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Immune escape phenomenon in molluscum contagiosum and the induction of apoptosis

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ABSTRACT

Background: Molluscum contagiosum (MC) may persist for many weeks, evading host immunity.

Objectives: We studied the mechanism of immune escape phenomenon in MC, and the possible inducer of apoptosis.

Methods: Using tissue samples of MC, we examined the numbers of epidermal Langerhans cells (LCs), the expression levels of MIP-3α and thymic stromal lymphopoietin (TSLP), and the apoptotic signals. After MCV genotyping, we studied the expression of MCV-encoded MC148mRNA and MC159mRNA which correspond to viral antagonist for CCR8 and viral Fas-linked interleukin-1β converting enzyme (FLICE)-like inhibitor protein (vFLIP), respectively. The nutlin-3-induced apoptosis in MC was observed ex vivo.

Results: The numbers of $CD1a^+$ or $Langerin^+$ epidermal LCs and the expression levels of MIP-3α were markedly decreased in MC. The expression of TSLP was enhanced in the lesional epidermis of atopic dermatitis and HPV-induced warts, whereas the expression was observed locally in MC. All 14 MC samples examined harbored MCV type 1. The MC148mRNA was detected in all 14 samples and the MC159 mRNA was detect in 13 samples. Apoptotic cells were absent or at a background level in the living layers of MC, but their numbers were increased in the molluscum bodies by overnight incubation with 5 μM nutlin-3 in culture medium.

Conclusions: Molluscum bodies are protected from host immune responses and apoptotic signals by being surrounded by LC-depleted epidermal walls and viral immunosuppressive molecules, but could be eradicated by reagents inducing p53-dependent apoptosis.

Key words: molluscum contagiosum virus, dendritic cell, Langerhans cell, MIP-3α, nutlin-3

Abbreviations: MC, molluscum contagiosum; MCV, molluscum contagiosum poxvirus; HPV, human papilloma virus; DC, dendritic cell; MIP-3α, macrophage inflammatory protein-3α; LC, Langerhans cell; TUNEL, terminal deoxydyl tranferase-mediated dUTP nick end labeling

INTRODUCTION

Molluscum contagiosum (MC) is a benign viral skin disease frequently observed in children with atopic dermatitis, organ transplant recipients, and patients with human immunodeficiency virus (HIV) infection. Molluscum contagiosum poxvirus (MCV) exists in epidermal keratinocytes, forming the so-called molluscum bodies in the living layers of epidermis. MC may develop in healthy individuals, and it persists for many weeks, evading the host immune response. Thus, in addition to the host immune suppression, remodelling of the immunological milieu of the skin may provide a susceptible condition for MCV infection. However, MC lesions can also spontaneously disappear, associated with inflammation. [1] A similar phenomenon was observed in human papilloma virus (HPV)-induced warts, in which the HPV-infected cells evade the host immune surveillance and sometimes regress following inflammation that is mediated mainly by CD8+T cells. [2, 3]

Previous investigations revealed the depletion of Langerhans cells (LCs) in MC lesions and HPV-induced warts, despite the presence of considerable numbers of LCs in the perilesional skin. [4, 5] Our previous observations indicated that the depletion of LCs in HPV-induced warts was associated with a down-regulation of the expression levels of macrophage inflammatory protein 3 alpha (MIP-3α) and E-cadherin in the lesional keratinocytes. [5] In the present study, we investigated the cellular and molecular mechanisms that allow MCV to evade host immune responses.

We first examined the density of epidermal LCs in MC lesions, and we determined the expression levels of MIP-3α, which is a chemokine for LCs. MIP-3α is a 10-kDa CC-chemokine expressed in intestinal epithelium and keratinocytes, capable of recruiting LCs, memory T cells, immature dendritic cells (DCs), and LCs via CCR6

expressed on the cell surfaces. [6, 7] A previous study found that MIP-3α expression is decreased in keratinocytes expressing E6/E7 encoded by HPV-16 and other HPV subtypes *in vitro*. [8]

Thymic stromal lymphopoietin (TSLP) is an epithelial cell-derived cytokine expressed in skin which drives DCs to generate T-helper (Th) 2 cytokine production. TSLP expression is up-regulated in epidermal keratinocytes of atopic dermatitis and bronchial epithelial cells in asthma. [9, 10] Because MC is frequently associated with atopic dermatitis, we examined the expression patterns of TSLP in MC, and we compared them with those of HPV-induced warts and other cutaneous neoplasms.

After performing the genotyping of MCV, we studied the expression of MCV-encoded immunoregulatory and antiapoptotic molecules. The viral CC chemokine, MC148 is a highly specific antagonist for the CCR8-mediated recruitment of monocytes and DCs. [11, 12] The protein, MC159 is a viral Fas-linked interleukin-1β converting enzyme-like protease (FLICE)-like inhibitor protein, or vFLIP. [13, 14]

We examined apoptotic and necrotic cells in tissue samples of MC, and we compared them with those in samples of inflammatory HPV-induced warts. From a therapeutic view point, we examined the possibility whether an MDM2 inhibitor, nutlin-3, can induce p53-dependent apoptosis in MC. [15]

MATERIAL

All tissue materials were obtained for diagnostic or therapeutic purposes. A total of 30 MC samples from 29 patients, six samples of HPV-warts, three skin biopsy specimens of atopic dermatitis, and one tissue sample of surgically removed tonsil were used for the present study with approval of the ethical committee at Okayama University Hospital (No. 1034). The diagnosis of MC was confirmed by the presence of molluscum bodies or keratinocytes with inclusion bodies of MCV. The diagnosis of HPV-induced warts was proven by immunostaining or polymerase chain reaction (PCR) amplification for HPV as described. [5]

METHODS

PCR and restriction fragment length polymorphism (RFLP) for MCV genotyping

Of the 12 samples used for the molecular analysis, the MCV genotype was determined by PCR amplification followed by restriction fragment length polymorphism (RFLP). Briefly, DNA was extracted from skin samples using a DNeasy tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR amplification was carried out using specific primer sets:

MCV,sense;5′-CCGATCTTTGCGAGCGTTCTTAA-3′ and antisense;

5′-TCCCATACAGCGAGGACAGCATA-3′, and *β-globin*, sense;

5′-CAACTTCATCCACGTTCACC-3′ and antisense;

5′-GAAGAGCCAAGGAAGGTAC-3′. [16] The PCR products were analyzed by electrophoresis on 2% agarose gels and visualized with ethidium bromide. The 167-bp PCR products were digested with the restriction enzyme Sac I and analyzed on agar rose gels. Since MCV type 1 contains the restriction site for Sac I, two DNA fragments with

molecular sizes of 109 bp and 58 bp could be detected after Sac digestion, but no cleavage was observed in MCV type 2.

