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ORIGINAL PAPER

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Cellular distribution of lens epithelium-derived growth factor (LEDGF) in the rat eye: loss of LEDGF from nuclei of differentiating cells

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Abstract Lens epithelium-derived growth factor (LEDGF) enhances the survival and growth of cells. To understand LEDGF's spatial localization and its putative function(s) during proliferation and differentiation, we localized LEDGF during terminal differentiation in whole rat lenses, lens epithelial cell (LEC) explants stimulated with FGF-2, and insulin, iris, human LECs with lentoids. In addition, intracellular localization of LEDGF was performed in other ocular tissues: ciliary body, retina, and cornea. We found the immunopositivity of nuclear LEDGF decreased in LECs of the equatorial region. In contrast, immunopositivity of LEDGF was detected in the cytoplasm of LECs and superficial fiber cells. After treating LEC explants with FGF-2 and insulin, which are known to be differentiating factors for LECs, the nuclei of these cells showed no LEDGF immunopositivity, but explants did express p57[kip2], a differentiation marker protein. Also, immunopositive LEDGF was not detected in the nuclei of differentiated cells, lentoid body, and corneal epithelial cells. This demonstrated that the loss of LEDGF from the nucleus may be associated with the process of terminal differentiation that might be in some way common with the biochemical mechanisms of apoptosis. The spatial and temporal distribution of LEDGF in the present study also provides a vision for further investigation as to how this protein is involved in cell fate determination.

Keywords LEDGF · Immunohistochemistry · Differentiation · In situ hybridization · Lentoid body · LEC explants

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Introduction

The ocular lens contains three principal cell types: central lens epithelial cells (LECs), germinative zone epithelial cells, and fiber cells. In the human lens, most central LECs survive for decades without dividing or differentiating, but approximately 14% of these cells die over the course of a 75-year lifetime, leaving gaps or areas covered by attenuated extensions of adjacent cells in the central epithelial monolayer (Balaram et al. 2000). The germinative LECs, found in the pre-equatorial region of the lens, are aptly named, for they divide and differentiate into elongated, fusiform, crystallin-rich, organelle-deficient, anucleate fiber cells. This transformation is called terminal differentiation (TD) or fiber differentiation. The TD begins in epithelial cells of the bow region and proceeds to completion in the deeper layers of the superficial cortex (Piatigorski 1981). Many investigations have greatly increased our understanding of the roles of growth factors in TD. For example, acidic and basic fibroblast growth factors (FGF-1 and FGF-2, respectively; Chamberlain and McAvoy 1987, 1989; Lovicu and McAvoy 1989; McAvoy and Chamberlain 1989), insulin growth factor-1 along with FGF (Chamberlain et al. 1991;

Klok et al. 1998), and epidermal growth factor (Ibaraki et al. 1995) all induce TD in LECs.

In contrast to the abundant research on growth factors and TD in the lens, there are few studies on factors involved in LEC survival and death. In 1992–3, Ishizaki et al. (1993) and Raff (1992) showed that LECs secreted survival factors, but they were not able to identify them. Renaud et al. (1994) showed that endogenous FGF-1 expression was higher in non-dividing LECs than in exponentially dividing cells and suggested that FGF-1 was not a mitogen but a survival factor. Also Stolen et al. (1997) demonstrated that FGF-2 modulated not only the late stages of differentiation, but also the survival of lens fiber cells. We have reported that lens epithelium-derived growth factor (LEDGF) enhances survival, adhesiveness, and growth of mouse LECs and other cell types in culture (Singh et al. 1999, 2000a; Nakamura et al. 2000) and we have shown that heparin even further enhances LEDGF's effects on cell growth and survival (Fatma et al. 2001). Furthermore, LEDGF is a novel transcriptional factor (Fatma et al. 2001; Singh et al. 2001a) and is identical with p75, a known co-activator of transcription (Ge et al. 1998a, b; Singh et al. 2000b). If overexpressed in LECs, LEDGF increased LEC resistance to various stresses (thermal, oxidative) and upregulated the expression of heat shock proteins, Hsp27, antioxidant protein 2, and αB-crystallin by binding to specific sites in each gene promoter (Singh et al. 1999, 2001a; Fatma et al. 2001). LEDGF was also upregulated when cells were exposed to thermal and oxidative stresses (Sharma et al. 2000). We have shown that the upregulation of stress proteins by LEDGF is one of the mechanisms by which LEDGF prolongs LEC survival (Singh et al. 1999).

This paper describes the spatial distribution of LEDGF protein and LEDGF mRNA in tissues of the rat eye using immunohistochemical and in situ hybridization techniques. We have also studied the effect of age on this distribution. The findings described herein link changes in the immunoreactivity of nuclear LEDGF levels to TD in four model systems: whole rat lens, rat LEC explants in which differentiation has been induced, lentoids produced in cultured human LECs, and cornea. The immunoreactivity of p57^{kip2}, a differentiation marker, was also examined in rat LEC explants.

Materials and methods

Animals

We used 3-, 7-, and 13-week-old Sprague-Dawley albino rats (Charles River, Wilmington, Mass., USA). All animals were handled in accordance with the ARVO Policies on the Use of Animals in Research. The rats were euthanized with CO_2 and then perfused through the left ventricle with 4% paraformaldehyde (PFA) in 20 mM phosphate-buffered saline (PBS), pH 7.4.

Antibody generation, production, purification, and neutralization

A polyclonal antibody (Ab) to a C-terminal peptide of human LEDGF (YNKFKNMFLVGEGDSVITQ) corresponding to the amino acid sequence (420–438) (Singh et al. 2001a) was raised in rabbits. This C-terminal region does not share homology with any known protein as determined by computer analysis program, BLASTp, the Basic Local Alignment Search Tool for Protein developed by NCBI, National Center for Biotechnology Information. The IgG fraction of the immune serum was affinity purified with Immunopure Immobilized Protein A (Pierce, Rockford, Ill., USA) column chromatography and concentrated with Centricon Plus 20 (Millipore, Bedford, Mass., USA). We neutralized the purified anti-LEDGF Ab by adding 250 μl keyhole limpet hemocyanin (KLH) and 500 μg GST-LEDGF to a 500-μl aliquot of purified Ab preparation. The techniques for producing and purifying the GST-LEDGF fusion protein have been published (Fatma et al. 2000). We controlled for non-specific Ab absorption by adding 250 μl KLH and 500 μg bovine serum albumin (BSA) to a second 500 μl aliquot of the purified Ab preparation, and both solutions were incubated overnight at 4°C and then centrifuged at 8,000 rpm for 5 min. The supernatants of these two solutions were designated the "GST-LEDGF-neutralized Ab" and the "anti-(C-terminal) LEDGF Ab" preparations. The concentration of protein in each Ab preparation was measured by the Bradford (1976) method and equalized at 1.28 mg/ml. P57^{kip2} antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) was used as differentiation marker.

