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Involvement of Interleukin 18 in Cataract Development in Hereditary Cataract UPL Rats

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Our previous studies have demonstrated that lens epithelial damage by excessive nitric oxide causes an elevation in lens opacification in UPL rats, and it has been reported that interferon-γ production in lens epithelial cells is involved in cataract development. In this study, we investigated the involvement of interleukin (IL)-18, which leads to interferon-γ, in UPL rat lenses. The opacification of UPL rat lenses starts at 39 days of age. The gene expression levels causing IL-18 activation (IL-18, IL-18 receptor and caspase-1) are increased at 32 days of age, and the expression of mature IL-18 protein in the UPL rat lenses also increases with ageing. On the other hand, the interferon-γ levels in UPL rat lenses are increased, and the increase in interferon-γ levels in UPL rat lenses reaches a maximum at 39 days of age. Mature IL-18 expression and interferon-γ production are achieved prior to the onset of lens opacification. In conclusion, the expression levels of IL-18 in the lenses of UPL rats are increased with aging. In addition, interferon-γ levels in the lenses of UPL rats are also increased. It is possible that interferon-γ generated by the activated IL-18 may induce cataract development in UPL rats.

Key words: cataract, interferon-γ, interleukin 18, interleukin 18 binding protein, UPL rat.

Abbreviations: GAPDH, glyceraldehyde-3-phosophate dehydrogenase; IFN-γ, interferon-γ; IL-18, interleukin 18; IL-18BP, interleukin 18 binding protein; IL-18R, interleukin 18 receptor; NO, nitric oxide; iNOS, inducible nitric oxide synthase; NFκB, nuclear factor kappa B; SDS, sodium dodecyl sulphate.

Any alteration in the optical homogeneity of the lens or decrease in its transparency is known as a cataract (*1*, *2*). Over the past several decades, there have been many studies exploring the mechanisms of cataract development (*3*). Currently, reactive oxygen species induced by UV rays in sunlight are considered to be important in perturbing lens homoeostasis. Therefore, exposure to reactive oxygen species results in a breakdown of lens homoeostasis, and the Ca^{2+} content in the lens becomes elevated. Elevated Ca^{2+} contents in the lens have been deduced to activate calpain, a Ca^{2+}-dependent protease. Furthermore, the degradation of lens proteins, such as crystallin proteins, would result in an opaque lens (*4*, *5*). On the other hand, Li *et al*. (*6*) reported that the oxidative stress induced by UV light triggers lens epithelial cell apoptosis, and that lens epithelial cell apoptosis causes cataract development. It is likely that epithelial cell damage in the ocular lens triggers the increase in Ca^{2+} content. These results suggest that inflammatory actions by various factors in the lens, such as reactive oxygen species, may play an important role in cataract development.

Interleukin (IL)-18, an interferon (IFN)-γ inducing factor, is a pleiotropic cytokine belonging to the IL-1 family (*7*, *8*). It is expressed as an inactive 24-kDa proform that is cleaved by caspase-1 (IL-1β converting enzyme, a cystine protease) to an 18-kDa active form (*7*, *8*). Released mature IL-18 exerts its effects upon binding to its cognate receptor (IL-18R, *9*). Two subunits of IL-18R have been characterized as belonging to the IL-1R family. The IL-1R related protein (IL-1Rrp or IL-1 R5) was identified as a low-affinity receptor for IL-18 and renamed IL-18 Rα (*9*). The second subunit, IL-18Rβ does not bind IL-18 directly, but the β chain increases IL-18 binding affinity and is necessary for initiating signal transduction in target cells (*10*). The binding of mature IL-18 to IL-18R leads to IFN-γ production (*11*), nuclear factor kappa B (NFκB) and inducible nitric oxide synthase (iNOS, *12*). IL-18 plays an important role in inflammatory action (*7*, *8*). Therefore, IL-18 may be related to cataract development.

