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ORIGINAL PAPER

Assessment of an imiquimod-induced psoriatic mouse model in relation to oxidative stress

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Abstract Psoriasis is a chronic inflammatory skin disease that is thought to be related to oxidative stress. Much progress has been made in understanding the pathophysiology of psoriasis in relation to the immunologic and antioxidant systems. However, this progress has been hindered by the lack of an appropriate animal model for psoriasis. Recently, imiquimod (IQM)-induced psoriasis-like cutaneous inflammation has been reported in mice and humans. We verified the usefulness of an IQM-induced mouse model in relation to the antioxidant system. BALB/C female mice at 8–10 weeks of age were treated with IQM cream in this study. We analyzed clinical and histopathological changes. Increased reactive oxygen species production was measured by glutathione assay. Levels of myeloperoxidase (MPO) and superoxide dismutase-1 (SOD1) were determined by western blotting and immunohistochemical analyses. The activity of SOD was measured by a SOD activity assay kit. Application of IQM-induced skin inflammation similar to psoriasis in clinical and histopathological aspects. Accumulation of immune cells was confirmed. Oxidative stress was increased, the antioxidant enzyme MPO levels were increased, and both SOD levels and activity were decreased. In conclusion, the IQM-induced mouse model showed an aberrant antioxidant system. Levels of MPO and oxidative stress were increased, and the level and activity of SOD were decreased. Since this model seemed to be an appropriate model for psoriasis, it can be used to further study the pathogenic role of redox imbalance in psoriasis.

Keywords Psoriasis · Mouse model · Antioxidant · ROS · Imiquimod · SOD

Introduction

Psoriasis is a chronic inflammatory skin disease that is thought to be related to oxidative stress. It is triggered or aggravated after mechanical, chemical, psychological, or microbial stress [16]. The pathogenesis of psoriasis is not fully understood, but recent progress in studying its complex mechanisms revealed that cross talk among T cells, epidermal keratinocytes, dendritic cells (DCs), neutrophils, endothelial cells and fibroblasts, together with growth factors, chemokines, and cytokines, is important in the development and maintenance of the disease [17, 23]. Among these immune cells, plasmacytoid DCs (pDCs) have been suggested to play an important role in the induction of early psoriasis. pDCs are considered to be key effector cells in innate immunity because they can produce high levels of type I interferon upon stimulation. Activation of pDCs by sensing self-DNA coupled with antimicrobial peptides in lesional skin produces massive amounts of interferon-α (IFN-α), which then leads to expansion of pathogenic T cells [16, 21].

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It has been suggested that increased reactive oxygen species (ROS) production and an aberrant antioxidant system may be main factors in the pathogenesis of psoriasis [27]. Generation of ROS from neutrophils and fibroblasts can activate neutrophils, which indicates a possible role of ROS in psoriasis [18, 30]. Supplementation of antioxidants in severe erythrodermic and arthropathic forms of psoriasis has proven to be effective [15], and the anti-psoriatic drug dimethylfumarate mediates the up-regulation of antioxidative pathways such as glutathione (GSH) [20, 39]. However, there are still conflicting thoughts on the roles of ROS and the antioxidant system in psoriasis.

Research in psoriasis has been hindered by the lack of an appropriate animal model. An ideal psoriatic model includes epidermal hyperproliferation and altered differentiation, papillomatosis, presence of inflammatory cells including T cells, altered vascularity, and response to anti-psoriatic agents [4]. There have been a large number of genetically engineered (transgenic or knockout) or induced (by immune cell transfer or by xenotransplantation) murine models for psoriasis [5]. These models exhibit skin conditions that are similar to psoriasis in humans, and each model has its strong points, but many discrepancies exist between mouse skin and human skin. Mouse skin has a denser follicular distribution, thinner epidermis and dermis, and an entire cutaneous muscle layer [2, 14]. Additionally, mice have immune cells that are different from humans and are usually inbred [1, 13]. One of the representative psoriatic mouse models is the xenograft model, which uses human psoriatic skin transplanted onto immunodeficient mice. It is a very close approximation of psoriasis in humans in terms of genetic, phenotypic, and immunologic changes, but is expensive and time-consuming and requires specialized laboratory skills [3, 22].

