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ARTÍCULO

Over-expression of $P2Y_2$ receptor after silencing in corneal wound healing

Aránzazu Mediero [§], Almudena Crooke [§], Jesús Pintor [*]

Departamento de Bioquímica y Biología Molecular IV.
Escuela Universitaria de Óptica. Universidad Complutense de Madrid.
[§] Estos autores contribuyeron de la misma manera al trabajo.
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ABSTRACT

Diadenosine polyphosphates are a family of dinucleotides with relevant properties in the eye and in other tissues. Diadenosine polyphosphates can activate P2Y and P2X receptors present on the ocular surface, anterior segment and retina. In the cornea, the presence of a $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor has been identified. Both diadenosine polyphosphates and other purinergic agonists modified corneal wound healing depending on the receptor that is activated by these substances. To confirm the involvement of the $P2Y_2$ receptor in the wound healing process after the challenge with Ap_4A, we have designed siRNA against $P2Y_2$ receptor. We have observed that $P2Y_2$ is localized in the most external layer of the corneal epithelium. The pre-treatment with siRNA produced a disappearance of the receptor at 12 and 24 hours after the wound, being the location for $P2Y_2$ restored 36 hours after the wound. We have also observed that in half of the tested corneas, there was an increase in the $P2Y_2$ expression after silencing compared to control and Ap_4A treated corneas, being this receptor localized both in corneal epithelium and stroma.

Key Words: Ap_4A; $P2Y_2$; siRNA; Corneal wound healing.

RESUMEN

Sobreexpresión del receptor $P2Y_2$ tras su silenciamiento durante el proceso de cicatrización corneal

Los diadenosina polifosfatos son una familia de dinucleótidos con gran relevancia en las propiedades del ojo y de otros tejidos. Estos diadenosina polifosfatos pueden activar los receptores P2Y y P2X presentes en la superficie ocular, en el segmento anterior y en la retina. En la cornea, se ha identificado la presencia de receptores $P2Y_2$, $P2Y_4$ y $P2Y_6$. Tanto los diadenosina polifosfatos como los receptores purinérgicos modifican el proceso de cicatrización corneal dependiendo del tipo de receptor activado por los distintos dinucleótidos. Para localizar el receptor $P2Y_2$ en córneas lesionadas y tratadas con Ap_4A en presencia o ausencia de un siRNA para el receptor $P2Y_2$, hemos realizado un ensayo de inmunohistoquímica. Hemos observado que el receptor $P2Y_2$ se localiza en el epitelio tras la lesión corneal y el consecuente tratamiento con Ap_4A. El pre-tratamiento con el siRNA produce la desaparición de la señal para este receptor tanto a las 12 como a las 24 horas de la lesión corneal, siendo la localización de este receptor $P2Y_2$ recuperada a las 36 horas de la lesión en presencia del siRNA. Además, hemos observado que en la mitad de las córneas analizadas, existía un incremento en la expresión del receptor $P2Y_2$ tras el silenciamiento del mismo comparado con las córneas control y con las tratadas con Ap_4A, localizando la presencia de este receptor tanto en el epitelio como en el estroma corneal.

Palabras clave: Ap_4A; $P2Y_2$; siRNA; Cicatrización corneal.

1. INTRODUCTION

The cornea is one of the most important components of the optical pathway. It is a multilayered tissue characterized by its transparency, avascularity, the ability to refract light and to filter out incoming ultraviolet radiation. Within the five layers that compound the cornea —epithelium, Bowman's membrane, stroma, Descemet's membrane and endothelium— the epithelium is the outer layer and the one that is easily damaged due to diverse factors. These include

the entry of a foreign body, any traumatic process, a defect in contact lenses or the use of refractive surgery to correct refractive alterations.