Immunohistochemistry

Before staining, the sections were deparaffinized, rehydrated, and pretreated by microwaving in 0.01 M citrate buffer or by boiling at 97°C for 35 min in antigen retrieval solution (Dako, Carpinteria, CA) in a pressure cooker. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide. The sections were incubated at 4°C overnight with murine monoclonal antibodies (mAbs), and further reacted with rabbit-peroxidaselabeled anti-mouse IgG Ab (IgG; Nichirei Biosciences, Tokyo) or alkaliphosphatase-labeled anti-mouse IgG Ab (LSAB/AP, K0678; Dako) at room temperature. The enzyme activities were visualized using 3-amino-9-ethylcarbazole (AEC) substrate, or Fuchsin substrate-chromogen, respectively.

Murine monoclonal antibodies used for the present study were as follows: antibodies against CD1a (Bioscience, San Jose, USA), human Langerin (Novocastra, Newcastle, UK), and TSLP (Abcam, Cambridge).

The numbers of LCs were determined in three randomly selected fields (original magnification ×400) per section. Each count was performed on three sections taken from different parts, and the results are expressed as the mean ±SD of cell numbers per three fields.

Terminal deoxydyl transferase-mediated dUTP nick end labeling (TUNEL) assay

Samples from patients were assessed for apoptosis on deparaffinized sections using a

terminal deoxydyl transferase-mediated dUTP nick end labeling (TUNEL) assay according to the manufacturer's protocol (Promega**,** Madison, WI). TUNEL positivity was examined in skin biopsy specimens of MC and HPV-warts.

Assay for nutlin-3-induced apoptosis

An MDM2 inhibitor, nutlin-3, was purchased from Cayman Chemical (Ann Arbor, MI), and stocked in 10% ethanol solution until use. Removed MC lesions were incubated with Dulbecco's Modified Eagle Medium (DMEM) containing 5–20 μM of nutlin-3 overnight, and the tissue samples were processed for paraffin embedding. The numbers of apoptotic cells in the living layers, determined by the TUNEL method, were enumerated in the tissue samples without incubation and in samples incubated with DMEM containing 5% ethanol (vehicle control) and DMEM containing nutlin-3.

The numbers of TUNEL-positive cells in MC and normal-appearing epidermal keratinocytes were determined in three randomly selected fields (original magnification×400) per section. Each count was performed on three sections taken from different parts, and the results are expressed as the mean ±SD of cell numbers per three fields.

***In situ* hybridization for MIP-3α**

Coupled primers were used to amplify the majority of the open reading frame (ORF) of the MIP-3α gene by PCR. *20/MIP-3α/CCL20* (sense5′-AATCAGAAGCAGCAAGCAAC-3′) and *29/MIP-3α/CCL20* (antisense5′-CGCACACAGACAACTTTTTC-3′) [17] were used with an annealing temperature of 61°C. The digoxigenin-labeled RNA probe (Nco I probe) was

transcribed in antisense orientation, using a DIG RNA Labeling kit (SP6/T7, Roche, Mannheim, Germany). “Labeled control RNA” (the same kit) was used as a negative control probe.

As the amount of MIP-3α mRNA was small, we optimized the tyramide signal amplification (TSA) using the GenPoint™ Catalyzed Signal Amplification System (Dako). TSA has been adapted for the *in situ* hybridization (ISH) detection of cellular mRNAs.[18] After deparaffinization and rehydration, the sections were immersed in a Coplin jar containing Target Retrieval Solution (Dako) at 95.0°C. The sections were treated with 2 μg/mL proteinase K. After the dehydration, the sections were treated with pre-hybridization solution (50% deionized formamide, 2.5 × SSC, 1× blocking reagent, 0.01% SDS) at 37°C. Hybridization mixtures were made by mixing each probe (0.5 pg/μL Nco probe and negative control probe) with mRNA ISH solution (Dako). The hybridization solution was applied and hybridized overnight at 37°C in a moist chamber. The sections were rinsed with 5 × SSC and 0.5 × SSC at 45°C. Anti-DIG-AP conjugate was used instead of anti-DIG-HRP conjugate. Biotinyl tyramide (BT) was applied to slides for 30 min at RT. Streptavidin AP solution was applied to the sections for 30 min at RT. The sections were then counterstained with hematoxylin.

The numbers of MIP-3α mRNA+ dots were determined in three randomly selected fields (original magnification ×400) per section. Each count was performed on three sections taken from different parts, and the results are expressed as the mean ±SD of dot numbers per three fields.

Detection of the MCV-encoded immunoregulatory molecules MC148 and MC159L

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to detect the

expression of the MCV-encoded immunoregulatory molecules MC148 mRNA and MC159L mRNA. For the RNA extraction from tissues, samples soaked in TRIzol (Invitrogen, Carlsbad, CA) reagent were cut into fine pieces. cDNA transcription was carried out in 10-μL reaction mixtures containing 1 μg RNA, 10 mM deoxyribonucleoside triphosphates (dNTP) (Promega), 18.75 pmol random hexamer primer (6mer) (100 pmol/μL) (Takara, Kyoto, Japan), 5 × buffer, 0.1 M DTT, 50 U SuperScript™III reverse transcriptase, and 2.5 U RNasin™ (Invitrogen).

