PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Expression of prostaglandin E receptor subtype EP4 in conjunctival epithelium of patients with ocular surface disorders
AUTHORS	Ueta, Mayumi ; Sotozono, Chie; Yamada, Keiko; Yokoi, Norihiko; Inatomi, Tsutomu; Kinoshita, Shigeru

VERSION 1 - REVIEW

REVIEWER	Yoshitsugu Inoue Professor and Chairman Division of Ophthalmology and Visual Science Faculty of Medicine, Tottori University, Japan I have no competing interests.
REVIEW RETURNED	01-Jun-2012

RESULTS & CONCLUSIONS	This paper has proven the expression of EP4 is different from various ocular surface diseases, however the order of description is confusing. In the present form, it looks like immunohistological study has revealed the difference, and real-time RT-PCR study confirmed that. But actually real-time RT-PCR study have not confirmed immunohistological study of this paper, but confirmed that of reference paper No. 8. The authors should mention real-time RT-PCR study first, which further confirm that EP4 is downregulated in chronic type of SJS/TEM and OCP, and then proceed to the study of other types of ocular surface diseases. If the authors have already checked the transcription level of acute type of ocular surface diseases, they can provide these data, and present form of order is fine, however if they have not checked yet, they should avoid the present form.
GENERAL COMMENTS	Major comments This paper has proven the expression of EP4 is different from various ocular surface diseases, however the order of description is confusing. In the present form, it looks like immunohistological study has revealed the difference, and real-time RT-PCR study confirmed that. But actually real-time RT-PCR study have not confirmed immunohistological study of this paper, but confirmed that of reference paper No. 8. The authors should mention real-time RT-PCR study first, which further confirm that EP4 is downregulated in chronic type of SJS/TEM and OCP, and then proceed to the study of acute types of ocular surface diseases. If the authors have already checked the transcription level of acute type of ocular surface diseases, they can provide these data, and present form of order is fine, however if they have not checked yet, they should avoid the present form. Minor comments

	P7L8 ‘nor from patients with severe GVHD’ should be rewritten to ‘nor from a patient with severe GVHD’, because only one patient of GVHD is included in this study. P7L11-12 ‘it was strongly down-regulated’ should be rewritten to ‘the immunoreactivity of EP4 was not detected’, which is the really observed result. P9L10 ‘because as in the acute or sub-acute stage’ What does that mean?

REVIEWER	Joshua Barnett, PhD Vanderbilt University United States of America
REVIEW RETURNED	18-Jun-2012

GENERAL COMMENTS	Study Section Comments: In regards to the study question, the authors state that they will examine the expression of EP4 in conjunctival epithelium of patients with various ocular surface disorders. There is no clear research question or thesis statement in the presented manuscript. It would help the reader, if the authors would clarify their study question and the reason they are looking for an answer to their question. Since there is no clearly defined study question, I cannot ascertain whether or not there study design is appropriate for their question. From some of what was included in their discussion, it appeared that the authors think that EP4 may be involved in inflammation and immune regulation via epithelial barrier function or TH1 and TH17 lymphocytes. It might have been appropriate for the authors to look at the expression of epithelial barrier proteins in their samples, if this was part of their question. The authors may have looked at the presence and numbers of TH1 and TH17 cells, since they mentioned EP4’s potential regulation of these cells in their discussion. Additionally, the authors could have better quantified the EP4 levels in the samples that they examined using an EP4 ELISA method. Additionally, there were very few patients used in each of several different diseases chemical eye burn (N=3), sub-acute SJS/TEN (N=2), severe GVHD (N=1), Moore’s ulcer (N=4), chronic SJS/TEN (N=4) and chronic stage OCP (N=4). The authors should attempt to explain to the reader why these patient types were examined together; this should preferably be done in the introduction. Why was each disease state examined? Why did the authors examine them together or what similarities existed in each disease state that the authors were trying to compare or contrast? Why were so few patient samples used in each category? Do the authors feel that they have enough samples from each of these disease states to be able to make a claim about EP4 expression that is representative of all patients with that disease? In regards to the description of the methods, the immunohistochemistry and qRT-PCR were described adequately, but the tissue samples and their collection were not. Some of the questions about these samples are in the preceding paragraph.