Recently, imiquimod (IQM)-induced psoriasis-like inflammation in mice and humans has been reported [12, 26, 29, 37]. IQM is a ligand for toll-like receptor 7 (TLR7) and TLR8, and is a potent immune activator. IQM's immunomodulatory effects in triggering psoriasis are attributed to stimulation of TLR7 on pDCs and an upregulated type I interferon pathway. IQM can also interfere with adenosine receptor signaling and augment inflammation [31]. Application of IQM has been shown to increase the number of pDCs in the skin of mice [25]. van der Fits et al. [37] demonstrated that IQM-induced skin inflammation in mice was immunologically mediated via the IL-23/IL-17 axis, and showed psoriasis-like histopathologic changes (acanthosis, parakeratosis and neoangiogenesis), including infiltration of $CD4^+$ T cells, $CD11c^+$ DCs and pDCs in this model. However, ROS system, which might be important part of psoriasis pathogenesis, has not been tested in this simple psoriasis-mimicking mouse model so far.

The aim of this study was to recreate an IQM-induced mouse model for psoriasis and examine the usefulness of this model in relation to oxidative stress-related enzyme systems in order to determine the role of ROS and the antioxidant system in psoriasis.

Materials and methods

Reagents and antibodies

Primary antibodies against mouse T cells: CD3 (clone KT3), CD4 (GK1.5), CD8 (YTS169), and DCs: CD11c (N418), MHC-II (M5/114), (BD Pharmingen, San Diego, CA, USA), pDC (120G8) (Imgenex Corporation, San Diego, CA, USA) markers and rabbit polyclonal antibodies to myeloperoxidase (MPO) and SOD-1 were purchased (Abcam, Cambridge, UK). The superoxide dismutase (SOD) activity kit was purchased from Enzo Life Science (Assay Design Inc., MI, USA).

Mouse model of psoriasis

BALB/C female mice at 8–10 weeks of age were used in this experiment. Mice in each of the experimental groups (disease free-control group and diseased group), containing at least five mice, were shaven along their backs, and the remaining hairs were completely removed with depilatory cream (Veet, Reckitt Benckiser, Cedex, France). To induce the psoriasis model, we used the method originally developed by van der Fits et al. [37] only with a slightly increased dose of IQM to generate a maximal effect. Briefly, mice were applied commercially available IQM cream (5 %) (Aldara; 3M Pharmaceuticals, UK), at a daily dose of 80 mg which contained 4 mg of the active compound, on the back skin and the right ear for 6 consecutive days. This dose was determined to cause the most optimal and reproducible skin inflammation in mice in our previous study. Control mice were treated similarly with a vehicle cream (Petrolatum).

GSH assay

We determined increases in ROS in the tissues, according to the principle that more GSH disulfide (GSSG), an oxidized form of GSH, in the experimental group than in the control group, indicates increased amounts of ROS. The GSH assay kit was purchased from BioVision (San Diego, CA, USA). GSH is the major intracellular low molecular weight, thiol-containing antioxidant that plays an essential role in cellular defense against ROS, reactive oxygen intermediates and oxidative stress in tissues and cells. Once GSH is oxidized by reactive molecules, the reduced form is

regenerated in the presence of GSH reductase. The balance between GSH and GSSG may be used to determine the redox status within the cell. Thus, if there is more GSSG in the experimental group than in the control group, it indicates that the cells or tissues have increased amounts of ROS. The GSH assay was performed according to the manufacturer's instructions. Briefly, tissues were ground with mortar and pestle in liquid nitrogen and suspended in GSH assay buffer. The suspensions of raw tissue were briefly (15 s) sonicated at 3,000 Hz, centrifuged for 5 min at 13,000*g* at 4 °C, and the supernatants were collected in prechilled tubes. Protein concentrations were measured by BCA assay, and the protein amounts in the samples were equilibrated by diluting 10 mg/mL protein in 100 μL assay buffer. The next stages were performed as described in the assay protocol. The black well plates containing samples were read by a fluorescence plate reader (Perkin Elmer LS50B) at excitation wave length 485 nm and emission wave length 530 nm with excitation split of 15. The GSSG, an oxidized GSH, in the unknown samples was calculated by the reduction of GSH, compared to the standard samples and normal control samples.

