Genome-Wide Expression Analysis of Human *In Vivo* Irritated Epidermis: Differential Profiles Induced by Sodium Lauryl Sulfate and Nonanoic Acid

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The pathogenesis of irritant contact dermatitis (ICD) is poorly understood, and genes participating in the epidermal response to chemical irritants are only partly known. It is commonly accepted that different irritants have different mechanisms of action in the development of ICD. To define the differential molecular events induced in the epidermis by different irritants, we collected sequential biopsies ($\frac{1}{2}$, 4, and 24 hours after a single exposure and at day 11 after repeated exposure) from human volunteers exposed to either sodium lauryl sulfate (SLS) or nonanoic acid (NON). Gene expression analysis using high-density oligonucleotide microarrays (representing 47,000 transcripts) revealed essentially different pathway responses $\frac{1}{2}$ hours after exposure: NON transiently induced the IL-6 pathway as well as a number of mitogen-activated signaling cascades including extracellular signal-regulated kinase and growth factor receptor signaling, whereas SLS transiently down-regulated cellular energy metabolism pathways. Differential expression of the cyclooxygenase-2 and matrix metalloproteinase 3 transcripts was confirmed immunohistochemically. After cumulative exposure, 883 genes were differentially expressed, whereas we identified 23 suggested common biomarkers for ICD. In conclusion, we bring new insights into two hitherto less well-elucidated phases of skin irritancy: the very initial as well as the late phase after single and cumulative mild exposures, respectively.

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INTRODUCTION

Irritant contact dermatitis (ICD) in its most frequent form—i.e., chronic ICD—most commonly results from repeated exposures to weak irritants. Chronic ICD is a common problem in occupational dermatology, and its pathogenesis remains largely unknown. The clinical expression and course depends on the irritant and mode of exposure: The picture of acutely irritated skin is divergent from that seen in cumulative skin irritation (Malten, 1981). Investigators have induced ICD experimentally in various ways to examine the natural history of the disease, and while most data on the pathogenesis of ICD derive from acute irritation studies (most frequently a patch test), knowledge of mechanisms of action in cumulative skin irritation is sparse (De Jongh *et al.*, 2006). However, cumulative skin irritation models are judged to mimic everyday exposure for patients more closely than a patch test.

It has been established that different chemicals have diverse actions on the skin (Willis *et al.*, 1989; Wilmer *et al.*, 1994; Holliday *et al.*, 1997). Sodium lauryl sulfate (SLS) is an irritant of the corrosive type and may—even in subclinical reactions—result in impairment of the water barrier function of the skin (Tupker *et al.*, 1997), whereas nonanoic acid (NON) induces irritancy of the noncorrosive type—i.e., the water barrier is not always affected (Fullerton *et al.*, 2002; Wahlberg and Lindberg, 2003). The diversity of skin reactions caused by SLS and NON is recognized clinically (Reiche *et al.*, 1998), instrumentally (Agner and Serup, 1989), histopathologically and ultrastructurally (Willis *et al.*, 1989; Forsey *et al.*, 1998), immunohistochemically (Lindberg *et al.*, 1991), and transcriptionally (Grangsjo *et al.*, 1996). Moreover, treatment responses may be different for cumulative skin exposure to SLS compared with NON (Andersen *et al.*, 2007).

Gene expression studies in ICD have focused on pro-inflammatory cytokines responsible for initiation of the

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Abbreviations: AP-1, activator protein 1; COX-2, cyclooxygenase-2; DUSP1, dual-specific phosphatase 1; FDR, false discovery rate; ICD, irritant contact dermatitis; LME, linear mixed effect; MAPK, mitogen-activated protein kinase; MMP3, matrix metalloproteinase 3; NON, nonanoic acid; PPAR, peroxisome proliferator-activated receptor; SLS, sodium lauryl sulfate; TEWL, transepidermal water loss

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inflammation cascade (Corsini and Galli, 2000; Lisby and Baadsgaard, 2006). However, given the complex cellular response mechanisms and multiple interactions of resident skin cells, genes involved specifically in ICD are supposedly only partly known. The technique of microarray gene chips offers expression analysis of a large number of genes simultaneously. Until now, studies using this technique have shed new light on skin irritation in humans (Marionnet *et al.*, 2003; Wong *et al.*, 2004), rats (Rogers *et al.*, 2003; McDougal *et al.*, 2007), and *in vitro* skin cultures (Fletcher *et al.*, 2001; Borlon *et al.*, 2007).

The purpose of this study was to use microarrays representing 47,000 transcripts to analyze the epidermal transcriptional changes on irritation as it may occur in real life with the main focus on differences between SLS and NON. By collecting skin biopsies from three early and one late time point, transcriptional profiles of cumulative skin irritancy over time were investigated. In this study, we show different gene expression profiles between SLS- and NON-irritated epidermis as well as highly changed transcripts equivalently regulated by the two irritants after cumulative exposure.

RESULTS

Skin irritation obtained by SLS and NON

The cumulative course of irritation is shown in Supplementary Figure S1 online showing a comparable level of clinical irritation for the two irritants. The mean clinical score at day 11 was within the target level of irritation (score of at least 2, but not higher than 4 corresponding to a moderate reaction). Biometric measurements are summarized as changes at day 11 compared with baseline values in Supplementary Table S1 online. Although Δ clinical score and Δ TEWL (transepidermal water loss) was comparable for the two irritants, NON had a more drying effect than SLS as shown by the larger decrease in stratum corneum hydration. Erythema as determined by colorimetry was increased by SLS but hardly affected at all by NON. After the single exposure (i.e., at time points $\frac{1}{2}$, 4, and 24 hours), skin hydration was the only affected parameter for both irritants, as it was substantially decreased after $\frac{1}{2}$ hour, remained low at 4 hours, and then nearly restored at 24 hours.

