Loricrin Protects against Chemical Carcinogenesis

TO THE EDITOR

Cutaneous squamous cell carcinoma is the second most prevalent skin cancer worldwide (Que et al., 2018). Although UV exposure is the most common cause of cutaneous squamous cell carcinoma, polycyclic aromatic hydrocarbons, such as 7,12-dimethylbenz[a]anthracene (DMBA), remain causative agents. Epidermal Langerhans cells (LCs) capture DMBA that can penetrate the stratum corneum and generate the highly carcinogenic compound DMBA-3,4-diol-1,2-epoxide (Modi et al., 2012). The aryl hydrocarbon receptor/cytochrome P-450 pathway mediates the metabolic conversion of DMBA in the epidermis (Modi et al., 2012). The hard electrophile DMBA-3,4-diol-1,2-epoxide not only forms DNA adducts but also perturbs cellular reduction–oxidation balance in keratinocytes that are adjacent to LCs (Modi et al., 2012). KEAP1 senses oxidative damage through reactive cysteine residues and unleashes NRF2-mediated anti-oxidative responses in keratinocytes (Schäfer and Werner, 2015). Loricrin (LOR) is a cytoskeletal-rich cornified envelope (CE) protein that shows biochemical and biological properties specific to the stratum corneum (Mehrel et al., 1990). LOR stabilizes the cytoskeleton through e-(γ-glutamyl) lysine/
disulfide (−S−S−) cross-linkages and promotes cornification of the epidermis (Mehrel et al., 1990). Therefore, CEs from LOR-knockout (LKO) epidermis show significantly impaired cross-linkages of the differentiated keratins, keratin 1/keratin 10, and FLG (Ishitsuka and Roop, 2018; Rice et al., 2016) and exhibit delayed maturation (Koch et al., 2000). Previously, we found that NRF2-dependent compensatory responses underlie mild LKO phenotypes; the adaptive response replenishes the CEs with antioxidative LOR mimics, namely, SPRRs (Huebner et al., 2012) and late cornified envelope proteins (Ishitsuka et al., 2016). Given the indispensable role of LOR in cornification and its potential antioxidative properties identified from previous research, we hypothesized that LOR may protect against the chemical carcinogenesis, LKO mice developed significantly more papillomas than wild-type mice subjected to low- and high-dose protocols (Figure 2a). N-Acetyl cysteine treatment in LKO mice but not in wild-type mice significantly reduced the tumor burden compared with the vehicle-treated control group (Figure 2b).

LKO mice were susceptible to DMBA-induced oxidative DNA damage, which is dependent on the cytochrome P-450-mediated metabolic activity of LCs (Lewis et al., 2015; Modi et al., 2012). In contrast, 12-O-tetradecanoylphorbol-13-acetate–induced irritant responses, which involve the activation of LCs and dendritic epidermal T cells, were not substantially altered in LKO mice (Supplementary Figure S1 and unpublished observation). On the basis of these findings, we formed the following tentative conclusions: first, LOR is not essential for the outside-in or inside-out chemical carcinogenesis model using LKO mice in FVB background, which is particularly sensitive to this procedure (Hennings et al., 1993).

The two distinct stages include the initiation stage in which oxidative DNA damage is induced and the promotion stage in which irritant reactions are evoked. In accordance with postnatal NRF2 activation in FVB LKO mice (Ishitsuka et al., 2016), single topical DMBA application increased the cytosolic accumulation of NRF2 in the LKO epidermis compared with that in the wild-type epidermis (Figure 1a). The levels of DNA with double-strand breaks, determined by detecting phosphorylated histone H2AX (i.e., γ-H2AX), were significantly higher in the LKO epidermis than in the wild-type epidermis (Figure 1b). The increased levels of γ-H2AX expression in the LKO epidermis were rescued through oral intervention by administering the glutathione precursor N-acetyl cysteine (Figure 1b). In contrast, steady-state expression of the cytochrome P-450 enzyme–coding genes, Cyp1a1 and Cyp1b1 (data not shown), or acanthotic/ear-swelling responses induced by the administration of 12-O-tetradecanoylphorbol-13-acetate were comparable between the two genotypes (Supplementary Materials and Methods). In the two-stage chemical carcinogenesis, LKO mice developed significantly more papillomas than wild-type mice subjected to low- and high-dose protocols (Figure 2a). N-Acetyl cysteine treatment in LKO mice but not in wild-type mice significantly reduced the tumor burden compared with the vehicle-treated control group (Figure 2b).

