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S100A8/A9, a Key Mediator for Positive Feedback Growth Stimulation of Normal Human Keratinocytes

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Abstract S100A8 and S100A9 are known to be up-regulated in hyperproliferative and psoriatic epidermis, but their function in epidermal keratinocytes remains largely unknown. Here we show that (1) S100A8 and S100A9 are secreted by cultured normal human keratinocytes (NHK) in a cytokine-dependent manner, (2) when applied to NHK, recombinant S100A8/A9 (a 1:1 mixture of S100A8 and S100A9) induced expression of a number of cytokine genes such as IL-8/CXCL8, CXCL1, CXCL2, CXCL3, CCL20, IL-6, and TNFα that are known to be up-regulated in psoriatic epidermis, (3) the S100A8/A9-induced cytokines in turn enhanced production and secretion of S100A8 and S100A9 by NHK, and (4) S100A8 and S100A8/A9 stimulated the growth of NHK at a concentration as low as 1 ng/ml. These results indicate the presence of a positive feedback loop for growth stimulation involving S100A8/A9 and cytokines in human epidermal keratinocytes, implicating the relevance of the positive feedback loop to the etiology of hyperproliferative skin diseases, including psoriasis. J. Cell. Biochem. 104: 453–464, 2008.

Key words: S100; MRP; psoriasis; inflammation

Psoriasis is one of the most prevalent skin diseases. The incidence of psoriasis is ~2% in Western countries, and the incidence in Japan, though less than that in Western countries, has been increasing [Yip, 1984; Menter and Barker, 1991; Kawada et al., 2003]. The disease is characterized by red, thickened, and flaking epidermis resulting from hyperproliferation and deteriorated differentiation of epidermal keratinocytes and marked infiltration of immune cells such as T cells, macrophages, and neutrophils. Psoriasis is rarely fatal and the lesion is readily manageable. However, symptoms often recur throughout life and psoriasis therefore remained a serious challenge to quality of life of the patients. Despite recent intensive studies on the etiology, molecular and cellular mechanisms underlying the onset of psoriatic lesions remain unclear [Chamian and Krueger, 2004; Pittelkow, 2005; Clark and Kupper, 2006]. To obtain better insights into the pathogenesis of psoriasis, much effort has been focused on developing appropriate animal models. A critical role of T cells in the onset of the disease was demonstrated by induction of psoriatic lesions in normal or non-lesional psoriatic skin tissues transplanted into SCID mice by injecting activated T cells isolated from patients [Wrone-Smith and Nickoloff, 1996; Nickoloff and Wrone-Smith, 1999]. Sano et al. [2005] revived a classical view of the disease, that is, the primary involvement of keratinocytes in triggering psoriasis, by showing that expression of

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Abbreviations used: NHK, normal human keratinocytes; GFP, green fluorescent protein; S100A8/A9, a 1:1 mixture of S100A8 and S100A9.

Takamasa Nukui and Ritsuko Eham contributed equally to this work.

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constitutively active STAT3 in mouse epidermis resulted in induction of psoriasis-like skin lesions with concomitant up-regulation of several molecules linked to the pathogenesis of psoriasis. Recently, Zenz et al. [2005] found that epidermis-specific deletion of JunB and c-Jun in adult mice led to psoriatic skin phenotype. The mice also had arthritis, which is often observed in human psoriatic cases and rarely brought forth in animal models. Thus, the JunB/c-Jun-ablated mouse model is considered to be the best currently available model of human psoriasis. Intriguingly, induction of S100A8 and S100A9 was one of the earliest events after deletion of the Jun proteins.

S100A8 and S100A9 belong to the EF-hand-type calcium-binding S100 protein family composed of more than 20 members [Marenholz et al., 200]. Both proteins are secreted by neutrophils, activated monocytes, and macrophages and act as chemotactic molecules for those cells [Roth et al., 2003; Ryckman et al., 2003; Foell et al., 2004], comprising a positive feedback loop with respect to recruitment of inflammatory cells [Roth et al., 2003]. S100A8- and S100A9-positive myeloid cells are the first cells infiltrating into an inflammatory region [Odink et al., 1987]. Increased serum levels of S100A8 and S100A9 have been found in a number of human inflammatory diseases, including rheumatoid arthritis [Liao et al., 2004], multiple sclerosis [Bogumil et al., 1998], Crohn's disease [Lugering et al., 1995], and connective tissue diseases [Kuruto et al., 1990]. Thus, S100A8 and S100A9 play a key role in the induction and propagation of inflammation.

Production of S100A8 and S100A9 is not restricted to inflammatory cells but has also been observed in some epithelial cells, particularly in activated or transformed conditions [Brandtzaeg et al., 1987a,b; Wilkinson et al., 1988]. In the epidermis, S100A8 and S100A9 are up-regulated in hyperproliferative keratinocytes like those in wound healing [Thorey et al., 2001] and in psoriatic lesions [Madsen et al., 1992; Broome et al., 2003]. The genes are encoded in the psoriasis susceptibility region PSORS4 [Semprini et al., 2002]. In spite of these observations, biological functions of S100A8 and S100A9 in epithelial cells remain largely unknown except for possible chemotactic action on inflammatory cells [Lackmann et al., 1992, 1993]. The aim of this study was therefore to determine whether S100A8 and S100A9 have a direct effect on epidermal keratinocytes. Exogenous S100A8/A9 (a 1:1 mixture of S100A8 and S100A9) stimulated cultured normal human keratinocytes (NHK) to produce cytokines that are up-regulated in psoriatic lesions, and the S100A8/A9-induced cytokines in turn stimulated the production and secretion of S100A8 and S100A9 in NHK. Furthermore, S100A8/A9 itself enhanced the growth of NHK. These results indicate the presence of a positive feedback mechanism for growth of NHK involving S100A8/A9 as a principal mediator and the possible relevance of the positive feedback mechanism to the onset of psoriasis.