SLS and NON induce asymmetrical overall expression profiles

Sequentially obtained RNA was hybridized to Affymetrix (Santa Clara, CA) GeneChips to characterize mechanisms induced by NON and SLS over time. Significantly changed transcripts at different time points were identified for both irritants separately using a linear mixed effect (LME) model approach with a false discovery rate (FDR) correction. The overall expression is summarized in Supplementary Figure S2 online showing different patterns by the two irritants. Fold changes and *q*-values for top 50 uppermost changed (both up- and downregulated) transcripts are shown for both irritants at all time points in Supplementary Table S2 online.

Significant irritant-discriminative genes are predominantly found at day 11

Identification of genes that were regulated differently by exposure to the two irritants was one of the main aims of the experiment. A *t*-test comparison between irritants both separately for each time point and combined for time points $\frac{1}{2}$, 4, and 24 hours disclosed no discriminative genes when FDR correction was applied. The top-differentially regulated genes for combined early time points are shown in a heat map in Supplementary Figure S3 online. The most differentially expressed early gene was matrix metalloproteinase 3, *MMP3* (uncorrected *P*-value = 0.000096) that was more highly expressed in response to SLS. Among the 10 uppermost early differentially expressed genes, two attracted special interest because they were each represented with two different probe sets: the insulin-like growth factor (IGF) binding protein *IGFBP3* ($P = 0.0003$ and 0.0005) and the noncoding maternally imprinted gene *H19* ($P = 0.0002$ and 0.0006) that were both less expressed in response to SLS.

An LME analysis including all chips in the experiment identified 883 irritant-discriminative transcripts (FDR < 0.05), and visualization of these 883 genes in a heat map showed that the day 11 response was by far the most powerful compared with the early time points (Figure 1a). In summary, looking at the overall expression pattern in the whole data set, day 11 was identified as the main contributor not only of significantly changed genes but also of genes that would pinpoint differences between the two irritants.

Most upregulated early response genes at $\frac{1}{2}$ hour are quickly turned off

The top 15 significantly upregulated genes (as identified by LME with FDR) for both irritants at $\frac{1}{2}$ hour are shown in Figure 2 allowing the reader to follow their expression over time. Many genes are upregulated in parallel for both irritants; the majority are rapidly upregulated and then quickly switched off from 4 hours and onward. The transcripts *FOS, DUSP1* (dual-specific phosphatase 1), and the Affymetrix probe set *240053_x_at* were actually among the top 10 upregulated transcripts for both irritants at $\frac{1}{2}$ hour and may thus be upregulated in skin irritation irrespective of the irritant applied. Conversely, transcripts such as *AMBRA1* and *XIRP2* (upregulated by NON and SLS, respectively) were dissimilarly regulated at $\frac{1}{2}$ hour, and for the transcript *207737_at* a divergent pattern was sustained over time.

Gene expression at the end of cumulative irritation (day 11)

Table 1 show 26 transcripts that were among the top 50 regulated (up or down) transcripts for both SLS and NON at day 11. Thus, these transcripts (representing 23 genes) may represent common biomarkers for cumulative irritant dermatitis irrespective of the chemical nature of the irritant. The occurrence of several probe sets per gene and overlapping genes between irritants serves as an internal validation of the study.

To characterize differences between irritant responses further, we subjected the 883 irritant-discriminative genes identified by LME (Figure 1a) to a cluster analysis (Figure 1b). Three clusters were formed: cluster A identified genes upregulated by NON and downregulated by SLS. The same was the case for cluster B but only one NON-treated subject seemed to contribute substantially to this pattern. The last

