differentiation. This artificially in-
duced cell differentiation raises questions as to what other cellular phenomena are altered by introduction of the temperature-sensitive SV40 virus. Kannan et al. demonstrated the ability to culture primary cells that achieve high TER; however, because these are primary cells derived from fetal human tissue, their usefulness as a standard model may be limited. Each of these cell models also has its strengths, resulting in the necessity for researchers to pick models based on their own particular interests. These results also demonstrate the necessity for the development of an RPE cell line with barrier characteristics similar to those found in vivo.

Regardless, the lack of complete junction formation in our cell model may help explain the inability of KGF to protect these cells from barrier permeability changes caused by oxidative stress. This is different from the situation in vivo, where the RPE has been shown to have complete tight junctional formation. Because KGF did prevent oxygen-induced DNA damage and cytoskeletal rearrangement, it is possible that in vivo, with complete tight junction formation, KGF will prevent permeability changes caused by oxidative insult.

We have successfully shown through a variety of experiments that the ARPE-19 cell line is responsive to KGF and that KGF is able to prevent oxygen-mediated DNA damage and cytoskeletal rearrangement. KGF was unable to prevent permeability changes in cultured cell monolayers, but this might have been because of the incomplete tight junction formation shown by occludin staining and freeze-fracture analysis. In vivo, these cells do have complete junction formation, and KGF may be effective in preventing permeability increases resulting from oxidative stress in this system.

Acknowledgments

The authors thank Melody Swartz, David Dean, and Dave Kelso for use of their facilities and equipment.

References


48. Schrautstatter IU, Hinshaw DB, Hyslop PA, Spragg RG, Cochrane CG. Oxidant injury of cells. DNA strand-breaks activate polyade-