	Additionally, the authors should more clearly define what they mean by the term “nearly normal” that they use to describe their control tissues. Why do the authors feel that these were appropriate controls for their study? How many control samples were used? In the patient samples with chemical eye burns, what types of burns did these patients receive? How long ago were the burns? The answers to these questions might affect the expected results of these studies. Since the authors seem to be interested in inflammation, it may matter that the patients are in the process of healing from their chemical burns or have long since healed. There is some issue with the way that the qRT-PCR was conducted. The authors normalize their data to the housekeeping gene, GAPDH. A large number of recent studies including two listed below have identified large variability in housekeeping genes, making the use of a single gene for normalization inappropriate for tissue sample normalization. This is a particular problem for the authors of this submitted work because they have so few samples used in their study. In order to address this, the authors should normalized their target get to several housekeeping genes as suggested by these listed references. Keertan Dheda, Jim F. Huggett, Stephen A. Bustin, Margaret A. Johnson, Graham Rook, and Alimuddin Zumla. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *BioTechniques*. 37:112-119. July 2004. Carmela Tricaricoa, Pamela Pinzania, Simonetta Bianchib, Milena Paglieranib, Vito Distantec, Mario Pazzaglia, Stephen A Bustind, Claudio Orlandoa. Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeepinggenes is inappropriate for human tissue biopsies. *Analytical Biochemistry*. 309(2): 293-300. 15 October 2002. The statistical methods used to describe the significance level determined in the qRT-PCR experiments shown in figure 2 are not described in the manuscript. As they are not described, they cannot be evaluated for appropriateness. <u>Results and Conclusion Section Comments:</u> Since there is no clear question presented by the authors, it is difficult to assess whether or not they met their goals for the study or answered they question that they were attempting to answer. As far as looking for differences in EP4 levels in the variety of tissues that the authors examined, the authors did find some reduced protein levels and mRNA expression of EP4, however, their sample numbers are so small and their sample types are so varied, it is difficult to draw any real or meaningful conclusions from their results. The results that the authors present are credible, however, the extrapolation to inflammation in general or the specific disease states that the authors are examining is difficult with such small

	samples sizes and the varied types of samples. The presentation of the results and their clarity could be improved through better context and more thorough examination. The authors never clearly state why they are interested in EP4 in their tissue samples. Without this knowledge the reader is unsure of how to consider the results presented by the authors. Additionally, the authors look at immunohistochemical staining for EP4 protein in a variety of samples with differing disease states, and EP4 mRNA expression in a separate set of samples with yet again, different disease states. How are the readers supposed to be able to interpret these results, especially with so few samples with seemingly varied disease histories? The authors could have looked more specifically at the quantity of the EP4 protein or other effects that EP4 may have had a role in within the tissue. (See the discussion on this in the Study Section Comments). Without more data it is unlikely that the authors or any reader could draw any significant conclusions from these cursory results. <u>Reporting and Ethics Section Comments:</u> While there were no immediate concerns in this area, it would benefit the manuscript to have a more thoroughly description of how each of the patient samples was obtained, why they were obtained and what they contribute to the study. Some of the aspects of the samples that could be more thoroughly addressed are the concerns about the chemical eye burn samples mentioned in the Study Section Comments. <u>Further Comments:</u> In general the authors need to start by better describing their experimental plan and setting up the background for the reader in the Introduction Section. The gene PTGER4 should be mentioned and described. Each of the disease states that will be examined in the study should be mentioned, described and evaluated in terms of its importance in the presented study. All of this should be done in the introduction, so that the reader is appropriately oriented to the history of the project and the thought process of the investigators before being presented with the results.

VERSION 1 – AUTHOR RESPONSE

Reviewer(s)' Comments to Author:

Reviewer: Yoshitsugu Inoue
Professor and Chairman
Division of Ophthalmology and Visual Science Faculty of Medicine, Tottori University, Japan

I have no competing interests.