In studies to identify the mechanisms of cataract development, the selection of an experimental animal is very important. The UPL rat is a dominant hereditary cataract model derived from Sprague-Dawley rats (*13*). Opacification of the lenses of UPL rats starts at 35–42 days of age, and by 50 days of age the lenses are almost entirely opaque. The incidence of cataracts in adult UPL rats is 100% (*13–16*). Previous investigations have revealed that oxidized glutathione concentrations in the lenses of UPL rats are increased, and reduced GSH values are decreased (*17*). The proteolyses of some crystallins and cytoskeletal proteins are enhanced in the lenses of UPL rats (*14*, *15*). Ca^{2+} concentrations in the lenses of UPL rats rise markedly with ageing in comparison to normal rats, and the autolytic product of

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calpain is also detected in the lenses of UPL rats (*15*). These changes in the biological characteristics of the lenses of UPL rats may correspond to those of human cataracts. Therefore, UPL rats may provide a useful model for studies on the mechanism of cataract development. In this study, we investigated the involvement of IL-18 in cataract development in UPL rats.

MATERIALS AND METHODS

Animals and Materials—The rats used were normal, male, late onset type UPL rats aged 25 to 53 days. They were housed under standard conditions [12 h/day fluorescent light (07:00–19:00); 25°C room temperature] and fed a commercial diet (CE-2, Clea Japan Inc., Osaka, Japan) and water. The animal experiments were performed in accordance with the ARVO Resolution on the Use of Animals in Research. Methylcellulose was obtained from Kanto Chemical (Tokyo, Japan). All other chemicals used were of the highest purity available.

Image Analysis for Cataract Development in UPL Rats—The pupils of rats were dilated by the instillation of 0.1% pivalephrine (Santen Pharmaceutical Co., Osaka, Japan) without anaesthesia. Changes in the transparency of the lenses were monitored using an EAS-1000 equipped with a CCD camera (Nidek, Aichi, Japan). The outline of the lens image was determined by selecting 4 points on the image, and then the transparent area within the outline and thread level were set automatically by the software. The total area of opacity of the lenses, expressed in pixels, was calculated using computerized image analysis software connected to the EAS-1000 system (*18*).

RNA Preparation—Total RNAs were extracted from the lenses of normal and UPL rats by the acid guanidium thiocyanate-phenol-chloroform extraction method (*19*) using Trizol reagent (Life Technologies Inc., Rockville, USA) according to the manufacturer's instructions.

Semi-quantitative Reverse Transcriptase-PCR (RT–PCR)—The RT reaction was performed using an RNA PCR kit (AMV Ver 2.1, Takara Bio Inc., Shiga, Japan). One microgram of total RNA was mixed with 3 μl of 10 mM Tris–HCl buffer (pH 8.3) containing 5 mM $MgCl_2$ and 50 mM KCl. The following components were then added to give a final volume of 10 μl: 1 U/μl RNase inhibitor, 10 mM deoxynucleotide triphosphate, 2.5 U/μl reverse transcriptase, and 0.125 μM oligo dT-adaptor primer. The RT reaction was performed at 42°C for 15 min, followed by 5 min at 95°C. The PCR reactions were performed by adding 10 μl of cDNA to 40 μl of the reaction mixture containing 3.125 mM $MgCl_2$, 12.5 mM Tris–HCl, pH 8.3, 1.563 U of *Taq* DNA polymerase and 25 pmol IL-18- or glyceraldehyde-3-phosophate dehydrogenase (GAPDH)-specific primers. The specific primers were: 5′TGGAGACTTGGAATCAGACC-3′ and 5′GGCAA GCTAGAAAGTGTCCT-3′ for IL-18 (*20*); and 5′GGTGCT GAGTATGTCGTGGAGTCTAC-3′ and 5′CATGTAGGCC ATGAGGTCCACCACC-3′ for GAPDH (*16*). The conditions for PCR were: 94°C for 2 min, 30 cycles of 94°C for 30 s (denaturing), 59°C for 45 s (annealing) and 72°C for 1 min (extension), and a final extension of 72°C for 10 min. The PCR products were separated in 1.5% agarose gels, visualized by staining with ethidium bromide, and then photographed with an ImageMaster-CL (Amersham Biosciences Corp., Piscataway, NJ, USA).