SOD activity assay

The SOD activity assay is a colorimetric assay in which superoxide anion converts chromogen into a water-soluble formazan dye that absorbs light at 450 nm in the absence of SOD. The activity of SOD is thereby determined as the inhibition of chromagen reduction in experimental samples, as previously described [24]. The measurement of the SOD was performed using a commercial kit according to the manufacturer's provided protocol. SOD activity in the tissues was determined by optical density (OD) using an ELISA plate reader at 450 nm in the kinetic mode at 2-min interval for 30 min; the last reading was chosen as a determination of the rate change. The active units were determined by comparing the values of the % inhibition rate of standard samples to the % inhibition rate of unknown samples. The average values of triplicates were considered as representative of each experimental group.

Protein extraction and western blotting

The back skin tissues from the mice were ground with mortar and pestle in liquid nitrogen. Equal amounts of tissue extract were sonicated briefly and lysed with PRO-PREP^{TM} protein extraction solution (iNtRON biotechnology Inc, Kyonki-Do, Korea) according to the manufacturer's protocol. The protein concentrations were determined using the BCA assay. Thirty μg of proteins were loaded by 10 % SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA, USA). The membranes were incubated with rabbit polyclonal anti-mouse-MPO and anti-mouse-SOD1 antibodies at 4 °C overnight. They were then washed, incubated further with HRP-conjugated, anti-rabbit IgG (1/1,000 dilution), and detected using an ECL detection system (Amersham Biosciences, Piscataway, NJ, USA). An anti-actin antibody (Sigma) was used as a loading control at 1:5,000 dilution.

Immunohistochemistry (IHC)

Six micrometer skin tissue sections were fixed in ice-cold acetone and 4 % formaldehyde for 10 min and incubated with rat anti-mouse antibodies listed in "Reagents and antibodies" or rat IgG1 isotype control antibody (eBioscience) for 1 h at room temperature, then incubated with an appropriate peroxidase-conjugated secondary Abs and streptavidin–biotin complex. Thereafter, the samples were developed with 3-amino-9-ethylcarbazole as substrate and counterstained with hematoxylin (HistoMouse-MAX kit; Invitrogen). Six micrometer skin sections were also stained with hematoxylin (Fisher Scientific, Pittsburgh, PA, USA) and eosin (Shandon, Pittsburg, PA, USA) for elucidation of epidermal and dermal alteration. Images were captured using a DP71 digital camera (Olympus) attached to an Olympus BX41 microscope.

Statistical analysis

Results are expressed as mean ± standard deviation of $n = 5$ mice/group. Data were analyzed using two-tailed unpaired Student's t test or one way ANOVA with Mann–Whitney U tests. Values of $p < 0.05$ were considered to be significant.

Results

Macroscopic features and histopathological alterations of IQM-applied mouse skin

Application of IQM for 6 days induced skin inflammation similar to psoriasis, showing redness, thickness and scale in BALB/c mice (Fig. 1a). Therefore, we adopted the clinically used psoriasis area and severity index (PASI) score to evaluate the similarity of IQM-induced skin inflammation to human psoriasis. One of the indications, skin erythema, appeared at day 1 after applying IQM and increased until day 4. The thickness started at day 3 after application of IQM and increased gradually until day 6, while the scaling started at day 3 and increased until day 7. Another sign that indicated specific immune response was delayed ear swelling after application of IQM. Ear swelling started in some mice at day 3 and rapidly increased through day 5

