Relationship Between Heat Shock Protein Induction and the Binding of Antibodies to the Extractable Nuclear Antigens on Cultured Human Keratinocytes

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The importance of environmental factors such as ultraviolet light and temperature in the pathogenesis of cutaneous lupus erythematosus is well recognized. Recent evidence suggests the presence of autoantibodies to heat shock proteins (HSP) in the sera and enhanced expression of the HSP70 gene in peripheral blood mononuclear cells of patients with systemic lupus erythematosus. We designed experiments to determine how HSP or stress protein inducers affect the cell surface binding of IgG antibodies from sera containing anti-SS-A/Ro and anti-ribonuclear protein (RNP) antibodies to keratinocytes because these antibodies are considered to be one of the immunologic triggers of cutaneous lupus erythematosus.

Immunofluorescence and immunoblot analysis using a monoclonal antibody to the 72 kDa of HSP revealed that an 18-h incubation with 10 μg/ml of Δ12-PGJ2, one of cytotoxic prostaglandins, induced HSP72 formation in cultured human keratinocytes. Δ12-PGJ2 augmented the binding of IgG antibodies from sera containing anti-U1RNP and anti-SS-A/Ro antibodies to cultured keratinocytes, but produced no enhancement of the binding of IgG antibodies from sera containing anti-Sm or anti-DNA antibodies. Similar results were also obtained by using flow cytometry analysis. HSP was also induced by ultraviolet B irradiation.

These results suggest that exposure of keratinocytes to stressors such as Δ12-PGJ2 and ultraviolet light increases the binding sites for U1RNP, SS-A/Ro, and SS-B/La antibodies. The association between HSP induction and the appearance of extractable nuclear antigens may provide a better understanding of why environmental stimuli can promote the development of erythematous lesions in the skin. J Invest Dermatol 101:191–195, 1993

Cutaneous lesions of systemic lupus erythematosus (SLE) and the subsets of LE are well known to be associated with ultraviolet light (UVL) exposure and they are aggravated by exposure to sunlight [1]. In neonatal LE (NLE), the skin lesions consist of annular, erythematous scaly plaques that are most prominent on the head and neck [2,3]. Subacute cutaneous LE (SCLE), another subset of cutaneous LE, has a characteristic distribution that suggests a photo-aggravated disease process [4]. Other environmental factors play a role in the pathogenesis of LE in addition to UVL and sunlight [5]. Genetics, sex hormones such as estrogens, virus, and stress play a crucial role in the etiopathogenesis of SLE [6] and a variety of forms of trauma ranging from insect bites to X-radiation have been implicated in the production of chronic cutaneous LE reactions [7]. Chilblain-like lesions due to the local action of cold may occur in discoid LE and SLE [8].

Recent investigations on the pathogenesis of photosensitive cutaneous LE have focused on the strong association of antibodies to SS-A/Ro with the development of clinical disease [4,9,10]. Immunoblotting methods revealed high incidences of anti-SS-A/Ro and anti-SS-B/La autoantibodies in SLE patients [11]. Anti-ribonuclear protein (RNP) antibody is also associated with photosensitive LE, milder SLE with limited renal disease, and often with mixed connective tissue disease [12–15].

The epidermal keratinocytes, especially the basal cell, are the major targets of immunologic damage in cutaneous lupus. Recent reports suggest that the sera of LE patients contain IgG antibodies for SS-A/Ro, SS-B/La, or U1RNP that bind to UVL-irradiated cultured keratinocytes [16,17]. Similar findings were demonstrated for cultured keratinocytes stimulated from estradiol-β [18,19].

The heat shock or stress response may be induced in cells of any living organism when exposed to heat or other stimuli such as hormones, UVL, chemical agents, and certain viruses [20]. These stressors are well known to induce heat shock proteins (HSP), mainly 83 to 90 kDa protein (HSP90 family) and 68 to 73 kDa protein (HSP70 family) [20]. Most of the environmental factors that affect cutaneous LE also induce these HSP. Recently we demonstrated the induction of 72-kDa HSP and cytoskeleton changes in cultured, transformed human epidermal cells by Δ12-PGJ2, one of the cytotoxic prostaglandins (PG) [21]. Therefore, we designed experiments to determine if Δ12-PGJ2, a stressor, can augment the bindings of IgG antibodies from sera containing anti-SS-A/Ro, anti-SS-B/La and anti-U1RNP antibodies to cultured keratinocytes.