Quantitative Real–Time RT–PCR—The composition and conditions of the RT reaction were the same as those described for the semi-quantitative RT–PCR method. The PCR reactions were performed using LightCycler FastStart DNA Master SYBR Green I according to the manufacturer's instructions (Roche Diagnostics Applied Science, Mannheim, Germany). Briefly, 2 μl of cDNA was mixed with 2 μl of reaction mixture, LightCycler FastStart DNA Master SYBR Green I Reaction Mix, containing FastStart *Taq* DNA polymerase, reaction buffer, $MgCl_2$, SYBR Green I dye and deoxynucleotide triphosphate mix. The following components were then added to give a final volume of 20 μl containing specific primers for IL-18, IL-18Rα, IL-18Rβ, IL-18-binding protein (IL-18BP), caspase-1 or GAPDH- (10 pmol each). The primers used are summarized in Table 1, and the conditions for PCR are shown in Table 2. The quantities of PCR products were measured fluorometrically in a real-time manner using a LightCycler DX 400 (Roche Diagnostics Applied Science, Mannheim, Germany). After the completion of the PCR reactions, dissociation curves of the PCR products were generated using the LightCycler Software Version 4.0 program to detect non-specific amplification, including primer-dimers, and to ascertain the quality of the amplification data. The differences in the threshold cycles for GAPDH and other groups (IL-18, IL-18Rα, IL-18Rβ, IL-18BP and caspase-1) were used to calculate the levels of mRNA expression in the UPL rats.

Measurement of Protein—Protein levels in the UPL rats lenses were determined according to the method of Bradford (*21*) using a Bio-Rad Protein Assay Kit (BIO-RAD, CA, USA) with bovine serum albumin as the standard.

Western Blot Analysis—Lenses from normal and UPL rats at 25, 32, 39, 46 and 53 days of age were homogenized in saline on ice. The lens homogenates were suspended in a buffer comprising 100 mmol/l Tris–HCl, pH 6.8, 4% sodium dodecyl sulphate (SDS), 10% glycerol, 5% 2-mercaptoethanol and 0.002% bromophenol blue, and boiled for 3 min. A sample of total protein (20 μg) was separated in an 8% polyacrylamide SDS gel. The proteins were then transferred to polyviniylidene difluoride membranes (BIO-RAD, CA, USA), using a semi-dry transfer cell (Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell, BIO-RAD, CA, USA). The transfer buffer used in the system contained 25 mmol/l Tris–HCl, 191 mmol/l glycine, 20% methanol, and 0.0375% SDS. After transfer, non-specific sites on the membranes were blocked with 5% non-fat dry milk in Tris-buffer (20 mmol/l Tris–HCl, and 500 mmol/l NaCl, pH 7.5). The blots were probed with 0.5 mg/l goat anti-rat IL-18 polyclonal antibody (Promega, Wisconsin, USA) for 2 h at room temperature. After washing with Tris-buffer containing 0.1% Tween-20, the membrane was incubated with a secondary alkaline-phosphatase conjugated anti-goat IgG (1:1000 dilutions, Promega, WI, USA) for 2 h at room temperature, washed with Tris-buffer containing 0.1% Tween-20, and incubated with a stabilized substrate for alkaline-phosphatase (Promega, WI, USA).