**Figure 1. Exaggerated oxidative DNA damage in LKO mice.** (a) Cytosolic NRF2 expression assessed 24 h after DMBA (40 nmol) treatment. Fold induction levels of NRF2 normalized to those of β-actin relative to the NT WT group. n = 3; *P < 0.05, **P < 0.01; two-way ANOVA. (b) γ-H2AX expression after 7 days of DMBA (40 nmol) treatment with or without oral administration of NAC dissolved in DW. Fold induction levels of γ-H2AX normalized to those of H2AX relative to the vehicle-treated (CTRL) DW group. n = 3; *P < 0.05; two-way ANOVA. Data are representative of at least three independent experiments with similar results. CTRL, control; DMBA, 7,12-dimethylbenz[a]anthracene; DW, deionized water; h, hour; H2AX, histone H2A.X; LKO, LOR knockout; NAC, N-acetyl cysteine; NT, nontreated; TBHQ, tert-butylhydroquinone; WT, wild type.

**Figure 2. Susceptibility to chemical carcinogenesis in LKO mice.** (a) Papilloma formation after low- or high-dose chemical carcinogenesis protocol (DMBA [200 or 400 nmol] initiation and weekly TPA [10 or 40 nmol] promotion). n = 10; ***P < 0.01, ****P < 0.005, *****P < 0.001; two-way ANOVA. (b) Papilloma formation after high-dose chemical carcinogenesis protocol along with NAC treatment. Open labels denote WT mice, and closed labels denote LKO mice. n = 7; **P < 0.01, ***P < 0.005, ****P < 0.001; two-way ANOVA (LKO DW vs. LKO NAC). Data are representative of at least two independent experiments with similar results. DMBA, 7,12-dimethylbenz[a]anthracene; DW, deionized water; LKO, LOR knockout; NAC, N-acetyl cysteine; TPA, 12-O-tetradecanoylphorbol-13-acetate; WT, wild type.
stratum corneum permeability barrier function as previously determined on the basis of Lucifer yellow dye diffusion or transepidermal water loss levels, respectively (Koch et al., 2000); second, although LKO activates NRF2 and upregulates the expression of the LOR mimics (e.g., SPRRs and late cornified envelope proteins), LOR is essential for inducing antioxidative defenses in the epidermis, such as the prompt formation of disulfide (S-S-) cross-linkages at the cell periphery on terminal differentiation (Huebner et al., 2012; Ishitsuka and Roop, 2018; Ishitsuka et al., 2016) or exposure to thiol-oxidizing electrophiles (Huebner et al., 2012); and third, neither the cytochrome P-450 activity of LCs nor irritant responses are affected in the LKO epidermis (Lewis et al., 2015).

The results of this study may contrast with those obtained using another mouse model lacking the earlier CE component such as envoplakin, periplakin, and involucrin (EPI) (Sevilla et al., 2007). EPI-deficient mice exhibited an ichthyosiform skin phenotype resembling atopic dermatitis; in these mice, the proportion of dendritic epidermal T cells was decreased, and CD4⁺ T cells infiltrated the dermis under steady-state conditions (Sevilla et al., 2007). In EPI-deficient mice, topical irritation responses are affected in the EPI-deficient mice (Sevilla et al., 2007)—but the reinforce component—the scaffolding CE protein, LOR, is dispensable for nonredundant functions of each CE (Sevilla et al., 2007)—but the reinforcement CE protein, LOR, is dispensable for nonredundant functions of each CE component—the scaffolding CE proteins, such as EPI, are indispensable (Sevilla et al., 2007)—but the reinforcement CE protein, LOR, is dispensable for nonredundant functions of each CE component—the scaffolding CE proteins, such as EPI, are indispensable (Sevilla et al., 2007)—but the enforcement CE protein, LOR, is dispensable for stratum corneum permeability barrier function (Koch et al., 2000).