MATERIALS AND METHODS

Cell Culture Cultured human, neonatal foreskin keratinocytes (EPIC-PACK) were purchased from Clonetics (San Diego, CA). The cells were...
expanded and passaged in keratinocyte growth medium (KGM) (Clonetics). In the present studies, third-passage cells were used. The basic procedures were described previously [17–19]. Briefly, third-passage cells were cultured in KGM on Lab-Tek chamber slides (Miles, Naperville, IL) or 60-mm plastic dishes for 4 to 5 d. When the cells grew to 70% to 80% confluency, they were treated with agents.

Chemicals $\Delta^{12}$-PGJ$_2$ was a generous gift from Ono Pharmaceutical Co. Ltd. (Osaka, Japan). Anti-HSP72 monoclonal antibody (MoAb) and fluorescein isothiocyanate (FITC)-labeled sheep anti-mouse IgG were obtained from Becton Dickinson (Buckinghamshire, England). Anti-HSP72 MoAb (code RN1197) reacts only with the induced HSP72. All other reagents were of analytic grade.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblot Analysis These procedures were performed according to our recent report [21]. Following the addition of $\Delta^{12}$-PGJ$_2$, cell suspensions were centrifuged at 800 rpm for 10 min and washed three times with 2 ml of phosphate-buffered saline (PBS). The cell pellets were precipitated with 2 ml of 5% trichloroacetic acid and washed three times with 2 ml of 1 M NaOH. To analyze the effect of $\Delta^{12}$-PGJ$_2$ on cellular protein patterns, 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was carried out, as described previously [21]. Proteins separated on the gels were transferred to nitrocellulose paper and processed with Western blotting using an anti-HSP72 MoAb.

Visualization of HSP72 Cells grown on Lab-Tek chamber slides after the addition of $\Delta^{12}$-PGJ$_2$ (37°C, 18 h) were fixed with methanol (−20°C, 2 min). Following treatment with normal sheep serum for 20 min at room temperature to block non-specific binding, the cells were stained with anti-HSP72 MoAb followed by FITC-conjugated sheep anti-mouse IgG antibody [21].

Cell Viability Cell viability was tested by the acridine orange/ethidium bromide (AO/EB) test [17]. Cytotoxicity was determined by subtracting the values for untreated cells from the values for treated cells. Each test was run in duplicate.

Serum Specimens Selection of antisera was similar to that of our previous reports [17–19]. Anti-U, RNP antisera, anti-Sm antisera, anti-SS/A-Ro antisera, anti-SS-B/La antisera, and anti-ssDNA antisera were obtained from four SLE, three SCLE, 3 Sjögren syndrome, and four mixed connective tissue disease patients. They were kindly provided by the Department of Internal Medicine of Kyoto University [19]. The specificity of all 14 sera was determined by the routine indirect immunofluorescence (IF) technique for antigen antibodies, double immunodiffusion, counter immunoelectrophoresis, and immunoblotting analysis using methods described previously [17]. Anti-SS-B/La antibodies were well known to be found only when anti-SS-A/Ro antibodies were present, which strengthens the association between anti-SS-A/Ro antibodies and anti-SS-B/La antibodies [22]. Anti-SS-A/Ro antibodies were demonstrated to be most closely linked with anti-SS-B/La antibodies by enzyme-linked immunosorbent assay (ELISA) with recombinant SS-B/La [23]. However, we used anti-SS-B/La antisera based on our previous determination of specificity because of comparison with our previous studies [17–19]. Specificity of anti-DNA binding was determined by ELISA according to our previous report [24]. MoAb against ss/dsDNA was obtained from autoimmune-prone mice by the hybridoma technique [19].

Indirect Immunofluorescence Method This method has been described previously [18] and is briefly summarized. An aliquot (1.5 ml) of a third-passage keratinocyte suspension (6.25 × 10$^6$ cells) was plated into each well of a Lab-Tek chamber slide (Miles) and cultured for 4 to 5 d in KGM. In most experiments, 18 h after adding $\Delta^{12}$-PGJ$_2$, each specimen on the glass slide was washed with PBS and incubated with diluted 1:100 antiserum or normal human serum (NHS) at 4°C for 1 h. Post-fixation by cold acetone was then performed. Positivity was determined by subtracting the values for untreated cells from the values for treated cells. Each test was run in duplicate.

