Cytokine production by human retinal pigment epithelial cells

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CHAPTER 6

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Expression of multiple forms of IL-1 receptor antagonist (IL-1ra) by human retinal pigment epithelial cells: identification of a new IL-1ra exon

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SUMMARY

The eye is considered an immunologically privileged organ and is separated from the rest of the body by blood-ocular barriers. Part of the blood-retina barrier consists of the retinal pigment epithelium (RPE). In addition to the physical barrier the monolayer of RPE cells forms, these cells contribute to ocular immune privilege by producing anti-inflammatory molecules that down-regulate potential damaging immune reactions. In this study the mRNA expression of interleukin-1 receptor antagonist (IL-1ra) by RPE cells was studied in 15 donor derived cell lines. Expression of both the intracellular and secreted IL-1ra was detected in unstimulated and IL-1β or phorbol 12-myristate 13-acetate-exposed RPE. Analysis of IL-1ra protein in RPE cell lysates and cell culture supernatants indicated that these cells produce mainly intracellular IL-1ra. No correlation between IL-1ra expression levels and the IL-1ra gene polymorphism could be detected. In addition to the two known intracellular IL-1ra variants (intracellular IL-1ra type I and type II) evidence is provided for the expression of a hitherto unknown splice variant of the IL-1ra mRNA by RPE cells. Expression was not confined to RPE cells and could also be detected in cultured human fibroblasts and macrophages. This variant, which we have tentatively named intracellular IL-1ra type III, encodes a C-terminally truncated protein of only 27 amino acids.

INTRODUCTION

The immune privileged nature of the human eye requires a strict regulation of immune reactions to prevent damage to the adjacent fragile neural tissue and to maintain transparency of the optic media. Immune privilege is obtained through various mechanisms like the absence of lymphatic drainage, the constitutive or induced expression of several immune modulating factors and the presence of a physical barrier between the eye and the blood. Part of the blood-eye barrier is formed by the retinal pigment epithelium (RPE) cell layer, strategically situated between the photoreceptors and the choriocapillaris. RPE cells further contribute to the unique microenvironment of the
posterior part of the eye by producing various cytokines like TGF-β2 and Fas ligand. The pro-inflammatory cytokine IL-1 is one of the most potent multifunctional cell activators and is also a strong stimulator of RPE cells. In reaction to IL-1β RPE cells are known to produce high levels of immunomodulatory molecules. To maintain retinal-tissue integrity during ocular inflammation the stimulation of RPE cells by IL-1β should therefore be precisely regulated and requires tight physiological control. One of the mechanisms to modulate the effects of IL-1 is via its naturally occurring antagonist the IL-1 receptor antagonist (IL-1ra). IL-1ra binds to the IL-1 receptors with similar affinities as do IL-1α and IL-1β, but without evoking intracellular signal transduction. Two variants of IL-1ra have been described, namely secreted IL-1ra (sIL-1ra) and intracellular IL-1ra (iIL-1ra). Secreted IL-1ra contains an N-terminally located signal sequence for secretion and was found as a secretory product of monocytes, but is now known to be expressed by a variety of cell types. iIL-1ra lacks the signal sequence and is found mainly in the cytoplasm of epithelial cells, including ocular tissues such as cornea and RPE cells. Although the function of iIL-1ra is not fully elucidated it is suggested that it inhibits IL-1 signalling either by release during tissue damage or by binding to the IL-1 receptor before presentation on the cell surface. Furthermore, IL-1ra has been shown to alter IL-1 induced gene expression by influencing messenger RNA stability of several cytokines.

The gene structure of IL-1ra is relatively complex. There are four so-called common exons (used by both the sIL-1ra and iIL-1ra) and two distinct first exons. The signal sequence for sIL-1ra is encoded by sequences immediately upstream of the first common exon (actually forming one exon). The first common exon contains a splice acceptor site within the coding region. This splice site is used to produce the intracellular form of which the first exon is separated from the common exons by a first intron, with an approximate size of 9.6 kb. More recently a splice variant of the iIL-1ra has been described (iIL-1ra type II), which contains an additional exon situated within the first intron.