Table 1. **Sequences of primers used for quantitative RT-PCR analysis.**

Primer		Sequence (5′–3′)	GenBank accession number
IL-18	FOR	CGCAGTAATACGGAGCATAAATGAC	NM_019165
	REV	GGTAGACATCCTTCCATCCTTCAC	
IL-18Rα	FOR	AGCAGAAAGAGACGAGACACTAAC	XM_237088
	REV	CTCCACCAGGCACCACATC	
IL-18Rβ	FOR	GACCACAGGATTTAACCATTCAGC	AJ550893
	REV	AGCAGGACCTAGTGTTGATGATG	
IL-18BP	FOR	TTGGTGGGTCCTGCTTCTATATG	AF154569
	REV	GGTCAGCGTTCCATTCAGTG	
Caspase-1	FOR	TGAAGATGATGGCATTAAGAAGGC	NM_012762
	REV	CAAGTCACAAGACCAGGCATATTC	
GAPDH	FOR	ACGGCACAGTCAAGGCTGAGA	NM_017008
	REV	CGCTCCTGGAAGATGGTGAT	

Table 2. **Parameters used for quantitative RT-PCR analysis.**

Primer	Hot start (10 min) (°C)	Denaturation (10 s) (°C)	Annealing (10 s) (°C)	Extension (5 s) (°C)
IL-18	95	95	53	72
IL-18Rα	95	95	57	72
IL-18Rβ	95	95	55	72
IL-18BP	95	95	53	72
Caspase-1	95	95	55	72
GAPDH	95	95	60	72

Assay of IFN-γ Levels in UPL Rat Lens—Lenses from normal and UPL rats at 25, 32, 39, 46 and 53 days of age were homogenized in ultrapure water. The lens homogenates were centrifuged at 1500 r.p.m. for 10 min at 4°C, and the supernatants were used for the measurement of IFN-γ levels. The IFN-γ levels were measured using Endogen® Rat IFNγ ELISA kits according to the manufacturer's instructions (Pierce Biotechnology Inc., WI, USA). Briefly, monoclonal antibodies specific for rat IFN-γ were pre-coated onto microplates. Standards and samples were pipetted into the wells, and the microplates were incubated at room temperature for 1 h. After washing to remove unbound materials, biotinylated antibodies were added to the wells. After washing, the streptavidin-HRP solutions were added, and washed with unbound materials. After that, substrates were added. The enzyme reactions yielded blue products that turned yellow when the stop solutions were added. The absorbance was measured with a microplate reader (BIO-RAD, CA, USA) at 450 nm.

Statistical Analysis—The data are expressed as means ± S.E. Statistical differences were determined by the unpaired Student's or Aspin-Welch's *t*-test. *P* values <0.05 were considered significant.

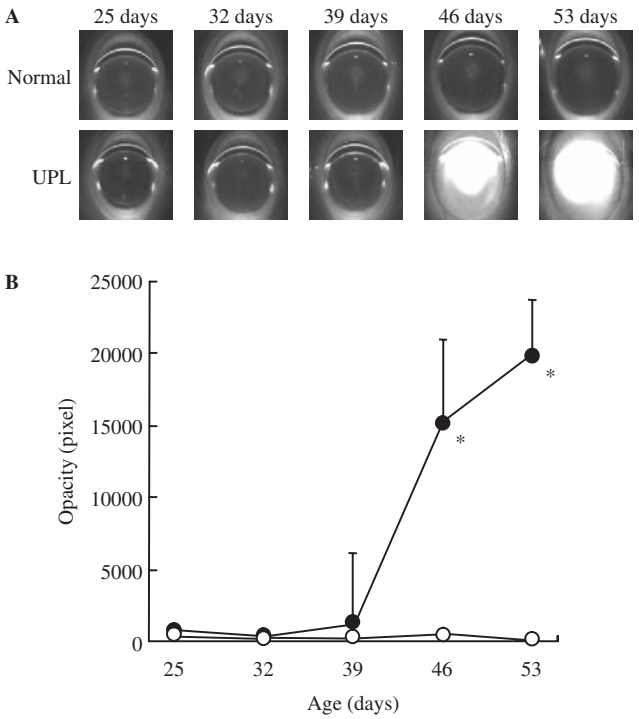

Fig. 1. **Cataract development in UPL rats with increasing age.** (A) Scheimpflug slit images of lenses from normal and UPL rats at 25–53 days of age. The Scheimpflug slit images were obtained by an anterior eye segment analysis system (EAS-1000). (B) Lens opacity of normal and UPL rats 25–53 days of age. The area of opacity (pixels) was analysed by image analysis software connected to the EAS-1000. Normal, normal rat lens (open circle); UPL, UPL rat lens (filled circle). The numbers above the photographs show the ages of the rats (days). The data are presented as means ± SE of five independent rat lenses. $^{*}P < 0.05$ vs. normal rat.

