

Significance of MDR1-Gene and P-Glycoprotein (P-gp) Expressions in the Lesional Skin of Psoriasis Vulgaris

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We have examined P-gp (P-glycoprotein) and multi-drug resistance 1 (MDR1) gene expressions in the lesional skin of psoriasis vulgaris and atopic dermatitis some of those showed a decreased clinical response to topical corticosteroid ointment during the clinical course.

The patients were subdivided into four groups; S (-): responder to topical steroid without steroid ointment for one month at the time of biopsy, S (+): responder to topical steroid and under steroid therapy at the time of biopsy, R (-): low or non-responder to topical steroid without steroid therapy for one month at the time of biopsy, R (+): low-or non-responder to topical steroid and under steroid therapy at the time of biopsy.

P-gp was mainly expressed in the cytoplasm of some epidermal keratinocytes and most of infiltrating cells in the dermis of the lesional skin of psoriasis or atopic dermatitis. Scores of P-gp protein-expression was significantly higher in the patients under steroid ointment both in psoriasis vulgaris and atopic dermatitis. While R (+) group showed much more intense expression of P-gp than S (+) group in psoriasis vulgaris but this was not the case for atopic dermatitis.

MDR1 gene was expressed in the lesional skin of R (+) psoriasis but not in S (+) psoriasis or normal skin.

These results suggest that steroid-resistance occasionally experienced in psoriasis vulgaris might be related to the overexpression of P-gp which is possibly induced after topical steroid ointment. This might provide a new insight for the mechanism of steroid -insensitivity in inflammatory skin disorders especially in psoriasis vulgaris.

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Introduction

Although several side effects were recognized, topical glucocorticosteroids (GC) ointment is the most widely used medication in the treatment of skin disorders; psoriasis vulgaris or atopic dermatitis.

Cellular factors that have significant roles in chemotherapy resistance include at least the following five mechanism : (1) changes in drug uptake or drug efflux across cell membrane or between cytoplasm and nucleus (transport-mediated resistance), (2) changes in the activation or inactivation of drugs within the cell (metabolic resistance), (3) changes in targeted enzymes through altered levels of those targets within cells or through the altered affinity of cellular enzymes for the drug (target resistance), (4) changes in DNA repair processes, (5) changes in the ability of cells to execute programmed cell death or apoptotic mechanisms¹.

P-Glycoprotein (P-gp), encoded by multi-drug resistance 1 (MDR1)² gene, is a transmembrane efflux pump for different lipophilic drugs including GC³. Thus upregulation of P-gp may provide a mechanism for reduced GC responses and GC induced cell apoptosis. To clarify whether P-gp plays a part in low responses to ordinal GC ointment in the patients with inflammatory skin disorders, we have examined P-gp expression and apoptosis in skin specimens from chronic inflammatory skin disorders such as psoriasis vulgaris or atopic dermatitis using a standard immunohistochemical technique. Furthermore, we examined MDR1 gene expression using RT-PCR technique in skin specimens from psoriasis vulgaris.

Patients and Methods

Patients

Twenty-eight patients with psoriasis vulgaris and 30 patients with atopic dermatitis were enrolled in this study. The samples and their assessment were shown in Table 1. Clinical assessment of psoriasis was

Table 1. Numbers of samples and thier assessment

	Psoriasis vulgaris	Atopic dermatitis
samples	28 specimens (22 patients)	30 specimens (30 patients)
assessment	Psoriasis area and severity index(PASI)	Severity index (Japanese Dermatological Association)

made using PASI score⁴ and atopic dermatitis using Severity Index proposed by Japanese Dermatological Association.

Nobody but one patient had received vitamin D3 ointment therapy at biopsy time in psoriasis vulgaris patients. Before and after one month ointment with group I or II class GC⁵, clinical assessment was made. Responders were defined as patients with reducing scores below 75% compared with pretreatment scores⁴, retrospectively. Low responders were defined as patients not corresponded to above. Patients were classified into four groups as described in summary.

Biopsied specimens were obtained from the patients under the informed consent before and after the treatment with topical GC. Paraffin-embedded tissues for immunostaining had been routinely fixed in 10% neutral buffered formalin for 8-48 hours. Cryosections were preserved in -30°C until RNA extraction.