In addition to the heterogeneity of the IL-1ra mRNA, the IL-1ra gene has been shown to be polymorphic. The second intron contains an 86 bp tandem repeat which occurs in different copy numbers. This repeat contains two consensus transcription silencer elements and has been shown to affect IL-1ra expression levels in monocytes.

Although RPE cells have been shown to constitutively express iIL-1ra type I, the possible expression of iIL-1ra type II and sIL-1ra has not been reported, and was
therefore the subject of the present study. We demonstrate that RPE cells are capable of expressing sIL-1ra and both types of icIL-1ra. In addition, a new splice variant of IL-1ra (icIL-1ra type III) was identified in RPE cells. This splice variant is predicted to give rise to a C-terminally truncated peptide of 27 amino acids.

RESULTS

Expression of IL-1ra by RPE cells

RPE cell lines from 15 different human donors were investigated by reverse transcription (RT)-PCR for IL-1ra gene expression. Cultures were assayed for constitutive expression and after exposure to IL-1β or PMA, both of which have been shown to be potent inducers of the IL-1ra gene\(^2\). In all cell lines tested constitutive expression of icIL-1ra could be detected using PCR primers 1 and 2 (Figure 1). Stimulation with IL-1β resulted in a maximal 55-fold increase of icIL-1ra mRNA while PMA stimulation led to a maximal 17-fold increase (Figure 2, Table 1). IL-1ra mRNA expression levels were normalized by RT-PCR analysis of mRNA levels for a household gene, porphobilinogen deaminase (PBGD)\(^2\). Remarkable differences between cell lines could be observed. While some cell lines reacted more strongly on exposure to IL-1β (Table 1, e.g. cell line 15), others produced highest icIL-1ra mRNA levels after PMA stimulation (Table 1, e.g. cell line 2). The number of passages of the RPE cell lines had no influence on the levels of cytokine expression (data not shown).

![Figure 1](image-url) Schematic representation of the human IL-1ra gene. Boxes represent the exons. The part of exon 1 specific for sIL-1ra is shown in black. The exons specific for the intracellular splice variants are icl, the first exon of icIL-1ra type I; icll, the second exon of icIL-1ra type II and the iclll, the third exon of the icIL-1ra type III. The lengths of the exons and introns are not to scale. The position of the PCR primers (numbers 1-5) are indicated with arrows and the positions of the in-frame stop codons are indicated with dots.
Table 1  
Relative expression and fold increase of expression of iclL-1ra type I and sIL-1ra by 15 donor RPE cell lines unstimulated or treated with IL-1β or PMA.

<table>
<thead>
<tr>
<th>donor #</th>
<th>alleles(b)</th>
<th>iclL-1ra type I</th>
<th>sIL-1ra</th>
<th>fold increase(a)</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>control</td>
<td>IL-1</td>
<td>PMA</td>
</tr>
<tr>
<td>1</td>
<td>A1A2</td>
<td>4.3</td>
<td>24</td>
<td>57</td>
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<td>2</td>
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<td>7.5</td>
<td>15</td>
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<tr>
<td>4</td>
<td>A1A1</td>
<td>5.4</td>
<td>15</td>
<td>23</td>
</tr>
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<td>A2A3</td>
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<td>4.3</td>
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<td>A1A1</td>
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<td>7.2</td>
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\(a\)The overall mean ± SEM of the fold increase for the iclL-1ra after IL-1 or PMA stimulation is 6.8 ± 3.8 and 4.7 ± 1.1, respectively and for the sIL-1ra 29.5 ± 23.6 and 15.3 ± 8.9, respectively.  
\(b\)The IL-1ra genotype of the cell.

The expression of mRNA coding for the sIL-1ra was determined by using a PCR primer corresponding to part of the mRNA specific for the signal sequence in combination with a primer in exon 4 (PCR primer 2 and 3, Figure 1). Constitutive expression of sIL-1ra was detected in 13 out of 15 cell lines tested. After stimulation expression of sIL-1ra was detectable in all RPE cell lines. Again, the effect of IL-1β on IL-1ra expression on some cell lines was stronger as compared to treatment with PMA (Figure 2).