Preparation of tissue sections

The lens was fixed for 48 h in 4% PFA in PBS, and other ocular tissues were dissected and immersed in the same solution for 24 h. Cornea, lens, iris, ciliary body, and retina were embedded in paraffin (Paraplast Plus; Oxford Labware, St. Louis, Mo., USA) and sectioned at 4 μm. The sections were deparaffinized in xylene and immersed in a graded series of ethanol and xylene. For cryosectioning, lenses were fixed for 48 h in 4% PFA in PBS and embedded in OCT compound (Tissue-Tek; Sakura Finetek, Torrance, Calif., USA), frozen, and then sectioned on a cryostat at 10 μm. The frozen sections were dried, washed in PBS, and then immersed in cold 100% methanol at −20°C for 2 min.

Flat preparations of rat lens epithelial cells

Lenses from 3-week-old rats were placed on a polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore). The posterior lens capsule was opened and the lens fibers were removed from the epithelial layer and capsule *en masse*. The remaining capsule epithelium was spread on the PVDF membrane, and the membrane was transferred to 4% PFA in PBS, pH 7.4, for 30 min at 4°C.

Preparation of paraffin sections of cultured human lentoids

Paraffin blocks containing human LECs (HLECs) and lentoid bodies were prepared as follows. The initial monolayer of cells obtained from anterior capsule explants after 2 weeks were trypsinized and subcultured twice to obtain HLECs as described previously (Arita et al. 1988, 1990). After the second subculture (passage 2) 1×10^5 cells were plated on Millipore CM 10-mm inserts (Millipore) in 12-well plates. The HLECs were cultured in DMEM medium (Gibco BRL, Grand Island, N.Y., USA) containing 20% fetal bovine serum. The medium was changed twice each week. By the 4th day, lentoids had formed on the surface of the membrane. The lentoids were fixed with 3% PFA in PBS after 2 and 3 weeks of incubation, embedded in paraffin, and sectioned at approximately 4 μm.

Preparation of rat LEC explants

Lens epithelial explants were cultured, and paraffin sections were prepared as previously described (Rampalli and Zelenka 1995). Briefly, lenses were removed from 4-day-old rats, and the lens epithelium was separated from the fiber mass through a tear in the posterior capsule. A square explant was cut from the central region of the lens epithelium and pinned down to the surface of the culture dish with the capsular surface toward the plastic. Differentiation was initiated by adding 1 μg/ml porcine insulin (Gibco) and 100 ng/ml human FGF-2 (Gibco) to Medium 199 (Gibco) containing penicillin and streptomycin and incubating the cells for 6, 24, and 48 h. Control explants (0 h) were fixed immediately without culturing. All epithelia were fixed in 4% PFA in PBS at room temperature for 2–3 h. The epithelia were embedded in paraffin and sectioned at 7 μm thickness.

Immunohistochemical staining

After washing in PBS, all sections were placed in 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. Paraffin sections from rat eye tissues and flat preparation of rat lens were immersed in 0.1% trypsin for 25 min, and paraffin sections of cultured LECs and explants were immersed in 0.1% trypsin for 2 min. After washing in PBS, all tissue sections and flat preparations were incubated for 30 min with 10% normal goat serum (Dako, Carpinteria, Calif., USA). Then they were exposed overnight at 4°C to the anti-(C-terminal) LEDGF Ab in PBS containing 5% dry milk or 10% goat serum. Antibody preparations were diluted 1:500 for use with frozen lens sections, and 1:750 for use with paraffin sections and flat preparations. After washing in PBS, these tissue sections were exposed to biotinylated anti-rabbit immunoglobulins in PBS containing carrier protein (Dako) for 2 h at room temperature and then washed again in PBS. The specimens were incubated in avidin–peroxidase-conjugated streptavidin (Dako) at room temperature for 2 h. Visualization of the antibody complex was carried out by adding a 0.02% solution of 3,3′-diaminobenzidine (DAB; BioRad Laboratories, Hercules, Calif., USA) and 0.05% hydrogen peroxide in TRIS-buffered saline, pH 7.6, containing 1 mM $CaCl_2$. Negative controls were incubated with the GST-LEDGF-neutralized Ab preparation overnight at 4°C. All sections, except the frozen sections and explants, were counterstained with Mayer's hematoxylin (Sigma, St. Louis, Mo., USA). In rat LEC explants, sections for fluorescence probes were also immunostained with anti-LEDGF Ab diluted 1:1,000 with 10% goat serum and anti-goat $p57^{kip2}$ Ab (Santa Cruz Biotechnology) diluted 1:200 with 5% BSA in PBS. As secondary Abs we used anti-rabbit IgG-HRP or anti-goat IgG-HRP (Santa Cruz Biotechnology). Sections were visualized with cyanin-3 tyramide fluorescence probe (NEN Life Science, Boston, Mass., USA).

Protein blot analysis

Lens capsule epithelium and whole retina were obtained from each of fifteen 3-week-old rats. The capsule epithelia and retinas were pooled and suspended in 1.0-ml aliquots of ice-cold RIPA buffer (1% Igepal CA-630; Sigma), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS; Gibco) containing 1,000 U/ml heparin (Gibco), 1 mM phenylmethylsulfonyl fluoride (Sigma), and one protease inhibitor cocktail tablet (Complete; Roche, Mannheim, Germany) per 50 ml to protect LEDGF from degradation (Fatma et al. 2000). Tissue specimens were homogenized with a Dounce homogenizer, centrifuged at 12,000 rpm for 15 min at 4°C, and the supernatant was collected. The protein concentration of each supernatant was determined by the Bradford (1976) method. The protein lysate was mixed with SDS polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (1:1, v/v), and samples were run on a 7.5% SDS-PAGE gel, transferred to a PVDF membrane (Immobilon-P; Millipore), and blocked with PBS-T (PBS containing 0.05% Tween 20), 7% dry milk, and 10% goat serum. The blots were incubated overnight at 4°C with affinity-purified rabbit anti-(C-terminal) LEDGF IgG (diluted 1:2,000 in PBS-T containing 5% dry milk and 5% goat serum. After washing in PBS-T, the membranes were incubated with goat anti-rabbit IgG labeled with horseradish peroxidase (diluted 1:2,000; Santa Cruz Biotechnology) for 2 h at room temperature and visualized by enhanced chemiluminescence according to the company's protocol (Western Blotting Luminol Reagent; Santa Cruz Biotechnology). Negative controls were incubated overnight with the GST-LEDGF-neutralized Ab preparation at 4°C. Full-size GST-LEDGF (MW 89 kDa) was run with these samples as a positive control.