The PCR was performed in a thermal cycler with a 25-μL reaction mixture containing 2.5 μL cDNA samples, 1.25 U Go Taq™ DNA polymerase, 5 × buffer, 25 mM MgCl2 (Promega), 2.5 mM dNTP (Takara), and each of the sense and antisense primers. Amplification was achieved by 30 cycles of denaturing at 94°C for 1 min, annealing at 57°C (MC148) or 64°C (MC159) for 30 s, and extension at 72°C for 3 min.

We used specifically designed primer sets for the majority of the ORFs of the MC148 gene (322 bp) and MC159 gene (443 bp): MC148, sense 5'-ATTAGCTAGCATGGGGAGGGGCGGAGAC-3', antisense 5'-ATTAGGATCCTTACCAGAGACTCGCACCC-3' [19]; MC159; sense5'-GACTACGCATCCGACTCCAAGGAGGTCCCTAG C-3', antisense 5'-CGGAATTCTCAAGTCGTTTGCTCGGGGCT-3'. [20] Beta 2-microgrobulin was used as the internal control, with primers of sense 5'-TACATGTCTCGATCCCACTTAACTAT-3' and antisense 5'-AGCGTACTCCAAAGATTCAGGTT-3'.[21] Each experiment was performed with water as a negative PCR control. The PCR products were analyzed by electrophoresis on 2% agarose gels and visualized with ethidium bromide.

Statistical analysis

We analyzed the results using the non-parametric Student's t-test. Differences were considered significant when $p<0.05$.

RESULTS

The depletion of LCs in MC

The numbers of both $CD1a^+$ and $Langerin^+$ cells were significantly decreased in the epidermal walls surrounding molluscum bodies compared to those in the perilesional normal-appearing epidermis (Fig. 1a, 1b). The mean numbers of $CD1a^+$ and $Langerin^+$ cells in the perilesional normal-appearing epidermis were $9.53 \pm 4.29/m^2$ and $11.70 \pm 7.34/mm^2$, respectively, whereas those in the epidermal walls surrounding the molluscum bodies were significantly decreased: $0.13 \pm 0.17/mm^2$ ($p<0.001$) and $0.54 \pm 1.22/mm^2$ ($p<0.001$), respectively (Fig. 1c, 1d).

MIP3α mRNA expression in MC

The expression levels of MIP-3α mRNA, a chemokine for LCs, were determined by *in situ* hybridization. In control materials, MIP-3α mRNA was expressed in the epithelial crypts of tonsil properly (Fig. 2a, b). As compared to the perilesional epidermis, the expression levels of MIP-3α mRNA were significantly decreased in the epidermal walls surrounding molluscum bodies (Fig 2c). The expression levels revealed approximately one-fourth of those in the perilesional normal-appearing skin ($p<0.001$) (Fig 2d).

TSLP expression

The expression of TSLP was enhanced in the whole prickle-cell layers of the atopic dermatitis and HPV-induced warts, whereas the expression was weak in the MC lesions. However, the TSLP expression in the MC lesions was induced on restricted areas such as the MCV-infected cells and the inner surfaces of the epidermal walls surrounding the molluscum bodies (Fig. 3).

Expression of MCV-encoded immunoregulatory and antiapoptotic molecules

The PCR products with an expected molecular size of 167 bp were amplified in all 14 MC lesions examined. The restriction enzyme Sac I cleaved the products into two fragments of 109 bp and 58 bp, indicating the presence of a DNA sequence of MCV type 1 (data not shown). Of the 14 MC samples examined by RT-PCR, the mRNA of MC148, an antagonist for CCR8, was detected in all samples. The mRNA of MC159, a vFLIP with an antiapoptotic property, was present in 13 of the 14 MC samples (Fig. 4).

Apoptotic cells in MC

No or only a few TUNEL+ cells were observed in the living layers of MC (Fig. 5a) and HPV warts (data not shown). Therefore, both noninflammatory HPV warts and molluscum bodies in the living layers were not in an apoptotic process. The number of TUNEL+ apoptotic cells was increased in molluscum bodies after stimulation with 5–20 μM nutlin-3. A concentration of 5 μM nutlin-3 was enough to induce apoptosis (Fig 5b). Unlike the inflammatory HPV warts (Fig 5c), the nutlin-3-induced apoptotic cells were observed in the molluscum bodies, but not in the basilar areas of living epidermis.

The increase of TUNEL+ cells was also observed in the vehicle control containing 5% ethanol. The TUNEL+ cell numbers were significantly increased in the nutlin-3-treated and vehicle-control groups compared to those of the freshly removed materials ($p<0.01$) (Fig. 5d). The mean number of TUNEL+ cells in the nutlin-3-treated group was higher than that of the vehicle-control group, but there was no statistical significance ($p>0.1$). Compared to the normal-appearing epidermal keratinocytes, TUNEL+ cells were more frequently observed in the molluscum lesions (Fig 5d).

DISCUSSION

From our study, five major findings were obtained in the immunological milieu in MC: 1) the depletion of LCs in the lesional epidermis, 2) the down-regulation of MIP-3α expression, 3) localized TSLP expression, 4) the generation of MCV-encoded immunoregulatory and antiapoptotic molecules, and 5) no or few apoptotic cells in the MC lesions.

The depletion of LCs in the epidermal walls surrounding the molluscum bodies was remarkable in the MC samples compared to the LC numbers in the perilesional epidermis. The depletion of LCs was also observed in HPV-induced warts such as common warts and plantar warts.[4] The down-regulation of the expression of MIP-3α, a specific chemokine for LCs, was also observed in the MC samples studied here, as was shown previously in HPV warts. [22] Therefore, the low expression of MIP-3α with a subsequent depletion of LCs is a common cellular and molecular event in both MC and HPV-induced warts.

However, we observed some differences in the structures and molecular levels between MC and HPV-induced warts. First, the histopathologic structure of MC differed from those of the HPV warts: the nests of molluscum bodies were surrounded by LC-depleted acanthotic epidermal walls, whereas papillomatous proliferation of the epidermis was remarkable in the HPV warts. The LC-depleted epidermal walls may therefore provide a barrier function to protect molluscum bodies from the host immune surveillance.