Flow-Cytometry Analysis A fluorescence-activated cell sorter (FACS) technique was developed that allowed identification of cell-surface antibody binding but limited subsequent internalization. Third-passage cells were cultured in KGM on 60-mm plastic dishes for 4 to 5 d. When the cell had grown to 70% to 80% confluency, they were treated with PG. Eighteen hours later, the cells were harvested and stained for FACS analysis as previously described [17,18]. In each experiment, negative controls included non-treated cells that were stained with the same antiserum plus second antibody and also treated cells that were stained with NHS and the second antibody. This FACS analysis was performed in triplicate.

Ultraviolet Light Irradiation Before irradiation, the medium in 60-mm plastic dishes was replaced with warmed PBS (30°C) free of any photoactive compounds. At least one dish of cells was not exposed to UVL (but was handled in the same fashion as the irradiated cells), whereas the remainder were exposed to 100 mJ/cm$^2$ or 200 mJ/cm$^2$ of UVB. Light sources and other details were described previously [25]. During UVB irradiation, the temperature was monitored; the decrease of temperature was less than 0.5°C.

Statistical Analysis Student t and paired t tests for paired samples were used and probability values less than 0.05 were considered significant.

RESULTS

HSP72 in Cultured Keratinocytes After $\Delta^{12}$-PGJ$_2$ Treatment Using an indirect IF technique, HSP72 was localized mainly to the cytoplasm of the cultured human keratinocyte (Fig 1A). After exposure to 10 µg/ml of $\Delta^{12}$-PGJ$_2$ for 18 h, HSP72 appeared mainly in the nucleus (Fig 1B).

Immunoblot analysis using anti-HSP72 MoAb, revealed an absence or very little HSP72 in control cells, even though 0.1% ethanol was also added to the medium. HSP was markedly induced, however, by the 18-h treatment with 5 µg/ml or 10 µg/ml of $\Delta^{12}$-
PGJ$_2$. HSP was also induced by 100 mJ/cm$^2$ UVB or 200 mJ/cm$^2$ UVB irradiation.

**Binding of IgG Antibodies from Sera Containing Anti-ENA Antibodies to Cultured Human Keratinocytes** Because $\Delta^{12}$-PGJ$_2$ has cytotoxic effects, cell viability was examined. As shown in Fig 2, $\Delta^{12}$-PGJ$_2$ (10 m$\mu$g/ml) had cytotoxic effects on cultured keratinocytes. These effects were dependent on the time of exposure. Binding of IgG of anti-U$_1$RNP antibodies was induced in a time-dependent manner. Cultured cells to this concentration of $\Delta^{12}$-PGJ$_2$ for 18 h were alive and continued to grow after $\Delta^{12}$-PGJ$_2$ was replaced with KGM. A similar percentage of binding of IgG of anti-SS-A/Ro antibodies was observed, in which the optimal binding was also induced in the cells treated with 10 m$\mu$g/ml $\Delta^{12}$-PGJ$_2$ for 18 h (data not shown). Based on the results of this experiment, the optimal exposure time was determined to be 18 h. The binding of IgG of anti-extractable nuclear antigen (ENA) antibodies to cultured keratinocytes was then examined. Figure 3 shows binding of IgG of anti-U$_1$RNP antibody mainly on the cell surface, and also some binding was found in the cytoplasm and the nucleus after exposure to 10 m$\mu$g/ml of $\Delta^{12}$-PGJ$_2$ for 18 h. Table I shows the percentage of keratinocytes labeled by various antibody probes to nuclear antigens. Ten, but not 1, micrograms per milliliter of $\Delta^{12}$-PGJ$_2$ induced the apparent binding of anti-U$_1$RNP and anti-SS-A/Ro antibodies to over 20% of the cultured cells. The percentage of cells expressing Sm antigen and ssDNA was low and not significant. PGE$_1$, PGE$_2$, PGF$_2$ and PGF$_{2\alpha}$ did not induce binding of anti-U$_1$RNP or anti-SS-A/Ro antibodies. The binding mode of IgG from sera containing anti-SS-B/La antibodies was almost similar to that of IgG of anti-SS-A/Ro antibodies. Cytotoxicity of PGE$_1$, PGE$_2$, PGF$_2$ and PGF$_{2\alpha}$ at the concentration of 5 or 10 m$\mu$g/ml for 18 h or 48 h was less than 15%, which was compatible with our previous report of transformed human epidermal cells in culture [26].