Antibodies

JSB-1 (NOVOCASTRA, Newcastle upon Tyne, UK), anti-P-gp antibody; it recognizes an internal epitope of PGP molecule⁶; the ascites was used in a dilution of 1:20. Anti CD3 antibody (DAKO, Copenhagen, Denmark) or anti CD20 antibody (DAKO) was used in a dilution of 1:50 or 1:100. Terminal deoxynucleotidyl transferase (Tdt) enzyme (INTERGEN, Oxford, UK) was used for labelling apoptotic cells⁷.

Phosphate-buffered saline (PBS) (pH 7.4) containing bovine serum albumin (BSA) (1%) was used for the dilution of JSB-1.

Immunohistochemistry

Paraffin tissue sections of 5 μ m were placed on siliconized glass slide and deparaffinized in xylene, 100% ethanol, 90% ethanol for P-gp, anti CD3, anti CD20 staining or Tdt-mediated Unscheduled Nick End Labelling (TUNEL).

Staining with anti P-gp staining: The antigen retrieval was performed by treatment with the slides in 90°C citrate buffer for 20 minutes. After washing in aqua for 10 minutes, endogenous peroxidase activities were blocked in aqua bidest, containing 3% H₂O₂ for

5 minutes. Then the slides were washed in PBS for 10 minutes. All tissue sections and positive controls⁶ were incubated with JSB-1 for overnight at 4°C; negative controls, however, were incubated only with 1% BSA. After washing in PBS for 10 minutes, all slides were covered with biotinylated goat antimouse IgG (DAKO), for 10 minutes at room temperature. The slides were then washed again for 10 minutes in PBS. Finally they were incubated for 10 minutes at room temperature with a streptavidin- biotin-conjugated peroxidase complex (DAKO).

Staining with anti-CD3 or CD20 staining: The antigen retrieval was performed by treatment in 0.1% trypsin for 30 minutes at room temperature for anti CD3. After washing in PBS for 10 minutes, endogenous peroxidase activities were blocked in aqua bidest, containing 3% H₂O₂ for 5 minutes. After washing in PBS for 10 minutes, the slides were incubated in 5% normal goat serum for 5 minutes. All tissue sections and positive controls were incubated with anti CD3 or CD20 antibody for 1h at 4°C; negative controls, however, were incubated only with 1% BSA. After washing in PBS for 10 minutes, all slides were treated same as P-gp staining.

TUNEL method: The slides were treated with 20 μ g/ml proteinase K in PBS for 1 hour at room temperature. Endogenous peroxidase activities were blocked in aqua bidest, containing 3% H₂O₂ for 5 minutes, same as P-gp staining. Then the slides were washed in PBS for 10 minutes and then treated with EQUILIBRATION BUFFER (INTERGEN, New York, USA) for 10 minutes. Then the slides were incubated with Tdt enzyme for 1 hour at 37°C and further incubated with WORKING STRENGTH STOP/WASH BUFFER (INTERGEN) for 10 minutes at room temperature. Finally they were incubated for 30 minutes at room temperature with anti-digoxigenin peroxidase conjugate (INTERGEN).

The peroxidase label was developed for tissues using 0.03% H₂O₂ and 3,3'-diaminobenzidine tetrachloride (DAB), which results in a brown staining product. The tissue sections were mounted with Permunt (Fisher Scientific, New Jersey, USA), and counterstaining with hematoxylin and methyl green for immunohistochemistry and TUNEL method, respectively.

Semiquantitative immunohistochemical scores were defined; 0: no staining, 1: weakly positive, 2: moderately positive, 3: strongly positive. Sweat gland of normal skin defined as score 1⁶ for P-gp.

RT-PCR

Total cellular RNA was isolated using RNeasy Mini Kit (QIAGEN, California, USA) as described by Meller et al⁸ RNA concentration and purity were determined spectrophotometrically

Total cellular RNA (500 ng) was amplified by RT PCR using Ready To Go™ RT-PCR Beads (Amersham Pharmacia Biotech, New Jersey, USA) with primers described by Chen et al⁹ (42°C for 30 minutes to reverse transcribe and PCR cycles, an initial step of 95°C for 5 minutes, 45 cycles of a 3-temperature PCR [95°C for 1 minute, 62°C for 1 minute, 72°C for 1 minute] and end with 72°C for 5 minutes Each PCR product was then size-fractionated through a 1.8% agarose gel The amplified cellular fragment (target) was 544 bp

Further, RT PCR products were extracted and digested with VSP I (Takara, Kyoto, Japan), digested almost 400bp and 150bp (GENE BANK ACCESSION M14758)