MATERIALS AND METHODS

Cells and Materials

NHK derived from the neonatal epidermis (NHK; KURABO, Osaka, Japan) were cultured in Epilife medium (Cascade Biologics, Portland, OR) containing calcium at a low concentration of 0.03 mM and HKGS Growth Supplement (Cascade Biologics). Keratinocytes were expanded by serial passage and used for experiments between passages 2 and 4. For monitoring DNA synthesis, tritiated thymidine (1 μCi/ml; ARC, St. Louis, MO) was added to the cultures 1 h before cell harvest. Recombinant human EGF, TNFα and IL-6 were purchased from PEPROTECH EC (London, England). Recombinant human IL-1F9, IL-8/CXCL8 and CXCL1 proteins and a neutralizing antibody against human IL-8/CXCL8 were from R&D Systems (Minneapolis, MN).

Preparation of Recombinant Proteins

cDNAs of S100A8 and S100A9 were amplified by PCR and cloned into pGEX6P1 (GE Healthcare Bio-Sciences, Piscataway, NJ: *Bam*H1–*Xho*1 site). The nucleotide sequences of the inserts were confirmed by DNA sequencing. *Escherichia coli* (BL21-Codon Plus-(DE3)-RIL; STRATAGENE, La Jolla, CA) cells were transformed by the vectors (pGEX6P1, pGEX6P1-S100A8 and pGEX6P1-S100A9). The recombinant GST-fusion proteins were purified by glutathione-agarose affinity chromatography using a Sepharose 4B column (GE Healthcare Bio-Sciences) under conventional conditions. GST was released by cleaving with PreScission protease (GE Healthcare Bio-Sciences) and removed from the final preparations using the Sepharose 4B column. For growth stimulation

experiments, S100A8 and S100A9 were further purified using ion-exchange columns (TSK gel DEAE-Toyopearl 650M and 650S, respectively; TOSOH, Tokyo, Japan) by eluting with increasing concentration of NaCl from 0 to 420 mM. Buffers used for the purification steps of S100A8 and S100A9 were calcium-free. To facilitate heterodimer formation, purified S100A8 and S100A9 were mixed at a 1:1 molar ratio in Hanks' balanced salt solution (HBSS) containing 1.3 mM $CaCl_2$ for 15 min at 37°C and dialyzed twice against excess volume of calcium free HBSS before use. LPS and endotoxin contaminated in the preparations purified by glutathione beads were determined by outsourcing to SRL (Tokyo, Japan) and with a Limulus test kit (Seikagaku Corporation, Tokyo, Japan), respectively.

Western Blot Analysis

Western blot analysis was performed under conventional conditions. Antibodies used were as follows: anti-human tubulin antibody (Sigma, St. Louis, MO), rabbit anti-human S100A8 (Calgranulin A) antibody (FL-83; Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-human S100A9 (Calgranulin B) antibody (H-90; Santa Cruz Biotechnology). The signals were visualized by horseradish-peroxidase-conjugated anti-mouse and anti-rabbit IgG antibody (Cell Signaling Technology, Beverly, MA), followed by a chemiluminescence system ECL plus, GE Healthcare Bio-Sciences). To monitor secretion of S100A8 and S100A9 into the culture medium, protein in the medium was precipitated with methanol and reconstituted into the same volume of buffer as that of the cell extracts. Equivalent aliquots of preparations from the cell extract and culture medium were applied for Western blot analysis to enable direct comparison of the relative amounts.

DNA Microarray

Logarithmically growing NHK were exposed to a mixture of S100A8 and S100A9 (10 μg/ml each) with 2 mM calcium chloride for 3 h. Total RNA was extracted with ISOGEN (Nippon Gene Co. Ltd., Tokyo, Japan) and purified using RNeasy spin columns (Qiagen, Valencia, CA). OD260/280 spectrophotometric ratios of the preparations were consistently higher than 2.0. RNA samples were labeled with Cy3 and Cy5 and hybridized to Human Whole Genome Oligo Microarray (Agilent Technologies) according to the protocol recommended by the manufacturer. The array was scanned with a Microarray Scanner Bundle G2565BA (Agilent Technologies). The TIFF array image was analyzed by feature extraction software (Agilent Technologies). Elements with a flag of gIsFeatNonUnifOL, rIsFeatNonUnifOL, gIsBGNonUnifOL, and rIsBGNonUnifOL were excluded from subsequent analysis. Two independently prepared mRNA samples were subjected to the array analysis (twice for each sample), and genes up-regulated more than 1.8-fold were shown in Table I.

TABLE I. Genes Up-Regulated in NHK Exposed to S100A8/A9 for 3 h

		Fold increased	
Genbank accession	Names of genes	Mean	SD
NM_000584	Interleukin 8 (IL-8)/CXCL8	5.84	2.79
NM_002089	Chemokine (C-X-C motif) ligand 2 (CXCL2)	4.42	2.24
NM_001511	Chemokine (C-X-C motif) ligand 1 (CXCL1)	4.11	0.84
NM_019618	Interleukin 1 family, member 9 (IL-1F9)	3.86	1.19
NM_006291	TNFα-induced protein 2 (TNFAIP2)	3.45	0.97
NM_004591	Chemokine (C-C motif) ligand 20 (CCL20)	3.11	0.73
NM_002090	Chemokine (C-X-C motif) ligand 3 (CXCL3)	2.95	1.76
NM_025079	FLJ23231	2.85	1.13
NM_002852	Prototypic long pentraxin (PTX3)	2.53	0.17
NM_000758	Colony stimulating factor 2 (CSF2)	2.42	0.28
NM_000594	Tumor necrosis factor (TNF)	2.21	0.19
NM_001165	BIRC3	1.96	0.17
A_23_P247	A_23_P247	1.93	0.76
NM_031419	NFKBIZ	1.93	0.61
NM_006945	Small proline-rich protein 2C (SPRR2B)	1.92	0.76
NM_000600	Interleukin 6 (IL-6)	1.91	1.08
NM_004428	Ephrin-A1 (EFNA1), transcript variant 1	1.88	0.33
NM_004417	Dual specificity phosphatase 1 (DUSP1)	1.87	0.63
M21539	Small proline-rich protein 2C (SPRR2C)	1.86	0.72