Our results further support the notion that LOR is a major effector of cornification (Eckhart et al., 2013), which confers the epidermis with tissue-intrinsic antioxidative defenses (Huebner et al., 2012; Ishitsuka and Roop, 2018; Ishitsuka et al., 2016; Koch et al., 2000; Mehrel et al., 1990; Rice et al., 2016).

Data availability statement
No datasets were generated or analyzed during this study.

ETHICS STATEMENT
All procedures were approved by the University of Tsukuba Ethics Committee.

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2021.12.015.

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Defining Psoriasis Remission Based on Histopathologic and Molecular Criteria: A Systematic Literature Review

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TO THE EDITOR

Tremendous progress has been made in the treatment of psoriasis owing to the successful use of translational medicine in the last two decades (Reid and Griffiths, 2020). Using targeted biologics, nearly 90% of patients with psoriasis can achieve a clear or almost clear response (Hawkes et al., 2018). Thus, it is timely to start thinking about the use of advanced endpoints for psoriasis clinical trials to drive the next level of innovation toward disease remission and, even more ambitiously, a cure. Yet, little information is available on how to measure or define such disease states for psoriasis (Koo and Lebwohl, 1999).

In support of a National Psoriasis Foundation initiative to develop consensus-based definitions for psoriasis remission and cure, we conducted a larger systematic literature review to identify definitions of psoriasis remission/cure. In this study, we focus on the portion of the remission/cure definitions based on histopathologic and molecular features of psoriasis. Definitions of remission/cure based on clinical criteria will be reported separately.

MEDLINE/PubMed (National Library of Medicine), Embase (Embase.com/Elsevier), and The Cochrane Central Register of Controlled Trials (cochranelibrary.com) were searched on July 22, 2020, for full-text studies in English, Spanish, or Dutch providing definitions for psoriasis remission/cure. Controlled vocabulary terms (i.e., Medical Subject Headings, EMTREE) were included when available and appropriate (Supplementary Text). The search strategies were designed by an experienced librarian from the Countway Library, Harvard University (Boston, MA). The systematic literature review protocol was registered with the International Prospective Register of Systematic Reviews (registration number CRD42021225212).

Eligible for inclusion were interventional and observational studies as well as consensus studies providing definitions of remission and/or cure for psoriasis in adult (aged ≥18 years) patients. Commentaries; reviews; conference abstracts; case reports; case series and observational studies involving <10 patients; articles in languages other than English, Spanish, or Dutch; or articles that were unavailable in full text were excluded.

Seven eligible articles were identified, and three articles were retrieved by hand searching reference lists. A total of 10 articles were included (Figure 1).

All studies were clinical trials, published between 2005 and 2020. Sample sizes ranged from 10 to 116 patients. Most studies assessed a biologic intervention: etanercept (three studies), brodalumab (two studies), alefacept, ixekizumab, and secukinumab. Other interventions evaluated were cyclosporine A and narrow-band UVB phototherapy. All studies included analyses of lesional skin biopsies taken at baseline and at later time points up to 13 weeks of treatment. The definitions were based on expert opinion using nonvalidated criteria. Importantly, no studies providing consensus-based definitions of histological/molecular psoriasis remission were found. These factors need consideration for potential bias when interpreting the results.

All studies provided a definition of remission or related terms such as resolution, normalization, and disease reversal on the basis of histological criteria (Table 1 and Supplementary Table S1). The histological features considered for psoriasis remission involved (i) normalization of keratinocyte differentiation as measured by keratin 16 immunostaining; (ii) normalization of keratinocyte proliferation as measured by Ki-67 immunostaining; (iii) reduction of epidermal hyperplasia; (iv) restoration of a granular layer and orthokeratosis toward approximating nonlesional skin; and (v) reductions in pathologic inflammatory cell population counts, in particular T cells and dendritic cells, Suárez-Fariñas et al. (2011) specifically defined histological resolution by >75% improvement of CD3+ T cells, CD11c+ myeloid dendritic cells, and CD163+ macrophages, among others.

