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Immunosuppressive effect of prolactin-induced protein: a new insight into the local and systemic role on chronic allergic contact dermatitis

Immunosuppressive effect of prolactin-induced protein

Tolerance, prolactin-induced protein, chronic contact dermatitis, atopic dermatitis

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Summary

Background Prolactin-induced protein (PIP) has been shown to bind to CD4 and is speculated to block CD4-HLA-DR interaction. However, the immunomodulatory effect of PIP on chronic allergic contact dermatitis (ACD) remains to be elucidated. **Objectives** The aim of this work was to define the role of PIP during the immunoresponse. **Materials and Methods** Using a low dose oxazolone-induced mouse chronic ACD model, expression of PIP was immunohistologically examined. Furthermore, effects of continued exposure of a peptide mimicking the major binding site of PIP (amino acids 106-132) for CD4 was examined in a mouse chronic ACD model. **Results** We clarified that keratinocytes, dermal infiltrating cells and infiltrating mononuclear cells into the spleen are positively stained with anti-PIP antibody. The PIP peptide significantly down-regulated oxazolone-induced mouse ACD compared to the controls. We also found that inflammation of PIP-non-applied control ear was also suppressed in a synchronized manner in late phase of the PIP peptide applied mouse. **Conclusions** These findings suggest that PIP might have a local and systemic immunosuppressive effect in mouse chronic ACD.

Introduction

Prolactin has been shown to stimulate T cells, B cells, natural killer cells and antigen-presenting cells and thereby act in maintaining immune system homeostasis.¹ Prolactin and growth hormone causes the induction of prolactin-induced protein (PIP) in a human breast cancer cell line.² PIP has also been independently identified as a secretory actin-binding protein (SABP), gross-cystic-disease fluid protein-15 (GCDFP-15) and gp17.³ PIP is detected in exocrine organs such as the bronchial epithelium and sweat, salivary and lachrymal glands as well as in the seminal vesicles and corresponding fluids. Human amniotic fluid contains high concentrations of PIP.⁴ PIP was also found in breast milk from normal women.⁵ Although the physiological role of this protein is still unknown, PIP specifically binds to CD4 and is a specific inhibitor of T lymphocyte programmed cell death.³ Autiero et al reported that PIP specifically binds to the CD4 domain 1.⁶ A major CD4 binding site is located between amino acids 134-148 in the β 2 domain of HLA-DR, and the β 2 domain of HLA-DR primarily binds to the CD4 domain 1.⁷ Autiero M et al. suggested that binding between PIP and CD4 blocks the CD4-HLA-DR interaction.⁸

In allergic contact dermatitis (ACD), immunocompetent cells express HLA-DR molecules and CD4 molecules during their active phase.^{9,10} Therefore we reasoned that PIP might modulate ACD. However, the distribution and immunological effect of PIP on ACD remain to be elucidated.

Skin lesions of atopic dermatitis (AD) consist of many CD4 positive T cells and Langerhans cells.¹¹ AD is a chronic and recalcitrant form of dermatosis. During childhood some AD patients spontaneously recover whereas others relapse and suffer skin lesions into adolescence.^{12,13} At present, the mechanisms of this natural resolution (tolerance) have not been clarified. Thus, induction of remission or tolerance to atopic dermatitis is considered highly desirable.

The aim of this work was to define the role of PIP during the immunoresponse. Firstly we observed PIP expression in a low dose oxazolone-induced mouse chronic ACD model. We also observed the effect of a synthetic peptide mouse PIP 106-132, corresponding to residues 106-132

of human PIP that constitute the major binding site for CD4, on the mouse model. We show that PIP attenuates local chronic inflammation response. Our results also indicate that extrinsic mouse PIP peptide can systemically intensify the original immunosuppressive role of intrinsic PIP.

Materials and Methods

Animals

Male C57BL/6 (Japan Clea, Hamamatsu, Japan) mice were used between 2 and 3 months of age. All experimental procedures were performed in accordance with the guidelines of the Shiga University of Medical Science for the use of living animals in scientific studies.