Real-Time Quantitative PCR

NHK were exposed to S100A8/A9 under conditions similar to those described for DNA microarray, and mRNA were extracted with a MagNA^{TM} Pure mRNA extraction kit and MagNA Pure^{TM} instrument (Roche Diagnostics, Tokyo, Japan). The mRNA preparation was reverse-transcribed with SuperScript^{TM}II (Invitrogen Corporation, Carlsbad, CA). Real-time quantitative PCR was performed on a LightCycler rapid thermal cycler system using a LightCycler FastStart DNA master SYBR green I kit (Roche Diagnostics) according to the manufacturer's instructions. Information on primers used is shown in Supplementary Material Table S1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a reference gene. Specificity of amplified fragments was confirmed by melting curve analysis. Expression level of each gene was analyzed quantitatively with LightCycler analysis software [Morrison et al., 1998]. Amounts of mRNA were normalized to those of GAPDH and finally presented as ratios to those of untreated control.

Quantitation of Cytokine Proteins Secreted Into Medium

Amounts of IL-8/CXCL8 and TNFα were determined using Quantikine Human IL-8/CXCL8 and DuoSet Human TNFα/TNFSF1A (R&D Systems, Minneapolis, MN), respectively, after appropriately diluting the culture media under the conditions recommended by the manufacturer.

RESULTS

Secretion of Endogenous S100A8 and S100A9 From NHK

At first we examined whether S100A8 and S100A9 are produced and secreted by NHK. NHK were routinely cultivated in a basal medium EpiLife supplemented with 1% human keratinocyte growth supplement (HKGS; Cascade Biologics). Under these conditions, NHK produced substantial amounts of S100A8 and S100A9 and readily secreted them into the medium (Fig. 1). The greater parts of S100A8 and S100A9 were recovered from the medium when analyzed 24 h after the replacement of medium (20–150 ng of S100A8 and S100A9 in 10 ml medium). HKGS, which contains 0.2 ng/ml EGF, 5 μg/ml insulin, 0.18 μg/ml hydrocortisone, 5 μg/ml tansferrin, and 0.2% (v/v) bovine pituitary extract, dose-dependently induced the production and resulting secretion of the proteins. Either EGF or high calcium alone also enhanced the production and secretion of S100A8 and S100A9 (Fig. 1).

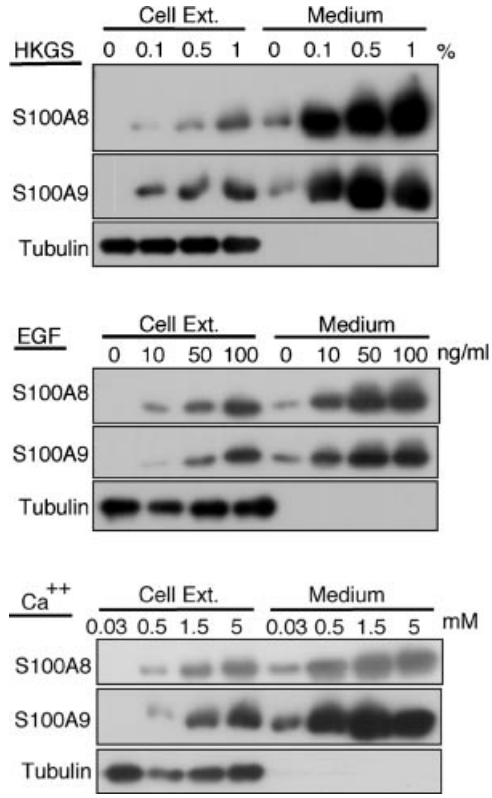

Fig. 1. Production and secretion of S100A8 and S100A9 by NHK. NHK were cultured in EpiLife medium with increasing amounts of human keratinocyte growth supplement (HKGS), EGF, or calcium chloride for 24 h. Equivalent aliquots of whole protein preparations from the cell extract and the culture medium were applied for Western blot analysis. Tubulin was used as a control for applied amount of the protein preparations.

Effect of Exogenous S100A8/A9 on Gene Expression of NHK

S100A8 and S100A9 are generally considered to act as activators and chemoattractants for neutrophils and monocytes/macrophages [Lackmann et al., 1992, 1993]. When human epidermal keratinocytes secrete S100A8 and S100A9, however, the keratinocytes themselves are inevitably exposed to the proteins. To explore possible direct effects of S100A8 and S100A9 on epidermal keratinocytes, we applied

a mixture of purified recombinant S100A8 and S100A9 proteins (S100A8/A9) onto NHK. The protein preparations purified with glutathione-beads were reasonably pure as shown in Figure 2A, and we further purified the proteins with ion-exchange columns for some experiments (Fig. 2B). The latter preparations showed a growth stimulation capacity similar to the preparation purified with glutathione beads alone (Fig. 6B). Treatment of NHK with LPS at a concentration equivalent to the contaminated amount in the S100A8/A9 preparations showed no effect (data not shown). Amount of endotoxin was determined by a Limulus test to be 0.0065 ng/ml for GST, 0.0070 ng/ml for S100A8, and 0.0061 ng/ml for S100A9 in the respective 10 μg/ml protein samples. GST was used as a control (Figs. 3B and 6A). The results described below indicate that the observed biological responses were due to the action of S100A8/A9 per se and not due to contaminants in the protein preparations.