In situ hybridization

After killing the 3-week-old rats, the eyes were rapidly removed and fixed with 4% PFA in 0.1% diethyl pyrocarbonate-treated PBS. Lenses were embedded in paraffin and sectioned at 4 μm. The following 48-bp antisense LEDGF oligonucleotide probe was used: 5′-GCT CTC CCC GTT ATG TTG TGG CTG ATT ACC ATC TTG AGC ATC AGA TCC-3′ (Life Technologies, Rockville, Md., USA). The GC content of this probe was 50%. This sequence corresponded to nucleotides 1751–1798 of the LEDGF cDNA sequence (Singh et al. 2000b), which is specific for LEDGF and would not pick up p52-specific transcripts. Control hybridizations were conducted using a corresponding 48-bp sense probe. An antisense mouse GAPDH RNA probe provided by the company (Ambion, Austin, Tex., USA) was used as a positive control. All probes were labeled with biotin using a Psoralen Biotin conjugate according to the protocol in the Ambion's BrightStar Psoralen-Biotin Nonisotopic Labeling kit. Briefly, the probes were diluted in TE (10 mM TRIS-HCl, pH 8, 1 mM EDTA) at a concentration of 50 ng/μl and then transferred to a well in the microtiter plate. Psoralen-Biotin reagent, 1 μg/10 μl, was added to each well containing the probes. A 365-nm UV light source was placed on the plate directly over the samples, and the samples were irradiated for 45 min. Non-crosslinked Psoralen-Biotin reagent was removed with distilled H_2O saturated with n-butanol. The following in situ hybridization procedure was performed using Ambion's mRNAlocator-Hyb kit. The labeled antisense and sense probes were diluted to a final concentration of 10 ng/100 μl (per each slide) with kit hybridization buffer. The labeled GAPDH probes were diluted to a final concentration of 1 ng/100 μl (per slide) with hybridization buffer. Sections were hybridized with the labeled probes at 65°C for 5 min followed by hybridization at 42°C overnight (for antisense and sense LEDGF probes) and at 50°C overnight (for the GAPDH probe). The slides were then washed with the kit's in situ wash buffer at 55°C for 30 min. Biotin detection procedure was performed using Ambion's mRNAlocator-Biotin kit. Briefly, sections were incubated with streptavidin-alkaline phosphatase conjugate (1:300 dilution with 1×TRIS buffer) at 37°C for 1 h. After washing in 1×TRIS buffer, sections were incubated with nitro blue tetrazolium salt/5-bromo-4-chloro-3-indolyl phosphate in bimethylformamide solution at 37°C for 1 h. We monitored the color development, then dehydrated and mounted the sections. Photomicrographs were taken with brightfield microscopy.

Results

Protein blot analysis of LEDGF in rat lens and retina

We first monitored the specificity of anti-LEDGF antibody to GST-LEDGF recombinant protein, and the presence of LEDGF in LECs and retina using protein blot analysis. Figure 1 presents western blots of purified GST-LEDGF (Fig. 1 *lanes A*, *B*), LEDGF from rat lens capsule epithelium (Fig. 1 *lanes C*, *D*), and rat retina (Fig. 1 *lanes E*, *F*) using the anti-(C-terminal) Ab preparation

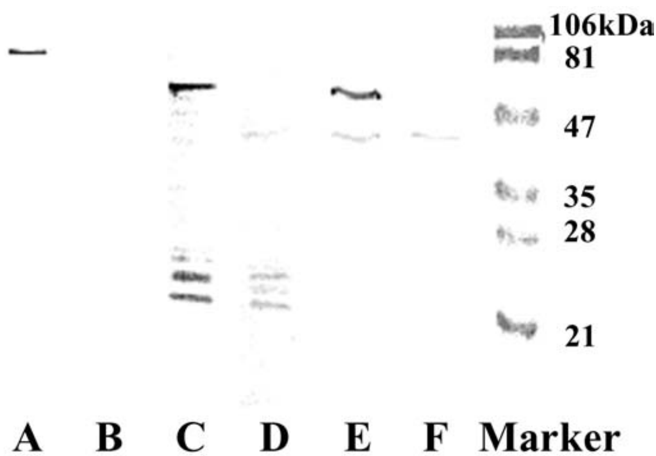

Fig. 1 Protein blot analysis of lens epithelial cell (LEC) and retinal proteins with anti-lens epithelium-derived growth factor (LEDGF) antibody. Purified GST-LEDGF (*lanes A*, *B*), total rat LEC preparation (*lanes C*, *D*), and rat retinal cell preparation (*lanes E*, *F*) were immunostained with anti-LEDGF antibody (Ab; *lanes A*, *C*, *E*) and with the GST-LEDGF-neutralized LEDGF Ab (*lanes B*, *D*, *F*). Stained protein bands at 89 and 60 kDa denote GST-LEDGF and native LEDGF, respectively. *Marker lane* contains molecular weight markers in kilodaltons

(Fig. 1 *lanes A*, *C*, *E*) and the GST-LEDGF-neutralized Ab preparation (Fig. 1 *lanes B*, *D*, *F*). In Fig. 1 *lane A* note the strong staining at 89 kDa, the MW of the GST-LEDGF conjugate (GST MW=29 kDa; LEDGF MW=60 kDa). In Fig. 1 *lane C*, the strong band at 60 kDa (endogenous LEDGF) and some minor bands were observed. Figure 1 *lanes B*, *D*, *F* shows the disappearance of the 89 and 60 kDa bands. Some of the minor bands at approximately 28 and 50 kDa (capsule epithelium) and 43 kDa (retina) that stain non-specifically did not disappear in the absorbed Ab preparation.

Immunohistological localization of LEDGF in rat lens

Cross-sections

The immunohistological localization of LEDGF in the 3-week-old rat lens is presented in Fig. 2 (paraffin section). The nuclei and cytoplasm of LECs in the germinative zones (Fig. 2A, B) were stained strongly. The nuclei of LECs in the central epithelium were also stained (data not shown). As one approaches the equator, the intensity of nuclear staining diminishes markedly (Fig. 2B), and at or near the postequator, the nuclei do not stain. The cytoplasm of the superficial fibers in the anterior cortex is only weakly immunopositive for LEDGF while the cytoplasm of the superficial fibers in the posterior cortex is strongly immunopositive (Fig. 2A, B). The intense staining of cytoplasmic LEDGF evident in the most superficial equatorial and posterior lens fibers, appears diminished in the deeper cortical fibers (Fig. 2A). The intensity of immunostaining was significantly reduced in the sections stained with the GST-LEDGF-neutralized Ab (Fig. 2C). The pattern of LEDGF immunostaining of the lens did not vary with age among rats at 3, 9, and 13 weeks of age (data for 9- and 13-week-old rats not shown). These results indicate that the pattern of expression of LEDGF varies with the region in the lens. We chose to confirm this by looking at LEDGF expression in flat preparations of lens epithelium.