As previously proposed,[23] the disruption of the barrier may induce inflammation by activation of a complement pathway upon the exposure of molluscum bodies to the tissue fluids, and by the release of cytokines such as interleukin (IL)-8 and growth-

regulated oncogene alpha (GROα).

Second, we observed the strong expression of TSLP in the whole epidermis of the samples of atopic dermatitis and HPV-induced warts, but the expression was weaker in the MC epidermis. In MC, TSLP was expressed in restricted areas including the molluscum bodies and inner surfaces of the epidermal walls surrounding them. These observations suggest that unlike atopic dermatitis, [9] Th2-mediated inflammation induced by TSLP or TSLP signaling is no longer essential for the development of MC.

We found that MCV-encoded molecules such as MC148 mRNA and MC159 mRNA were expressed in MC lesions. MC148 is a highly specific antagonist for CCR8, and it interferes with the recruitment of monocytes and dendritic cells. [11, 12] Therefore, in addition to the low expression of MIP-3α, the biological property of MC148 might be related to the depletion of LCs in MC. The MC159 protein is a viral FLIP that inhibits TNFR1-induced NF-κB activation and apoptosis.[13, 14]

In the present study, no or few TUNEL+ cells were observed in the molluscum bodies, even though they look like cells with a cytopathic effect morphologically. However, in our *in vitro* study, the incubation of MC tissue samples with either DMEM containing 5–20 μM nutlin-3 or DMEM containing 5% ethanol (vehicle control) induced apoptosis in the molluscum bodies, but apoptosis was observed less frequently in the normal-appearing epidermal keratinocytes. Furthermore, the localization of nutlin-3-induced apoptotic cells in noninflammatory MC was different from that of inflammatory HPV-warts and MC, where the apoptotic cells were mainly observed in the basilar layers of the epidermis.[5,24] These data suggest that the mechanism in inflammation-induced apoptosis is different from that in the nutlin-3-induced apoptosis in which the p53-dependent apoptotic pathway is implicated.[15]

Our observations indicate that molluscum bodies harboring many MCV are separated by LC-depleted epidermal walls immunologically as well as structurally, and MCV generate immunoregulatory and antiapoptotic molecules such as MC148 and MC159. The topical application of nutlin-3 in ethanol solution might be a therapeutic option to eradicate MC.

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Figure legends

Figure 1. Epidermal Langerhans cells in MC

CD1a+ (a) and Langerin+ (b) cells in noninflammatory MC lesions (original magnification: x100). Mean numbers of $CD1a^+$ (c) and $Langerin^+$ (d) cells in the epidermal walls surrounding the molluscum bodies ($0.13 \pm 0.17/mm^2$ and $0.54 \pm 1.22/mm^2$, respectively) are significantly decreased than those in the perilesional epidermis ($9.53 \pm 4.29 /mm^2$ and $11.70 \pm 7.34 /mm^2$, respectively) ($p<0.001$).

Error bars indicate SD.

Figure 2. MIP3α mRNA expression in MC

MIP3α mRNA+ dots are detected properly in the epithelial crypts of surgically removed tonsil (a, b: negative control probe). (original magnification: x400). The expression of MIP3α mRNA is significantly decreased in MC lesion, compared with that in the perilesional epidermis ($p<0.001$) (c, d). Error bars indicate SD.

Figure 3. TSLP expression in MC

In the MC lesions, the TSLP expression is induced on the restricted areas such as the MCV-infected cells, and the inner surfaces of epidermal walls surrounding molluscum bodies (a,b). In HPV-warts, the expression of TSLP was enhanced in the whole prickle cell layers (c, d) (original magnification: a,c x100, b,d x400)

Figure 4. MC148 and MC159 mRNA expression in MC

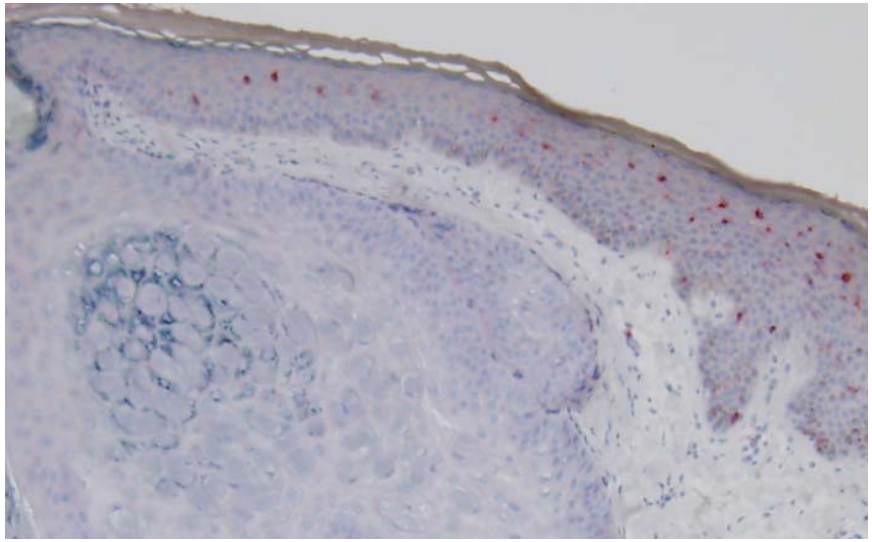
Representative data on samples 1 and 2. MC148 mRNA (322bp) and MC159 mRNA (443bp) are detected (a: MC148mRNA, b: MC159mRNA) in reverse transcriptase (RT)

+ lanes, but negative in the RT- lanes. M: molecular markers

Figure 5. Apoptotic cells in MC and HPV-warts, and nutlin-3-induced apoptosis

No or only a few TUNEL+ cells are observed in the living layers of noninflammatory MC (a). After stimulation with nutlin-3, the number of TUNEL+ cells increase in MC (b). The localization of TUNEL+ cells are mainly present in the molluscum bodies, whereas they exist at the boundary of dermoepidermal junction in the inflammatory HPV-warts (c: no counter staining). Apoptotic cell numbers are significantly increased in the nutlin-3-treated and vehicle control (5% ethanol) groups than those of freshly removed materials ($p<0.01$) (d). A mean number of apoptotic cells is higher in the nutlin-3-treated group than that of the vehicle control group, but there is no statistic difference ($p=0.117$). As compared to the normal-appearing epidermal keratinocytes, TUNEL+ cells are more frequently observed in molluscum bodies in the nutlin-3-treated group ($p<0.01$).