Major comments

This paper has proven the expression of EP4 is different from various ocular surface diseases, however the order of description is confusing. In the present form, it looks like immunohistological study has revealed the difference, and real-time RT-PCR study confirmed that. But actually real-time RT-PCR study have not confirmed immunohistological study of this paper, but confirmed that of reference paper No. 8.
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Response: In accordance with the reviewer's suggestions we changed the order of description. We address the real-time RT-PCR study first. It confirmed that EP4 was down-regulated in the chronic type of SJS/TEN and OCP. We then present our study of SJS/TEN in the sub-acute stage and of other ocular surface diseases.

Minor comments

P7L8 'nor from patients with severe GVHD' should be rewritten to 'nor from a patient with severe GVHD', because only one patient of GVHD is included in this study.

Response: We made the necessary change.

P7L11-12 'it was strongly down-regulated' should be rewritten to 'the immunoreactivity of EP4 was not detected', which is the really observed result.

Response: We complied.

P9L10 'because as in the acute or sub-acute stage' What does that mean?

Response: The revised text states: "We posit that the down-regulation of EP4 in conjunctival epithelium is associated with the ocular surface inflammation seen in patients with OCP, SJS/TEN, and Mooren's ulcer." (page 9, line 8-10 in our revised manuscript.)

Reviewer: Joshua Barnett, PhD
Vanderbilt University
United States of America

Study Section Comments:
In regards to the study question, the authors state that they will examine the expression of EP4 in conjunctival epithelium of patients with various ocular surface disorders. There is no clear research question or thesis statement in the presented manuscript. It would help the reader, if the authors would clarify their study question and the reason they are looking for an answer to their question.

Response: To comply we rewrote the text to read: "Here we examined the mRNA expression of EP4 in the conjunctiva of SJS/TEN and OCP patients in the chronic stage to confirm that EP4 is downregulated in their conjunctiva. We also examined the expression of EP4 protein in the conjunctival epithelium of patients with various ocular surface disorders such as chemical eye burn,

Mooren’s ulcer, severe graft versus host disease (GVHD), and of patients in the sub-acute stage of SJS/TEN." (page 4, line 16-21 in our revised manuscript.)

Since there is no clearly defined study question, I cannot ascertain whether or not there study design is appropriate for their question. From some of what was included in their discussion, it appeared that the authors think that EP4 may be involved in inflammation and immune regulation via epithelial barrier function or TH1 and TH17 lymphocytes. It might have been appropriate for the authors to look at the expression of epithelial barrier proteins in their samples, if this was part of their question. The authors may have looked at the presence and numbers of TH1 and TH17 cells, since they mentioned EP4’s potential regulation of these cells in their discussion.

Although Yao et al. reported that PGE2 acting on its receptor EP4 on T- and dendritic cells facilitated TH1 cell differentiation and TH17 cell expansion, we could not find the expression of EP4 protein in cells infiltrating subconjunctival tissues in any of the human conjunctival tissues we examined immunohistochemically.

Additionally, the authors could have better quantified the EP4 levels in the samples that they examined using an EP4 ELISA method.

As the amount of available tissue samples was very small, it would have been difficult to perform ELISA. Therefore we confirmed the expression of EP4 in mRNA by quantitative RT-PCR and the protein levels by immunohistochemistry.

Additionally, there were very few patients used in each of several different diseases chemical eye burn (N=3), sub-acute SJS/TEN (N=2), severe GVHD (N=1), Moore’s ulcer (N=4), chronic SJS/TEN (N=4) and chronic stage OCP (N=4). The authors should attempt to explain to the reader why these patient types were examined together; this should preferably be done in the introduction. Why was each disease state examined? Why did the authors examine them together or what similarities existed in each disease state that the authors were trying to compare or contrast? Why were so few patient samples used in each category? Do the authors feel that they have enough samples from each of these disease states to be able to make a claim about EP4 expression that is representative of all patients with that disease?