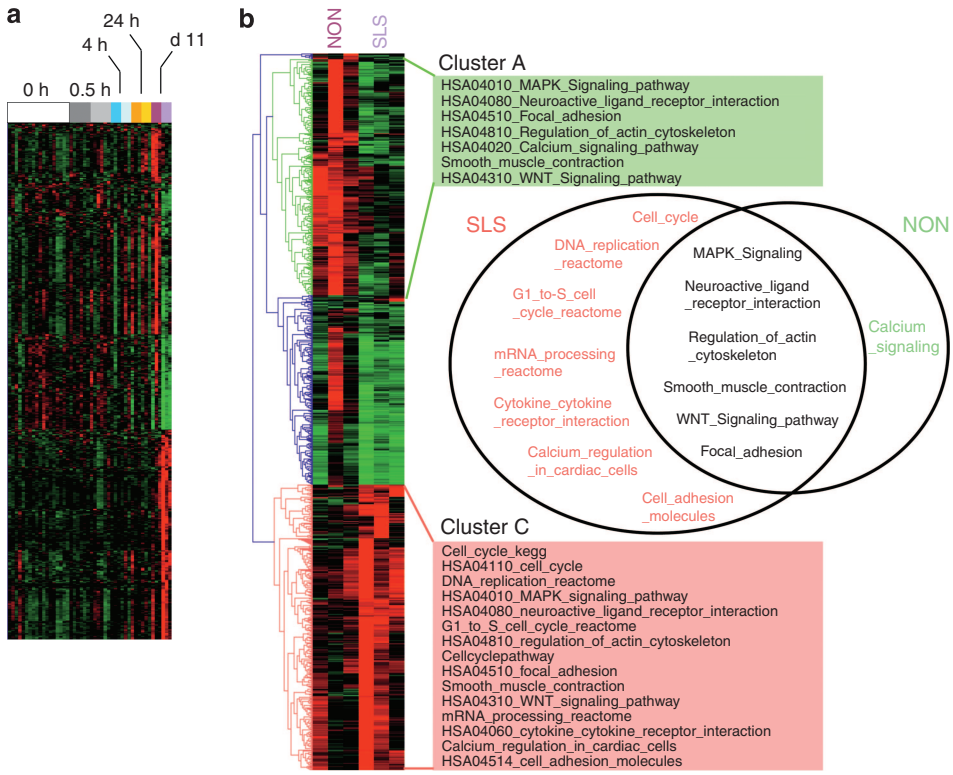

Figure 1. Differential gene expression induced by nonanoic acid (NON) and sodium lauryl sulfate (SLS). (**a**) Cluster of 883 significant irritant-discriminative transcripts identified by linear mixed effect (LME) (false discovery rate (FDR) < 0.05). The differences are predominantly found at day 11 (d11; purple color). Dark and light color tones within each time point indicate NON and SLS exposure, respectively. (**b**) Clustering of the same 883 transcripts as in **a** using d11 chips only. Clusters A (containing 290 transcripts) and C (containing 352 transcripts) are summarized by subsequent pathway overrepresentation analysis (MSigDB, canonical pathways; $P<0.05$).

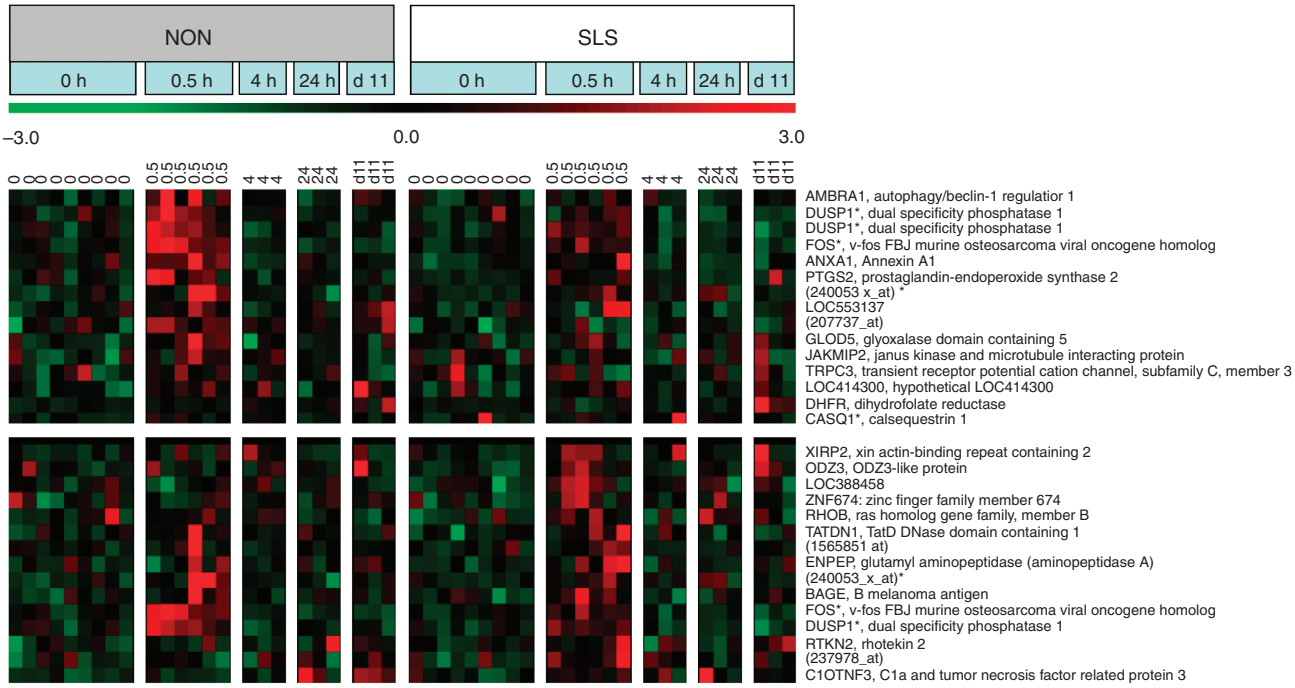

Figure 2. Significantly upregulated genes after $\frac{1}{2}$ hour compared for sodium lauryl sulfate (SLS) and nonanoic acid (NON) over time. Top 15 significantly changed genes for NON (upper part) and SLS (lower part) as determined by linear mixed effect (LME) with false discovery rate (FDR) correction. The expression is shown for all chips in the experiment. Up- and downregulation is depicted in red and green, respectively. Transcripts marked with *were also significantly upregulated (FDR<0.05) by the other irritant.