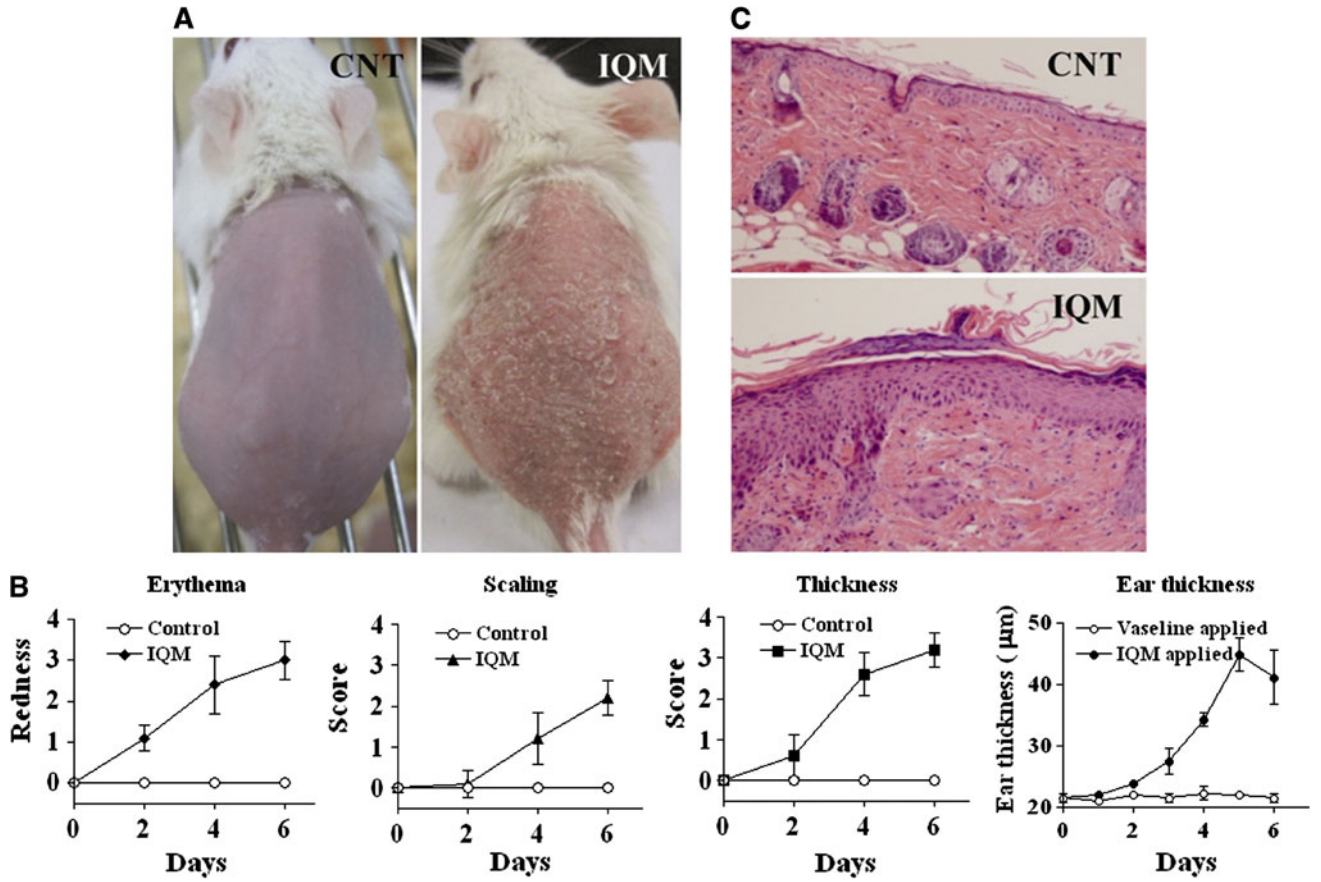

Fig. 1 Clinical and histopathological changes of the imiquimod (IQM)-applied mouse skin. **a** The control mice (*left*) showed no changes in skin lesions. The IQM-applied mice (*right*) showed psoriasis-like skin lesions with erythema, thickness and scale. The picture is representative of the five mice in the IQM group. **b** Clinical evaluation of psoriasis-like skin lesions was indicated by PASI scores; the erythema increased from day 1 until day 6, the scaling was observed on day 3 and gradually increased until day 6, and the thickness was observed from day 2 and increased until day 6. Ear swelling started from day 3 and increased until day 5. On the day of killing, the thickness of the IQM-applied ear (*left ear*) had increased threefold compared to the control (*right ear*). The line in the *X–Y* plot is the average of at least five independent experimental samples. **c** H&E staining of the IQM-applied back skin section showing psoriasis-like changes including scaling, epidermal thickness, keratinocyte hyperproliferation and granulocyte infiltration compared to control back skin. A representative picture of five mice is shown

and day 6 after application of IQM (Fig. 1b). On the day of killing, the ear thickness had increased threefold compared to pre-IQM application thickness.

Six consecutive days of IQM application induced epidermal changes, including hyperproliferation and altered differentiation of keratinocytes, which was revealed by H&E staining prepared from the ear and back skin samples. In H&E staining, scaling, epidermal thickening, papillomatosis and inflammatory cell infiltration into the epidermis and dermis were also observed in the IQM-applied mouse skin samples (Fig. 1c). The results were constantly reproducible in all mice.