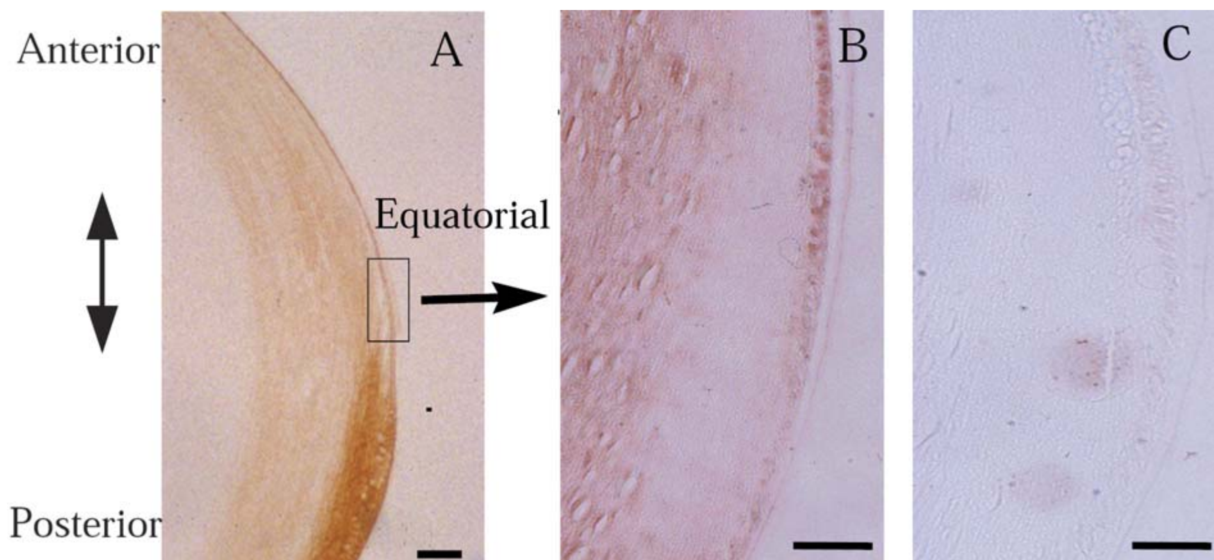

Fig. 2A–C Immunohistochemical staining of LEDGF in the 3-week-old rat lens. Paraffin sections of rat lens immunostained with anti-LEDGF Ab (**A**) or with LEDGF-neutralized Ab (**B**, **C**). The intense immunostaining of nuclei, the weak staining of the cytoplasm in the germinative LECs, and the gradual loss of staining in the nuclei of the equatorial bow region and the nucleated superficial fiber cells were observed. Cortical fibers in the posterior region were strongly immunostained with anti-LEDGF Ab. **A** *Bar* 120 μm, magnification ×40. **B**, **C** *Bar* 80 μm, magnification ×100

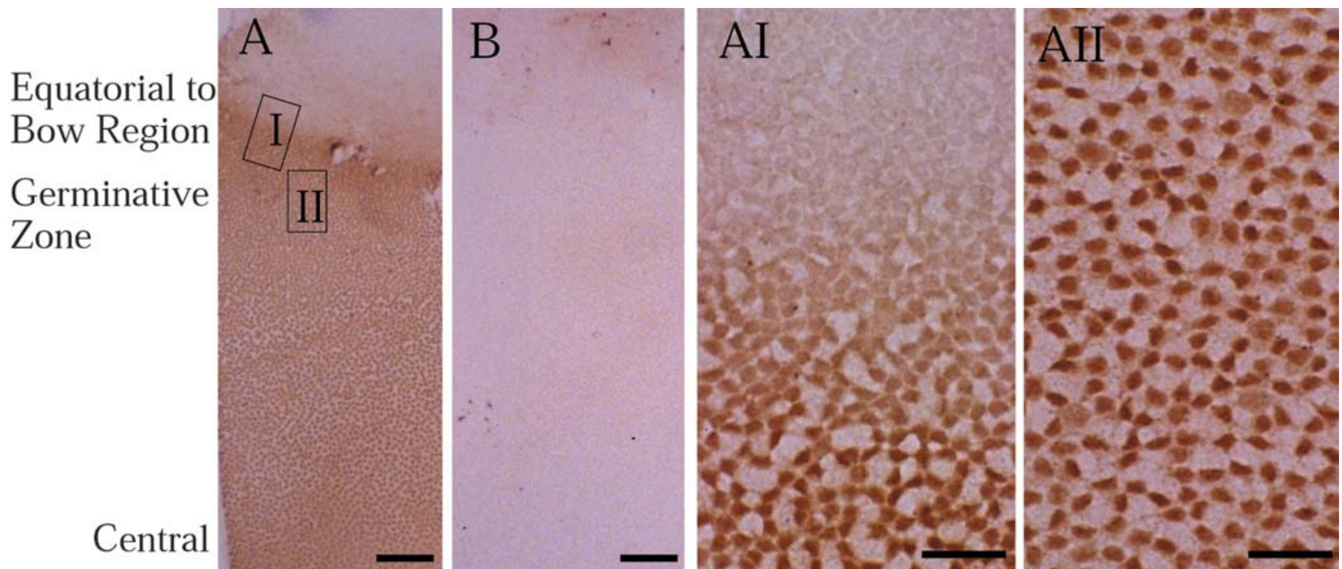

Fig. 3A, B Immunohistochemical staining of LEDGF and counterstaining with hematoxylin of flat preparations of the lens epithelium from a 3-week-old rat. The capsule epithelium stained with anti-LEDGF Ab (**A**). *Insets I* and *II* indicate the germinative zones (*I*) and anterior LECs (*II*). Anterior LECs showed intense immunostaining in nucleus. A gradual loss of nuclear LEDGF in cells of the equatorial bow region (*I*) is evident. In control sections stained with neutralized LEDGF Ab, LEDGF staining is significantly reduced (**B**). **A**, **B** *Bar* 160 μm, magnification ×25. *AI*, *AII* *Bar* 100 μm, magnification ×150

Flat preparations

The diminished immunoreactivity of nuclear LEDGF in cells of the equatorial bow regions, evident in cross-sections of whole lens (Fig. 2B), was even more apparent in flat preparations of LECs from 3-week-old rats (Fig. 3 *insets AI*, *AII*). The nuclei in the central epithelium, and in the sample from the germinative zone, all showed strong immunostaining with anti-LEDGF Ab. In the germinative zone, nuclear LEDGF immunostaining was significantly diminished (Fig. 3 *inset AI*). All magnified views of these zones (Fig. 3 *insets AI*, *AII*) were derived from the same flat preparation, and they demonstrate the rank order of nuclear staining intensity: central region>germinative region>bow region.

Furthermore, the staining of the cytoplasm in all these regions was generally much less intense and more varied than in the nuclei. In control sections stained with the GST-LEDGF-neutralized Ab preparation, LEDGF immunostaining was significantly reduced (Fig. 3B); this confirms the lack of non-specific staining and the specificity of the signal derived with the anti-(C-terminal) Ab preparation. These findings suggest that the loss of nuclear LEDGF in differentiating LECs may reflect the change in functional status of the cell.