Table 1. Gene transcripts regulated in parallel by SLS and NON at day 11

Affymetrix ID	Gene symbol	Gene title	Synonym	*q*-value		Fold change	
				NON	SLS	NON	SLS
Upregulated							
Epidermal differentiation							
211906_s_at	*SERPINB4*	Serpin peptidase inhibitor, clade B (ovalbumin), member 4	Squamous cell carcinoma antigen 2	0.01415	0.00065	7.38	20.76
203535_at	*S100A9*	S100 calcium-binding protein A9	Calgranulin B	0.00359	1.16E−05	3.69	13.95
224328_s_at	*LCE3D*	Late cornified envelope 3D	Small proline rich-like 6A	0.00203	3.20E−05	3.00	4.05
202917_s_at	*S100A8*	S100 calcium-binding protein A8	Calgranulin A	0.02806	8.68E−06	2.88	7.58
210413_x_at	*SERPINB4*	Serpin peptidase inhibitor, clade B (ovalbumin), member 4	Squamous cell carcinoma antigen 2	7.66E−06	8.75E−05	2.81	4.11
209720_s_at	*SERPINB3*	Serpin peptidase inhibitor, clade B (ovalbumin), member 3	Squamous cell carcinoma antigen 1	0.00092	0.00022	2.76	5.05
209719_x_at	*SERPINB3*	Serpin peptidase inhibitor, clade B (ovalbumin), member 3	Squamous cell carcinoma antigen 1	3.36E−06	3.08E−05	2.74	3.75
213796_at	*SPRR1A*	Small proline-rich protein 1A	Cornifin A	0.00050	0.00016	2.52	5.17
Cell cycle							
209891_at	*SPC25*	SPC25, NDC80 kinetochore complex component, homolog (*S. cerevisiae*)	—	0	0	2.29	3.6
201291_s_at	*TOP2A*	Topoisomerase (DNA) II alpha 170 kDa	—	3.37E−13	0.00089	2.27	3.58
202095_s_at	*BIRC5*	Baculoviral IAP repeat-containing 5	Survivin	0	9.17E−15	2.23	3.16
Miscellaneous							
212531_at	*LCN2*	Lipocalin 2	Neutrophil gelatinase-associated lipocalin	0.00924	0.0021	3.41	5.28
1569428_at	*WIBG*	Within bgcn homolog (*Drosophila*)	—	0.00683	0.0003	2.29	2.83
1553396_a_at	*CCDC13*	Coiled-coil domain containing 13	—	0.00574	0.00186	2.31	2.99
Downregulated							
Cell adhesion							
228706_s_at	*CLDN23*	Claudin 23	—	0.00300	0.00190	−2.89	−10.24
228707_at	*CLDN23*	Claudin 23	—	0.02558	0.00016	−2.41	−10.16
Cell growth							
231148_at	*IGFL2*	IGF-like family member 2	—	0.01306	0.00054	−2.32	−4.14
Enzymatic activity							
215554_at	*GPLD1*	Glycosylphosphatidylinositol-specific phospholipase D1	Glycoprotein phospholipase D	0.00017	3.87E−12	−2.39	−6.05
209460_at	*ABAT*	4-Aminobutyrate aminotransferase	GABA transferase	0.0085	8.66E−13	−2.51	−4.77
1553734_at	*AK7*	Adenylate kinase 7	—	0.01259	1.65E−05	−2.36	−4.96
Epidermal differentiation							
1554921_a_at	*SCEL*	Sciellin	—	0.04683	0.00081	−2.5	−11.45
Sterol biosynthesis							
213562_s_at	*SQLE*	Squalene epoxidase	Squalene monoxidase	1.69E−05	0.000144	−2.17	−4.24

Table 1 continued on the following page

Table 1. Continued

Affymetrix ID	Gene symbol	Gene title	Synonym	*q*-value		Fold change	
				NON	SLS	NON	SLS
Unknown							
233045_at	*LOC286126*	Hypothetical protein LOC286126	—	0.00025	0	−2.54	−4.31
1556220_at	—	—	—	7.62E−11	1.07E−05	−2.51	−4.5
240242_at	—	—	—	0.00073	1.39E−08	−2.44	−6.19
227480_at	*SUSD2*	Sushi domain containing 2	—	0	4.44E−05	−2.19	−5.17

Abbreviations: NON, nonanoic acid; SLS, sodium lauryl sulfate.
These genes were selected among the top 50 up- and downregulated transcripts at day 11. Genes occur in the list only if they were changed in the same direction by both irritants. The top 50 genes were selected as those with the highest fold change among all significant (FDR <0.05) genes. Full top 50 lists are shown in Supplementary Table S2 online.

pattern represented by cluster C showed a general picture of upregulated transcripts in response to both irritants; however, the upregulation was much more prominent in response to SLS.

We analyzed cluster A versus C to functionally summarize the transcripts and to enable biological interpretation of the changed transcripts. A hypergeometric overrepresentation analysis using the MSigDB database overlap calculation for canonical pathways showed both overlapping and distinct pathways for the two irritants (Figure 1b).

Pathway analysis (Gene Set Enrichment Analysis)

Pathway analysis in large-scale gene expression experiments is based on small but consistent changes in expression of a group of genes with related function (Curtis *et al.*, 2005). We identified significantly regulated pathways associated with skin irritation using paired Gene Set Enrichment Analysis. Twenty pathways were found after $\frac{1}{2}$ hour (Supplementary Table S3 online). All were upregulated in response to NON, and these mainly inflammatory (e.g., IL-6 and T-cell receptor), mitogenic (e.g., IGF1 and platelet-derived growth factor), and even cancer-related pathways share many upregulated genes (Supplementary Figure S4 online). These genes are primarily associated with intracellular signaling, such as the mitogen-activated protein kinase (MAPK) pathways (*RAF1*, *MAP2K1+2*, *MAPK1*, *MAPK8*), signaling molecules downstream to membrane-bound growth factor/cytokine receptor activation (*GRB2*, *SOS*, *PIK3R1*, *PRKCB1*, *RAC1*), intracellular calcium signaling (*CALM1+2*, *PPP3CC*), and MAPK-activated transcription factors (*Elk-1*, and the activator protein 1 (AP-1) subunits *FOS* and *JUN*). In contrast, SLS did not induce the significant regulation of any pathways by paired analysis after $\frac{1}{2}$ hour despite the overweight in significantly changed transcripts identified by LME (Supplementary Figure S2 online). At time points 4 hours, 24 hours, and day 11, paired analysis did not result in any significantly regulated pathways for any of the irritants, which we ascribe the small number of chips (3 vs. 3). However, an unpaired analysis with the inclusion of more chips (Supplementary Table S4 online) indicated that the $\frac{1}{2}$ hour response to SLS is associated with downregulation of cell energy metabolism (such as tricarboxylic acid cycle, fatty acid β-oxidation and protein metabolism) as well as cholesterol biosynthesis. Significantly regulated pathways at time point 4 and 24 hours were very sparse with focal adhesion/extracellular matrix receptor interaction being upregulated by NON at both 4 and 24 hours possibly indicating keratinocyte dyscohesion.