In addition to the intracellular IL-1ra as described by Haskill et al.\(^{18}\) a splice variant of icL-1ra has been identified\(^{23}\) (IL-1ra type II). The mRNA encoding this variant could selectively be detected by RT-PCR using a primer situated in the icL-1ra type II specific exon (PCR primer 4, Figure 1). The expression of icL-1ra type II specific mRNA was tested in two RPE cell lines and could be readily detected in unstimulated and stimulated cells. Up-regulation after IL-1β or PMA stimulation, as observed for the sIL-1ra and icL-1ra type I, was not apparent for the icL-1ra type II specific mRNA in RPE cells. Interestingly, an additional PCR product was observed which migrated slightly above the expected product of 443 basepairs (Figure 3).
Expression of IL-1ra by RPE cells

Figure 2 RT-PCR analysis of icIL-1ra type I and sIL-1ra expression by human RPE cells. Total RNA isolated from unstimulated cells (C) and cells exposed to IL-1β (I; 100U/ml) or PMA (P; 20 ng/ml) for 4 hours, was reverse transcribed followed by PCR amplification of icIL-1ra type I (PCR primers 1 and 2, Figure 1), sIL-1ra (PCR primers 2 and 3, Figure 1) and PBGD (Table 2). PCR products were size-fractionated on agarose gel, blotted and hybridized with an IL-1ra or PBGD specific probe. The expression by cell lines of 6 different donors are shown (numbers 1-6), as well as a negative (-) and a positive (+) control. Note that the exposure time of the Southern blots of the different gene products is not identical.

Figure 3 RT-PCR analysis of icIL-1ra type II expression. RPE cells from 2 different donors (numbers 1 and 2) were analyzed before (C) and after IL-1β (I) or PMA (P) stimulation (4 hours) for icIL-1ra type II expression by RT-PCR using primer combination 2 and 4 (Table 2). The band corresponding to the expected product of the expected length is indicated (icIL-1ra type II), the additional amplified product is indicated by an asterisk.

Identification of a new IL-1ra exon

To identify the origin of the additional PCR product, both fragments of the icIL-1ra type II PCR were cloned and subjected to sequence analysis. The icIL-1ra type II PCR product of the expected length appeared to be identical to the sequence as reported by Muzzio et al.23 (data not shown). The longer PCR product also contained the icIL-1ra type II specific exon sequence but in addition had an 171 bp insertion, located exactly between the splice donor site of icIL-1ra type II specific exon and the splice acceptor site of the icIL-1ra common exon 1, corresponding to part of the large intron upstream of the first
common exon (Figure 1 and 4). The remaining part of the messenger RNA (common exons 1 to 4) was identical to iclL-1ra type I and type II. In the genomic sequence the 171 nucleotides sequence was flanked by intron-exon junction consensus AG and GT(AAGT) nucleotides and was preceded by a polypyrimidine-rich tract (Figure 4, top sequence). Remarkably, this new exon contains an in-frame stop codon after eight nucleotides. The presence of this stop codon was confirmed by sequencing 4 independent clones and was also present in the IL-1ra gene sequence of the EMBL nucleotide sequence database (accession number U65590, unpublished). Translation of this new variant of the IL-1ra messenger would lead to a C-terminally truncated peptide of only 27 amino acids. Nine of these 27 amino acids are glycine residues, six of which are consecutive (Figure 4, bottom sequence). We named this alternatively spliced form of intracellular IL-1ra, iclL-1ra type III (Figure 1). Constitutive expression of iclL-1ra type III was not confined to RPE cells, but could also be detected in cultured human fibroblasts and macrophages, indicating little cell type-specificity (results not shown).
IL-1ra protein expression

To investigate whether RPE cells are capable of producing IL-1ra protein, cell lysates and culture supernatants were assayed by ELISA. In cell lysates of all four RPE cell lines tested IL-1ra protein could be detected, ranging from 223 pg to a maximum of 2.1 ng per 5x10^6 cells (Figure 5). The induction of the IL-1ra mRNA levels by IL-1ß or PMA was not reflected by an apparent increase on the IL-1ra protein level. The IL-1ra protein levels in the cell culture supernatants appeared to be low as compared to the protein levels in cell lysates, ranging from non detectable to 31 pg per 5x10^6 cells.