Induction of oxazolone-induced contact sensitivity

Mice were sensitized with a single application of 10 μ l of a 1.6% solution of oxazolone (Sigma-Aldrich, St Louis, MO) in ethanol on their left ear 6 days before challenge. In the experiment of repeated oxazolone challenges, each of the six mice were challenged with 10 μ l of a 0.05% or 0.1% solution of oxazolone on the left ear (mice challenged on days: 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20). Another four mice were challenged for a total of 25 days with 10 μ l of a 0.05% solution of oxazolone on both ears (mice challenged on days: 0, 2, 4, 6, 7, 9, 11, 13, 14, 16, 18, 20, 21, 23, 25).

Immunohistological study of the ear and spleen with anti-PIP antibody in oxazolone challenged mice

At 20 days after challenge, the ear samples from twelve mice were fixed with 10% neutral formaldehyde. At 25 days after challenge, tissue samples of spleen from another four mice were collected. The four mice were transcardially perfused as described previously.^{14,15} Paraffin sections (4 μ m) were incubated with a rabbit polyclonal antibody to mouse PIP/GCDFP-15 (dilution 1:1000; Santa Cruz Biotech., Santa Cruz, CA) at 4°C overnight. Sections were then incubated with peroxidase-labeled anti-rabbit or anti-mouse antibody (Histofine Simplestain Max PO; Nichirei) for 30 min at room temperature. Peroxidase activity was detected with 3,3'-diaminobenzidine (DAB; Sigma-Aldrich). Sections were counterstained with hematoxylin.

Construction of mouse PIP peptide

One of the strongest CD4 binding domains of human PIP has been determined as RTVQI AAVVDVIREL GICPDAAVI PI.⁸ From an alignment of the mouse and human PIP gene products,¹⁶ RTVTF ATVIDIVREK NICPNDMAVV PI was identified as a CD4 binding domain for the mouse protein (59.3 % homology at the amino acid level). The mouse PIP gene peptide was synthesized and used in the present mouse chronic ACD model study. As a control peptide, QD NTRKIIKNF DIPKSVRPND EVTAVLAVQT ELK was used in the study.

Animals and Induction of oxazolone-induced contact sensitivity

Male C57BL/6 mice were challenged with 10 µl of a 0.05% solution of oxazolone on both ears four times per week (mice challenged on days: 0, 2, 4, 6, 7, 9, 11, 13, 14, 16, 18, 20, 21, 23, 25) as described before.

Induction of an immunomodulatory effect by PIP peptide

1 µg/ml of PIP peptide assay (six mice):

A modulatory effect was induced by repeated topical application of PIP peptide (10 µl of a 1 µg/ml stock solution with 0.0124 % DMSO in normal saline) on the right ear. An individual mouse was treated on the left ear with 10 µl of the vehicle (0.0124% DMSO in normal saline) alone. Application of PIP to the right ear and vehicle to the left ear was performed a few minutes after each application of oxazolone.

25 µg/ml of PIP peptide assay (six mice):

Using the same protocol as described for the 1 µg/ml of PIP peptide assay, 10 µl of a 25 µg/ml of PIP peptide with 0.31% DMSO in normal saline was applied to the right ear and 10 µl of vehicle (0.31% DMSO in normal saline) to the left ear of six mice.

1 µg/ml and 25 µg/ml of control peptide assay (six mice each):

In the experiment involving repeated oxazolone challenges, mice were challenged with oxazolone and 10 µl of 25 µg/ml or 1 µg/ml control peptide in normal saline solution on the right ear and oxazolone and control vehicle (normal saline) on the left ear four times per week (mice challenged days, 0, 2, 4, 6, 7, 9, 11, 13, 14, 15).

Measurement of ear thickness and tissue sample collections

Ear thickness was measured with a dial micrometer (Mitsutoyo Co., Tokyo,

Japan) 24 h or 48 h after each challenge. For the 1 µg/ml and 25 µg/ml PIP peptide group, tissue samples from twelve mice were collected at day 18 (4 mice) after challenge on the ascending phase of ear thickness, day 21 (4 mice) on peak plateau of ear thickness and day 25 (4 mice) on the descending phase of ear thickness. The results of the time course experiment were defined by comparison to the thickness of oxazolone alone applied ears. Tissue samples were collected 24 h or 48 h after each challenge.