Increased numbers of DCs and T cells in IQM-applied mouse skin

To determine the accumulation of antigen presenting cells and T cells, we performed IHC staining on the IQM-applied mouse skin. $CD11c^+$ DC (15-fold), pDCs (tenfold), $CD3^+$ (10.5-fold), $CD4^+$ (10.7-fold) and $CD8^+$ T cells (8.2-fold) were increased in IQM-applied mouse skin compared to normal mouse skin (Fig. 2). All of these results indicate that IQM-induced skin alteration in mice is very similar to human psoriasis.

Oxidative status in IQM-induced psoriasis-like skin inflammation in mice

We next assessed whether the IQM-induced mouse model of psoriasis had a redox imbalance. An oxidative status was determined by measuring GSSG/GSH ratio in IQM-induced psoriasis-like mouse skin using the GSH assay kit. In the IQM-induced psoriasis-like skin homogenate, the GSSG/GSH ratio was increased by 1.5-fold compared to that of normal mouse skin (Fig. 3).

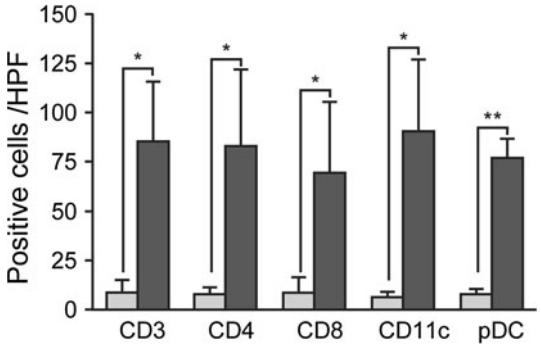

Fig. 2 Accumulation of immune cells in IQM-applied mouse skin. Immunohistochemical (IHC) staining on control and IQM-applied back skin sections revealed increased $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD11c^+$ myeloid dendritic cells (DC), and plasmacytoid DCs. Cell numbers were obtained by two individuals who independently counted positive cells in high power fields (HPF) for five experimental samples from each group. $*p < 0.05$; $**p < 0.01$

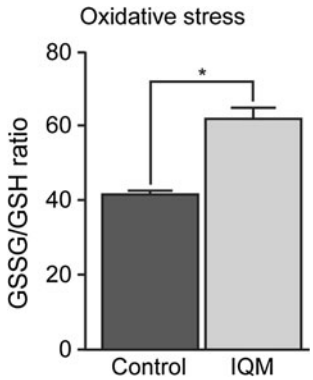

Fig. 3 Oxidative stress in IQM-applied mouse skin. Skin tissue homogenates containing equal amount of proteins from normal and IQM-applied skin were assayed with a glutathione assay kit. The assay revealed that oxidized GSH level was 1.5 times higher in the skin of IQM-applied mice than in the control skin homogenate, which was statistically significant ($p < 0.05$). $*p < 0.05$