The differential early response between the irritants was confirmed by a 6 vs. 6 chips comparison using all $\frac{1}{2}$ hour chips in the experiment (Supplementary Table S5 online). The core message being that central cell energy metabolism pathways and steroid/cholesterol biosynthesis are differentially regulated in response to the two irritants.

Taken together, the above analyses indicate that growth, pro-survival, and inflammation-related pathways are significantly upregulated in the very early epidermal response to NON, whereas in SLS irritation a downregulation in cellular catabolic as well as anabolic pathways could be shown.

Histopathological changes are absent at early time points and sparse at day 11

There were hardly any histopathological changes from baseline through the time points $\frac{1}{2}$, 4, and 24 hours. At day 11, both irritants induced slight acanthosis, hyperkeratosis (Figure 3a-c), and perivascular lymphocytic infiltration (Figure 3d-f), and the increase in all three parameters was more pronounced in response to SLS than NON. Occasional sparse parakeratosis was seen ($n=4$) and was not restricted to any irritant. The increased transcription of cell-cycle genes at day 11 was supported indirectly by an increased immunostaining with the mitosis-specific antibody MIB-1. However, the anticipated difference between irritants as determined by canonical pathways (Figure 1b) could not be confirmed (Figure 3g-i).

Altogether, the histopathological changes at day 11 were smaller than might have been expected from clinical appearances.

Immunohistochemical analysis confirmed dissimilar expression of MMP3 and COX-2

From transcriptional results one might hypothesize that S100A7 were induced by SLS (FDR = 0.021, Supplementary Table S2 online) and not by NON (FDR = 0.145) after cumulative exposure. However, both irritants induced an

equal S100A7 upregulation visualized immunohistochemically at day 11 (Figure 3j-l). No changes in antibody staining were observed during early time points.

Differential cyclooxygenase 2 (COX-2) protein expression between the two irritants was confirmed. The most intense changes in immunostaining were observed in stratum spinosum (Figure 3m-o) and stratum granulosum (day 11, not shown). Induction of COX-2 protein could be shown after $\frac{1}{2}$ hour for NON, whereas SLS was only able to induce this protein after cumulative exposure. Thus, at least in the early irritation phase does COX-2 expression seem to correlate with the transcription of its encoding gene *PTGS2*.

Matrix metalloproteinase 3 (MMP3) was the most differentially expressed transcript for combined early time points (Supplementary Figure S2 online) and showed diffuse epidermal staining that was not altered at any time points for any of the irritants (not shown). Nuclear staining was, indeed, significantly induced after $\frac{1}{2}$ hour exclusively by SLS ($P=0.02$), and this change was sustained—although insignificantly—for 4 and 24 hours (Figure 3p-r).

DISCUSSION

Serial sampling in a cumulative irritation study enabled us to examine genome-wide transcriptional changes over time in response to SLS and NON. Focus on the epidermal response was given priority to minimize ''noise'' from the various different cell types of the dermis, and analysis of the dermal transcriptional response is thus excluded from this study. Two essentially different situations were studied: (1) cumulative irritation at a stage where clinical signs are observable, epidermal homeostasis and barrier function are disturbed, and healing processes are active; and (2) the early epidermal response to a subclinical mild irritative insult where no perturbation of barrier function could be shown (as determined by TEWL, Supplementary Table S1 online).

On the basis of sequential measurements, we observed a difference in the number of transcripts induced by the two irritants indicating that SLS may induce a transcriptional response that is more enhanced, more protracted, and possibly with an earlier onset compared with NON (Supplementary Figure S2 online). Whether the observed differences are caused by (1) irritant reaction kinetics, (2) irritant concentrations, or (3) actual differences in the mechanisms of action, cannot be answered unequivocally from this study design. Therefore, these aspects must be kept in mind when interpreting the results.

Interpretation of the initial epidermal response to mild irritant exposure

Although a relatively small number of transcripts was initially induced by NON compared with SLS, the response seems to be a relevant one not far from responses induced by a variety of other short-term exogenous stimuli such as UV light, hyperosmotic stress, oxidative stress (Bender *et al.*, 1997), chemically induced carcinogenesis (Cheepala *et al.*, 2009), and jet fuel-induced skin irritancy in rats (McDougal *et al.*, 2007). Significantly upregulated pathways by NON include inflammatory, proliferation/pro-survival pathways where many of the contributing genes exhibit substantial overlap. The overall picture is consistent with activation of MAPK cascades (especially the extracellular signal-regulated kinase (ERK) branch) that are well-known stress signal transduction pathways crucial for keratinocyte proliferation, apoptosis, and survival (Iversen *et al.*, 2005). Selective activation of the IL-6 pathway by NON, but not by SLS, is in line with previous findings (Grangsjo *et al.*, 1996).

Consistent with the involvement of MAPK signaling is the robust upregulation of the *DUSP1* and *FOS* transcripts. *DUSP1* negatively regulates MAPK by inactivation of JUNK, ERK and p38, and thus, has an important role in the termination of MAPK signaling (Abraham and Clark, 2006). *FOS* is a transcription factor that heterodimerizes with members of the JUN family to form the transcription factor complex. AP-1 is located downstream of MAPK signaling, and its activation results in the transcription of a wide array of genes important for epidermal homeostasis, inflammation, and pathology (Zenz *et al.*, 2008).