Nuclear LEDGF was undetected in lentoid body and explants of cells

We extended our study to include two well-defined fiber cell differentiation systems: (1) lentoid bodies comprised of aggregated LECs grown on agar-coated plates (Arita et al. 1988, 1990) and (2) LEC explants exposed to differentiating factors (Rampalli and Zelenka 1995). LECs were cultured for 2–3 weeks on Millipore CM inserts (Millipore) to generate lentoid bodies (Fig. 4A–C). Single LECs were also cultured in 96-well plates as a control vehicle (Fig. 4D, E). Subsequently, they were subjected to immunostaining with anti-LEDGF Ab to localize LEDGF. Prior to immunostaining, the sections of lentoid body were stained with hematoxylin and eosin

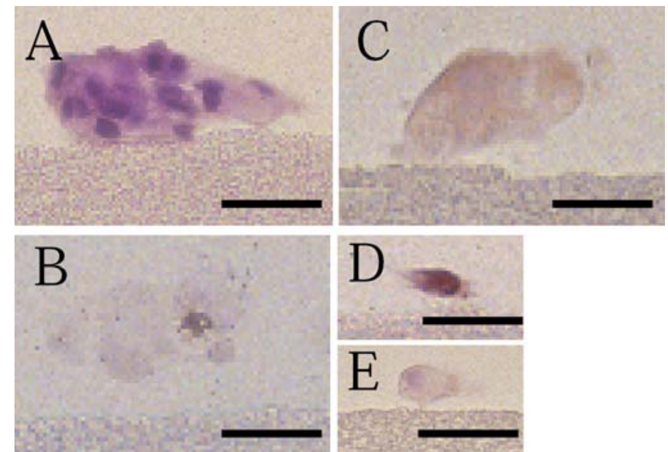

Fig. 4A–E Immunohistochemical staining and counterstaining with hematoxylin and eosin-stained lentoid bodies and single LEC. Thin paraffin sections of the lentoid body were stained with hematoxylin and eosin (**A**), with anti-LEDGF Ab (**B**), and with neutralized LEDGF Ab (**C**). Single LEC stained with anti-LEDGF Ab (**D**) or with LEDGF-neutralized Ab (**E**). **A–E** *Bar* 20 μm, magnification ×150

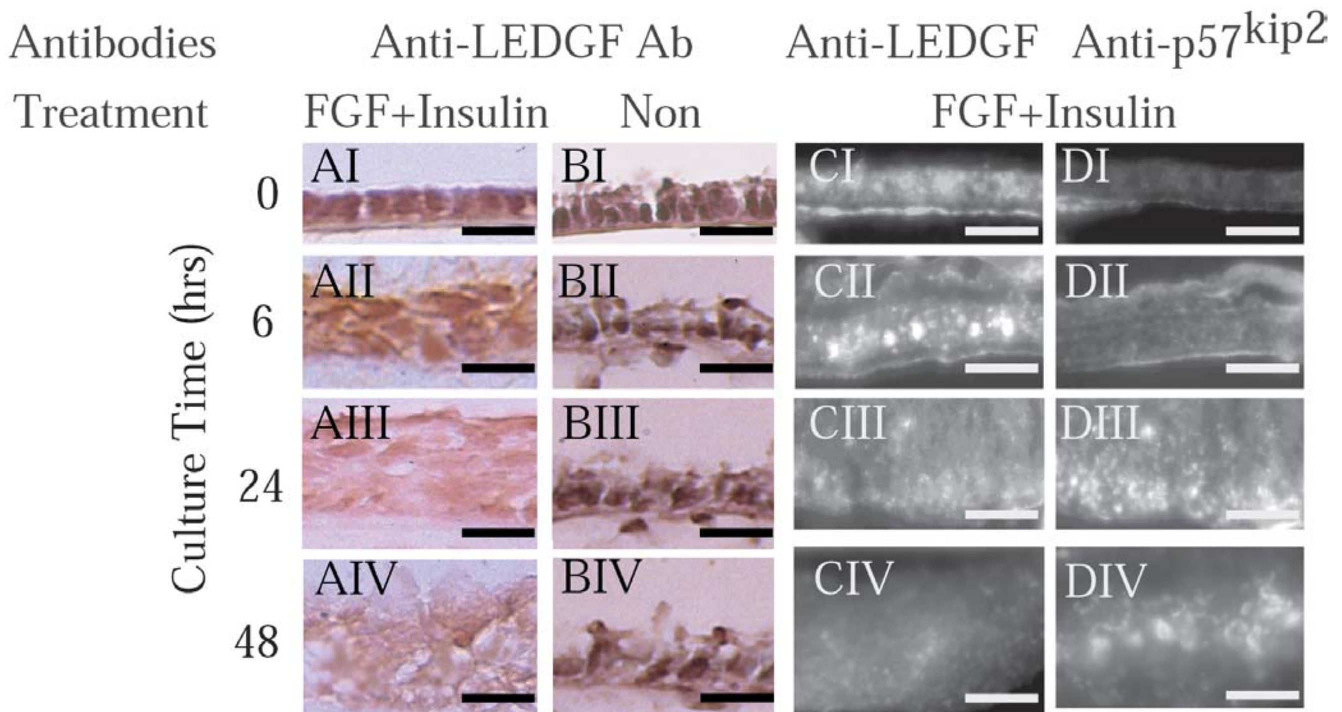

Fig. 5A–D Immunohistochemical staining of LEDGF and p57^{kip2} in rat LEC explants. The explants were fixed with 4% paraformaldehyde in phosphate-buffered saline either after 0 (*I*), 6 (*II*), 24 (*III*), and 48 (*IV*) h exposure to basic fibroblast growth factor (FGF-2) and insulin or no exposure. The explants were stained with the anti-LEDGF antibody (**A–C**) or the anti-p57^{kip2} Ab (**D**). The explants were visualized with DAB-colorimetric assay (showing *brown color*; **A***I–IV*, **B***I–IV*) and counterstained with hematoxylin (**A***I–IV*, **B***I–IV*). Sections were also visualized with cyanin-3 fluorescence probes (showing with *white color*; **C***I–IV*, **D***I–IV*). Cells exposed to FGF-2 and insulin differentiated into elongated fibers-like cells (*III*, *IV*), and untreated cells were single layered (**B**). The section stained with neutralized LEDGF Ab (photograph not shown) and with anti p57^{kip2} Ab (**D***I–IV*) showed significant reduced immunostaining. *Bars* 20 μm, magnification ×150

which showed the presence of nuclei (Fig. 4A). In the section of lentoid bodies, immunostaining of LEDGF in the cell's nucleus was almost negative, where as it was positive in cytoplasm (Fig. 4B); by contrast, immunostaining of a single LEC with anti-LEDGF Ab, showed strong immunopositivity for LEDGF in nucleus and weak in the cytoplasm (Fig. 4D). In control sections immunostained with the neutralized Ab, LEDGF immunostaining was significantly diminished in the lentoid bodies as well as in the single LEC (Fig. 4C, E).