RESULTS

Expression of IL-18 mRNA and Protein in UPL Rat Lens during Cataract Development—Figure 1 shows Scheimpflug slit images (A) and opacity levels (B) of normal and UPL rat lenses as documented by EAS-1000. The lenses of normal rats aged 25–53 days were transparent. Opacification of UPL rat lenses started at 39 days of age, and the UPL rat lenses at 46 days of age were almost entirely opaque. Figure 2 shows the agarose gel electrophoresis of IL-18 mRNA in UPL rat lenses aged 25–53 days as determined by the semi-quantitative RT–PCR method. In normal rats, the expression of IL-18 mRNA was not detected during this period. In contrast to the results in normal rats, the expression of IL-18 mRNA in the lenses of UPL rats increases with aging. Figure 3 shows the expression of mature IL-18. Mature IL-18 was not detected in normal rat lens. On the other hand,

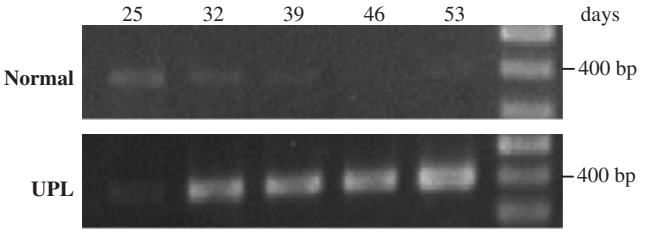

Fig. 2. **Semi-quantitative RT–PCR analysis of IL-18 mRNA in the lenses of UPL rats during cataract development.** Agarose gel electrophoresis of IL-18 mRNA in the lenses of 25–53-day-old normal and UPL rats. The numbers above the photographs show the ages of the rats (days).

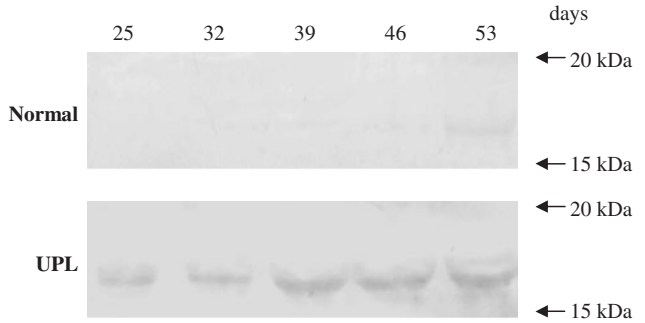

Fig. 3. **Expression of the mature IL-18 protein in the lenses of 25–53-day-old normal and UPL rats.** Mature IL-18 protein was detected by western blot analysis. The numbers above the photographs show the ages of the rats (days).

the expression of mature IL-18 protein was observed in the lenses of UPL rats, and the expression levels increased with age.

Changes in IL-18, IL-18Rα, IL-18Rβ, IL-18BP and Caspase-1 mRNA and IFN-γ Levels in UPL Rat Lens during Cataract Development—Figure 4 shows the expression levels of the mRNAs for IL-18 (A), IL-18Rα (B), IL-18Rβ (C), IL-18BP (D) and caspase-1 (E) in the lenses of normal and UPL rats aged 25 to 53 days as determined by the quantitative real-time PCR method. The expression levels of the mRNAs for IL-18, IL-18Rα, IL-18β, IL-18BP and caspase-1 did not change with aging in normal rats, while the mRNA expression levels of all the above mRNAs increased with aging in UPL rats. Although the lenses of UPL rats at 25 days of age were transparent, the expression levels of the mRNA for IL-18Rβ was already elevated, and the expression levels of the mRNAs for IL-18, IL-18Rα and caspase-1 were increased at 32 days of age. The expression level of the mRNA for IL-18BP was found to be increased at 46 days of age when the lenses were already opaque. No changes in the IFN-γ levels in the lenses of 25–53-day-old normal rats were observed. The IFN-γ levels in the lenses of UPL rats were increased from 25 to 39 days of age, reaching a maximum at 39 days of age, and then subsequently decreasing. On the other hand, the IFN-γ levels in the lenses of UPL rats at 53 days of age were significantly decreased compared with the level in normal rats (Fig. 5).