After applying S100A8/A9 to NHK in the presence of 2 mM calcium, we analyzed possible change in the gene expression profile by a DNA microarray covering 41,765 genes and found that 19 genes were up-regulated (>1.8-fold) by exposure of the cells to S100A8 and S100A9 for 3 h (Table I). The up-regulated gene group includes IL-8/CXCL8, CXCL1, CXCL2, CXCL3, CCL20, IL-1F9, IL-6, SPRRs, and TNFα and its related proteins. What particularly attracted our attention was that most of the up-regulated genes are those known to be up-regulated in psoriatic epidermis [Bowcock et al., 2001; Oestreicher et al., 2001; Zhou et al., 2003]. To confirm the induction of genes, we performed quantitative RT-PCR of NHK exposed to S100A8/A9 for 3 h (Fig. 3A) in the presence of 0.06 mM or 2 mM calcium. mRNA levels of IL-8(CXCL8), CXCL1, CXCL2, CXCL3, CLC20, IL-1F9, and TNFα increased depending on the dose of S100A8/A9 (Fig. 3A,B). Calcium concentration in the medium was found to affect the efficiency of induction IL-8/CXCL8 and TNFα, but the induction itself was observed irrespective of calcium concentration. GST prepared under the same conditions with those for S100A8 and S100A9 showed no effect (Fig. 3B). The extents of up-regulation of the genes examined varied from less than 2 fold (TNFα) to up to ninefold (IL-8/CXCL8), being roughly in accordance with the results of DNA microarray analysis. S100A8 and

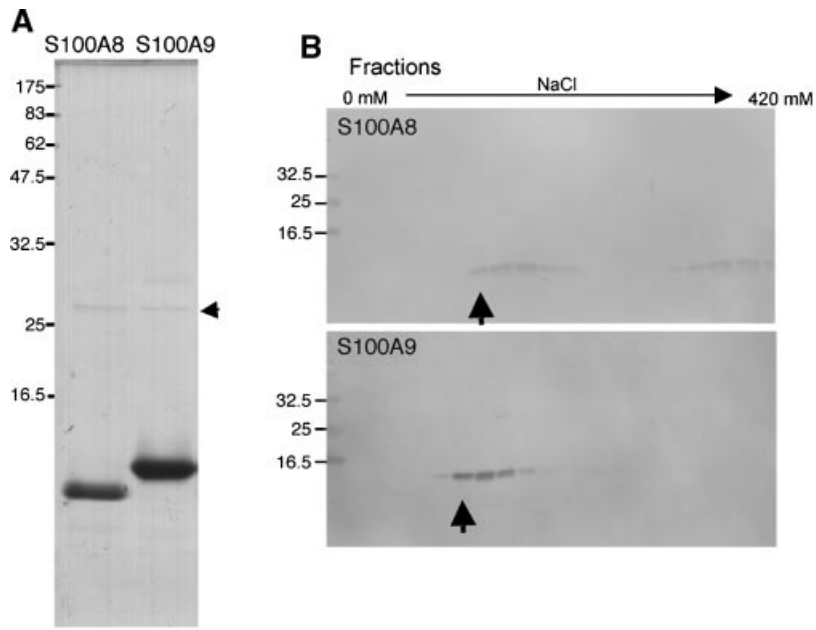

Fig. 2. Preparation and purification of recombinant S100A8 and S100A9 proteins. **A**: The proteins were produced as GST-fusion proteins and purified using glutathione-beads after elimination of GST with PreScission protease. Arrowhead indicates the position of GST. **B**: S100A8 and S100A9 were further purified using ion-exchange columns. The proteins were eluted with increasing concentration of NaCl from 0 to 420 mM. Arrows indicated the fractions used for the experiment shown in Figure 6B. The gels were stained with Coomassie Brilliant Blue.