Although the immunomodulator *DUSP1* was upregulated for both irritants, other inhibitory effector arms in immune/inflammatory response pathways (such as, peroxisome proliferator-activated receptor α (PPAR-α), ubiquitin-dependent proteolysis, and the upregulation of the anti-inflammatory homeostatic protein annexin A1) were exclusively associated with the NON response, thereby offering a possible explanation to why NON induced the regulation of relatively few transcripts compared with SLS within the first 24 hours after exposure. Of particular interest is the PPAR-α pathway that was not only upregulated by NON but was also among the most differentially involved pathways when comparing NON and SLS. PPARs are ligand-activated transcription factors of the nuclear hormone receptor superfamily that behave as negative regulators of the inflammatory phase of skin repair/homeostasis (Icre *et al.*, 2006). Topically applied PPAR-α agonists have been shown to reduce 12-*O*-tetradecanoylphorbol-13-acetate-induced skin irritation in mice (Sheu *et al.*, 2002). Noteworthy, PPAR ligands include fatty acids (such as NON, speculatively) and eicosanoids that might explain the solitary NON-induced activation of PPAR-α signaling.

The transcription of both *MMP3* and *PTGS2* correlated with their protein expression. To our knowledge, we are the first to show *in vivo* induction of these innate immune-related proteins just 30 minutes after irritant exposure in human skin. The non-extracellular (i.e., nuclear) location of MMP3 has previously been shown in UVA photoprovoked skin (Jarvinen *et al.*, 2007), and this protein has important functions in cellular proliferation, migration, apoptosis, and inflammation through enzymatic cleavage of non-matrix substrates (McCawley and Matrisian, 2001). Interestingly, one of its substrates, IGFBP3, was less transcribed on exposure to SLS compared with NON. Because MMP3-catalyzed cleavage of IGFBP3 increases the bioavailability of IGF (Fowlkes, 1997), the combined upregulation of MMP3 and downregulation of IGFBP3 may act in concert to increase IGF bioactivity by SLS but not by NON.