DISCUSSION

IL-18, which induces IFN-γ, NFκB and iNOS, is a well-known pleiotropic cytokine that regulates innate and acquired immune responses, and plays an important role in inflammatory action (*7*, *8*). In this study, we investigated the involvement of IL-18 in cataract development in UPL rats.

Our study demonstrates for the first time the expression of IL-18 mRNA and protein in the lenses of normal and UPL rats. The levels of IL-18 mRNA expression in normal rats do not change with aging, and the levels are lower than in the lenses of UPL rats. The expression of mature IL-18 was not detected in normal rat lens. In contrast to the results in normal rats, the levels of IL-18 mRNA and protein expression in the lenses of UPL rats increased with ageing (Figs 2 and 3). This result suggests that IL-18 may play an important role in cataract development in the lenses of UPL rats. Therefore, we investigated the relationship between IFN-γ production and the expression levels of genes involved in the regulation of IL-18 activation (IL-18, IL-18Rα, IL-18Rβ, IL-18BP and caspase-1) in the lenses of UPL rats during cataract development. Initial opacification of the lenses in UPL rats appears between 42 and 49 days of age. IL-18Rβ gene expression levels are already increased at 25 days of age, and the expression levels of the other genes involved in IL-18 activation (IL-18, IL-18Rα and caspase-1) are increased at 32 days of age. The IFN-γ levels in UPL rat lenses are also increased at 32 days of age, and reach a maximum at 39 days of age. However, IFN-γ levels in the lenses of UPL rats decrease thereafter from 46 to 53 days of age, and IFN-γ levels in the lenses of UPL rats are significantly lower in comparison with normal rats at 53 days of age with elevated IL-18 expression observed in the lenses of UPL rats. Soluble IL-18BP has recently been purified and cloned from serum and urine. It shows no homology to IL-18R, and is a specific, functional inhibitor of IL-18, which may act as an endogenous inhibitor of circulating IL-18 (*22*, *23*). Hurgin *et al*. (*24*) reported that the elevation in IL-18BP expression is induced by IFN-γ. We show that the expression of IL-18BP mRNA, which inhibits the activation of IL-18, in the lenses of UPL rats is also increased at 46 days of age. These facts suggest that the elevation in IL-18BP expression in the lenses of UPL rats may be caused by IFN-γ production through the induction of IL-18 activation. And the elevated IL-18BP levels may suppress IFN-γ production in UPL rat lenses.

At present, there is no direct evidence for a role of IL-18 in cataract development. However, Awasthi and Wagner (*25*) reported that IFN-γ production in lens epithelial cells is involved in cataract development. As mentioned above, our previous studies indicated that lens epithelial cells damage leads to lens opacification in UPL rats (*17*). Moreover, we demonstrated that mature IL-18 expression and IFN-γ production occur prior to the onset of lens opacification. Taking these findings together, it seems likely that IFN-γ generated by activated IL-18 is involved in cataract development in UPL lens. The lens epithelium is a single layer of cuboidal cells on the anterior surface of the lens.