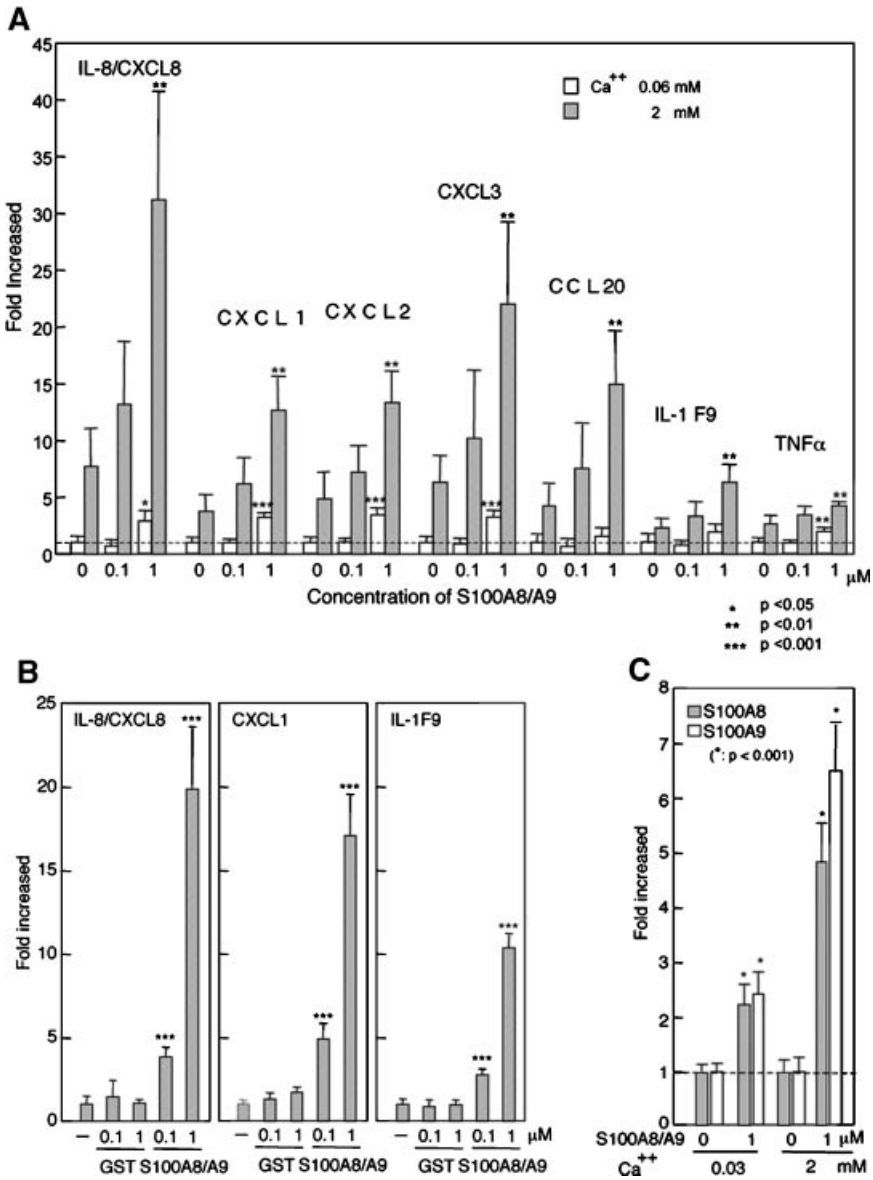

Fig. 3. Genes induced by S100A8/A9 in NHK as assayed by quantitative real-time RT-PCR. **A**: NHK were exposed to a mixture of S100A8 and S100A9 in the presence of 0.06 mM or 2 mM calcium for 3 h and the extracted RNA was analyzed for the designated transcripts by quantitative RT-PCR. Amounts of the transcripts are expressed as ratios to the value of untreated controls at 0.06 mM calcium. Statistical significance in difference was assessed comparing to respective untreated groups at the same calcium concentration. **B**: RT-PCR performed under the conditions similar to those shown in (A) in the presence of 2 mM calcium. GST was purified under the same conditions with those for S100A8 and S100A9. **C**: Induction of S100A8 and S100A9 by S100A8/A9. The experiments were performed under the conditions similar to those described in (B) except for the treatment time of 24 h. Significance in difference was assessed by Student's *t*-test.

S100A9 themselves were also induced by S100A8/A9 24 h after the application (Fig. 3C). S100A8/A9 enhanced amounts of IL-8/CXCL8 and TNFα proteins secreted into medium at 2 mM calcium but not at 0.03 mM calcium (Fig. 4). The cytokine levels obtained by 1 μM S100A8/A9 were about one fifth of those produced by NHK stimulated with 10 ng/ml IL-1α.

Enhanced Production and Secretion of S100A8 and S100A9 by S100A8/A9-Induced Cytokines in NHK

We next examined whether S100A8/A9-induced cytokines affect production of S100A8 and/or S100A9 in NHK. NHK were incubated in the basal EpiLife medium without HKGS but

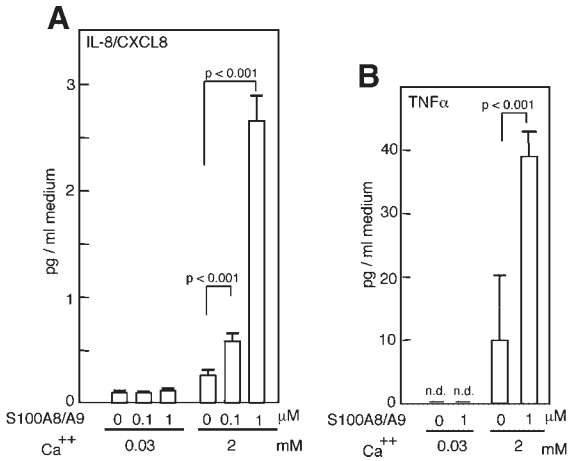

Fig. 4. Induction of cytokine protein production in NHK by S100A8/A9. NHK cells were treated with S100A8/A9 in the presence of different concentrations of calcium under the conditions similar to those described in Figure 3A. The amount of IL-8/CXCL8 and TNFα were determined by ELISA. Significance in difference was assessed by Student's *t*-test.

with the cytokines for 24 h at a calcium concentration of 0.03 mM. Equivalent aliquots of whole protein preparations from the cell extract and the culture medium were applied for Western blot analysis so as to roughly estimate the secreted fraction of whole protein. As shown in Figure 5A, all of the S100A8/A9-induced cytokines examined, that is, CXCL1, IL-8/CXCL8, IL-1F9, IL-6, and TNFα stimulated production and secretion of S100A8 and S100A9 in a dose-dependent manner. Expressions of receptors for these cytokines were reported in human epidermal keratinocytes [Schulz et al., 1993; Debets et al., 2001; Dhawan and Richmond, 2002]. Interferon(IFN)-α and -γ also showed a similar effect. IFNα appears to induce intracellular accumulation of S100A8 and S100A9 rather than their release, suggesting possible presence of differential regulation mechanism in NHK for production and secretion of the proteins. Figure 5B shows the time course of induction of S100A8 and S100A9 in NHK exposed to 100 ng/ml TNFα. Production and secretion of S100A8 and S100A9 were enhanced 16–24 h after the onset of exposure. These results indicate that S100A8 and S100A9 and the cytokines induced by S100A8/A9 comprise a positive feedback loop with respect to their production and secretion in NHK. Mork et al. [2003] reported that a combination of TNFα and IFNγ efficiently increased mRNA level of S100A8 and S100A9 in NHK.