(a)



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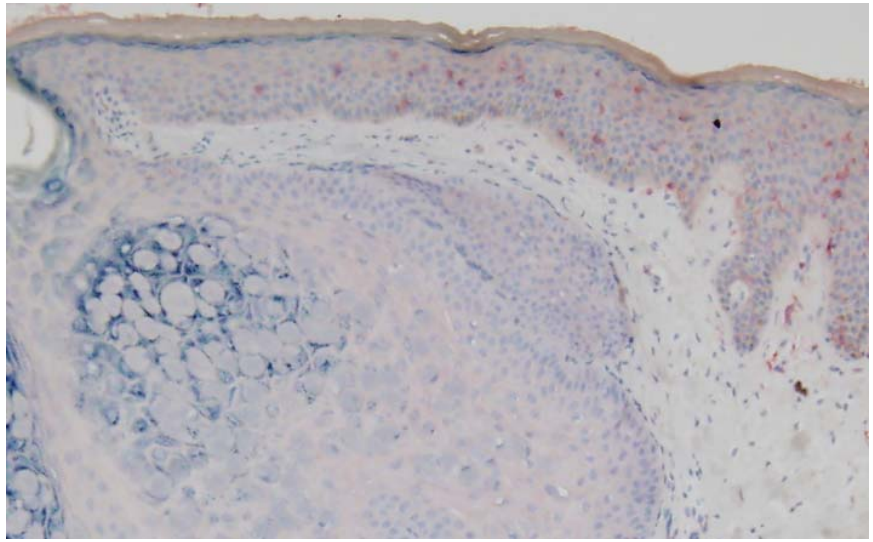

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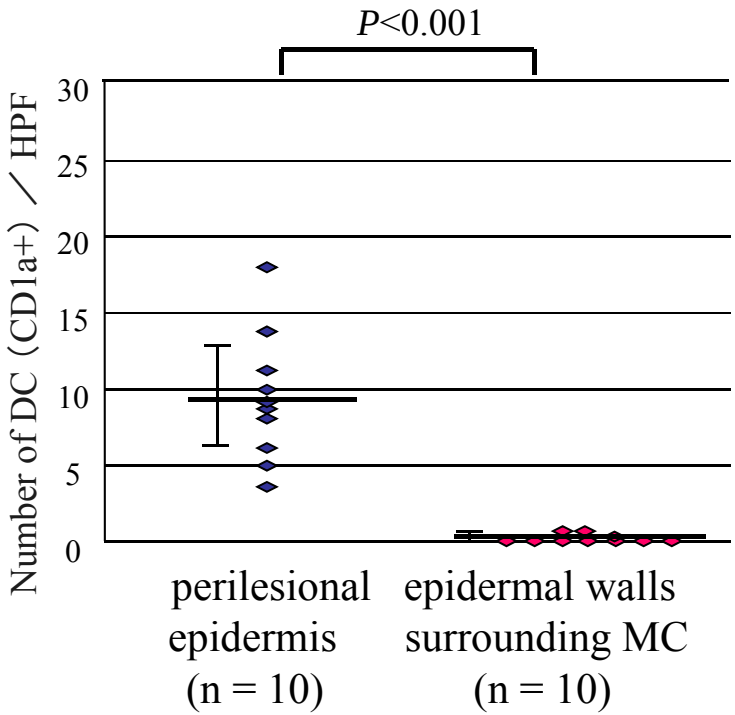

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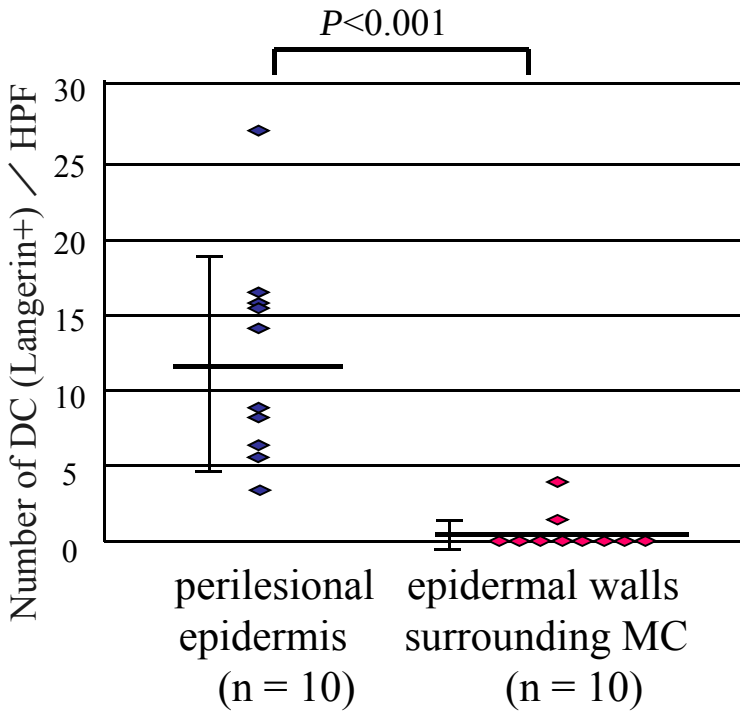
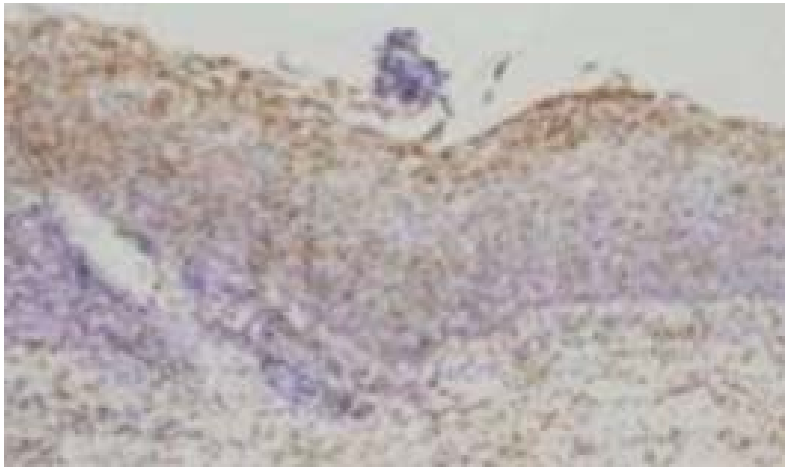
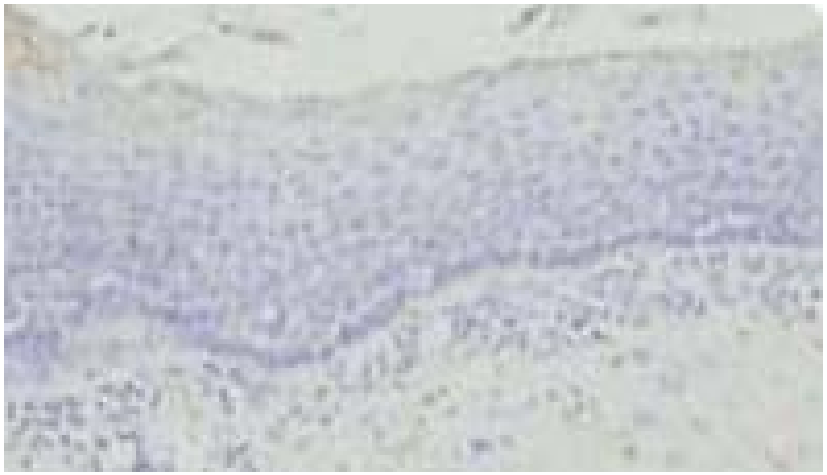