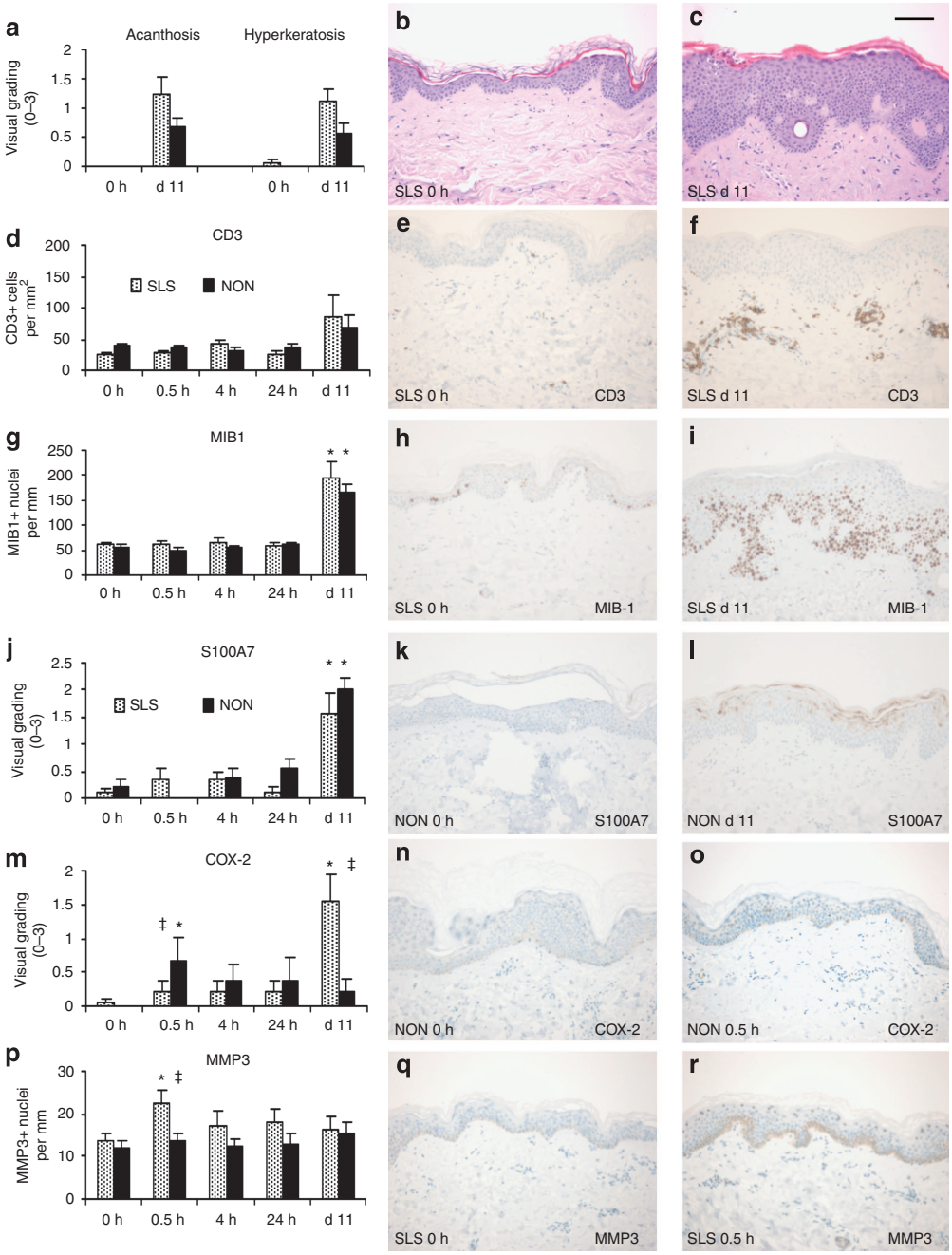

Figure 3. Histopathological changes at various time points for sodium lauryl sulfate (SLS) and nonanoic acid (NON). Acanthosis and sparse *ortho*-hyperkeratosis is induced after cumulative exposure at day 11 (d11) for both irritants (**a**) shown for SLS at 0 hour (**b**) and d11 (**c**). The slight inflammatory response, which is dominated by CD3 + T cells is hardly demonstrable until d11 (**d**) for both SLS and NON. Barely any CD3 + T cells at 0 hour (**e**), but a perivascular increase at d11 (**f**). The change in MIB1 staining shows an identical pattern with an increase at d11 for both SLS and NON (**g**) and is shown for SLS at 0 hour (**h**) and d11 (**i**). The increase in nuclear staining of the basal and suprabasal cells indicates a proliferative response. S100A7 is upregulated for both NON and SLS at d11 (**j**) in the upper epidermis, shown for NON at 0 hour (**k**) and d11 (**l**). There is a differential increase in cyclooxygenase 2 (COX-2) staining for NON and SLS with a peak at $\frac{1}{2}$ hour and at d11, respectively (**m**). The slight increase in staining of the keratinocytes in stratum spinosum $\frac{1}{2}$ hour after NON exposure is shown in **o**. There is no spinous staining at 0 hour, but a slight staining of the basal layer (**n** and **o**), equal for both irritants. The increase of matrix metalloproteinase 3 (MMP3) staining of the stratum granulosum half an hour after SLS exposure is shown by many positive nuclei at $\frac{1}{2}$ hour (**p**, **r**), but hardly any at 0 hour (**q**). Significant changes from baseline (0 hour) and from the other irritant are designated by *and ‡, respectively. The magnification of all microphotographs is identical; scale bar in **c** = 0.1 mm.

Epidermal response to repeated skin irritation

The observed degree of cumulative ICD at the end of a 5 + 4 days exposure model was histologically very modest, and microscopic examination is not an applicable tool for distinguishing between SLS and NON cumulative reactions. Thus, our results confirm what others have found previously:

diverse morphology in acute challenges cannot be corroborated in cumulative situations (Moon *et al.*, 2001; Willis, 2006).

The 23 suggested biomarkers for cumulative ICD (Table 1) were robustly regulated genes observed for both irritants at day 11. Gene members of the epidermal differentiation complex (Mischke *et al.*, 1996) were upregulated. These genes of the epidermal differentiation complex colocalize within 2.05 kb on the 1q21 chromosome and are important for terminal differentiation of human epidermis. Noteworthy, other prominent epidermal differentiation complex gene members, such as, loricrin, involucrin, and profilaggrin, were not among the significantly regulated transcripts at any time point in our experiment (not shown). The apoptosis-regulating protein survivin (encoded by *BIRC5*) and the putative antimicrobial peptide lipocalin 2 were not previously associated with ICD but have both been shown to have a role in maintenance of skin homeostasis: Survivin is upregulated in the epidermis in response to UVB (Dallaglio *et al.*, 2009), and lipocalin is upregulated in hyperproliferative skin diseases such as psoriasis and chronic eczema (sic), but not acute allergic contact dermatitis (Mallbris *et al.*, 2002).

To our knowledge, loss of function of the tight junction protein claudin 23, the cornified envelope precursor sciellin, and the rate-limiting enzyme in sterol biosynthesis squalene epoxidase (encoded by *SQLE*) is previously unreported in the context of skin irritation. Although the expressions of other claudins have been shown to be influenced by phorbol ester exposure to murine skin (Arabzadeh *et al.*, 2008), expression of claudin 23 in skin was never previously reported. The expression of sciellin has not been described in any pathological skin conditions; in contrast, sciellin null mice have normal skin development and structure (Baden *et al.*, 2005). Downregulation of *SQLE* indicates altered epidermal cholesterol biosynthesis. Although *SQLE* has not previously been associated directly with skin disease, the oral administration of a squalene epoxidase inhibitor to dogs resulted in dermatitis (Chugh *et al.*, 2003). Thus, new genes of potential diagnostic, therapeutic, and prognostic value have been identified in the context of ICD and skin homeostasis.

In summary, we present new insights into the molecular events in the epidermal response to two different experimental irritants. Relevant genes/pathways were identified already 30 minutes after exposure as was protein expression. Early pathways involved in the initial phase of skin irritancy were very different when comparing SLS and NON, and differential expression for selected genes could be confirmed at the protein level. The differences reported may depend on experimental design and dose of irritants applied. Twenty-three possible common biomarkers for cumulative skin irritancy are accessible for further validation.

In conclusion, although SLS and NON irritation had more similar than dissimilar macro- and microscopic morphological profiles in the initial and late exposure phase, gene expression profiles are much different and worth exploring further in future studies.

MATERIALS AND METHODS

Subjects

A total of 36 healthy human volunteers gave their written, informed consent to participate. The study was performed in accordance with the Declaration of Helsinki Principles and approved by the local ethics committee (Videnskabsetisk komité for Region Syddanmark; S-20070012). UV exposure, washing, and treatment of test sites were not allowed during the study.

Irritant exposure

Eighteen volunteers (10 men, 8 women; mean age 28.7 years) were exposed to 2% SLS (purity >99.9%; Sigma Chemicals, St Louis, MO) aqueous, and 18 volunteers (7 men, 11 women; mean age 25.9 years) were exposed to 10% NON (purity 99.3%; ABCR, Karlsruhe, Germany) in n-propanol. Skin irritation was accomplished in a cumulative open application test as previously described (Andersen *et al.*, 2006). Briefly, the volar forearms were exposed 10 minutes for 5 + 4 days using a 20 mm diameter open chamber.

In addition, three sites were noncumulatively irritated (10 minutes single exposure) and named $\frac{1}{2}$, 4, or 24 hours according to the elapsed time from end of exposure till biopsies were taken.

Evaluation of skin irritancy

Clinical assessment. Visual scoring was performed in accordance with the European Society of Contact Dermatitis guideline on cumulative/subacute SLS irritation (Tupker *et al.*, 1997). The target level of irritation at day 11 was a score of at least 2 but preferably not higher than 4 corresponding to a moderate reaction.