Histology and Immunohistochemistry in oxazolone and PIP peptide induced ear and spleen of mouse

Perfusion fixation

Twelve mice were transcardially perfused with an ice-cold fixative using the same method as described previously.^{14,15} Pinnae were sliced from base to apex. The slices of pinnae and spleens were processed into 4 µm paraffin sections and stained with hematoxylin and eosin. The stained sections were then examined by light microscopy.

Staining with anti-PIP antibody

All samples obtained from the ear or spleen of each mouse was stained with anti-mouse PIP antibody as described above.

Statistical Analyses

Differences of ear thicknesses between mice treated and untreated with PIP peptides were evaluated by using paired t-test. Differences less than the false discovery rate¹⁷ of 0.05 were considered significant.

Results

Expression of PIP in skin lesions and the spleen of mouse chronic ACD model

PIP expression was absent in normal keratinocytes of the normal control mouse (Fig. 1a). In skin lesions of the ear obtained at 20 days after challenge of the oxazolone-induced chronic ACD mouse model, cell surface of hyperplastic epidermal cells was positively stained as a reticular pattern with anti-PIP antibody (Fig. 1b). PIP expression of infiltrating cells of the spleen was absent in the normal control mouse (Fig. 1c), and some of

positive cells were seen in the T cell area of the periarteriolar lymphoid sheath (Fig. 1d) and in the vessels (Fig. 1e) of the spleen sample at 25 days after challenge in the mouse chronic ACD model.

Immunosuppressive effect of PIP peptide on mouse chronic ACD model

1 µg/ml of PIP peptide assay (six mice):

Fig 2a shows the average ear thickness from day 0 to day 21. Repeated application of 1 µg/ml PIP peptide and oxazolone significantly ($p < 0.05$) inhibit the ear swelling from day 6 to day 21 compared to oxazolone alone. Ear thicknesses of individual mice examined are shown in Fig. 2a-1 (oxazolone alone) and Fig. 2a-2 (1 µg/ml PIP peptide and oxazolone). As shown in Fig. 2a-1, thickness of the ear treated with oxazolone alone starts to decrease 21 days after the initial challenge. By contrast, as shown in Fig 2a-2, thickness of the ear treated with 1 µg/ml PIP peptide and oxazolone starts to decrease after day 18.

25 µg/ml of PIP peptide assay (six mice):

Fig 2b shows the average ear thickness from day 0 to day 21. Repeated application of 25 µg/ml PIP peptide and oxazolone significantly inhibit the ear swelling from day 7 to day 21 compared to oxazolone alone. Ear thicknesses of individual mice examined are shown in Fig. 2b-1 (oxazolone alone) and Fig. 2b-2 (25 µg/ml PIP peptide and oxazolone). Both ears from each mouse treated with oxazolone alone (Fig. 2b-1) or 25 µg/ml PIP peptide and oxazolone (Fig. 2b-2) simultaneously started to decrease in thickness 14 days after the initial challenge.

Thus, the time course of right and left ear thickness of each mouse was strictly synchronized in 25 µg/ml PIP peptide and oxazolone applied mice. The turning point of ear thickness on the oxazolone alone applied side occurred shortly after challenging the mice with 25 µg/ml PIP peptide and oxazolone (day 14 in Fig. 2b-1) compared to mice in which 1 µg/ml PIP peptide and oxazolone (day 21 in Fig 2a-1) was applied. These findings strongly indicate that mouse PIP peptide not only has a local immunosuppressive role but also systemically influences the levels of ACD.

1 µg/ml and 25 µg/ml of control peptide assay (six mice each):

Significant difference of total ear thickness was not detected between the control peptide applied side and non-applied side in the repeated oxazolone induced mouse ACD model.

Local inhibitory effect of PIP peptide observed by histopathology

Thick parakeratosis, epidermal thickening, spongiosis and infiltrates of inflammatory cells were observed in ears treated with oxazolone alone on day 18 (ascending phase) (Fig. 3a) to day 21 (peak plateau) after the initial challenge. A similar, but much less intense, reaction was seen for ears treated with oxazolone and 1 µg/ml (Fig. 3b) or 25 µg/ml of PIP peptide by comparison with the oxazolone alone control. At day 25 (descending phase) after challenge, both the 1 µg/ml of PIP peptide group and 25 µg/ml PIP peptide group displayed very mild epidermal hyperplasia and few dermal infiltrating cells.