Figure.1

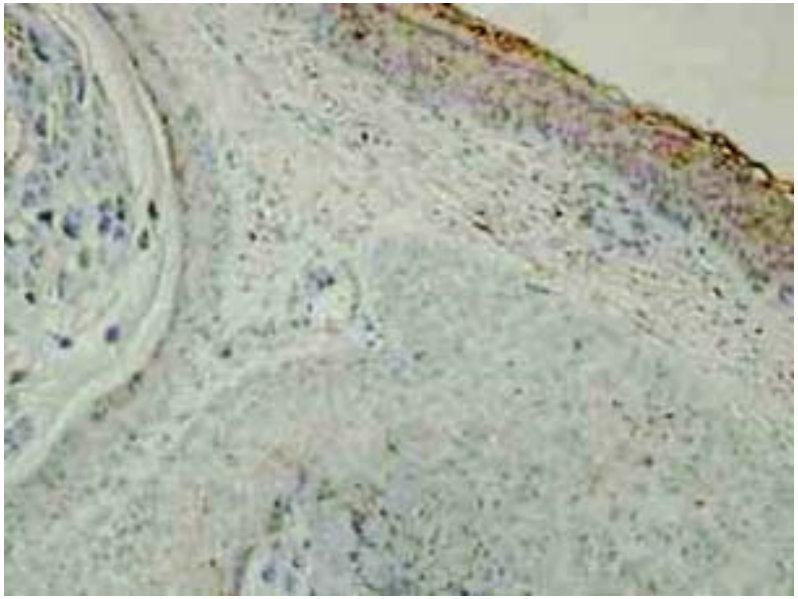
(a)



(b)



(c)



(d)

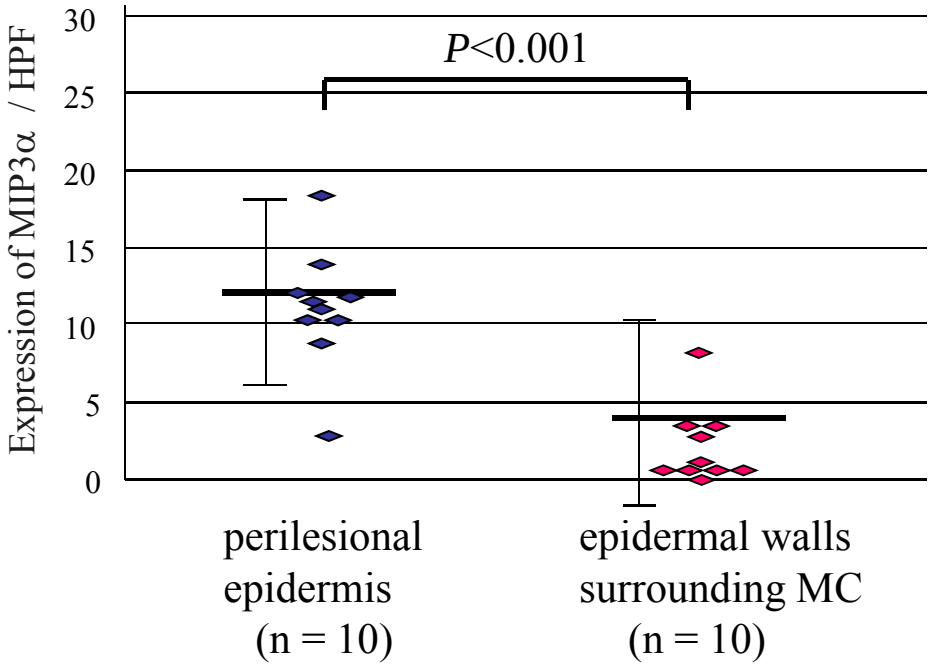
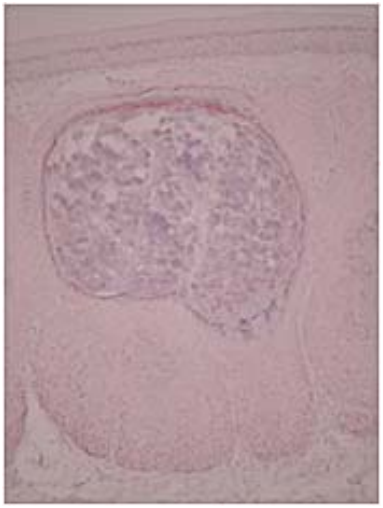
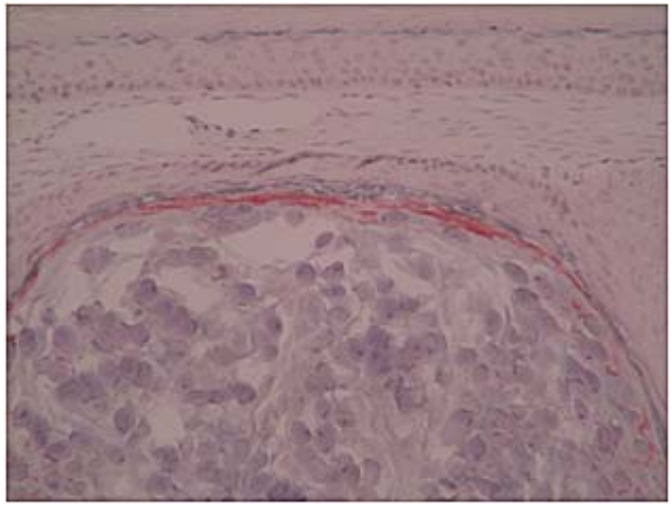