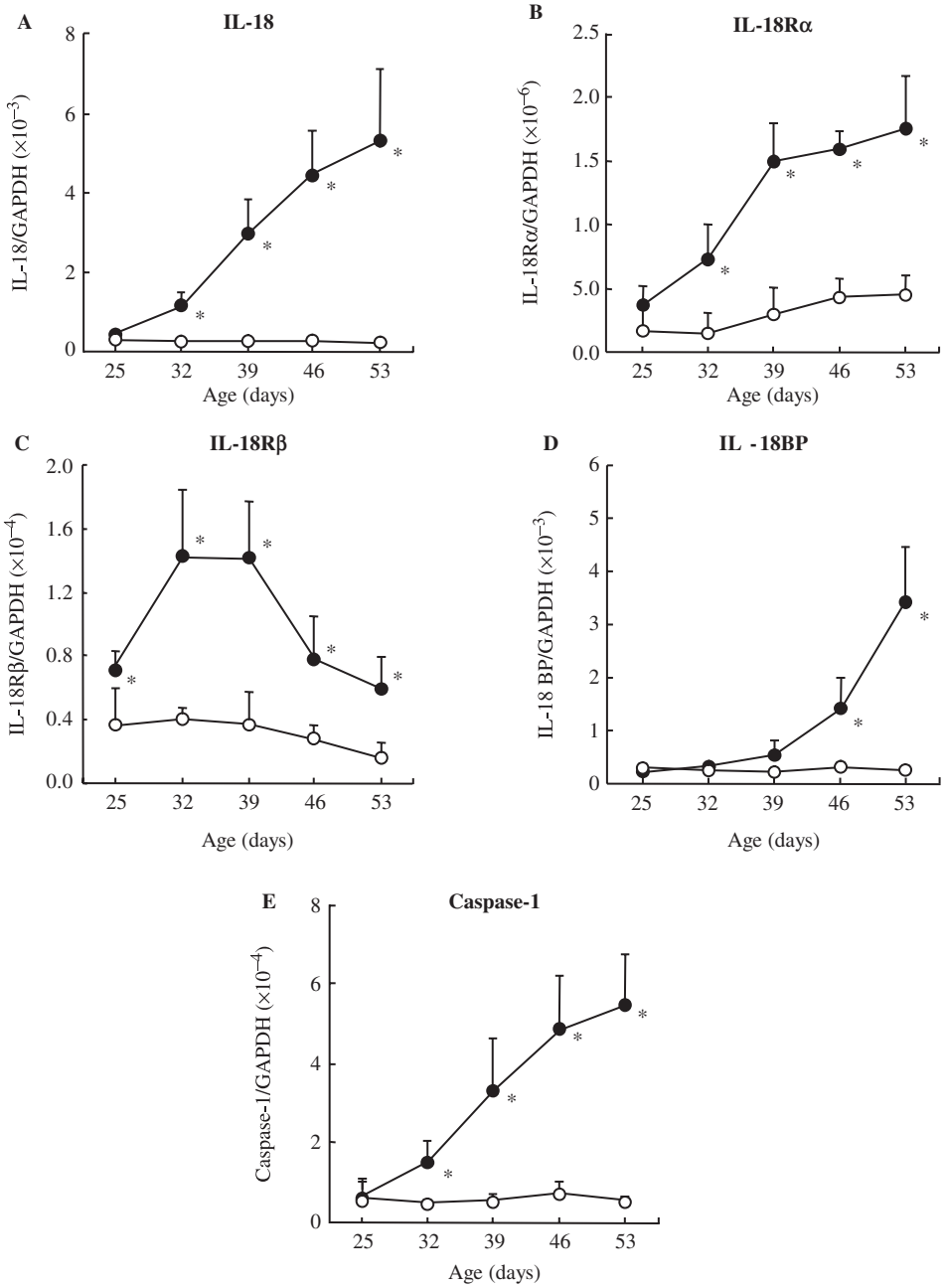

Fig. 4. **Expression levels of the mRNAs for IL-18, IL-18Rα, IL-18Rβ, IL-18BP and caspase-1 in the lenses of 25–53-day-old normal and UPL rats.** mRNA expression levels were determined using the quantitative real-time RT–PCR method. A, IL-18; B, IL-18Rα; C, IL-18Rβ; D, IL-18BP; E, caspase-1. Normal rat (open circle), UPL rat (filled circle). The data are presented as means ± SE of 6–11 independent rat lenses. $^{*}P < 0.05$ vs. UPL rat.