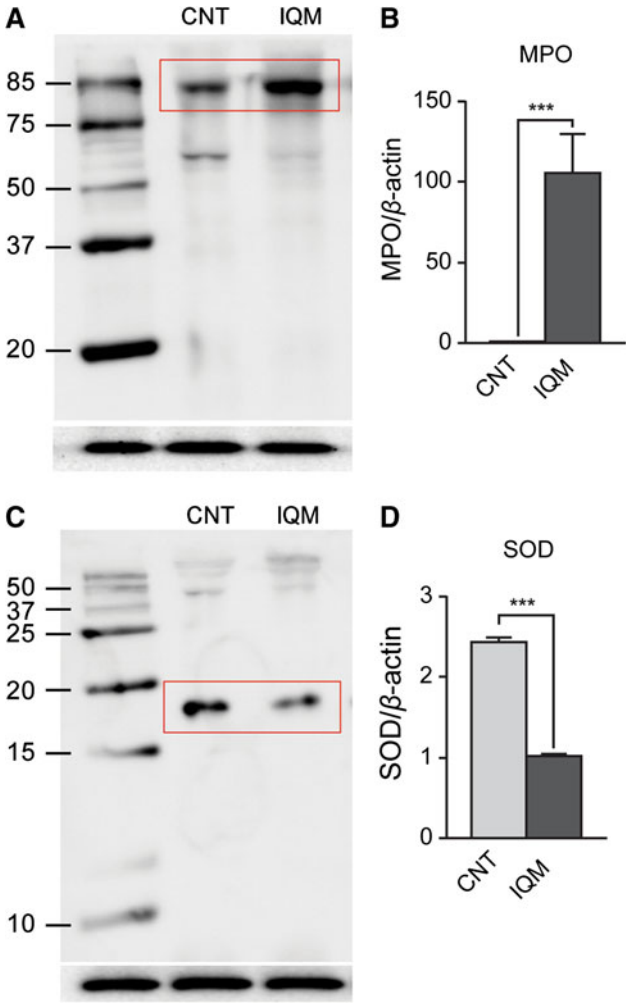

Fig. 4 Imbalanced antioxidant enzymes in the IQM-applied mouse skin. Western blot analysis on the tissue protein from normal and IQM-applied mouse back skin revealed that **a** MPO was increased and **c** SOD level was decreased. The *bar graphs* show relative intensities of the protein bands of three independent experiments. **b** MPO level increased by 74-fold ($p < 0.001$) and **d** SOD level decreased by 2.5-fold ($p < 0.05$) when compared to that of control skin homogenate. $***p < 0.001$

Involvement of ROS generating enzymes in IQM-induced psoriasis-like skin inflammation in mice

After demonstrating that IQM-applied psoriasis-like mouse lesional skin has increased oxidative levels, we then further investigated the relevance of some enzymes associated with ROS generation, such as MPO and SOD1. IHC staining revealed that MPO levels were increased, while SOD1 level was decreased in IQM-induced psoriasis-like mouse skin compared to that of control mouse skin (data not shown). We also confirmed the above results by western blot analysis. There was a 74-fold increase of MPO level and a 2.5-fold decrease of SOD1 level in IQM-induced psoriasis-like mouse skin lesions when compared to those of control mouse skin (Fig. 4a–d).

Impaired SOD activity in IQM-induced psoriasis-like mouse skin lesions

Given that the expression level of SOD1 was decreased in the psoriasis-like skin lesions, we sought to determine SOD activity as a functional analysis. Activity of SOD was measured by the SOD activity assay kit, which found that the activity of SOD was decreased by 1.7-fold in protein extract from IQM-applied psoriasis-like mouse skin compared to control mouse skin (Fig. 5).

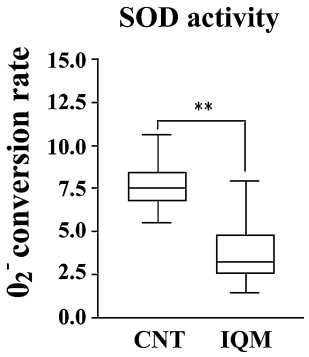

Fig. 5 Impaired SOD activity in IQM-applied mouse skin. Skin tissue homogenates containing equal amount of proteins from normal and IQM-applied skin were assayed with a SOD activity kit. The activity of SOD1 was decreased significantly (1.7-fold) ($p < 0.05$) in the skin of IQM-applied mice. The *bar graph* showed that superoxide conversion rate was lower in IQM-applied skin tissue than that in control skin tissue. The *horizontal line* represents the mean of five independent experimental results. $^{**}p < 0.01$

Discussion

Though definitive conclusions are difficult to make because oxidative stress and a deficient antioxidant system can simply be consequences of chronic inflammatory conditions, there has been some evidence that supports the role of ROS and an impaired antioxidant system in the pathogenesis of psoriasis. Increased ROS production may affect lipid peroxidation, DNA modification, protein and carbohydrate metabolism, and inflammatory cytokine production in cells. Lipid peroxidation results in deactivation of adenyl cyclase and activation of guanyl cyclase, leading to a decreased cAMP/cGMP ratio, which is responsible for epidermal hyperproliferation [27]. ROS from neutrophils, keratinocytes, and fibroblasts have chemotactic effects on neutrophils, which play an important role in psoriasis [18, 30]. Several redox sensitive cellular signal transduction pathways such as mitogen-activated protein kinase/activator protein 1 (MAPK/AP-1), nuclear factor κB (NF-κB) and Janus kinase-signal transducers and activators of transcription (JAK-STAT) are involved in the pathogenesis of psoriasis through up-regulation of proinflammatory cytokines and chemokines [42]. TNF-α, as a major pathogenic cytokine in psoriasis, generates ROS in primary human keratinocytes, and ROS mediate TNF-α induced cytokine production [40]. Cross talk between oxidants and cytokines plays a crucial role in the pathophysiology of a large variety of skin disorders, including psoriasis [28].