Results

Immunostaining

In immunohistochemically, only sweat gland cells showed weakly positive staining of P gp in the normal skin (Fig 1a) In contrast, P gp was mainly expressed in the cytoplasm of infiltrated cells as well as keratinocyte and vascular endothelial cells in each samples obtained from psoriasis vulgaris or atopic dermatitis patients The TUNEL positive cells showed a nuclear staining pattern in the lesional skin of psoriasis vulgaris and atopic dermatitis

Patients profiles of psoriasis vulgaris are summarized in Table 2 The mean age of S () group younger than other groups, but no significant differences were seen in other parameters

There was no significant correlation between immunohistochemical scores of P gp and ages, PASI scores, PUVA therapy, biopsy site nor complication of

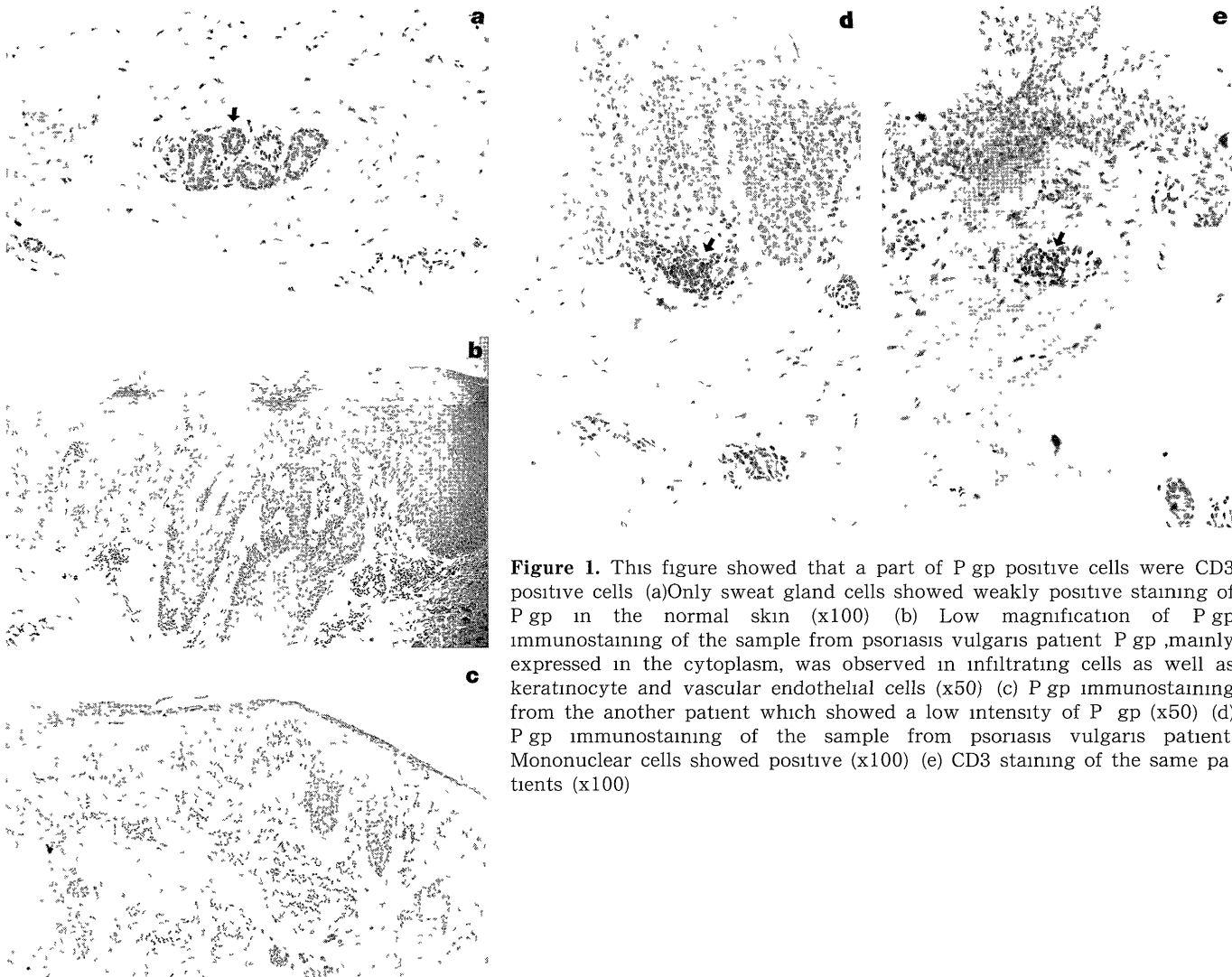


Figure 1. This figure showed that a part of P gp positive cells were CD3 positive cells (a)Only sweat gland cells showed weakly positive staining of P gp in the normal skin (x100) (b) Low magnification of P gp immunostaining of the sample from psoriasis vulgaris patient P gp ,mainly expressed in the cytoplasm, was observed in infiltrating cells as well as keratinocyte and vascular endothelial cells (x50) (c) P gp immunostaining from the another patient which showed a low intensity of P gp (x50) (d) P gp immunostaining of the sample from psoriasis vulgaris patient Mononuclear cells showed positive (x100) (e) CD3 staining of the same patients (x100)