We used differentiation factors to differentiate LECs into lens fiber cells, by culturing explants of LECs in presence of differentiation factors (Rampalli and Zelenka 1995). Rat LEC explants were cultured immediately after removing the cortex and nucleus through a posterior opening in the lens and treated with or without FGF-2 and insulin for 6, 24, and 48 h. Thereafter, the samples were fixed with 4% PFA in PBS and thin sections were prepared and immunostained either with anti-LEDGF Ab or LEDGF-neutralized Ab or anti-p57^{kip2} Ab (Fig. 5). The explants in Fig. 5A*I–IV* and B*I–IV* have been visualized with DAB and were slightly stained with hematoxylin, and those in Fig. 5C*I–IV* and D*I–IV* were visualized with cyanin-3 fluorescence probes. With both staining systems, DAB and cyanin-3, LEDGF immunostaining was similar (compare Fig. 5A*I–IV* and C*I–IV*). Furthermore, we observed that the explanted LECs that had been exposed to FGF-2 and insulin had elongated, but there was no distinguishable change in untreated explants (Fig. 5B*I–IV*; Rampalli and Zelenka 1995). In explanted LECs cultured without or with 6 h exposure to FGF and insulin, positive LEDGF immunostaining was observed predominately in the nucleus and weakly in the cytoplasm (Fig. 5A*I*, A*II*, C*I*, C*II*). In the explants exposed to FGF and insulin for 24 h, nuclear LEDGF was reduced (Fig. 5A*III*, C*III*). In explants exposed to FGF and insulin for 48 h the cytoplasm was weakly immunopositive for LEDGF, but the nuclei were no longer LEDGF immunopositive (Fig. 5A*IV*, C*IV*). In contrast, p57^{kip2}, a marker for lens fiber differentiation (Zohang et al. 1997; Lovicu and McAvoy 1999), progressively and dramatically increased during the culture period (0–48 h; see Fig. 5D*I–IV*). In addition, p57^{kip2} was weakly immunopositive at 0 or 6 h (Fig. 5D*I*, D*II*) and strongly immunopositive in cells exposed for 24 and 48 h with FGF-2 and insulin (Fig. 5D*III*, D*IV*). Thus we found that more differentiated LECs in which p57^{kip2} was immunopositive did not demonstrate nuclear LEDGF immunostaining. In control sections stained with the GST-LEDGF-neutralized Ab preparation, LEDGF immunostaining was absent (Fig. 5B, D). Taken together, our data from immunohistochemical staining of tissue cross-sections, LEC flat

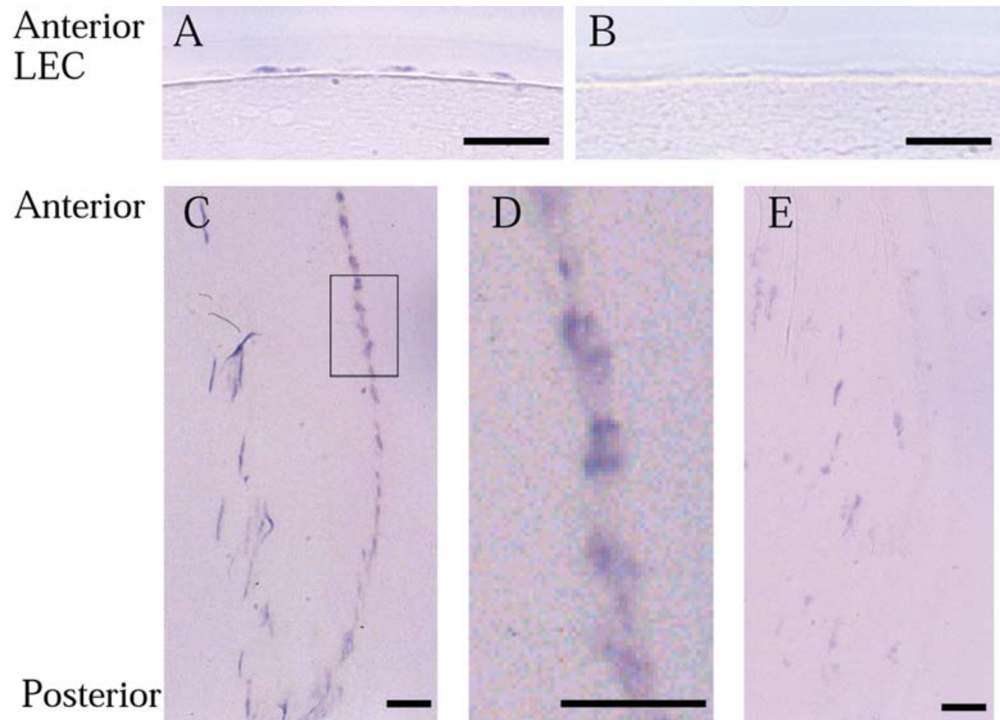

Fig. 6A–E In situ hybridization of LEDGF mRNA in the central LECs and bow region with biotinylated probes (*blue color*). In the antisense images there are positive signals overlying the anterior epithelial cells (**A**) and in the equatorial bow region (**C**), the positive signals overlying the nuclei and the cytoplasm of presumptive germinative epithelial cells (**C**). *Inset of box* indicates that the germinative zones (**D**) showed strong immunostaining in and/or around the nucleus. In the cells hybridized with sense probe, there were no apparent signals overlying the nuclei or cytoplasm in central (**B**) or equatorial LECs (**E**). **A–E** *Bar* 50 μm, magnification ×100

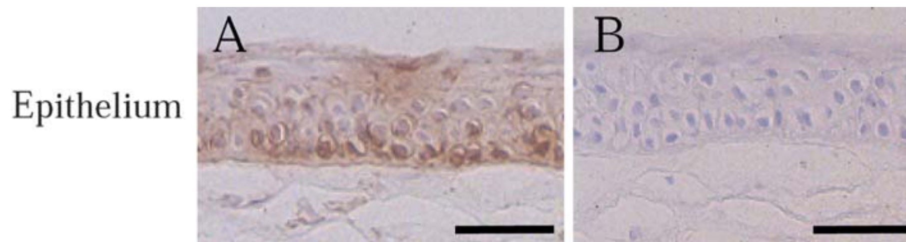

Fig. 7A, B Immunohistochemical staining of LEDGF and counterstaining in the 3-week-old rat cornea. **A** The nuclei in the basal layers of the corneal epithelium are strongly stained; the nuclei in the superficial epithelial cells are lightly stained. **B** Note the absence of specific staining in the negative control section of cornea that had been stained with the GST-LEDGF-neutralized LEDGF Ab. **A**, **B** *Bar* 80 μm, magnification ×100

preparations, lentoid bodies, and explanted LECs suggest that the process of differentiation of LECs is associated with progressive diminution of nuclear LEDGF.

Localization of LEDGF mRNA in rat lens

Next, we were keen to know LEDGF mRNA expression in the differentiating LECs. We found that LEDGF mRNA expression was decreased in the differentiating cells (Fig. 6). The hybridization of LEDGF mRNA to the LEDGF antisense probe is evident in the purple staining of the epithelial cells (Fig. 6A, C, D). In control sections (Fig. 6B, E) the hybridization of the LEDGF mRNA to the sense probe was not above the background staining. The LEDGF mRNA was present around the nucleus and the cytoplasm of epithelial cells, but it was less evident in the fiber cells (Fig. 6C). Thus, LEDGF mRNA expression also decreased in the differentiating cells.

In rat corneal epithelium, LEDGF localized in nuclei of basal cells, but not in nuclei of superficial cells

Since corneal epithelial cells are continually renewed, basal cells divide and superficial cells shed off, we were interested to monitor LEDGF distribution in these cells or tissue. We immunolocalized LEDGF and immunohistological localization of LEDGF protein in the 3-week-old rat cornea is presented in Fig. 7. Immunostaining of LEDGF was much more intense in the nuclei than in the cytoplasm of the suprabasal and basal epithelial cells (Fig. 7A). There was a decrease in the intensity of nuclear LEDGF immunostaining between the basal and superficial layers of the epithelium. Corneal stromal and endothelial cells were weakly immunopositive for LEDGF, but it was not clear if the staining was greater in the nuclei than in the cytoplasm. In control sections stained with the GST-LEDGF-neutralized Ab preparation, LEDGF immunostaining was markedly reduced (Fig. 7B).