Figure.2

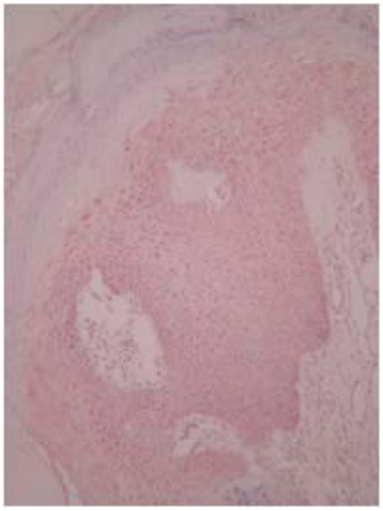
(a)



(b)



(c)



(d)

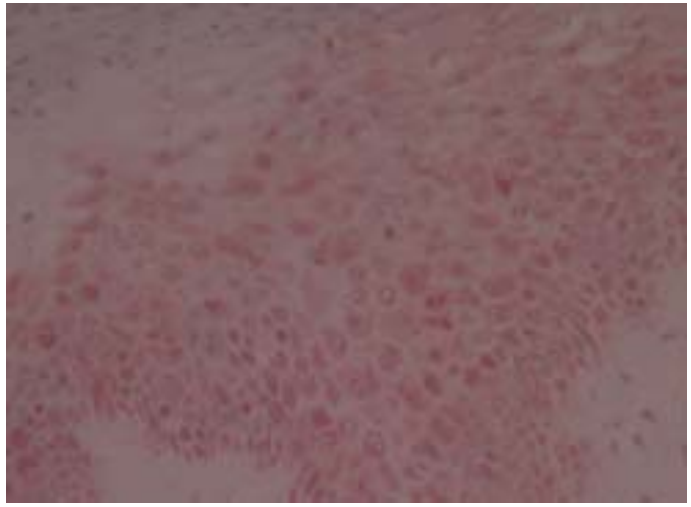


Figure.3

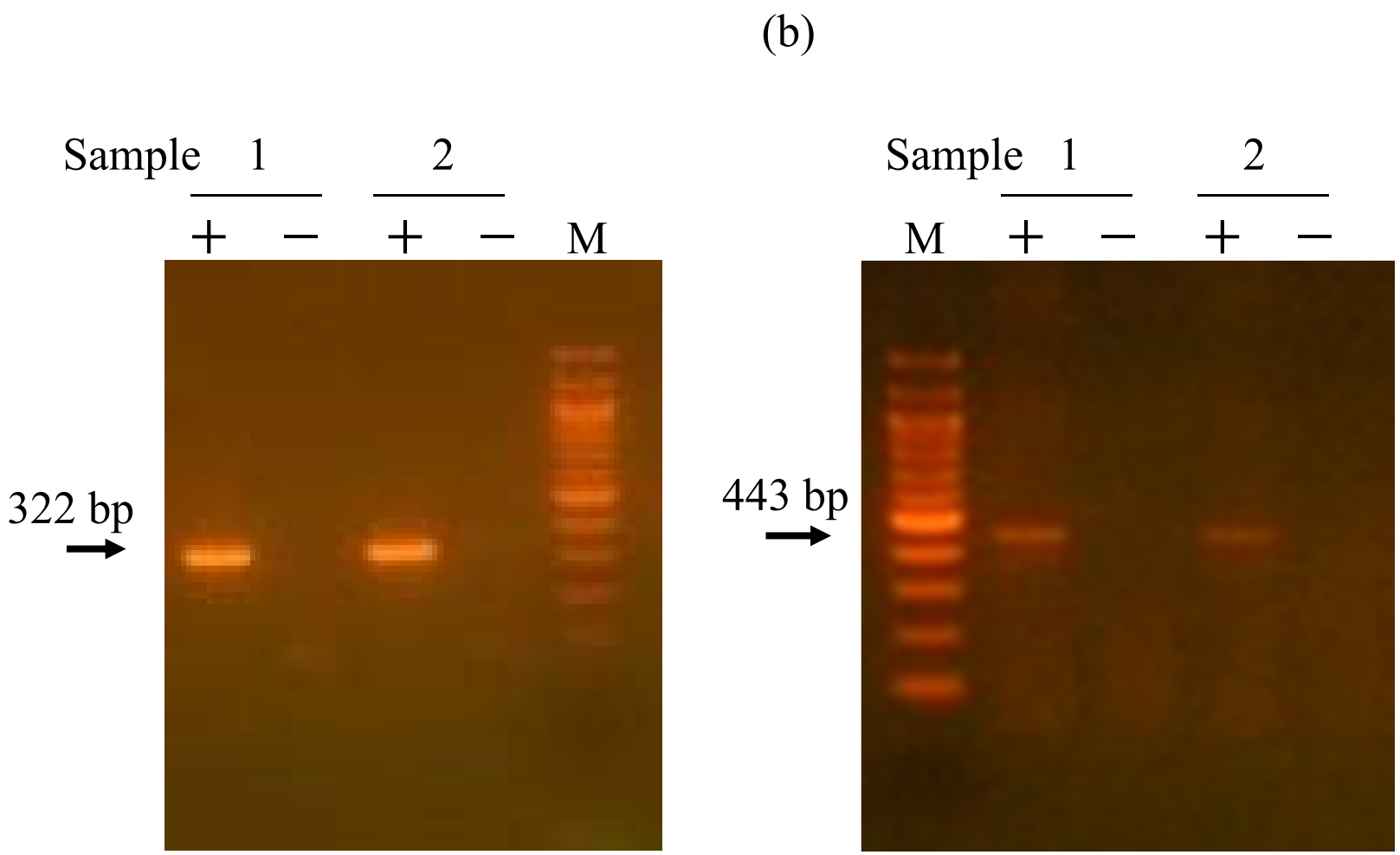
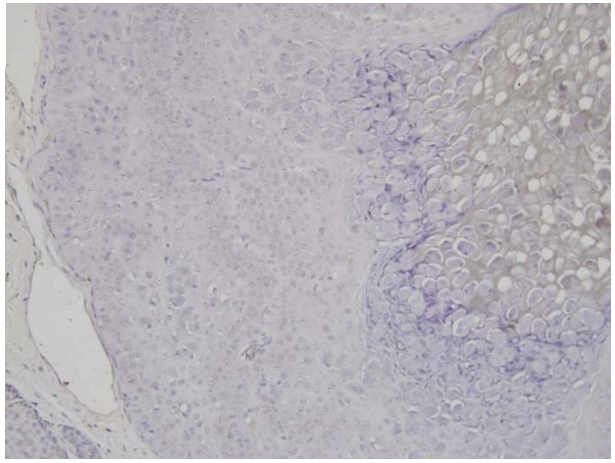
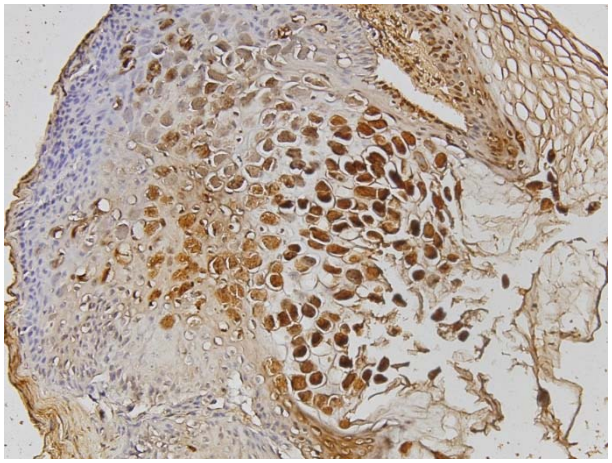