A dynamic change of intrinsic PIP expression during time course experiments of the oxazolone alone applied ear

Keratinocytes were not stained with anti-PIP antibody in normal ears of normal control mice. In oxazolone alone applied ears, keratinocytes were not stained with anti-PIP antibody at day 18 (ascending phase) (Fig. 4a1 and 4b1), but strongly stained at day 21 (peak plateau) (Fig. 4a2 and 4b2), and weakly stained at day 25 (descending phase) (Fig. 4a3 and 4b3) after challenge in both the 1 µg/ml and 25 µg/ml PIP applied mouse groups. These dynamic changes of PIP expression were commonly found in all 12 mice examined (4 mice each in the individual three phases). In addition, some of the mononuclear cells within the dermal vessels were positively stained with anti-PIP antibody (Fig. c; oxazolone alone applied ear).

Staining of spleen with ant-PIP antibody

In the late phase of chronic ACD at day 21 and day 25 after challenge, infiltrating cells into the spleen were positively stained with anti-PIP antibody. The appearance of anti-PIP antibody staining on infiltrating cells in the spleen and in the venules is consistent with our interpretation that PIP plays a systemic role in chronic ACD.

Discussion

In the present study we established that intrinsic PIP appears on keratinocytes in the mouse low dose oxazolone-induced chronic ACD model.

This is the first study to report that PIP is expressed on keratinocytes and infiltrating cells in the chronic phase of immunoresponse. These findings indicate that PIP might be involved in a delayed type hypersensitivity reaction in mouse. Intriguingly, the intrinsic PIP appeared on day 21, which represents the peak plateau of ear thickness, rather than day 18, which represents the ascending phase of ear thickness (Figure 4a-b). The delayed appearance of intrinsic PIP during the immunoresponse of ACD has not been previously noted in the literature. Indeed, we have been unable to find any publications that refer to the immunological role of PIP in ACD.

The most interesting finding in our study was to define the immunological role of PIP. Specifically, the local and systemic immunosuppression of PIP and our finding that extrinsic mouse PIP peptide intensifies the original immunosuppressive role of intrinsic PIP.

Firstly, to determine the local role of PIP in the delayed type hypersensitivity reaction, we applied a synthetic PIP peptide (amino acids 106-132) to the mouse chronic ACD model. From these experiments we established that PIP peptide attenuates local chronic inflammation (Figs. 2 and 3). Secondly, the present study suggests that PIP and/or PIP peptide exerts a systemic immunosuppressive effect. This conclusion is supported by three independent lines of evidence. 1) The descending phase decrease of ear thickness observed for PIP-non-applied ear was also suppressed in a synchronized manner with the PIP-applied ear (Fig. 2). 2) As shown in Figure 2, a high dose of PIP peptide brings about an earlier turning point of ear thickness on the oxazolone alone treated side than when using a low dose of PIP peptide. 3) As shown Figure 1e and 4c, PIP positive infiltrating cells appear in the spleen and dermal vessels. Thirdly, extrinsic PIP peptide could intensify the immunosuppressive role of intrinsic PIP. Indeed, PIP displays an immunosuppressive effect that increases in accordance with an increase of PIP peptide concentration as shown by Figure 2.

As speculated by Autiero et al.,⁸ intrinsic PIP observed in the present study might block CD4-HLA-DR interaction. Unfortunately, we could not verify whether the mouse PIP peptide displays binding capacity with the CD4 molecule. In addition, we were unable to determine the underlying mechanism of the immunosuppressive effect displayed by PIP in the mouse chronic contact dermatitis model system. Nonetheless, our data clearly shows that intrinsic mouse PIP does bind to dermal infiltrating mononuclear cells in the mouse chronic ACD model.

Gaubin M et al.³ clarified that PIP is a strong and specific inhibitor of CD4/TCR-mediated apoptosis, and they suggested PIP plays a role in host defense against infections and tumors. Considering the local and systemic immunosuppressive effect of PIP observed in the present study, we additionally speculate a physiological role for the potent inhibitory effect of CD4 apoptosis, which might play a part in the long lasting immunosuppressive effect and for preventing excessive immunoreaction.