Response: SJS/TEN, OCP, Mooren's ulcer, chemical burn, and GVHD are all ocular surface inflammatory diseases in which there is persistent inflammation on the ocular surface not only in the acute- but also the chronic stage. Few patients undergo ocular surface reconstruction surgery; in fact, our samples were collected in the course of more than 3 years. This explains the small number of available samples. The revised test states: "SJS/TEN, OCP, Mooren's ulcer, chemical burn, and GVHD are all ocular surface inflammatory diseases with persistent inflammation on the ocular surface not only in the acute- but also in the chronic stage.” (page 5, line 17-19 in our revised manuscript.)

In regards to the description of the methods, the immunohistochemistry and qRT-PCR were described adequately, but the tissue samples and their collection were not. Some of the questions about these samples are in the preceding paragraph. Additionally, the authors should more clearly define what they mean by the term “nearly normal” that they use to describe their control tissues. Why do the authors feel that these were appropriate controls for their study? How many control samples were used? In the patient samples with chemical eye burns, what types of burns did these patients receive? How long ago were the burns? The answers to these questions might affect the expected results of these studies. Since the authors seem to be interested in inflammation, it may matter that the patients are in the process of healing from their chemical burns or have long since healed.

Response: To address these issues we rewrote the text to read: "The controls for

immunohistochemical analyses were nearly normal conjunctival tissues obtained during surgery for conjunctivochalasis, a disease in which the conjunctiva relaxes due to aging, resulting in a foreign body sensation on the ocular surface. We also prepared human conjunctival tissues from samples obtained during surgery to reconstruct the ocular surface in 3 patients with chemical (alkali) eye burn (2 in the chronic- and one in the sub-acute stage), 2 patients with sub-acute SJS/TEN, one patient with severe GVHD, and from 4 patients with Mooren's ulcer undergoing resection of inflammatory conjunctiva." (page 5, line 10-17 in our revised manuscript.)

There is some issue with the way that the qRT-PCR was conducted. The authors normalize their data to the housekeeping gene, GAPDH. A large number of recent studies including two listed below have identified large variability in housekeeping genes, making the use of a single gene for normalization inappropriate for tissue sample normalization. This is a particular problem for the authors of this submitted work because they have so few samples used in their study. In order to address this, the authors should normalized their target get to several housekeeping genes as suggested by these listed references.
Keertan Dheda, Jim F. Huggett, Stephen A. Bustin, Margaret A. Johnson, Graham Rook, and Alimuddin Zumla. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. BioTechniques. 37:112-119. July 2004.
Carmela Tricaricoa, Pamela Pinzania, Simonetta Bianchib, Milena Paglieranib, Vito Distantec, Mario Pazzaglia, Stephen A Bustind, Claudio Orlandoa. Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeepinggenes is inappropriate for human tissue biopsies. Analytical Biochemistry. 309(2): 293-300. 15 October 2002.
The statistical methods used to describe the significance level determined in the qRT-PCR experiments shown in figure 2 are not described in the manuscript. As they are not described, they cannot be evaluated for appropriateness.

We also confirmed the expression of EP4 protein by immunohistochemistry.

Results and Conclusion Section Comments:
Since there is no clear question presented by the authors, it is difficult to assess whether or not they met their goals for the study or answered they question that they were attempting to answer. As far as looking for differences in EP4 levels in the variety of tissues that the authors examined, the authors did find some reduced protein levels and mRNA expression of EP4, however, their sample numbers are so small and their sample types are so varied, it is difficult to draw any real or meaningful conclusions from their results.
The results that the authors present are credible, however, the extrapolation to inflammation in general or the specific disease states that the authors are examining is difficult with such small samples sizes and the varied types of samples.
The presentation of the results and their clarity could be improved through better context and more thorough examination. The authors never clearly state why they are interested in EP4 in their tissue samples. Without this knowledge the reader is unsure of how to consider the results presented by the authors. Additionally, the authors look at immunohistochemical staining for EP4 protein in a variety of samples with differing disease states, and EP4 mRNA expression in a separate set of samples with yet again, different disease states. How are the readers supposed to be able to interpret these results, especially with so few samples with seemingly varied disease histories? The authors could have looked more specifically at the quantity of the EP4 protein or other effects that EP4 may have had a role in within the tissue. (See the discussion on this in the Study Section Comments). Without more data it is unlikely that the authors or any reader could draw any significant conclusions from these cursory results.