Definitions for psoriasis resolution based on molecular features measured by microarray gene expression profiling were also identified. These definitions were based on the extent to which mRNA transcript profiles of psoriasis-related differentially expressed genes in treated lesional skin returned to nonlesional skin levels. Differentially expressed genes are defined by fold
SUPPLEMENTARY MATERIALS AND METHODS

Animal procedures

LOR-knockout mice on the FVB background (Koch et al., 2000) and strain-matched wild-type control mice purchased from CLEA Japan (Tokyo, Japan) were maintained under specific pathogen-free conditions at the animal facility of the University of Tsukuba (Tsukuba, Japan). Age-matched mice (aged 8–12 weeks) were randomly selected from a pool for experiments. All procedures were approved by the University of Tsukuba Ethics Committee.

Two-stage chemically induced tumorigenesis

The back skin was carefully trimmed using a mechanical trimmer (Trimmer 8655, Wahl, Sterling, IL), followed by depilatory cream application (Kracie, Tokyo, Japan). After 7 days, initiation by application of 7,12-dimethylbenz[a]anthracene (D464500, Toronto Research Chemicals, Ontario, Canada) in 200 µl of acetone was done and was followed by twice weekly application of 12-O-tetradecanoylphorbol-13-acetate (P-1680, LC Laboratories, Woburn, MA) in 200 µl of acetone. For low- or high-dose two-stage chemically induced tumorigenesis, 200 nmol or 400 nmol 7,12-dimethylbenz[a]anthracene and 10 nmol or 40 nmol 12-O-tetradecanoylphorbol-13-acetate were used, respectively. Cutaneous tumors were counted weekly and evaluated visually by an observer blinded to the experimental groups (TO, YI, YN).

N-acetyl cysteine treatment

The mice received either deionized water or N-acetyl cysteine—containing water (013-05133, FUJIFILM Wako Pure Chemical, Osaka, Japan) for 12 weeks) were randomly selected from a pool for experiments. All procedures were approved by the University of Tsukuba Ethics Committee.

Western blotting

The whole-cell protein samples were prepared using SDS extraction buffer (Tris-hydrogen chloride [pH 6.8], 2% SDS, 0.86 M β-mercaptoethanol, and 10% glycerol). The histone protein samples were collected using the Histone/DNA Binding Protein Extraction Kit (cells) (P514-25, Bio-101, Sunnyvale, CA) and dissolved in the SDS buffer. An equal amount of protein was subjected to SDS-PAGE on Mini-PROTEAN TGX Precast Gels (4569036, Bio-Rad Laboratories, Hercules, CA) and transferred onto nitrocellulose membranes (10600001, GE Healthcare, Buckinghamshire, United Kingdom). The membranes were incubated overnight with the following primary antibodies: anti-NRF2 (1:200) (Huebner et al., 2012), anti-β-actin (5 µg/ml, 3662, BioVision, Milpitas, CA), anti–phosphorylated histone H2A.X (i.e., anti–γ-H2AX) (1:500, 9718, Cell Signaling Technology, Danvers, MA), and anti-H2AX (1:200, 2578, Cell Signaling Technology), followed by 60-minute incubation with horseradish peroxidase–labeled secondary antibodies against rabbit IgG (0.04 µg/ml, sc-2004, Santa Cruz Biotechnology, Dallas, TX). Antibody binding was visualized using the SuperSignal West Dura Extended Duration Substrate (34075, Thermo Fisher Scientific, Waltham, MA) and image analysis system (LAS4000 mini, Fujifilm, Tokyo, Japan). The intensities of NRF2 or phosphorylated histone H2A.X band were quantified by the densitometry (FUSION FX7.EDGE, Vilber Lourmat, Collégien, France), and the expression levels were normalized relative to those of β-actin or histone H2A.X band, respectively.

Statistical analysis

Data were presented as mean values ± SEM. Comparisons were performed with unpaired t-tests using Welch’s correction (between two groups) or two-way ANOVA (two independent variables). Analyses were performed using the GraphPad Prism 9 (GraphPad Software, San Diego, CA). For all studies, P < 0.05 indicated statistical significance.

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