PIP in the human amniotic fluid⁴ and in the mouse oral cavity¹⁸ is thought to provide some protective role by binding to any invading bacteria before activation of the immune system. Our results suggest that PIP, in addition to providing a host defense role, also exerts a physiological protective role for excessive delayed type hypersensitivity reaction in parts of the body where PIP is abundant, such as the oral cavity, bronchus, palms and soles.

From a clinical point of view, the itch-eliciting role of sweating in atopic dermatitis is generally accepted. Skin lesions usually deteriorate by rubbing one's eyes with tears, perioral area with saliva and antecubital and popliteal fossae of extremities with sweat, where PIP exocrines abundantly. These clinical findings are contrary to the immunosuppressive role of PIP. Currently, the reason for the discrepancy between the clinical features and immunosuppressive effect of mouse PIP is unclear. One possibility is that mouse PIP, unlike human PIP, lacks the residues essential for aspartic peptidase activity.¹⁹

Prolactin has been shown to stimulate many immunocompetent cells and act in maintaining immune system homeostasis.¹ The best described signaling pathway activated by prolactin is the JAK/Stat pathway.²⁰ The promoter region of PIP has an androgen responsive element and consensus Stat5 binding element.²¹ Prolactin has pleiotropic actions in different organs. In the present study, we observed an immunosuppressive effect of PIP. Thus, some immune system homeostasis functions, which are known to be introduced by prolactin, might be expressed *via* PIP. In addition to cutaneous delayed type reaction, PIP might act as an immunomodulator in other organs such as oral cavity, bronchus, nasopharyngeal area, intestine and lachrymal related orbital area. A comparative chemical genetic study between mouse PIP and human PIP will provide new insights into the immunological investigation of several organs and could help alleviate many immunological diseases in human.

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Legends for Figures1

Figure 1.

Expression of PIP in skin lesions and spleen of the mouse chronic ACD model

PIP expression was absent in normal keratinocytes of the normal control mouse (Fig. 1a). For skin lesions of the ear obtained at 20 days after challenge in the oxazolone-induced chronic ACD mouse model, cell surface of hyperplastic epidermal cells was positively stained in a reticular pattern with anti-PIP antibody (Fig. 1b). PIP expression of infiltrating cells of the spleen was absent in the normal control mouse (Fig. 1c). Some positive cells were seen in the T cell area of the periarteriolar lymphoid sheath (Fig. 1d) and in the vessels (Fig. 1e) of spleen samples 25 days after challenge in the mouse chronic ACD model. Original magnifications: ×200 (1a,1b,1e); ×100 (1c,1d).

Figure 2.

Immunosuppressive effect of PIP peptide on the mouse chronic ACD model

Repeated application of 1 µg/ml PIP peptide and oxazolone significantly ($p < 0.05$) inhibit the ear swelling from day 6 to day 21 compared to oxazolone alone (Fig. 2a). Ear thicknesses of individual mice examined are shown in Fig. 2a-1 (oxazolone alone) and Fig. 2a-2 (1 µg/ml PIP peptide and oxazolone). As shown in Fig.2a-1, thickness of the ear treated with oxazolone only starts to decrease 21 days after the initial challenge. By contrast, thickness of the ear treated with 1 µg/ml PIP peptide and oxazolone starts to decrease after 18 days (Fig 2a-2). Repeated application of 25 µg/ml PIP peptide and oxazolone significantly inhibit the ear swelling from day 7 to day 21 compared to oxazolone alone (Fig. 2b). Ear thicknesses of individual mice examined are shown in Fig. 2b-1 (oxazolone alone) and Fig. 2b-2 (25 µg/ml PIP peptide and oxazolone). Ear thickness in mice treated with oxazolone only on one side of the head (Fig. 2b-1) or 25 µg/ml

PIP peptide and oxazolone on the other side (Fig. 2b-2) simultaneously started to decrease 14 days after the initial challenge. Each value represents the mean $\pm$ S.E.M.* There was a significant difference between the two groups at 5 % false discovery rate for a paired t-test.