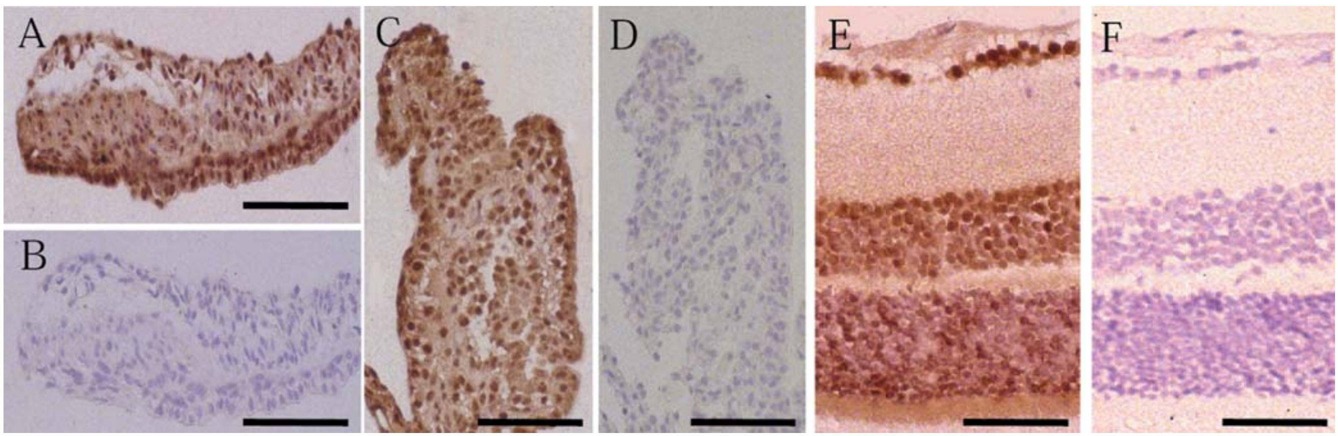

Fig. 8A–F Immunohistochemical staining of LEDGF and counterstaining in the 3-week-old rat iris (**A**, **B**), ciliary body (**C**, **D**), and retina (**E**, **F**). Positive staining for LEDGF in the nuclei of iris (**A**), ciliary body (**C**), and retina (**E**) were observed (*brown color*). In control sections stained with LEDGF-neutralized LEDGF Ab for iris (**B**), ciliary body (**D**), and retina (**F**), there was no detectable immunostaining of LEDGF. **A–F** *Bar* 80 μm, magnification ×100

There was no difference in the pattern of LEDGF immunostaining in the cornea of 7-week-old rat (data not shown).

Localization of LEDGF in iris, ciliary body, and retinal cells

In the iris (Fig. 8A, B) and ciliary body (Fig. 8C, D), intense immunostaining was evident in the nuclei of epithelial and stromal cells; cytoplasmic staining was less intense. However, cytoplasmic staining in the cells just anterior to iris pigment epithelium was stained somewhat darker than in the epithelial cells, but there was more cytoplasmic staining on the cells just anterior to the iris pigment epithelium. In the retina (Fig. 8E, F), intense staining was evident in the inner and outer nuclear layer, and homogenous/uniform staining was evident in these inner and outer nuclear layers. Furthermore, ganglion cells were strongly stained. However, the pattern of immunolocalization was indistinguishable between the retinas of 3- and 7-week-old rats. In control sections where LEDGF neutralized anti-LEDGF Ab was applied, immunostaining was knocked out (Fig. 8B, D, F).

Discussion

Immunohistochemistry of LEDGF in the rat eye revealed the presence of LEDGF in the ocular tissues, lens, cornea, iris, ciliary body, and retina, with strong LEDGF immunopositivity in the nuclei and weaker immunopositivity in the cytoplasm of these cells. Interestingly, there was no detectable level of LEDGF in the nucleus of the differentiating lens fiber cells and superficial corneal epithelial cells. We further confirmed this finding and we found that LEDGF is indeed diminished significantly in the fiber cells of lentoid body, a model of LEC differentiation. Similarly, LEDGF is also lost from the nucleus of explants of LECs after 24–48 h of treatment with insulin and FGF-2 and fiber-specific protein $p57^{kip2}$ was subsequently increased in elongating LECs of the explants. Furthermore, the intensity of nuclear LEDGF immunostaining in anterior and germinative LECs was much stronger than in the nuclei of differentiating LECs. The loss of nuclear LEDGF immunopositivity began in the postequatorial region and was complete before the cells reached the equatorial bow area. The nuclei of cells in the bow area and in the superficial cortex were not immunopositive for LEDGF. Similarly in rat cornea, the intensity of nuclear LEDGF immunostaining in the basal epithelial cells was higher than in the superficial epithelial cells. The changing intensity of nuclear LEDGF immunostaining occurred in tissues undergoing TD, and this suggested to us that a more comprehensive study of LEDGF in differentiating tissues was likely to be of interest. In two of the models of TD, epithelial explants (Piatigorski 1981; Chamberlain and McAvoy 1987, 1989; Lovicu and McAvoy 1989; Chamberlain et al. 1991; Klok et al. 1998) and lentoids (Arita et al. 1988, 1990), we showed that the immunopositivity of nuclear LEDGF decreased dramatically during TD. We do not have evidence yet that the decrease in immunopositivity is due to TD or that LEDGF itself plays a primary role in TD. Interestingly, however, there is a temporal association between the apparent decrease of nuclear LEDGF and the appearance of $p57^{kip2}$ in elongating rat LEC explants exposed to FGF and insulin. $P57^{kip2}$ is marker of differentiation in LECs (Zohang et al. 1997; Lovicu and McAvoy 1999; Carey et al. 2002) and is a member of the CIP/KIP family (Hatada and Mukai 1995; Lee et al. 1995; Matsuoka et al. 1995). Overexpression of $P57^{kip2}$ arrests cells in G1 (Hengst et al. 1994). The LECs in the equatorial region express high levels of $P57^{kip2}$ (Zohang et al. 1997), and $P57^{kip2}$ mRNA expression in adult mice is restricted to the transitional region where LECs begin