Finally, the binding of IgG antibodies to cultured cells was examined by FACS analysis. The positive binding of IgG of anti-U$_1$RNP antibodies (Fig 4b) was blocked by co-treatment with cycloheximide (1 m$\mu$g/ml) (Fig 4d). Similar bindings with FACS analysis were observed for cultured cells incubated with IgG of anti-SS-A/Ro antibodies, but not with IgG of anti-Sm antibodies (Table II, Fig 5a), in which cycloheximide blocked the positive binding of each antibody. The binding of IgG from sera containing anti-SS-B/La antibodies was almost similar to that of IgG of anti-SS-A/Ro antibodies (Fig 5b).

**DISCUSSION**

The significance of HSP72 in the pathogenesis of SLE is a hotly debated topic [27–29]. Autoantibodies to HSP90 [30,31] and enhanced expression of HSP70 gene in peripheral blood mononuclear cells [32] have been demonstrated in patients with SLE. These reports suggest SLE patients may respond to stress stimuli (i.e., increases in temperature, exposure to toxic chemicals, and UVL) with an induction of stress protein or HSP synthesis [20]. The binding of IgG antibodies from sera containing anti-ENA antibodies to cultured keratinocytes has been shown to be related to the pathogenesis of cutaneous LE [17–19]. Ultraviolet irradiation [17] and estradiol [18,19] have been shown to enhance the binding to cultured keratinocytes of IgG antibodies from sera containing anti-U$_1$RNP and anti-SS-A/Ro antibodies. The interaction of HSP with steroid hormone receptors for estrogen, progesterone, and glucocorticoid is well known [20] and, as shown in the present study, UBV irradiation induces HSP72. Because the skin is one of the major target organs in SLE, the pathogenesis of cutaneous lesions may be related to the effects of HSP inducers or stress protein inducers on the cultured keratinocytes. Relevant to this point, our study has shown that $\Delta^{12}$-PGJ$_2$ can induce HSP72 formation and augment binding of IgG antibodies from sera containing anti-U$_1$RNP and anti-SS-A/Ro antibodies in normal human cultured keratinocytes.

Some kinds of PG have been shown to induce HSP during PG-induced inhibition of cell proliferation [33]. One of cyclopentenone PG, $\Delta^{12}$-PGJ$_2$ induces several specific proteins, mainly those from the HSP family, prior to G$_1$ arrest [34]. Previously we reported that $\Delta^{12}$-PGJ$_2$ potently inhibited cell growth, induced protein synthesis (probably of one of the HSP), and affected the arrangement of actin filaments in PAM 212-transformed mouse epidermal cells in culture [35,36].

Recently we demonstrated the induction of HSP72 in transformed human epidermal cells in culture [21]. In this study, the concentration of $\Delta^{12}$-PGJ$_2$ that we used for the expression of HSP72 was 10 m$\mu$g/ml medium. An 18-h exposure to this concentration of $\Delta^{12}$-PGJ$_2$ caused 50% inhibition of cell growth of 80% confluent cells (data not shown). At 50% inhibition of cell growth, these cells...
were arrested at the G1 phase and were alive, as verified by AO/EB staining, whereas higher doses led to cell death [21,37]. Therefore, we consider that antibodies to cytoplasmic or nuclear constituents are unable to enter non-specifically into the cell. We also consider that the cytotoxic effect and induction of IgG of anti-ENA antibodies are independent.