Skin contains enzymatic antioxidants such as SOD, catalase, glutathione peroxidase and reductase and thioredoxin reductase [35]. SOD, one of the most essential antioxidant enzyme systems, catalyzes the conversion of the superoxide anion into hydrogen peroxide and molecular oxygen. Previous studies reported suppressed SOD activity in erythrocytes [36], neutrophils [7], tissue [36], and plasma [38], and increased SOD activity in plasma [36], fibroblasts and erythrocytes [34] in psoriasis. There are three types of SOD: Cu, Zn-SOD, Mn-SOD, and extracellular SOD. We chose to study SOD1 (Cu, Zn-SOD) in this study. Gerbaud et al. [11] reported that SOD1 activity was markedly increased in human dermal psoriatic fibroblasts and that SOD1 protein and mRNA levels were decreased after retinoic acid treatment of psoriatic fibroblasts. Another report found that SOD1 activity was decreased in human psoriatic epidermis but restored after supplementation with antioxidants [15]. Nitric oxide (NO) regulates the expression of SOD1 in human keratinocytes, and NO is regarded as a main cause of keratinocyte hyperproliferation in psoriasis. Our results showed decreased levels of SOD1 and activity of SOD in mouse skin, which may be consumed in oxidative stress conditions. The reduction in SOD activity could be also caused by an acute immune reaction in a relatively short period. Recently, Gabr et al. [9] reported decreased serum SOD activity in psoriatic patients and negative correlation between SOD activity and disease severity. They suggested decreased SOD activity could be caused by increased superoxide anion production. But in cumulative oxidative stress conditions, SOD activity can be higher due to compensatory activation. A similar phenomenon was reported in trichloroethylene-induced cutaneous irritation in BALB/C mice [32], and it could be an explanation for the conflicting results of the relationship between SOD and psoriasis in previous studies. pDCs are key effectors of innate immunity for induction of early psoriatic lesions. It has been reported that self-DNA coupled with cathelicidin LL-37 triggers TLR9 and enters pDCs to produce IFN-α in the initiation of psoriasis [16]. Similarly, self-RNA–LL37 complexes induce TLR7 activation in pDCs and trigger the secretion of IFN-α. Self-RNA–LL37 complexes also trigger the direct activation of myeloid DCs to secrete TNF-α and IL-6 and differentiate into mature DCs [10]. LL-37, one of the antimicrobial peptides, is not expressed by healthy keratinocytes, but only released following skin injury [8]. In addition, LL-37 induces the migration of neutrophils [6], suppresses the apoptosis of neutrophils [19] and mediates the generation of ROS [41]. LL-37 can be a touchstone for the link between the generation of ROS and triggering of an immune reaction in IQM-induced psoriasis-like lesions.

Recently, genome-wide expression profiling of five mouse models (K5-Tie2, IQM, K14-AREG, K5-Stat3C, K5-TGFbeta1) was reported [33]. K14-AREG, K5-Stat3C, and K5-TGFbeta1 exhibited heightened inflammation and increased TNF-α and IFN-γ relative to the other two models. The indication of T cell infiltration in genome-wide expression profiling was absent in the IQM-applied

C57BL/6 mouse model, although there were increased $CD4^+$ and $CD8^+$ T cells, proven by IHC stain [37]. At first, van der Fits et al. employed two types of mice, BALB/c and C57BL/6. Phenotypes such as erythema, scaling and induration were similar between the two groups. Because IL-23p19- and IL-17RA-deficient mice were generated on a C57BL/6 background, IL-23/IL-17 axis was proven only in C57BL/6 mice. Severity of skin inflammation in C57BL/6 mice was lower than that of BALB/c mice, and IL-22 was not induced in C57BL/6 mice, whereas transient induction of IL-22 was observed in BALB/c mice. Further investigation into standard types of mice, natural courses, responses to anti-psoriatic agents in this model, and the link between ROS and immune reactions is needed.

In conclusion, an aberrant oxidant–antioxidant system in the IQM-induced mouse model for psoriasis was revealed. Oxidative stress was increased, the antioxidant enzyme MPO levels were increased, and both SOD levels and activity were decreased. This is the first study of oxidative stress and the antioxidant system in a psoriatic mouse model. It is suggested that the IQM-induced mouse model is useful for investigating the pathogenic role of redox imbalance in psoriasis.

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Conflict of interest The authors have no conflict of interest to declare.

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