When this happens, a process named corneal wound healing starts to regenerate normal epithelium in order to maintain the correct refraction of light. Corneal wound healing involved three consecutive phases that are part of a continuous process. Animal studies have shown that these three stages are: lag phase (from 0 hours to 10 hours after the wound), cell migration (until 24 to 36 hours after the wound) and cell proliferation (lasting from 24-36 hours after the wound to weeks) (1).

There are many substances present in tears, aqueous humour or released from corneal nerves, that modified the wound healing process after ocular surface injuries (2, 3). Within these molecules we find nucleotides and dinucleotides (3, 4). In our previous works we demonstrated that the dinucleotides can modify rate of corneal re-epithelialization in New Zealand White Rabbits both *in vivo* and *in vitro* (3, 5). We have demonstrated, both pharmacologically and with the used of the RNA interference (RNAi) technology, that Ap_4A produces acceleration in the rate of corneal re-epithelialization by stimulating to $P2Y_2$ receptors. On the contrary other dinucleotides, Ap_3A and Ap_5A exert the opposite effect delaying corneal re-epithelialization by binding to a $P2Y_6$ receptor (5, 6).

The aim of this manuscript is to describe the presence of the $P2Y_2$ receptor in the cornea and to see the effect of a siRNA against the $P2Y_2$ receptor in the presence and in the absence of Ap_4A.

2. MATERIALS AND METHODS

2.1. Animals

Male, adult New Zealand White Rabbits were used. All the animals were kept in individual cages with free access to food and water, under controlled cycles (12 hours light:12 hours dark), and the experimental procedures were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the European Communities Council Directive (89/609/EEC).

2.2. $P2Y_2$ silencing

To design $P2Y_2$ receptor-specific siRNA duplexes, rabbit $P2Y_2$ receptor coding sequence (GenBank accession number **EU886321)** was submitted to the Ambion siRNA target Finder website (http://www.ambion.com/techlib/misc/siRNA_finder.html) for siRNA prediction. One sequence of nine (max. GC content 60%) suggested candidates was selected (6). Nucleotide sequence of the siRNA target sites was as follows: $P2Y_2$ siRNA #2, 5'-AACCTGTACTGCAGCATCCTC-3'. This siRNAs was obtained from Applied Biosystems, in annealed and lyophilized forms and were suspended in 0.9% NaCl before *in vivo* use.

2.3. *In vivo* delivery of $P2Y_2$ siRNA and wounding procedure

The siRNA was applied in one single eye in 10 nmol 0.9% NaCl drops (volume instilled 40 µl) along four consecutive days. The contralateral eye received the same volume of saline solution (0.9% NaCl). Slit-lamp biomicroscopy was performed during instillation process to evaluate possible changes in the cornea.

Corneal wounds were performed 10 hours before the fourth siRNA instillation. After topical anaesthesia (0.4% oxibuprocaine and 1% tetracaine, Alcon Cusi, Barcelona, Spain), corneal wound were made to the epithelium of both eyes by applying a 5-mm disc of Whatman no. 1 paper soaked in n-heptanol (Sigma-Aldrich, St. Louis, MO) as previously described (3). Briefly, discs were place in the centre of the cornea and left there for 30 seconds (7) and after removal of the disc, the eyes were washed with isotonic saline solution.

Ap_4A treatment was performed every six hours as described previously (3).

2.4. Immunohistochemistry

12, 24 and 36 hours after epithelium wounding (72, 84 and 96 hours after the first siRNA instillation), rabbits were euthanized with sodium pentothal and eyes were enucleated. Corneas were dissected