The epithelial cells remain quiescent in the central section, divide toward the equatorial area, and terminally differentiate into fibre cells in the equatorial region (*26*). This single layer of lens epithelial cells is important for maintaining metabolic homoeostasis and transparency of the lens (*25*). Apoptosis-like events play an important role in lens development (*27*, *28*), and it is known that the apoptosis of lens epithelial cells is associated with cataract development (*6*, *29*). It has been reported that IFN-γ leads to cataract development by causing the apoptosis of lens epithelial cells; therefore, it is conceivable that IFN-γ generated by activated IL-18 induces the apoptosis of lens epithelial cells resulting in cataract development in UPL lens. On the other hand, Chen *et al*. (*30*) reported that IFN-γ induces excessive nitric oxide (NO) production *via* iNOS. We also previously reported that the induction of iNOS mRNA expression and iNOS protein occur prior to the elevation in the lens Ca^{2+} content and opacification in UPL rats (*17*). The excessive production of NO *via* iNOS causes damage in lens epithelial cells (*31*). The oral administration of disulfiram, a dimer of diethyldithiocarbamate,

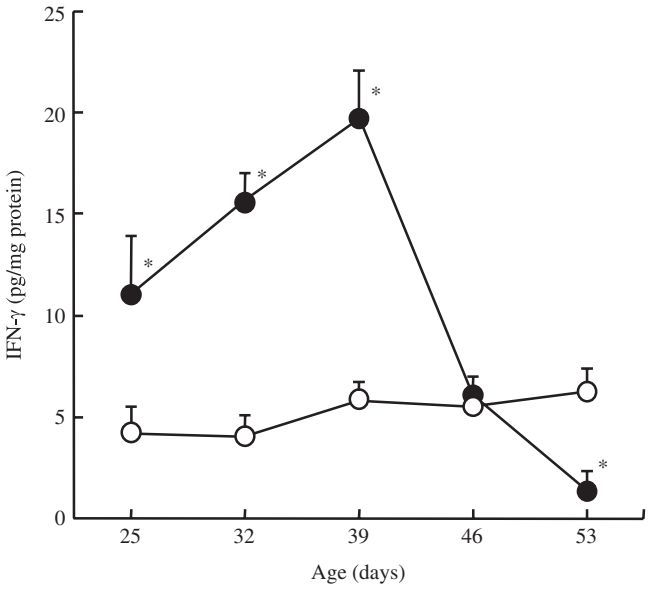

Fig. 5. **Changes in IFN-γ levels in the lenses of 25–53-day-old normal and UPL rats.** IFN-γ levels were determined using the ELISA method. Normal rat (open circle), UPL rat (filled circle). The data are presented as means ± SE of 6–11 independent rat lenses. $^{*}P < 0.05$ vs. UPL rat.

an inhibitor of NFκB (*32*, *33*), and aminoguanidine, a selective inhibitor of iNOS (*34*), prevents the elevation in Ca^{2+} content and lens opacification in the lenses of UPL rats (*17*). Taken together, IL-18 may also be concerned in excessive NO production *via* iNOS in cataractous lenses in UPL rats. Further studies are needed to elucidate the precise relationship between cataracts and IL-18. Therefore, we are now planning to investigate the effect of IL-18 inhibitors on UPL rats.

In conclusion, we demonstrate the involvement of IL-18 in the development of cataracts in UPL rats. The expression levels of the IL-18 mRNA and protein in the lenses of UPL rats increase with aging. In addition, IFN-γ levels in the lenses of UPL rats are also increased. It is possible that IFN-γ generated by activated IL-18 may play a role in cataract development in UPL lens. These findings provide information significant for designing further studies to develop potent anticataract drugs.

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