IL-1ra gene polymorphism and expression

In view of the variability of IL-1ra expression levels between individual RPE cell lines we tested whether expression was correlated with the IL-1ra genotype of the 15 donor derived RPE cell lines. Eight RPE cell lines were of the A1A1 genotype, six were of the A1A2 genotype and one was of the A2A3 genotype (Figure 6). This allele distribution is similar to the allele frequency for the Dutch population as reported earlier. Statistical analysis of the IL-1ra mRNA expression of untreated cells or cells exposed to IL-1ß or
PMA did not reveal any significant differences between the two groups with genotype A1A1 or A1A2 (Table 1). Of the four RPE cell lines analyzed for protein expression the two cell lines with the A1A1 genotype appeared to contain less icIL-1ra than the cell lines with the A1A2 genotype (Figure 5).

**DISCUSSION**

In this report we demonstrate that RPE cells express all three variants of IL-1ra mRNA and produce icIL-1ra protein and possibly secrete low levels of IL-1ra. Furthermore, a new IL-1ra exon is described which leads to the production of a C-terminally truncated protein of only 27 amino acids which is rich in glycine residues.

The RPE cell layer is an important barrier structure in the posterior segment of the human eye and is thought to play a central role in the immunological processes of the outer retina. These processes should be precisely regulated to prevent damage to the vulnerable neurosensory retinal tissue. RPE cells are armed with a variety of mechanisms to maintain retinal homeostasis and to modulate inflammatory reactions. The tight-junctions between RPE cells form a physical barrier and regulate the access of leukocytes to the retina. RPE cells express Fas ligand on their cell surface to locally inhibit activated T cells. More recently, we demonstrated that RPE cells further contribute to a highly
regulated ocular immune response by secreting pro-inflammatory cytokines preferentially in the direction of the choroid\textsuperscript{13}. Interleukin-1\textbeta is one of the most potent stimulators of RPE cells and induces expression of several proinflammatory cytokines and chemokines\textsuperscript{6,9,28}. Uveitic models confirm the inflammatory potential of IL-1\textbeta\textsuperscript{29,30} and suggest that strict regulation of this mediator is essential for maintaining retinal integrity during ongoing inflammatory processes. The regulation of IL-1 signalling is strikingly complex and emphasizes the need for precise control of IL-1 activity. IL-1 comprises two mediators, IL-1\textalpha and IL-1\textbeta and two high-affinity receptors IL-1 RI and RII. Only IL-1 RI has intracellular signalling activity while IL-1 RII has been proposed to be a 'decoy' receptor and to compete with IL-1 RI for IL-1\textsuperscript{31}. The IL-1 RII receptor occurs as a membrane bound form and as a soluble protein. The IL-1ra is a naturally occurring inhibitor of IL-1 that binds to the membrane bound IL-1 receptors without agonistic activity, but not to the soluble IL-1 RII\textsuperscript{32}. The IL-1 system is also active in human RPE cells since these cells are known to express the IL-1 RI\textsuperscript{33}, react strongly upon IL-1\textbeta stimulation\textsuperscript{6,9,28}, produce IL-1\textalpha\textsuperscript{5,14} and icIL-1ra\textsuperscript{5}.

In this paper we extended these observations by analysing the expression of the variants of IL-1ra in several human RPE cell lines. Using RT-PCR the expression of the icIL-1ra type I and type II could be detected, which was upregulated after exposure to IL-1\textbeta or PMA. Alternatively, the increase in IL-1ra mRNA after stimulation could also be explained by decreased mRNA degradation. icIL-1ra protein could be readily detected by ELISA in RPE cell lysates. Remarkably, the increase of icIL-1ra mRNA after IL-1\textbeta or PMA stimulation was not reflected by an increase of IL-1ra protein. A similar observation was reported by Jaffe et al.\textsuperscript{5} for stimulated RPE cells. Possibly, translation efficiency is affected by stimulation or the increase in IL-1ra mRNA is only transiently and not detected on protein level after 24 hours. Binding of icIL-1ra to an intracellular ligand, thereby making the protein undetectable for ELISA, could also explain the unchanged protein levels after stimulation. icIL-1ra is suggested to modulate intracellular IL-1\textalpha signalling in RPE cells\textsuperscript{5} since IL-1\textalpha is active in its intracellular precursor form\textsuperscript{34}. Other possible mechanisms by which icIL-1ra affects IL-1 signalling are blocking the IL-1 receptors before presentation on the cell surface and release of icIL-1ra during cell damage\textsuperscript{21}. Recently, it has been shown that human airway epithelial cells are capable of releasing icIL-1ra in their environment\textsuperscript{35}. 
Possibly, RPE cells are also capable of secreting their icIL-1ra under certain conditions. 