and fixed with 4% paraformaldehyde in PBS 0.15M at 4 ºC for 6 hours. After fixation, corneas were embedded in Jung Tissue Freezing Medium (Leica Microsystems, Barcelona, Spain) and 10 µm sections were done. $P2Y_2$ immunocytochemical assay was performed as previously described for cells. Briefly, sections were permeabilized with blocking solution (PBS 1X BSA 3% Triton X-100 FBS 5%) for 1 hour to block the non-specific binding, and after washing with PBS 1X BSA 3%, sections are incubated with primary goat polyclonal anti-$P2Y_2$ (1:50) or PBS 1X BSA 3% for negative controls overnight at 4 ºC. Sections were washed twice in PBS 1X BSA 3% and incubated with the secondary antibody donkey anti-goat IgG-FITC (1:200) for 1 hour at room temperature. Finally, after washing in PBS 1X slices were mounting with Vectashield mounting medium and observed under confocal microscope (Axiovert 200M; Carl Zeiss Meditec GmbH, Jena, Germany), equipped with a Pascal confocal module (LSM 5; Zeiss). All images were managed with the accompanying Pascal software.

3. RESULTS

3.1. $P2Y_2$ location in the cornea

Immunocytochemical analysis for $P2Y_2$ in the cornea reveals that this receptor is mainly localized in the outer layer of the epithelium (Figure 1), while the inner layers of the epithelium are barely marked. We have not found any $P2Y_2$ signal in the other layers of the cornea, neither in the stroma nor in the endothelium.

3.2. Inhibition of $P2Y_2$ receptor expression by siRNA

After performing the treatments described in Methods, corneas were wounded and the effect of the siRNA against the $P2Y_2$ receptor was tested by immunohystochemical analysis 12, 24 and 36 hours after the healing (72, 84 and 96 hours after the first siRNA instillation). As we can observe, after the corneas were wounded, the $P2Y_2$ receptor was still localized in the outer layer of the epithelium, being this signal higher in control corneas than in Ap_4A treated corneas,

both at 12 and 24 hours after wounding (Figure 2A and 2B). 36 hours after the wounds were performed, $P2Y_2$ staining was similar in the three different treatments, including the siRNA treated corneas, revealing a full recovery of the $P2Y_2$ receptor (Figure 2C).

These results indicate that silencing the $P2Y_2$ receptor in our model was detected 12 hours after the wound was performed. Nevertheless, $P2Y_2$ receptor signal was again visible 36 hours after the wound had been made.

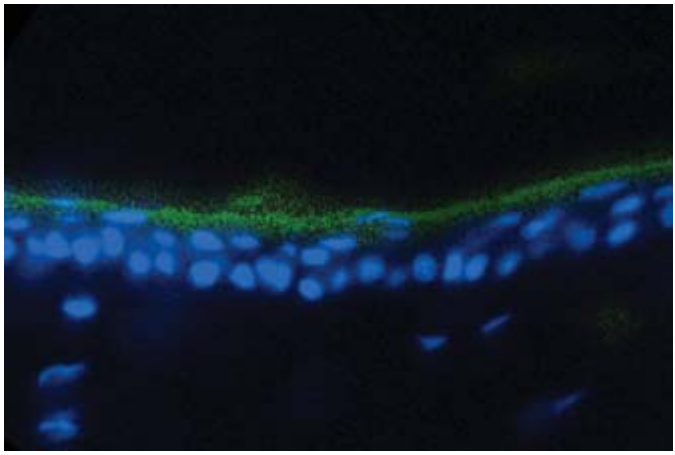

Figure 1. $P2Y_2$ receptor location in the cornea. Immunocytochemical analysis for the $P2Y_2$ in the cornea revealing the presence of the $P2Y_2$ receptor in the corneal epithelium (green fluorescence by FITC). Image managed with the Pascal software of the Axiovert 200M confocal microscope at 40X magnification.

3.3. Over-expression of $P2Y_2$ receptor after silencing

In half of the siRNA treated corneas, and 36 hours after the wounding (96 hours after the first siRNA instillation), we have observed an increase in the expression of $P2Y_2$ receptors compared with control and Ap_4A (Figure 3). In this case, the $P2Y_2$ signal was not constrained to the outer layer of the epithelium, and it was possible to localize $P2Y_2$ receptors in the whole epithelium and in the stroma (Figure 3).