early fiber differentiation (Lovicu and McAvoy 1999). The transformation of epithelial into fiber-like cells in the LEC explants and lentoids resembles the differentiation that occurs in the whole lens (Piatigorski 1981; Chamberlain and McAvoy 1987, 1989; Arita et al. 1988, 1990; Lovicu and McAvoy 1989; McAvoy and Chaberlain 1989; Chamberlain et al. 1991; Rampalli and Zelenka 1995; Klok et al. 1998). Lentoids also express several fiber-specific marker proteins (Arita et al. 1988, 1990). The cytoplasmic localization of LEDGF in lentoids also supports the data that nuclear LEDGF decreases in cells undergoing TD. The loss of nuclear LEDGF immunopositivity that accompanies the process of TD in LECs is similar to that which occurs in corneal epithelium (Kubo et al. 2002), where the immunopositivity of nuclear LEDGF decreases as the suprabasal cell differentiates into a superficial squamous cell. These results suggest that the loss of LEDGF from the cell nucleus might be one of the important events during the progression of lens cell fiber differentiation, and need to be investigated in detail. However, lens fiber cell differentiation represents an ideal system in dissecting the role of LEDGF, a survival factor. Moreover, it has been suggested that this could simply be due to triggering events, such as drop in cytoplasmic pH, a fall in oxygen tension, or build up of metabolic waste occurring at the center of the lens. These events could lead to the subsequent disintegration of cell organelles and would then lead to the initiation of the denucleation and degradation of proteins due to release of proteases. Furthermore, it has been also suggested that expression of proapoptotic cytokines such as members of the tumor necrosis factor group may initiate lens fiber cell organelles loss (Wride and Sanders 1998). LEDGF is a transcription factor and induced cell survival (Fatma et al. 2000, 2001; Singh et al. 2001a), and loss or degradation of this protein by proteases may be one of the prime events toward the differentiation of cells (Wu et al. 2002). Raff's group has shown that the general caspase inhibitor (Z-VAD-FMK) is able to abolish nuclear degeneration and could protect certain proteins in an in vitro model of rat lens fiber differentiation (Ishizaki et al. 1998).

Recently, it has been observed that the process of LECs differentiating into fiber cells has biochemical parallels with apoptosis (Wride 2000). Unlike the situation in apoptosis, the lens fiber cells that lose their organelles persist throughout the life time of the organism, suggesting that there may be some important biochemical differences between the way in which apoptotic cells die and lens fiber cells lose their organelles. In fact, it has been suggested that lens fiber cell organelle loss can be considered to be a kind of attenuated apoptosis (Dahm 1999), perhaps in which the nuclear changes are biochemically dissociated from the normal changes of apoptosis such as the cytoskeleton remains intact during organelle loss in lens fibers, while in apoptosis proper it is degraded (Bassnett and Beebe 1992; Dahm et al. 1998).

In our earlier studies we have shown that in the presence of LEDGF cells survive but they die in its absence (Singh et al. 1999, 2000a; Nakamura et al. 2000). LEDGF exerts its survival function by binding to heat shock elements (nGAAn) and stress response elements (A/TGGGGA/T) and activates the expression of stress-related proteins including Hsps in LECs (Singh et al. 2001a). Recently, several independent groups have identified that Hsp27, alpha B-crystallin, and Hsp70 exert direct, but negative influence on apoptosis signaling, and hence protect the cell (Beere et al. 2000; Bruey et al. 2000; Saleh et al. 2000). We believe that LEDGF may protect the cells via similar a mechanism as Hsps have been shown to be anti-apoptotic and LEDGF is a transactivator of Hsps (Fatma et al. 2001; Singh et al. 2001a), although further detailed investigation is required to establish the mechanism lying behind the interaction(s) and systemic regulation and expression of LEDGF and Hsps, and inhibition of caspases during lens cell fiber differentiation. However, it has been found that indeed caspase-3 triggered apoptosis in okadaic acid-induced lens epithelial cell and alpha B-crystallin could inhibit it (Li et al. 2001)

Furthermore, in the crystalline lens we found moderately intense immunostaining of LEDGF in the cytoplasm of epithelial cells and intense immunostaining of the cytoplasm in nucleated fiber cells at the equator. A most intriguing observation is that the cytoplasm of superficial cortical fibers in the anterior half of the lens is much less immunopositive for LEDGF than the cytoplasm of the superficial cortical fibers in the posterior half of the lens. It appears that there is an intracellular gradient (within the cytoplasm of superficial fibers) of LEDGF immunopositivity. Where fibers contact the capsule (posteriorly) LEDGF immunopositivity is intense, and where fibers are separated from the capsule by epithelial cells, LEDGF immunopositivity is low. Posterior fibers separated from the posterior capsule by other fibers are LEDGF immunonegative. This region of LEDGF immunonegativity corresponds roughly to the zone in which all, or almost all, cytoplasmic organelles are absent in the lens fiber. The significance, if any, of this observation is not yet clear, but it is interesting to consider that cytoplasmic LEDGF levels might also influence the later stages of TD. Furthermore, we found the intense nuclear staining of LEDGF in retina, ciliary body, and iris, where the cells are well-differentiated. We have reported that LEDGF promotes cellular survival of retinal photoreceptor cells and human retinal pigment epithelium (Nakamura et al. 2000; Machida et al. 2001; Matsui et al. 2001). However, the localization pattern of LEDGF indicates that it may have different functions in different cells, that may depend on the stage and type of cells and its intracellular distribution.

The in situ hybridization experiments with sections of rat lenses have shown that the mRNA for LEDGF is present, even abundant, in the epithelial cells of the pregerminative, germinative, and the most superficial equatorial zones. A reduction in the amount of LEDGF mRNA begins and progresses rapidly in more deeply located fibers, and in the deepest cortical fibers there is no

LEDGF mRNA. The presence of LEDGF mRNA in the superficial cortex confirms that LEDGF transcription is active there. This is intriguing for it suggests that there are extranuclear functions for LEDGF in the cell. The transcription and pre-mRNA splicing roles would be effected in the nucleus. LEDGF localizes in the cytosol of cultured LECs at 15°C and 28°C (Singh et al. 1999). However, we do not yet understand what might be the normal extranuclear functional roles of LEDGF. LEDGF localizes in nucleus (Singh et al. 2001b), and furthermore, phosphorylation of LEDGF might also modify its intracellular distribution. LEDGF is a phosphoprotein and phosphorylation alters the intracellular localization of many proteins. One example is basonuclin, a zinc finger protein (Iuchi and Green 1997) and transcription factor (Tseng et al. 1999). We might expect the subcellular distribution of LEDGF to change as a function of the phosphorylation status of LEDGF. We have recently begun a series of experiments focusing on how phosphorylation alters the localization and functional properties of LEDGF.

So far three known transcription factors are reported to determine the fate of LECs: (1) loss of LEDGF in the LECs using antisense LEDGF cDNA-induced cell death (unpublished data), (2) loss of retinoblastoma susceptibility gene, a negative regulator of the cell proliferation, also reported to induce cell death (Tan et al. 1997), and (3) Creb-2, an evolutionary conserved member of the CREB/ATF family of basic-leucin zipper transcription factor, is reported to be an important regulator of mammalian lens development (Hettmann et al. 2000).

Further detailed studies will be required to elucidate the mechanism involved in the expression and localization of this physiologically important gene. In summary, however, we established a temporal relationship between the loss of LEDGF from nucleus and fiber cell differentiation. In addition, our findings add weight to the hypothesis that LEDGF is one of the factors that may play a regulatory role in determining the fate of cells. We believe our continuing studies of LEDGF on the functional role of cytoplasmic or nuclear LEDGF within the cell, the fate of LEDGF during differentiation and apoptosis, and the effect of proteases on LEDGF, will provide a better understanding of the roles of LEDGF in cell survival and differentiation.

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