Figure.4

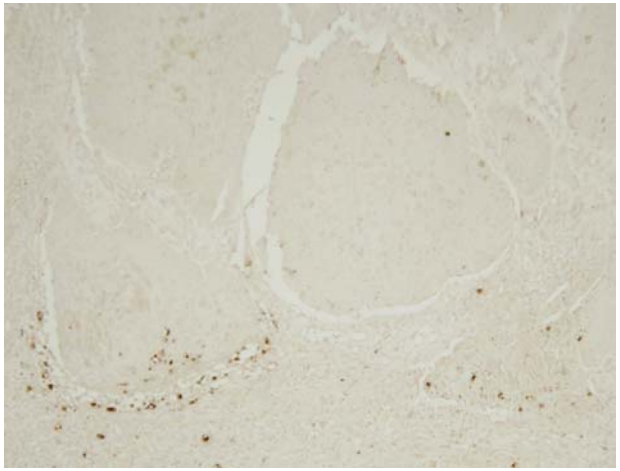
(a)



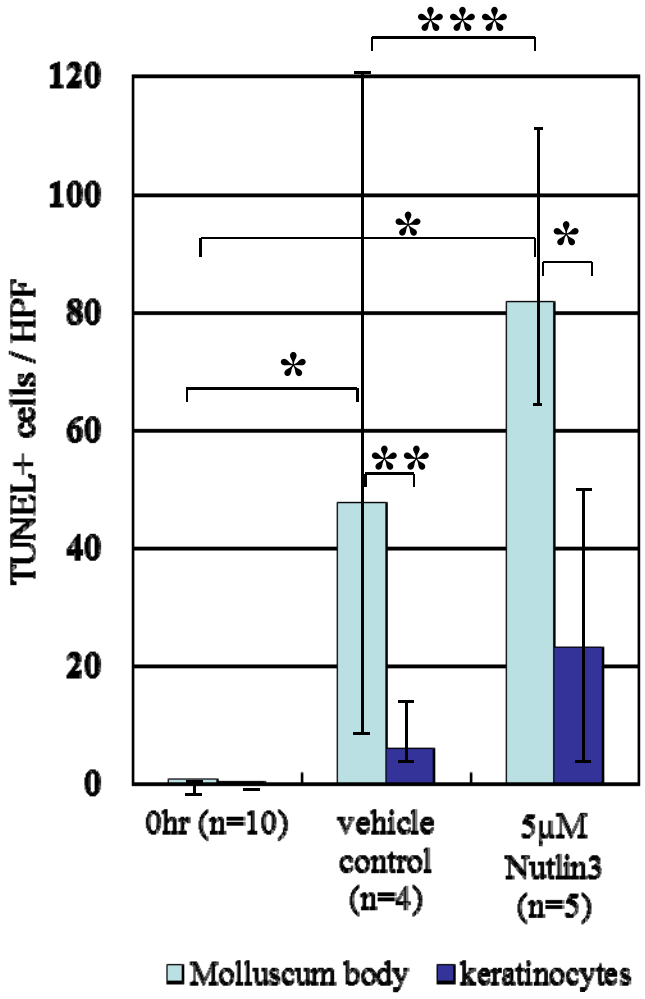
(b)



(c)



(d)



* P < 0.01 , ** P = 0.234 , *** P = 0.117

Figure.5