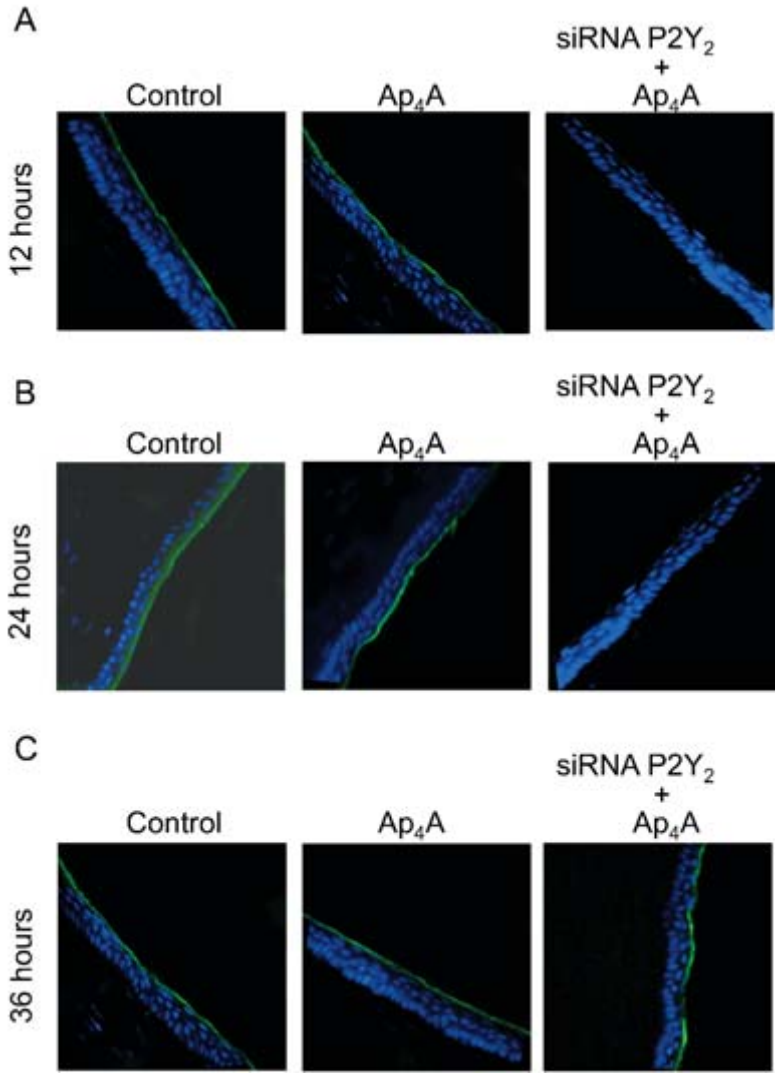

Figure 2. $P2Y_2$ immunostaining of treated corneas after wound. (A) A series of micrographs showing the $P2Y_2$ signal in corneas treated with saline 0.9%, Ap_4A 100 µM and siRNA + Ap_4A 100 µM, 12 hours after wound. **(B)** Immunostaining for $P2Y_2$ in treated corneas 24 hours after the wound. **(C)** A series of micrographs showing the $P2Y_2$ signal in corneas treated with saline 0.9%, Ap_4A 100 µM and siRNA + Ap_4A 100 µM, 36 hours after wound. Green fluorescence (FITC) localizes $P2Y_2$ receptor while in blue we can observe the nuclear staining for DAPI. Images are managed at a magnification of 40X.