Response: In accordance with the reviewer’s suggestions we changed the order of description. We address the real-time RT-PCR study first. It confirmed that EP4 was down-regulated in the chronic

type of SJS/TEN and OCP. We then present our study of SJS/TEN in the sub-acute stage and of other ocular surface diseases.

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While there were no immediate concerns in this area, it would benefit the manuscript to have a more thoroughly description of how each of the patient samples was obtained, why they were obtained and what they contribute to the study. Some of the aspects of the samples that could be more thoroughly addressed are the concerns about the chemical eye burn samples mentioned in the Study Section Comments.

Response: We state that: "We also prepared human conjunctival tissues from samples obtained during surgery to reconstruct the ocular surface in 3 patients with chemical (alkali) eye burn (2 in the chronic- and one in the sub-acute stage), 2 patients with sub-acute SJS/TEN, one patient with severe GVHD, and from 4 patients with Mooren's ulcer undergoing resection of inflammatory conjunctiva." (page 5, line 13-17 in our revised manuscript.)

Further Comments:
In general the authors need to start by better describing their experimental plan and setting up the background for the reader in the Introduction Section. The gene PTGER4 should be mentioned and described. Each of the disease states that will be examined in the study should be mentioned, described and evaluated in terms of its importance in the presented study. All of this should be done in the introduction, so that the reader is appropriately oriented to the history of the project and the thought process of the investigators before being presented with the results.

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VERSION 2 – REVIEW

REVIEWER	Yoshitsugu Inoue Professor and Chairman Division of Ophthalmology and Visual Science Faculty of Medicine, Tottori University, Japan I have no competing interests.
REVIEW RETURNED	09-Jul-2012

THE STUDY	There are no statements on statistical methods of quantitative PCR analyses.
GENERAL COMMENTS	Please describe the statistical method of quantitative PCR analyses.

REVIEWER	Joshua Barnett Vanderbilt University Medical Center Vanderbilt Eye Institute Nashville, Tennessee, United States
REVIEW RETURNED	23-Jul-2012

GENERAL COMMENTS	Study Section Comments: In regards to the study question, the authors state that they will

	examine the expression of EP4 in conjunctival epithelium of patients with various ocular surface disorders. There is no clear research question or thesis statement in the presented manuscript. It would help the reader, if the authors would clarify their study question and the reason they are looking for an answer to their question. Response: To comply we rewrote the text to read: "Here we examined the mRNA expression of EP4 in the conjunctiva of SJS/TEN and OCP patients in the chronic stage to confirm that EP4 is downregulated in their conjunctiva. We also examined the expression of EP4 protein in the conjunctival epithelium of patients with various ocular surface disorders such as chemical eye burn, Mooren's ulcer, severe graft versus host disease (GVHD), and of patients in the sub-acute stage of SJS/TEN." (page 4, line 16-21 in our revised manuscript.) Reviewer rebuttal: This is an adequate revision. Since there is no clearly defined study question, I cannot ascertain whether or not there study design is appropriate for their question. From some of what was included in their discussion, it appeared that the authors think that EP4 may be involved in inflammation and immune regulation via epithelial barrier function or TH1 and TH17 lymphocytes. It might have been appropriate for the authors to look at the expression of epithelial barrier proteins in their samples, if this was part of their question. The authors may have looked at the presence and numbers of TH1 and TH17 cells, since they mentioned EP4's potential regulation of these cells in their discussion. Although Yao et al. reported that PGE2 acting on its receptor EP4 on T- and dendritic cells facilitated TH1 cell differentiation and TH17 cell expansion, we could not find the expression of EP4 protein in cells infiltrating subconjunctival tissues in any of the human conjunctival tissues we examined immunohistochemically. Reviewer rebuttal: The previous comment was meant to suggest that the authors look at epithelial barrier proteins like ZO-1, ZO-2, or Occludin. This stems from the authors' suggestion in their discussion that EP4 was linked to epithelial barrier function. Can and would the authors look into some of these epithelial barrier proteins in their samples? Additionally, the authors could have better quantified the EP4 levels in the samples that they examined using an EP4 ELISA method. As the amount of available tissue samples was very small, it would have been difficult to perform ELISA. Therefore we confirmed the expression of EP4 in mRNA by quantitative RT-PCR and the protein levels by immunohistochemistry.