By differential splicing, two variants of the icIL-1ra protein are produced: icIL-1ra type I and type II. The icIL-1ra type II has a glycine-rich insertion of 21 amino acids within the N-terminal part of the protein as compared to the type I protein. Both intracellular forms of IL-1ra are biologically active and have similar capacity to inhibit IL-1 activity. Our results showed that RPE cells also express the mRNA coding for the secreted variant of IL-1ra, which contains an N-terminally located signal sequence. In a report by Jaffe et al., this variant could not be detected by RT-PCR in cultured RPE cells. This discrepancy is most likely due to the increased sensitivity of the assay used in this paper; Southern blot analysis using labelled probes are more sensitive and specific than ethidium bromide staining.

To determine whether RPE cells are capable of secreting IL-1ra, culture supernatants were assayed by ELISA. Only small amounts of IL-1ra were detected in these supernatants suggesting that RPE cells mostly produce icIL-1ra. Furthermore, the ELISA does not discriminate between intracellular and secreted IL-1ra and we can not exclude the possibility that the IL-1ra in the supernatant is derived from leakage of icIL-1ra from damaged cells. This implies that the mRNA coding for sIL-1ra is not efficiently translated by RPE cells. A similar mechanism was suggested for the observation that RPE cells expressed the IL-1β gene but contained only a small amount or no IL-1β protein. Alternatively, RPE cells which are known to express the IL-1 receptor I may efficiently bind their sIL-1ra or lack the mechanism to secrete sIL-1ra.

Considerable variation in IL-1ra expression levels among RPE cell lines of different donors was observed. Similar variations in levels of cytokine expression by RPE cell lines have been reported earlier, and may reflect intrinsic variability among donors. We were unable to link the expression of the IL-1ra gene to the polymorphism which is known to affect expression in monocytes. Of the four RPE cell lines analyzed for intracellular IL-1ra protein the two cell lines homozygous for allele A1 showed a somewhat reduced expression of icIL-1ra as compared to the two A1A2 heterozygous cell lines. This difference in expression between A1A1 and A1A2 IL-1ra genotypes is in accordance with production of IL-1ra protein by human monocytes related to this IL-1ra gene polymorphism in monocytes. Due to the paucity of different human RPE cell lines and the predominance of the IL-1ra A1 allele, small differences in expression between various
alleles would remain undetected on the mRNA level. Alternatively, the transcription silencing function ascribed to this polymorphic region in monocytes may not be operational to the same extent in RPE cells.