Interestingly, the disappearance of actin filaments and the disarrangement of keratin filaments were described in our recent investigations [38] as well as transformed cells in culture [21,35,36]. Consequently, the cell surface binding of IgG antibodies from LE or other sera was present in our sera as determined by immunoblot analysis and the antisera probes. We need further investigations to determine the association between HSP and the translocation of these antigens to the cell membrane. However, on the basis of previous reports and the present study, we believe that exposure of keratinocytes to various kinds of biologic stress translocates the nuclear antigens, U1 RNP, SS-A/Ro, and SS-B/La, to the cell surface of human keratinocytes. We have also found that heat-shock treatment at 43°C for 90 min induced binding to cultured human keratinocytes of IgG antibodies from sera containing anti-U1RNP and anti-SS-A/Ro antibodies (manuscript in preparation). These keratinocytes then experience lysis consequent to complement activation or more likely by antibody-dependent cell cytotoxicity mediated by lymphocyte or monocyte effectors [41-43]. This study provides new insights into the mechanisms by which erythematosus lesions of LE occur at skin sites susceptible to environmental factors including UV and other stressors.

### Table I. Binding of IgG Antibodies from Sera Containing Antinuclear Antibodies to Cultured Keratinocytes Stimulated with Δ12-PGJ2

<table>
<thead>
<tr>
<th>PG</th>
<th>U1 RNP</th>
<th>SS-A/Ro</th>
<th>Sm</th>
<th>ssDNA</th>
<th>Murine MoAb</th>
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<tbody>
<tr>
<td>Δ12-PGJ2</td>
<td></td>
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<tr>
<td>1 µg/ml</td>
<td>5.8 ± 6.1</td>
<td>7.0 ± 6.7</td>
<td>5.4 ± 3.1</td>
<td>3.8 ± 2.8</td>
<td>4.3 ± 5.2</td>
</tr>
<tr>
<td>10 µg/ml</td>
<td>26.5 ± 8.1</td>
<td>20.1 ± 8.8</td>
<td>5.2 ± 6.4</td>
<td>5.1 ± 5.0</td>
<td>5.8 ± 5.0</td>
</tr>
<tr>
<td>PGFα 10 µg/ml</td>
<td>4.0 ± 3.6</td>
<td>2.9 ± 2.6</td>
<td>2.4 ± 3.5</td>
<td>1.7 ± 2.2</td>
<td>1.8 ± 3.6</td>
</tr>
<tr>
<td>PGFβ 10 µg/ml</td>
<td>3.7 ± 4.8</td>
<td>3.1 ± 3.8</td>
<td>3.0 ± 2.9</td>
<td>3.3 ± 4.3</td>
<td>1.9 ± 2.8</td>
</tr>
</tbody>
</table>

Table II. FACS Analysis of Binding of IgG of Anti-ENA Antibodies to Cultured Keratinocytes Stimulated with Δ12-PGJ2 (10 µg/ml for 18 h)

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Binding (%)</th>
</tr>
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<tbody>
<tr>
<td>Anti-SS-A/Ro</td>
<td>25.8 ± 7.3</td>
</tr>
<tr>
<td>Anti-U1RNP</td>
<td>30.4 ± 11.2</td>
</tr>
<tr>
<td>Anti-Sm</td>
<td>5.8 ± 8.9</td>
</tr>
<tr>
<td>NHS</td>
<td>3.5 ± 3.7</td>
</tr>
</tbody>
</table>

* The results represent mean values from three experiments.

** Mean ± 1 SD.

*p < 0.05 versus control.

0.02 < p < 0.05.

Figure 4. Flow cytometry analysis of binding of IgG antibodies from sera containing anti-U1RNP antibodies to cultured keratinocytes. a, untreated cells. b, cells treated with 10 µg/ml of Δ12-PGJ2 for 18 h. c, cells treated with 1 µg/ml of cycloheximide. d, cells treated with both 10 µg/ml of Δ12-PGJ2 and 1 µg/ml of cycloheximide for 18 h.

Figure 4. Flow cytometry analysis of binding of IgG antibodies from sera containing anti-U1RNP antibodies to cultured keratinocytes. a, untreated cells. b, cells treated with 10 µg/ml of Δ12-PGJ2 for 18 h. c, cells treated with 1 µg/ml of cycloheximide. d, cells treated with both 10 µg/ml of Δ12-PGJ2 and 1 µg/ml of cycloheximide for 18 h.
This work was supported by a grant from the Japanese Ministry of Education, Science and Culture, by a grant in aid for Comprehensive Ten Year Strategy for Cancer Control from the Ministry of Health and Welfare of Japan, the Japanese Intractable Disease Research Foundation, and the Cosmetology Research Foundation.

REFERENCES