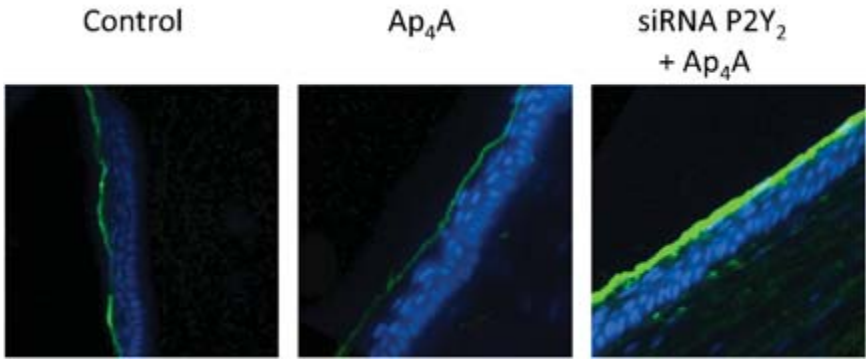

Figure 3. Over-expression of $P2Y_2$ receptor after silencing. Immunostaining for $P2Y_2$ 36 hours after wound (96 hours after the first siRNA instillation) where we can observed an increase in $P2Y_2$ expression after siRNA instillation compared with control and Ap_4A treated corneas. Green fluorescence (FITC) localizes $P2Y_2$ receptor while in blue we can observe the nuclear staining for DAPI. Images are managed at a magnification of 40X.

4. DISCUSSION

As we have previously mentioned, the cornea is formed by five to six different layers, including the outer one, the epithelium. The distribution of the purinergic receptors present in the cornea revealed that $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor are present this part of the eye (8).

The present experimental work confirms the location of the $P2Y_2$ receptor after corneal wound healing and how when using a siRNA against this receptor there is an initial disappearance of the receptor followed by an over-expression of this protein.

The presence of the $P2Y_2$ receptor in the epithelium is related to the ability of some nucleotides to increase the rate of re-epithelialization after a corneal wound (6). The involvement of metabotropic P2 receptors in corneal wound healing has been also reported by other groups and in all the cases the different researchers report that ATP, UTP and Ap_4A accelerate the rate of healing (9-11).

Our IHC results reveal that after wounded, the $P2Y_2$ staining in Ap_4A treated lessons is less intense that in control wounds. As happens

with many other agonists (for example insulin), when Ap_4A binds to its receptor $P2Y_2$ on the cell surface, the Ap_4A-$P2Y_2$ complex undergoes down-regulation and presumably endocytosis and is subsequently intracellular lysosomal/proteosomal degradation (12, 13). This down-regulatory mechanism together with the receptor rate of synthesis permits to maintain a minimal number of $P2Y_2$ receptor on epithelial cell surface. This is absolutely relevant since in case that an injury occur the cornea needs to trigger the wound healing mechanism to keep this ocular structure perfectly transparent.

All this equilibrium between the production and degradation of the $P2Y_2$ receptor is altered when a selective siRNA against the $P2Y_2$ mRNA is tested. When the siRNA starts it effect, there are still receptors both in their way to degradation and from the Golgi to the membrane. This fact produces a delay between the moment the siRNA is applied to the moment when it is possible to see a decrease in the $P2Y_2$ expression. There is a mechanism of repression of the protein synthesis that the epithelial cells try to resist, possibly by increasing the synthesis of $P2Y_2$-mRNA, but which is destroyed by the siRNA. Nevertheless, when the ability of the oligonucleotide decreases, it is possible that the overproduction of $P2Y_2$-mRNA can start to synthesize the protein reason by which we see an over-expression of $P2Y_2$ receptor 96 hours after the first siRNA instillation.

It is clear that more experiments should be done to confirm this hypothesis and also it would be interesting to see whether or not this effect is tissue selective or if this is a general feature of siRNA.

5. ACKNOWLEDGEMENTS

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*** Información de contacto:**
Doctor Jesús Pintor.
Dpto. de Bioquímica y Biología Molecular IV. Escuela Universitaria de Óptica. Universidad Complutense de Madrid.
C/ Arcos de Jalón, 118. 28037 Madrid (Spain).
Telf.: +34 91 394 68 59. Fax: +34 91 394 68 85.
Email: jpintor@vet.ucm.es