In addition to the three known molecular variants of IL-1ra (sIL-1ra, icIL-1ra type I and type II), we have identified an additional splice variant which was named intracellular interleukin-1 receptor antagonist type III (iclIL-1ra type III). This new transcript contains an additional 171 bp sequence situated between the icIL-1ra type II specific exon and the common exon 1. The presence of consensus splicing signals bordering this sequence and its presence in several sequenced RT-PCR products, indicated that this is a functional exon. When incorporated into the icIL-1ra mRNA the in-frame stop codon present in this icIL-1ra type III specific exon will lead to C-terminally truncated protein of only 27 amino acids. The insertion of an exon containing an in-frame stop codon or leading to a frame-shift resulting in a stop codon has been described for numerous alternatively spliced messenger RNAs, and in some cases was shown to lead to a physical separation of functional protein modules\textsuperscript{36-38}. Possibly, the glycine-rich sequence which is linked to the N-terminus of icIL-1ra type II represents such a module. The stop codon in the icIL-1ra type III, as presented in this paper, would then lead to the expression of this module without the icIL-1ra protein attached. The icIL-1ra type III is very unlikely to bind to the IL-1 receptor as it lacks the C-terminal IL-1ra sequences involved in receptor binding\textsuperscript{21}. Of the 27 amino acids for which the icIL1-ra type III mRNA encodes, 21 are from the type II specific exon which contains 9 glycine residues. Glycine-rich regions have been shown to be involved in protein-protein interactions of mainly nucleic acid binding proteins\textsuperscript{39}. Possibly, icIL-1ra type II and type III further add to the complexity of the IL-1 system by binding to intracellular regulatory proteins. Further research is, however, needed to clarify the function of these splice variants. Our analysis indicate that these splice variants can also be observed in fibroblasts and macrophages and are thus not unique to the RPE cell. The observation that the IL-1ra gene is highly active in RPE cells indicates that IL-1ra contributes to the unique microenvironment of the posterior segment of the human eye.
MATERIALS AND METHODS

RPE cell cultures

Human donor eyes (age of the donors: 12-23 years) obtained from the eye bank were used as a source of RPE cells. RPE cells were isolated within 24 hours post mortem, as described previously\(^{13}\). Briefly, after removal of the cornea (for transplantation purposes), iris and ciliary body, the optic nerve was cut and vitreous and neural retina were washed out of the eye cup with Hank's Balanced Salt Solution (HBSS, Gibco BRL, Breda, The Netherlands). RPE cells were detached from the eye cup by trypsin dissociation. Isolated cells were plated in 24-well plates (Costar, Cambridge, MA, USA) at \(10^5\) cells per well in Iscoves Modified Dulbecco's Medium (IMDM, Gibco BRL) supplemented with 20% Fetal Calf Serum (FCS, Gibco BRL), penicillin (100 U/ml, Gibco BRL) and streptomycin (100 \(\mu\)g/ml, Gibco BRL). Nonadherent cells were removed after two days by washing and refreshing the culture medium. RPE cell lines were analyzed morphologically and immunohistochemically to detect possible contamination with other cell types\(^{13}\). At confluency, cells were detached by trypsin treatment and seeded in culture flasks at approximately \(4 \times 10^4\) cells/cm\(^2\). For stimulation experiments, RPE cells between the second and eighth passage were used. IL-1ra expression was determined in both unstimulated or stimulated RPE cell cultures. Cells were stimulated with 100 U/ml IL-1\(\beta\) (Genzyme Diagnostics, Cambridge, MA, USA), or 20 ng/ml PMA (Sigma Chemical Co., St Louis, MO, USA) for 4 hours (messenger RNA analysis) or 24 hours (protein analysis). Earlier studies have shown that mRNA for IL-1ra was produced optimally by RPE cells after 4 hours of stimulation in RPE cells\(^5\) while IL-1ra protein was measured after 24 hours in various cell types\(^{16,18,19,25}\).

RNA isolation and cDNA synthesis

Before RNA isolation cells were washed with PBS (37\(^\circ\)C). RNA was isolated by a single step extraction method using RNAzol (Cinna Biotecx Laboratories Inc., Houston, Texas, USA) and dissolved in 10 \(\mu\)l sterile water. For cDNA synthesis, 2 \(\mu\)g of total RNA
Expression of IL-1ra by RPE cells

was incubated with 2.5 µg oligo(dT)₁₂₋₁₈ primer and 200 U Superscript RNaseH reverse transcriptase (Gibco-BRL, Eggenstein, Germany) according to the manufacturers' instructions. After incubation for 1.5 hour at 42°C, the reaction was terminated by heat inactivation at 65°C for 5 minutes.

Table 2 Characteristics of IL-1ra PCR primers.

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<th>primer sequence (5'-3')</th>
<th>primer position in genomic sequence*</th>
<th>primer position in IL-1ra gene</th>
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*The numbers corresponding to the position of the PCR primers were based on the genomic sequence of IL-1ra in the EMBL nucleotide sequence database (accession number U65590, unpublished).