Table 2. Patients profiles of psoriasis vulgaris

Parameters	S(-)	S(+)	R(-)	R(+)
Number of subjects*	6	5	5	12
Age (years)±S.D.**	38.5±16.3	50.8±16.9	62.8±21.7	61.6±16.2
Sex (m/f)	6/0	8/1	3/2	8/4
DM or hyperlipidemia (+/-)	3/3	0/5	2/3	4/8
Biopsy site (SEA***/non SEA)	1/6	2/3	4/1	1/11
PUVA (+/-)	1/5	2/3	0/5	5/7

*: mean value of subjects, **: p<0.05, ***: SEA=sun exposed skin

S (-): responder to topical steroid without steroid ointment for one month at the time of biopsy.
S (+): responder to topical steroid and under steroid therapy at the time of biopsy, R (-): low or non-responder to topical steroid without steroid therapy for one month at the time of biopsy, R (+): low-or non-responder to topical steroid and under steroid therapy at the time of biopsy.

Table 3. Atopic dermatitis patients characteristics

Parameter	S(-)	S(+)	R(-)	R(+)
Number of subjects*	8	13	0	9
Age (years)*±S.D.	20.1±4.5	22.8±7.3		26.7±12.5
Sex (m/f)	4/4	8/5		7/2
Number of eosinophils±S.D.	899.4±743.0	969.6±705.7		801.7±522.9
Biopsy site (SEA***/non SEA)	4/4	7/6		5/4
Red face** (+/-)	0/7	0/13		8/1

*: mean value of subjects, **: p<0.05***: SEA=sun exposed skin

S (-): responder to topical steroid without steroid ointment for one month at the time of biopsy, S (+): responder to topical steroid and under steroid therapy at the time of biopsy, R (-): low or non-responder to topical steroid without steroid therapy for one month at the time of biopsy, R (+): low-or non-responder to topical steroid and under steroid therapy at the time of biopsy.

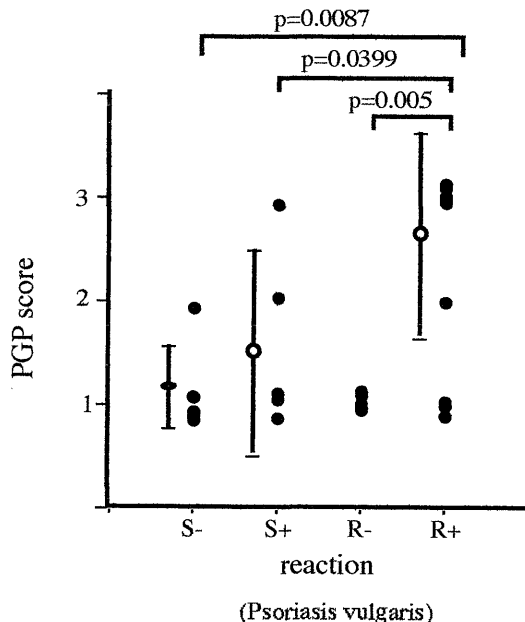


Figure 2. In psoriasis vulgaris patients, immunohistochemical scores of P-gp were significantly higher in R(+) group than in other groups.

DM or hyperlipidemia at biopsy times (data not shown).

The immunohistochemical scores of P-gp and GC ointment duration were significantly higher in R(+) group than in other groups (Fig.2). There was no significant differences between R(-) samples and S(+) nor S(-) samples. Expression of PGP was correlated with GC ointment duration (Correlation coefficient=0.584, $p<0.05$), but not ages at biopsy times.