Growth Stimulation of NHK by S100A8 and S100A8/A9

To examine the possible effects by S100A8 and S100A9 on the growth of NHK, NHK were incubated in the basal EpiLife medium without HKGS but with S100A8, S100A9 or a mixture of both proteins for 24 h. As shown in Figure 6A, S100A8 but not S100A9 remarkably enhanced the growth of NHK. The mixture of both proteins further enhanced of the growth of NHK compared with the effect of S100A8 alone. The optimum concentration of S100A8/A9 was ~100 ng/ml, and higher concentrations resulted in lower growth enhancement. One of the S100A8/A9-induced cytokines, IL-1F9, whose function is not well understood, also stimulated the growth of NHK. Preparations of S100A8 and S100A9 before and after purification with ion-exchange columns showed similar levels of growth stimulation (Fig. 6B). Lipopolysaccharide showed no effect at a concentration equivalent to that contaminated in the protein preparations before the ion-exchange column purification. These results indicate that the observed growth stimulation was due to the action of S100A8/A9 per se and not due to contaminants in the preparations. An antibody neutralizing IL-8/CXCL8 partially abrogated the growth of NHK stimulated by S100A8/A9 (Fig. 6C), indicating that the S100A8/A9-induced growth promotion takes place at least in part indirectly via IL-8/CXCL8.

DISCUSSION

In this study, we showed that (1) S100A8 and S100A9 were produced and secreted in a cytokine-dependent manner, (2) recombinant S100A8/A9 stimulated NHK to produce a number of cytokines that are known to be up-regulated in psoriatic epidermis, (3) the S100A8/A9-induced cytokines enhanced production and secretion of S100A8 and S100A9 by NHK, and (4) S100A8 and S100A8/A9 as well as the cytokines stimulated the growth of NHK. These results indicate the presence of a positive feedback loop for growth stimulation involving S100A8/A9 and the cytokines in NHK (Fig. 7).

The screening for genes induced by S100A8/A9 using a microarray resulted in identification of limited number of genes, which were mostly inflammatory cytokines (Table I). One of the reasons for the small number of genes identifies was probably because the incubation period was

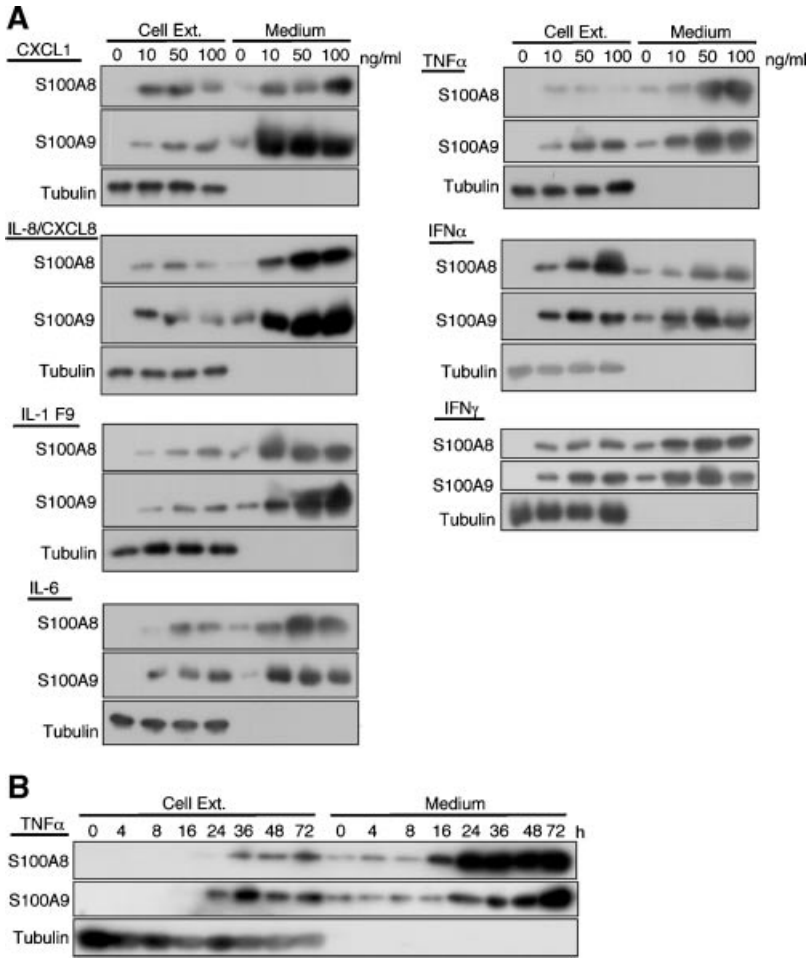

Fig. 5. Stimulation of production and secretion of S100A8 and S100A9 in NHK by cytokines. **A**: NHK were incubated in basal EpiLife medium without HKGS (Ca^{++}, 0.03 mM) but with the designated cytokines for 24 h. Equivalent aliquots of whole protein preparations from the cell extract and the culture medium were applied for Western blot analysis. Tubulin was used as a control for applied amount of the cell extracts. **B**: Time course of the induction of S100A8 and S100A9 in NHK by TNFα (100 ng/ml).

fixed as 3 h to analyze immediate reaction of the cells. S100A8/A9 and the cytokines comprising the positive feedback loop are known to act as chemoattractants for inflammatory cells and to be produced in large quantities by the recruited inflammatory cells. This implies that once inflammatory cells infiltrate into the epidermis, the positive feedback loop for growth stimulation of keratinocytes may function at a much higher level. This notion and its biological relevance, however, should be assessed more closely on quantitative basis, although direct comparison between doses used in vitro and those estimated in vivo is mostly difficult.