IL-1ra PCR and Southern blot analysis

PCR primer pairs for the amplification of sIL-1ra, icIL-1ra and porphobilinogen deaminase (PBGD) were designed using the OLIGO Primer Analysis Software (National Biosciences Inc, Plymouth, MN, USA) and are shown in Table 2. PCR primer pairs for icIL-1ra type II²³ and the IL-1ra gene polymorphism²⁴ have been described. Oligonucleotides were synthesized by Eurogentec (Seraing, Belgium). PCR products were cloned in pGEMTeasy (Promega, Madison, WI, USA) using standard procedures. For PCR amplification, 2 µl cDNA was added to a reaction mixture, consisting of PCR buffer (Promega, Madison, WI USA), 0.5 mM dNTP's, 0.2 pmol of each primer and 1 U Taq DNA polymerase (Promega, Madison, WI, USA) in a volume of 50 µl. After the PCR reaction was carried out in a thermocycler (Perkin Elmer Cetus, Norwalk, CT, USA). Part of the PCR reaction mixture was electrophorized on a 2% agarose gel and visualized by
ethidium bromide staining. To verify the identity of the PCR products, gels were blotted to Genescreen-plus membranes (NEN-Du Pont, 's Hertogenbosch, The Netherlands) after denaturation in 0.4 N NaOH for 20 min. The filters were incubated in hybridization mixture (6xSSC, 5xDenhardt’s, 0.1% SDS, 100 μg/ml sheared and denatured herringsperm DNA) at 65°C for 15 minutes. Purified cloned PCR products of IL-1ra and PBGD were used as templates for 32P-labelled probes using the multiprime DNA labelling system (Amersham, UK). After overnight hybridization, filters were washed in 0.2 X SSC, 0.1% SDS at 65°C for 10 minutes and exposed to X-ray film at -70°C.

Cloning and sequence analysis of icIL-1ra type III.

The PCR products of the icIL-1ra type II PCR (see Table 2 for primer combination) were excised from gel, purified and cloned in pGEMTeasy and transformed to E.coli JM109. From the lower band one clone and from the upper band, four clones were further analyzed by sequencing the complete icIL-1ra type III specific exon. Sequences were compared to database entries using the BLAST search tool[40]. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AJ005835.

Dotblot analysis

To quantify IL-1ra expression levels of unstimulated and IL-1β or PMA treated RPE cells, an aliquot of the PCR product was dotblotted on Genescreen-plus membrane and hybridized with random-primed labelled probes. After hybridization and washing the radioactivity of each dot was determined by scintillation counting. Signals were corrected for background and amount of input RNA by determining the RT-PCR signal for the PBGD household gene.

PCR analysis of the IL-1ra gene polymorphism

Genomic DNA was isolated from RPE cells according to the method described by Boom et al.[41]. For determining the IL-1ra alleles of the various RPE cell lines the region
containing the 86-bp tandem repeat of the IL-1ra gene was amplified by PCR using the primer combination as described\textsuperscript{24} (Table 2). PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. The size of the PCR products, indicative for the genotype, was determined using a molecular 100 bp ladder (Gibco-BRL). In addition several DNA preparations with known IL-1ra genotypes were analyzed in parallel.

**IL-1ra protein**

RPE cell culture supernatants were isolated whereafter the cells were washed three times with warm PBS (37°C). Cell lysates were obtained by three cycles of freezing and thawing. Supernatants and cell lysates were stored at -20°C until assay. IL-1ra protein was measured using a commercially available ELISA consisting of a matched antibody pair and recombinant human IL-1ra as a standard (Medgenix Diagnostics, Fleurus, Belgium) with a detection limit of 4 pg/ml. The ELISA does not discriminate between secreted or intracellular forms of IL-1ra. Cell lysates were tested undiluted, whereas supernatants of the stimulated RPE cells were concentrated 25 times using Ultrafree-MC centrifugal filter units (Millipore Co., Bedford, MA, USA) with a cutoff of 10 kDa, according to the instructions of the manufacturer.

**Statistics**

The difference in IL-1ra expression level of the two groups with different IL-1ra polymorphism was tested by the Mann-Whitney test. Differences were considered statistically significant when \(P=0.05\).

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