A part of P-gp positive cells were mast cells or CD3

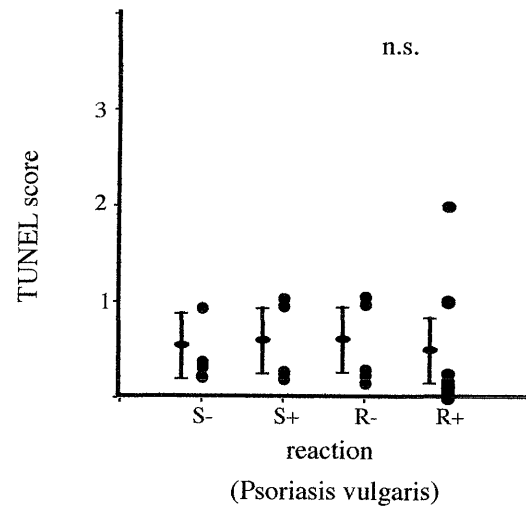


Figure 3. TUNEL scores of the samples from psoriasis vulgaris patients. There was no differences between 4 groups ; S (-): responder to topical steroid without steroid ointment for one month at the time of biopsy, S (+): responder to topical steroid and under steroid therapy at the time of biopsy, R (-): low or non-responder to topical steroid without steroid therapy for one month at the time of biopsy, R (+): low-or non-responder to topical steroid and under steroid therapy at the time of biopsy, in TUNEL scores.

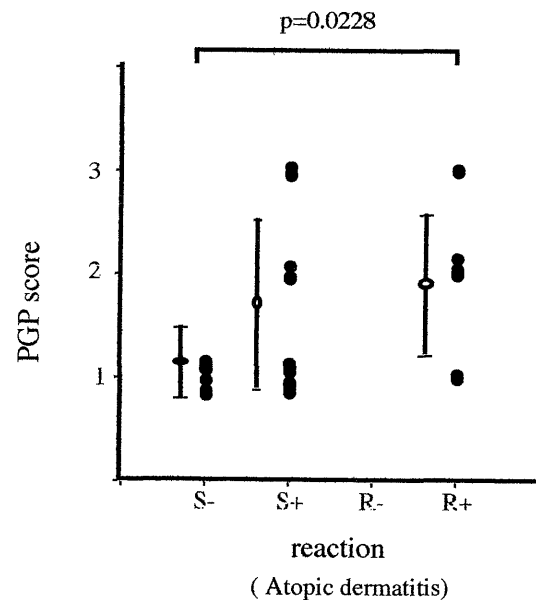


Figure 4. In atopic dermatitis patients, immunohistochemical scores of P-gp were significantly higher in R (+) group than S (-) group, but not S (+) group.

positive cells (Fig.1b-e) but not CD20 positive cell (data not shown).

There was no differences between 4 groups in TUNEL scores in psoriasis vulgaris (Fig.3). TUNEL scores of symptomless margin of the lesion had not differences from lesional scores.

In case of atopic dermatitis, clinical profiles are summarized in Table 3. No other parameters but atopic red face had significant differences between 4 groups.

There was no significant correlation between immunohistochemical scores and severity index, number of peripheral blood eosinophils, total IgE nor biopsy site (data not shown).

The immunohistochemical scores of P-gp were significantly higher in R (+) group than in S (-) group (Fig.4). There was no differences of P-gp scores between S (+) samples and R (-) nor R (+) samples. There was no significant differences between S (+) and R (+) group in GC ointment duration. Expression of P-gp was not correlated with GC ointment duration.

Same as in psoriasis vulgaris patients, a part of P-gp positive cells were mast cells or CD3 positive cells but not CD20 positive cell (data not shown).

There was no significant differences between 4 groups in TUNEL scores in atopic dermatitis same as psoriasis vulgaris (data not shown).

Expression of MDR-1 mRNA

Samples from which showed strong P-gp intensity in immunohistochemistry, demonstrated MDR1 gene expression in RT-PCR (Fig. 5). RT-PCR products were digested into almost 400bp and 150bp fragments by VSP-I (data not shown). MDR1 mRNA expression was upregulated in the patients with R (+) group but not in S (+) group of psoriasis vulgaris.