	Reviewer rebuttal: It may have been difficult, but it would be more reliable and quantifiable. Do the authors have the materials to do these experiments using ELISA? If so why not confirm the protein level results with ELISA? Additionally, there were very few patients used in each of several different diseases chemical eye burn (N=3), sub-acute SJS/TEN (N=2), severe GVHD (N=1), Moore's ulcer (N=4), chronic SJS/TEN (N=4) and chronic stage OCP (N=4). The authors should attempt to explain to the reader why these patient types were examined together; this should preferably be done in the introduction. Why was each disease state examined? Why did the authors examine them together or what similarities existed in each disease state that the authors were trying to compare or contrast? Why were so few patient samples used in each category? Do the authors feel that they have enough samples from each of these disease states to be able to make a claim about EP4 expression that is representative of all patients with that disease? Response: SJS/TEN, OCP, Mooren's ulcer, chemical burn, and GVHD are all ocular surface inflammatory diseases in which there is persistent inflammation on the ocular surface not only in the acute- but also the chronic stage. Few patients undergo ocular surface reconstruction surgery; in fact, our samples were collected in the course of more than 3 years. This explains the small number of available samples. The revised test states: "SJS/TEN, OCP, Mooren's ulcer, chemical burn, and GVHD are all ocular surface inflammatory diseases with persistent inflammation on the ocular surface not only in the acute- but also in the chronic stage." (page 5, line 17-19 in our revised manuscript.) Reviewer rebuttal: This is an adequate revision. In regards to the description of the methods, the immunohistochemistry and qRT-PCR were described adequately, but the tissue samples and their collection were not. Some of the questions about these samples are in the preceding paragraph. Additionally, the authors should more clearly define what they mean by the term "nearly normal" that they use to describe their control tissues. Why do the authors feel that these were appropriate controls for their study? How many control samples were used? In the patient samples with chemical eye burns, what types of burns did these patients receive? How long ago were the burns? The answers to these questions might affect the expected results of these studies. Since the authors seem to be interested in inflammation, it may matter that the patients are in the process of healing from their chemical burns or have long since healed. Response: To address these issues we rewrote the text to read: "The controls for immunohistochemical analyses were nearly normal conjunctival tissues obtained during surgery for conjunctivochalasis, a disease in which the conjunctiva relaxes due to aging, resulting in a foreign body sensation on the ocular surface. We also prepared human conjunctival tissues from samples obtained during surgery to reconstruct the ocular surface in 3 patients with chemical (alkali) eye burn (2 in the chronic- and one in the sub-acute stage), 2 patients with sub-acute SJS/TEN, one patient with severe GVHD, and from 4 patients with Mooren's ulcer undergoing resection of inflammatory conjunctiva." (page 5, line 10-17 in our revised manuscript.)

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VERSION 2 – AUTHOR RESPONSE

Reviewer's Comments to Author:

Reviewer: Joshua Barnett
Vanderbilt University Medical Center
Vanderbilt Eye Institute
Nashville, Tennessee, United States

Since there is no clearly defined study question, I cannot ascertain whether or not there study design is appropriate for their question. From some of what was included in their discussion, it appeared that the authors think that EP4 may be involved in inflammation and immune regulation via epithelial barrier function or TH1 and TH17 lymphocytes. It might have been appropriate for the authors to look at the expression of epithelial barrier proteins in their samples, if this was part of their question. The authors may have looked at the presence and numbers of TH1 and TH17 cells, since they mentioned EP4's potential regulation of these cells in their discussion.

Response: Although Yao et al. reported that PGE2 acting on its receptor EP4 on T- and dendritic cells facilitated TH1 cell differentiation and TH17 cell expansion, we could not find the expression of EP4 protein in cells infiltrating subconjunctival tissues in any of the human conjunctival tissues we examined immunohistochemically.