S100A8 and S100A9 are hardly detected in normal human epidermis. The genes are upregulated in hyperproliferative keratinocytes or epidermal tissues in wound healing, in stress, in Spitz nevi, and in psoriasis in particular [Brandtzaeg et al., 1987a,b; Thorey et al., 2001; Semprini et al., 2002; Broome et al., 2003; Grimbaldeston et al., 2003; Ribe and McNutt, 2003]. Within the hyperproliferative epidermis, S100A8 and S100A9 were detected mainly in the upper layers, that is, in the differentiating non-growing cell population, this being corroborated by the observation of preferential co-expression of S100A8 and S100A9 and the differentiation marker cytokeratin K10 [Thorey et al., 2001]. In accordance with this, induction of differentiation of NHK by high calcium and serum in culture resulted in increased levels of both proteins (Fig. 1). On the other hand, HKGS and EGF that support NHK to grow also enhanced production and secretion of S100A8 and S100A9. It is known that the

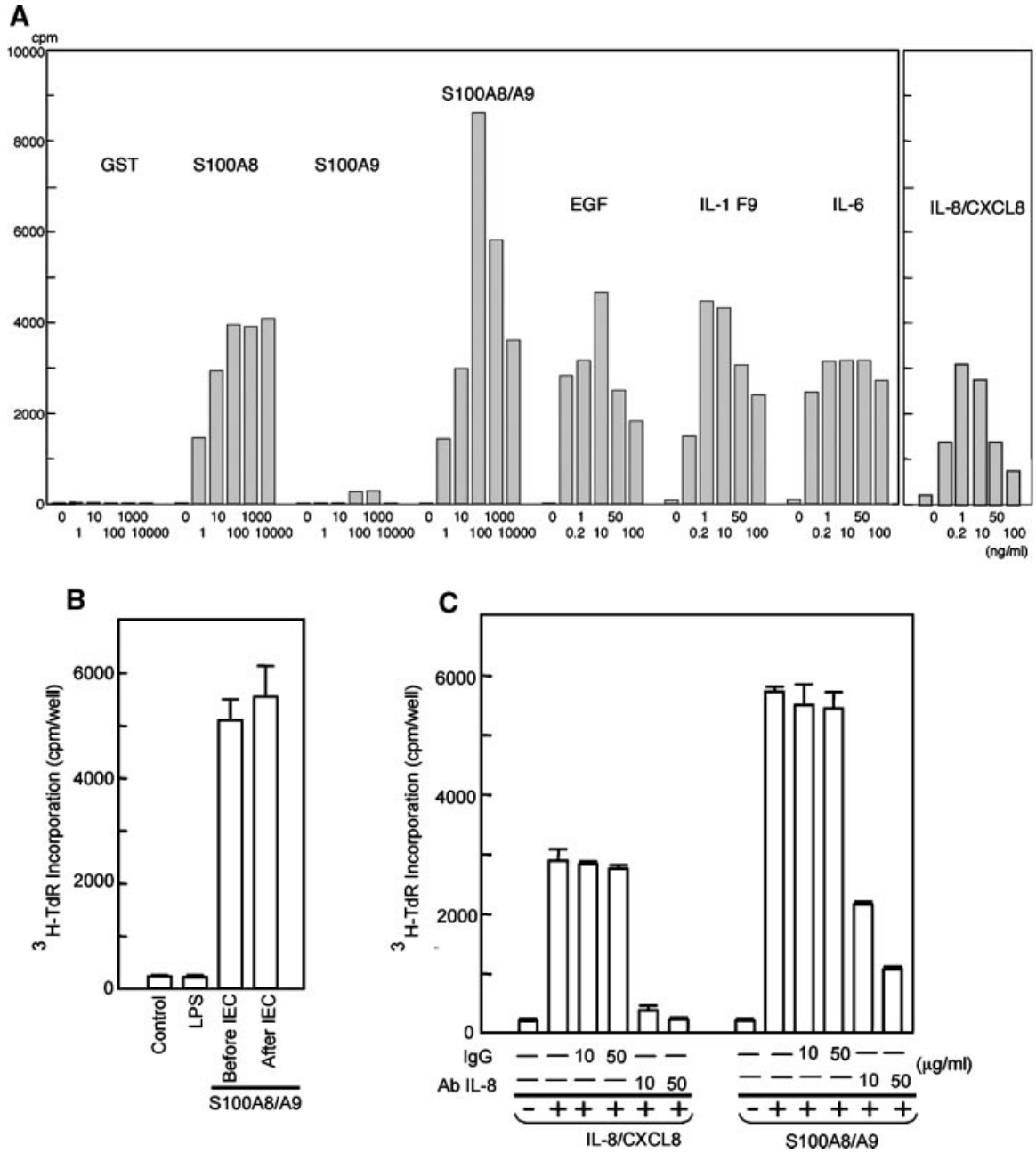

Fig. 6. Growth stimulation of NHK by S100A8 and S100A8/A9 and cytokines. NHK cells were incubated in basal EpiLife medium without HKGS but with the designated cytokines for 24 h. Calcium concentration of the medium was 0.03 mM. 3H-Thymidine (1 μCi/ml) was added to the medium 1 h prior to harvest, and radioactivity in an insoluble fraction was counted. **A**: GST was used as a control. The experiment was performed in triplicate, and the standard deviations observed were very limited and are not shown for simplicity. **B**: Effect of different S100A8/A9 preparations. IEC, ion-exchange column. Lipopolysaccharide (LPS) was added at a concentration of 0.3 pg/ml, an equivalent amount contaminated in the S100A8/A9 (100 ng/ml) preparation before IEC. **C**: Partial abrogation of S100A8/A9-induced growth of NHK by anti-IL-8/CXCL8 antibody. +: IL-8/CXCL8, 10 ng/ml; S100A8/A9, 100 ng/ml.