Figure 3

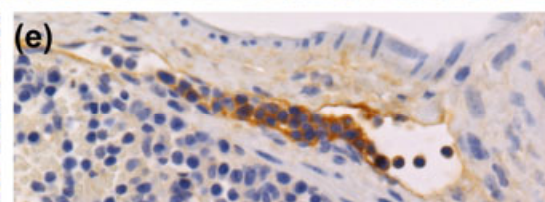
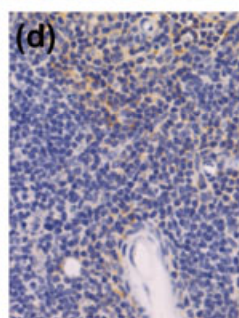
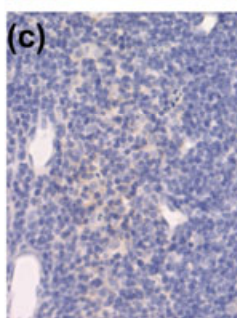
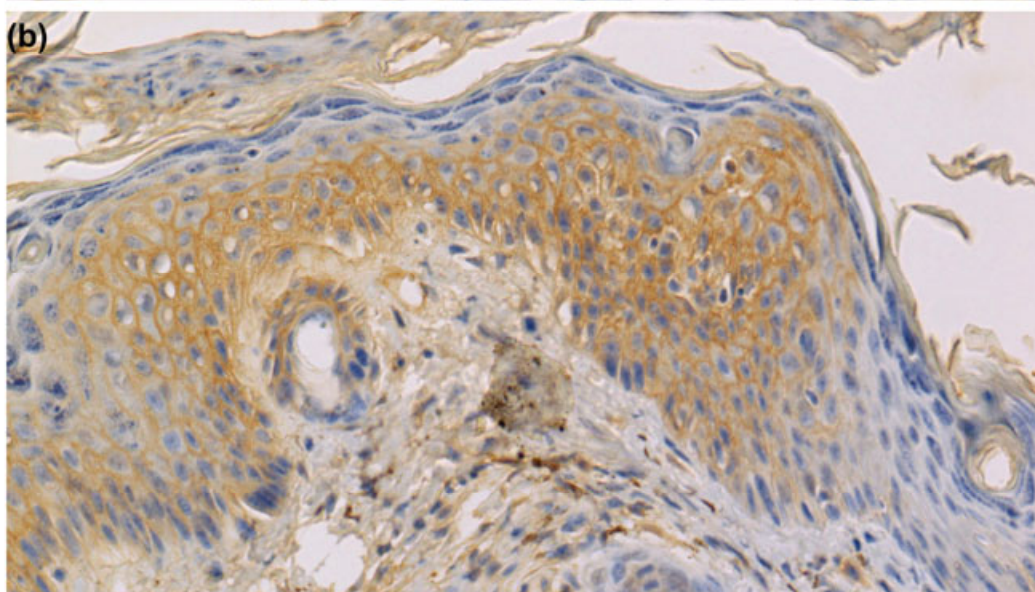
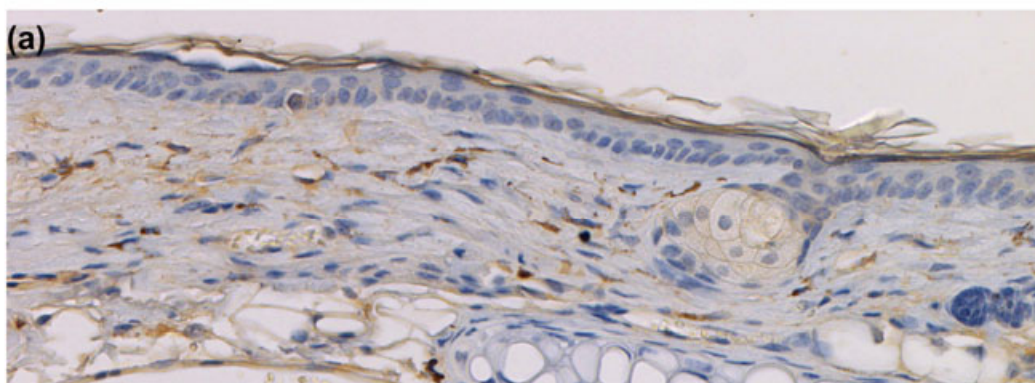
Local inhibitory effect of PIP peptide observed by histopathology