Reviewer rebuttal: The previous comment was meant to suggest that the authors look at epithelial barrier proteins like ZO-1, ZO-2, or Occludin. This stems from the authors' suggestion in their discussion that EP4 was linked to epithelial barrier function. Can and would the authors look into some of these epithelial barrier proteins in their samples?

Re-response: We appreciate the Reviewer's valuable suggestion for the improvement of our study. In accordance with the Reviewer's suggestion, we will perform the immunohistological analysis of epithelial barrier proteins like ZO-1, ZO-2, or Occludin in future, because we must submit our revised manuscript within 10 days.

Additionally, the authors could have better quantified the EP4 levels in the samples that they examined using an EP4 ELISA method.

Response: As the amount of available tissue samples was very small, it would have been difficult to perform ELISA.Therefore we confirmed the expression of EP4 in mRNA by quantitative RT-PCR and the protein levels by immunohistochemistry.

Reviewer rebuttal: It may have been difficult, but it would be more reliable and quantifiable. Do the authors have the materials to do these experiments using ELISA? If so why not confirm the protein level results with ELISA?

Re-response: We could not find the method of ELISA of EP4 protein using very small tissue samples.

There is some issue with the way that the qRT-PCR was conducted. The authors normalize their data to the housekeeping gene, GAPDH. A large number of recent studies including two listed below have identified large variability in housekeeping genes, making the use of a single gene for normalization inappropriate for tissue sample normalization. This is a particular problem for the authors of this submitted work because they have so few samples used in their study. In order to address this, the authors should normalized their target
get to several housekeeping genes as suggested by these listed references.
Keertan Dheda, Jim F. Huggett, Stephen A. Bustin, Margaret A. Johnson, Graham Rook, and Alimuddin Zumla. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. BioTechniques. 37:112-119. July 2004.
Carmela Tricaricoa, Pamela Pinzania, Simonetta Bianchib, Milena Paglieranib, Vito Distantec, Mario Pazzaglia, Stephen A Bustind, Claudio Orlandoa. Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeepinggenes is inappropriate for human tissue biopsies. Analytical Biochemistry. 309(2): 293-300. 15 October 2002.
The statistical methods used to describe the significance level determined in the qRT-PCR experiments shown in figure 2 are not described in the manuscript. As they are not described, they cannot be evaluated for appropriateness.

Respose: We also confirmed the expression of EP4 protein by immunohistochemistry.

Reviewer rebuttal: The authors do not address this comment adequately. 1) If the authors still have samples remaining, can they check their results against another, different housekeeping gene other than GAPDH? This would make for a more thorough examination of a small sample size of varied sample types. It would it would instill more confidence to the reader of the authors results. 2) The authors failed to describe their statistical methods as mentioned in the previous comment. Can the authors, please, resolve this by describing their statistical methods?

Re-response: We appreciate the Reviewer's valuable suggestion for the improvement of our study. In accordance with the Reviewer's suggestion, we will perform the quantitative RT-PCR using another housekeeping gene, actin beta, in future, because we must submit our revised manuscript within 10 days. Moreover, we have added the description of our statistical method as follows:

“Data analysis
Data were expressed as the mean ± SEM and evaluated by the Student’s t-test using the Microsoft Excel software program.” (page 6, line 17-19 in our revised manuscript.)

Results and Conclusion Section Comments:
Since there is no clear question presented by the authors, it is difficult to assess whether or not they met their goals for the study or answered they question that they were attempting to answer. As far as looking for differences in EP4 levels in the variety of tissues that the authors examined, the authors did find some reduced protein levels and mRNA expression of EP4, however, their sample numbers are so small and their sample types are so varied, it is difficult to draw any real or meaningful conclusions from their results.
The results that the authors present are credible, however, the extrapolation to inflammation in general or the specific disease states that the authors are examining is difficult with such small samples sizes and the varied types of samples. The presentation of the results and their clarity could be improved through better context and more thorough examination. The authors never clearly state why they are interested in EP4 in their tissue samples. Without this knowledge the reader is unsure of how to consider the results presented by the authors. Additionally, the authors look at immunohistochemical staining for EP4 protein in a variety of samples with differing disease states, and EP4 mRNA expression in a separate set of samples with yet again, different disease states. How are the readers supposed to be able to interpret these results, especially with so few samples with seemingly varied disease histories? The authors could have looked more specifically at the quantity of the EP4 protein or other effects that EP4 may have had a role in within the tissue. (See the discussion on this in the Study Section Comments). Without more data it is unlikely that the authors or any reader could draw any significant conclusions from these cursory results.