Biometric assessment. Skin barrier integrity was assessed by measuring the TEWL using a DermaLab (Cortex, Hadsund, Denmark) TEWL probe. Skin color reflectance was determined using a colorimeter (Minolta CR300, Osaka, Japan) quantifying erythema. Stratum corneum hydration was estimated by measuring conductance with the DermaLab (Cortex) moisture pin probe. Means from triplicate measurements were used for colorimetry and hydration.

Skin biopsies and separation of epidermal sheets

Double biopsies (one 3 mm and one 4 mm punch biopsy with a distance of approximately 3 mm) were taken from preselected sites according to an incomplete block design with each subject contributing with biopsies from three of the five possible sites, i.e., 0 hour (control), $\frac{1}{2}$ hour, 4 hours, 24 hours, and day11. Control biopsies from 0 hour sites ($n = 36$) were secured from all participants.

Biopsies (3 mm) were formalin fixed and paraffin embedded. Ammonium thiocyanate-induced dermo-epidermal separation of 4 mm biopsies was performed as previously described (Clemmensen *et al.*, 2009). The epidermal sheets were stored in RNA*later* (Applied Biosystems/Ambion, Austin, TX) at 4°C for 48 hours and then at −20°C until further processing.

RNA extraction and hybridization to microarrays

Epidermal specimens were homogenized in a TissueLyser (Qiagen, Valencia, CA) and RNA extracted using the RNeasy Micro Kit (Qiagen) following the manufacturer's instructions. RNA integrity was assessed on a Bioanalyzer (Agilent, Santa Clara, CA). RNA (500 ng) was amplified with the MessageAmp II Biotin Enhanced

kit (Applied Biosystems/Ambion) and hybridized to Affymetrix GeneChip HG U133 plus 2.0.

Selection of samples for the microarray experiment

RNA from 48 samples was hybridized to microarrays. Uninterrupted series of three samples per subject were preferred. The number of chips was expanded for the time point $\frac{1}{2}$ hour, which was of special interest with respect to identifying initial differences between the two irritants. The samples from time points 0 hour ($n=9$), $\frac{1}{2}$ hour ($n=6$), 4 hours ($n=3$), 24 hours ($n=3$), and day 11 ($n=3$) originated from 9 of the 18 SLS-irritated subjects and from 9 of the 18 NON-irritated subjects. The 18 subjects who contributed with samples hybridized to microarrays had a mean visual sum score at day 11 of 3.58 (range 1.5–10).

Histopathology

Sections (3 μm) were cut from the paraffin blocks and stained with hematoxylin and eosin and immunohistochemically with antibodies to COX-2 (NeoMarkers, Fremont, CA), S100A7 (Abcam, Cambridge, UK), MMP3 (Abcam), CD3 (Dako, Glostrup, Denmark), and MIB1 (Immunotech, Glendale, CA). Visual grading through four steps (0–3) was applied for hyperkeratosis, acanthosis, and perivascular inflammation. CD3-positive cells were counted in a subepidermal area of 1 mm^2. Epidermal cytoplasmic staining was graded by the visual four-step scale for S100A7 and COX-2, whereas nuclear staining was evaluated by counting positive nuclei along 1 mm of full-thickness epidermis (MIB1 and MMP3). The cytoplasmic staining of MMP3 was performed automatically on the ACIS III (Dako). The epidermal cytoplasmic staining with anti-COX-2 segregated into a diffuse, dense staining of the basal layer, a less intense sporadic staining of the spinous layer, and a totally different, distinct, paranuclear, granular staining of the granular layer. Consequently each of these three epidermal compartments was evaluated separately. All evaluations were blinded.

Data analysis

Microarray experiment. Raw data have been deposited at NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE18206. CEL files were normalized using the quantile method in R environment (http://cran.r-project.org/) using all genes in data sets. The design of our experiment was characterized by (1) a treatment effect between two groups of subjects receiving different irritants, (2) an effect of time, and (3) subjects contributed with samples from different time points creating an unbalanced data structure. During the experiment, the gene expression level for an individual was measured repeatedly over time such that the data for an individual were correlated although at different time points. Considering the specific experiment design and data structure, we applied the LME model to our data with fixed effects for the treatment and for the time effect together with possible interactions between them and random effect for individual differences; i.e., we model

$$\vec{y}=X\vec{\beta}+U\vec{\xi}+\vec{\varepsilon}$$

Here, $\vec{y}$ stands for the vector of measured gene expression levels, X and U are the known design matrices, $\vec{\beta}$ is the fixed effect vector to be estimated, $\vec{\xi}$ is the vector for the random effects, and $\vec{\varepsilon}$ is the vector of residuals. Nested models with only the fixed effect for one treatment were also fitted for specific time points.

LME and *t*-test calculations were performed in R using FDR to adjust for multiple testing. Hierarchical clustering was performed using TM4 Multi Experiment Viewer and Euclidian distance. Pathway analysis was performed using a collection of 639 canonical pathways in Gene Set Enrichment Analysis (http://www.broad.mit.edu/gsea) correcting for multiple testing with the family-wise error rate. Unpaired pathway analysis was performed separately for each irritant with 6 vs. 12 chips ($\frac{1}{2}$ hour) or 3 vs. 15 chips (4 hours, 24 hours, and day 11) omitting 0 hour samples for the examined time point. Hypergeometric test for canonical pathway overlap was conducted with MSigDB.

Histopathology. For identification of significantly changed expression from 0 hour samples, we used the Wilcoxon signed rank test. For direct comparison between SLS and NON groups, we used the Wilcoxon rank sum test. α Values < 0.05 were considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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