differentiation process is altered in the epidermis in hyperproliferative states as indicated by aberrant expression of genes, including hornerin [Takaishi et al., 2005] and cytokeratin K6 [Freedberg et al., 2001]. It is probable, therefore, that S100A8 and S100A9 are preferentially secreted in the epidermis by non-growing keratinocytes that have undergone an aberrant differentiation pathway and promote the growth of cells retaining growth potential located in the lower epidermal layers via a paracrine mechanism. In psoriatic lesions,

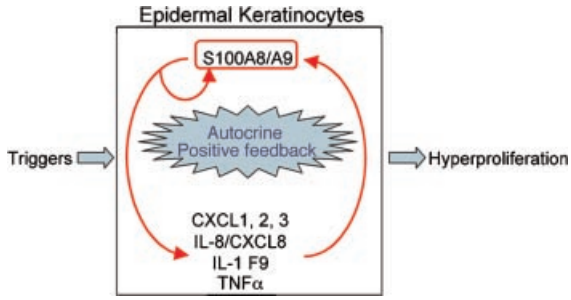

Fig. 7. A schematic representation of a possible positive-feedback loop for growth stimulation of epidermal keratinocytes. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

S100A8 and S100A9 were shown to be expressed in all layers of the epidermis [Broome et al., 2003]. Benoit et al. [2006] demonstrated that serum levels of S100A8 and S100A9 increase depending on disease activity and abnormal differentiation of keratinocytes in psoriasis. These lines of evidence indicate possible involvement of S100A8/A9 in physiological regulation and pathological dysregulation of keratinocytes, although it remains to be clarified whether epidermal keratinocytes are exposed to biologically significant levels of S100A8/A9 in vivo. When we titrated the amount of S100A8 and S100A9 secreted into the medium (~10 ml) by semi-confluent NHK using Western blotting, 20–150 ng of the protein was produced during 24 h incubation. This is somewhat underestimated because not all of the protein was precipitated with methanol during the sample preparation for Western blot analysis. Thorey et al. [2001] reported that HaCaT cells secreted ~5 ng S100A8/A9/10^6 cells/24 h, the amount being roughly comparable to that secreted by NHK. As shown in Figure 6A, 1 ng/ml of S100A8/A9 showed a distinct growth enhancing effect on NHK. The growth stimulation was observed over doses up to 10 μg/ml, though less efficiently at higher concentrations, that is, >10 μg/ml for S100A8 and >100 ng/ml for S100A8/A9. With respect to growth stimulation, therefore, the amount of S100A8/A9 produced by NHK appears sufficient to play a physiologically relevant role if similar secreting activity takes place in vivo. Chemotactic activity for neutrophils was observed also in a concentration range of 0.01~10 ng/ml of S100A8/A9 [Ryckman et al., 2003]. On the other hand, induction of cytokines in NHK appears to need higher concentration of S100A8/A9, that is, at least 1 μg/ml (~0.1 μM; Figs. 3 and 4). If these dose-dependent responses actually take place in vivo, the positive feedback loop illustrated in Figure 7 should be interpreted in a more exquisite way. In NHK under moderately stimulated conditions without massive inflammatory cell infiltration, S100A8 and S100A9 may be mainly produced by NHK to a level sufficient to stimulate growth of NHK and chemotaxis of inflammatory cells but not to induce cytokines in NHK. With infiltration of neutrophils and macrophages that can secret large amount of S100A8 and S100A9 [Roth et al., 2003; Foell et al., 2004], resulting higher amounts of S100A8 and S100A9 in the epidermis may act on NHK to produce various cytokines, many of which induce S100A8 and S100A9 in turn and also enhance the growth of NHK by themselves. Viemann et al. [2005] showed IL-8/CXCL8 was induced by ~3-fold in endothelial cells exposed to 200 μg/ml of S100A8/A9. Shibata et al. [2005] reported that S100A9 moderately enhanced the growth of normal rat kidney fibroblasts (NRK-49F] at a concentration of 300 nM (~4 μg/ml). These results indicate that amounts of S100A8 and S100A9 necessary for various biological responses largely differ depending on different types of cells and experimental conditions and that more intensive studies in terms of dose of S100A8/A9 are needed to thoroughly understand physiological and pathological relevance of S100A8/A9.

In summary, our results suggested that there are three different states of growth regulation in human epidermal keratinocytes, that is, (1) a state of normal turnover with no appreciable involvement of S100A8 and S100A9, (2) a state of hyperproliferation without remarkable infiltration of inflammatory cells, in which the positive feedback loop involving S100A8/A9 functions in keratinocytes, and (3) a state of excessive growth induced by the S100A8/A9-mediated positive feedback loop over-amplified by infiltrated inflammatory cells, involving S100A8/A9 and inflammatory cytokines. Potential functional significance of S100A8 and S100A9 has recently been indicated by elevated serum levels of the proteins in psoriatic patients in a severity-dependent manner [Benoit et al., 2006]. Although much work remains to be done for firmly establishing the presence of such growth regulation and for revealing detailed molecular mechanisms, implications of the

present study provide a new insight into the etiology of hyperproliferative skin diseases, particularly the etiology of psoriasis.

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