Response: In accordance with the reviewer’s suggestions we changed the order of description. We address the real-time RT-PCR study first. It confirmed that EP4 was down-regulated in the chronic type of SJS/TEN and OCP. We then present our study of SJS/TEN in the sub-acute stage and of other ocular surface diseases.

Reviewer rebuttal: The authors do not adequately address this comment. 1) Why are the authors interested in EP4 in their tissue samples? This explanation should be added to the manuscript. 2)How can a reader compare the samples used for analysis of mRNA expression to those used in the immunohistochemical protein analysis? They are from different disease states. Do the authors still have the samples to examine the mRNA from the samples used for protein analysis or to examine the protein in the samples used for mRNA analysis? Without this connection the results and data seem disjointed.

Re-response: We appreciate the Reviewer’s valuable suggestion for the improvement of our study. In accordance with the Reviewer’s suggestion, we have modified and added the descriptions in Introduction and Results section of our revised manuscript as follows:

“The ocular surface is also one of the mucosa that is in contact with commensal bacteria like the intestine. Therefore, we focused the expression of EP4 in human conjunctival epithelium and the difference of its expression between various ocular surface diseases.” (page 4, line 12-15 in our revised manuscript.)

“We previously documented that EP4 protein expression was down-regulated in conjunctival epithelium of devastating ocular surface inflammatory disorders such as chronic SJS/TEN and chronic OCP [8]. In this study,” (page 7, line 2-4 in our revised manuscript.)

“Moreover, we examined the expression of EP4 protein in the conjunctival epithelium of patients with other various ocular surface disorders.” (page 7, line 13-14 in our revised manuscript.)

Further Comments:
In general the authors need to start by better describing their experimental plan and setting up the background for the reader in the Introduction Section. The gene PTGER4 should be mentioned and described. Each of the disease states that will be examined in the study should be mentioned, described and evaluated in terms of its importance in the presented study. All of this should be done in the introduction, so that the reader is appropriately oriented to the history of the project and the thought process of the investigators before being presented with the results.

Response: In accordance with the reviewer’s suggestions we changed the order of description. We address the real-time RT-PCR study first. It confirmed that EP4 was down-regulated in the chronic type of SJS/TEN and OCP. We then present our study of SJS/TEN in the sub-acute stage and of other ocular surface diseases.

Reviewer rebuttal: The authors did not reply to this comment and did not address the suggestion of amending their manuscript’s experimental plan and background (introduction). Why don’t the authors mention PTGER4 in the introduction? The authors need to rewrite the introduction to clarify the importance of EP4 levels in chronic, conjuctival inflammation.
Additionally, what do the results of this study mean for patients with disease states causing persistent inflammation on the ocular surface? What do these results mean to physicians treating these patients? This should be included in the discussion. Why are these results significant? What are potential future directions of study? Why might EP4 be down-regulated in these study conditions and how might this knowledge be of use?

Re-response: We appreciate the Reviewer’s valuable suggestion for the improvement of our study. In accordance with the Reviewer’s suggestion, we have modified and added the descriptions in Introduction and Discussion section of our revised manuscript as follows:

“The ocular surface is also one of the mucosa that is in contact with commensal bacteria like the intestine. Therefore, we focused the expression of EP4 in human conjunctival epithelium and the difference of its expression between various ocular surface diseases.” (page 4, line 12-15 in our revised manuscript.)

“Our results suggest that it is possible that EP4 in conjunctival epithelium might contribute the ocular surface homeostasis, while the EP4 could not necessarily be down-regulated in all devastating ocular surface disorders.”(page 9, line 18-20 in our revised manuscript.)