Histopathology (hematoxylin and eosin staining) of mouse ears. Fig. 3a shows vehicle alone applied left ear of number 4 mouse in Fig. 2a-1. Fig. 3b shows 1 μ g/ml PIP peptide applied right ear of number 4 mouse in Fig. 2a-2 at day 21 after challenge. The thickness of the keratotic cell layer decreased in PIP peptide applied ears (Fig. 3b) compared with that of vehicle alone applied ears (Fig. 3a). Original magnifications: $\times$ 100 (3a,3b).

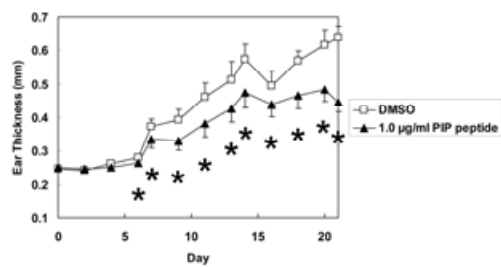
Figure 4

A dynamic change of intrinsic PIP expression during time course experiments from oxazolone alone applied ear

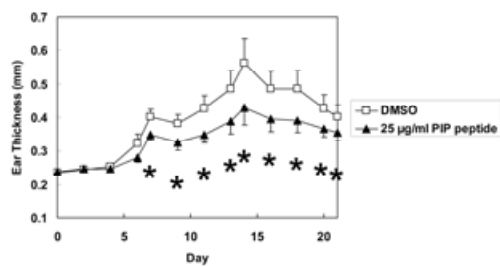
Figure 4 shows PIP stained sections from the oxazolone treated left ear. Fig. 4a-1, 4a-2, 4a-3 shows an ear from the 1 μ g/ml PIP peptide applied mouse. Fig.4b-1, 4b-2, 4b-3 shows an ear from the 25 μ g/ml PIP peptide treated mouse. For the oxazolone only treated ear, keratinocytes were not stained with anti-PIP antibody at day 18 (ascending phase) (Fig. 4a-1 and 4b-1), but strongly stained at day 21 (peak plateau) (Fig. 4a-2 and 4b-2), and then weakly stained at day 25 (descending phase) (Fig.4a-3 and 4b-3) after challenge in both the 1 μ g/ml and 25 μ g/ml PIP applied mouse groups. These dynamic changes of PIP expression were commonly found in all twelve mice examined (four mice each in the individual three phases). In addition, some of the mononuclear cells within the dermal vessels at day 21 were positively stained with anti-PIP antibody (Fig. 4c). Original magnifications: $\times$ 200 (4a-1,2,3,4b-1,2,3); $\times$ 400 (4c).



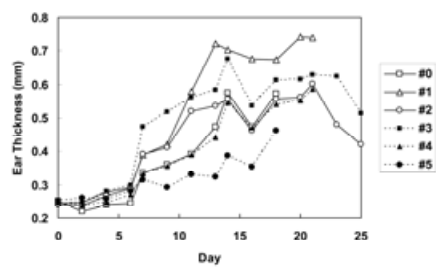
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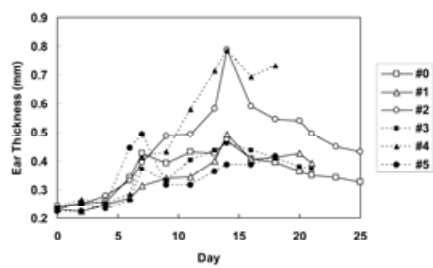
(b)



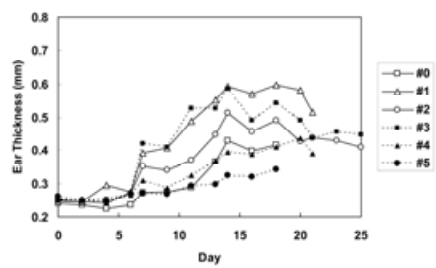
(a-1)



(b-1)



(a-2)



(b-2)