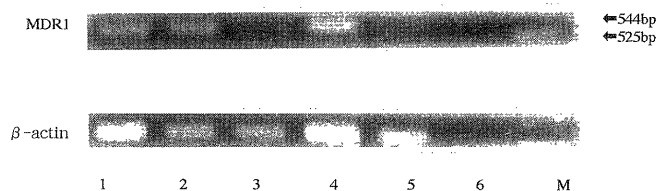


Figure 5. MDR1 gene expression of the lesional skin from the patients with psoriasis vulgaris by RT-PCR. Samples with strong P-gp intensity demonstrated significant MDR-1 gene expression. No.1,2,4: Sample from R (+) group, No. 3: Sample from S (+) group, No. 5: normal human non sun-exposed skin, No. 6: negative control, M: Bio Marker

Discussion

Although several side effects were recognized¹⁰, topical GC ointment is the most widely used medication in the treatment of skin disorders; psoriasis vulgaris¹⁰ or atopic dermatitis¹¹. In the last decade, new type of skin manifestations, i.e., persistent erythema of the face (atopic red face)¹² or

"poikiloderma-like lesion on the neck"¹³, have been recognized in patients with atopic dermatitis, especially in Japan. Poikiloderma-like lesion on the neck could be attribute to chronic inflammation and delay of wound healing process, possibly caused by long-standing topical GC therapy¹³. Topical GC preparations are categorized into different potency groups by vasoconstrictor (i.e., skin blanching) assay. However, this is subjective and dose not correlate perfectly with clinical antiinflammatory effects, percutaneous absorption, or propensity for adverse effects¹⁴. No correlation was seen between Lymphocyte steroid sensitivity (LSS) parameters: maximal percentage inhibition of thymidine incorporation achieved at the highest concentration of dexamethason (Imax) and the concentration of steroid at which thymidine incorporation is reduced to 50% of Imax value, and cutaneous vasoconstrictor response; suggested tissue differences in steroid sensitivity¹⁵. Currently, there is no reliable and accessible method available for expressing GC potency in a better way¹⁴.

Other problems have been implicated about topical GC ointment, i.e., GC insensitivity. GC insensitivity was observed in the subset of asthmatic³ or leukemic¹⁶ patients : they showed low responses to ordinal GC therapy, but others showed good responses. Low responders are resistant to the antiinflammatory effect of GC while simultaneously showing several other side effects known to reflect normal sensitivity¹⁷.

P-gp is a energy-derived plasma membrane transporter which pumps certain drugs including GC out of the cells¹⁸. The physiologic role of P-gp is speculative, and the possibilities include the protection of normal tissues from environmental and endogenous toxins, steroid secretion in the adrenal gland, secretion of bile salts in the bile canaliculi, and secretory functions in the kidney¹. Prolonged exposure of tumor cells to natural hydrophobic cytotoxic drugs may result in the acquisition of resistance by cells not only to the drugs used but also to a series of structurally and functionally unrelated drugs¹⁹.

Recently, several reports have described relation between GC and P-gp in patients with asthma or nasal polyp. Henriksson et al.²⁰ reported that nasal polyps from 5 of 17 patients treated with clinical dose of a topical nasal steroid showed a stronger staining intensity for P-gp than polyps from 13 untreated patients. Montano et al.³ showed that surface P-gp expression and Rh 123 efflux in B cells were decreased in GC sensitive asthma than in GC resistant patients or mild asthma, significantly. There was no significant differences between their GC sensitive and resistant patients in total daily steroid dose. Furthermore, they

observed reduced surface functional P-gp expression by B cells from normal volunteers after 48-h exposure of cells to 10 nM dexamethasone.

In our observation, intensity of P-gp and MDR1 gene expressions showed a positive correlation to the duration of GC application to the lesional skin in psoriasis vulgaris which might suggest that P-gp plays some role in induction of GC insensitivity as well as asthma or nasal polyp patients.

In contrast to psoriasis vulgaris, there was no significant correlation between P-gp scores and duration of steroid ointment in the patients with atopic dermatitis; suggested atopic dermatitis occurred not only by inflammation but by impaired barrier function²¹.

Several reports showed GC induces cell apoptosis^{22, 23}. However, in our patients, TUNEL scores were not significantly different between 4 groups, and scores of most patients showed 0. Our data suggested that clinical response to GC ointment in inflammatory skin diseases was caused not by apoptotic but by another mechanism; i.e., downregulation of the expression of cytokines and adhesion molecules²⁴.

Maillefert et al. demonstrated that larger dose of GC induced higher expression of MDR1 gene and P-gp in rheumatoid arthritis and suggested the possible resistance to other drugs²⁵. In a similar manner to their observation, our data on psoriasis vulgaris suggested that GC ointment induces the expression of P-gp to reduce the response to other drugs.

In conclusion, we should pay careful attention for the possible induction of GC insensitivity in the patients with inflammatory skin disorders under GC ointment. Combination of phototherapy, vitamin D3 ointment, or immunosuppressive agent with GC might prevent the induction of drug resistance as demonstrated in combination chemotherapy for cancer patients.

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