mAgK114 Suppresses Lymphocyte Infiltration into Epidermis in the Picryl Chloride-induced Atopic Dermatitis-like Skin Lesions of NC/Nga Mice

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Abstract. Background: We have previously shown that when mouse AgK114 (mAgK114, a glycosylphosphatidylinositol anchored membrane-associated protein) is applied to the wound area, the inflammatory responses in the early recovery phase of damaged tissue are enhanced and wound closure is accelerated. This suggests that mAgK114 has an important effect on skin wound repairing. Materials and Methods: Whether mAgK114 suppresses the development, in NC/Nga mice, of atopic dermatitis (AD)-like skin lesions induced by repeated application of 2,4,6-trinitrochlorobenzene (picryl chloride, PiCl) was examined under specific pathogen-free conditions. Results: Histopathologically, the application of mAgK114-ointment to the PiCl-treated NC/Nga mice remarkably suppressed severe lymphocytic infiltration into the epidermis, although total skin severity scores, histological changes in hypertrophy, erosion and infiltration of inflammatory cells into the corium and subcutaneous tissues were comparable between the mAgK114-treated group of mice and the control group. Conclusion: Our results suggest that mAgK114 would be beneficial for the treatment of atopic dermatitis by suppressing severe lymphocytic infiltration into the epidermis.

We have previously identified a novel glycosylphosphatidylinositol (GPI) anchored membrane-associated protein, AgK114, from hamster keratinocytes (GenBank Accession No. AB154828) (1). The AgK114 was restrictly expressed on the dermal sheath cells near bulged areas of the hair follicle, and on the differentiated sebocytes of normal adult hamster skin (1). Interestingly, AgK114 was induced in basal keratinocytes after UV exposure, and was also induced on edged keratinocytes adjacent to the incision area (1). Thereafter, homologues of AgK114 were identified in pigs, humans and mice at the DNA level (placenta-expressed transcript 1; PLET1, GenBank Accession Nos. AY351651, AY364431 and AY364436) (2). Furthermore, a splicing variant coding a presumptive soluble form was also registered for the mouse (NCBI Nucleotide Accession No. AK002767 or NCBI Protein Accession No. BAB22342). It seems likely that AgK114 is involved in various physiological and pathological processes. However, the biological functions of AgK114 remain to be revealed.

As an attempt to elucidate its biological functions, the effects of exogenously administered mouse AgK114 (mAgK114) were investigated on mouse cutaneous wound healing (3). It was observed that mAgK114 promoted the production of various inflammation-related proteins in the impaired skin, such as pro-matrix metalloproteinase 9 (pro MMP-9), transforming growth factor-β1 (TGF-β1), interleukin-6 (IL-6) and IL-1β. Furthermore, the production of vascular endothelial growth factor (VEGF) was also enhanced by AgK114. On histopathological examination, the development of blood vessels was observed. AgK114 seemed to enhance the inflammatory responses in the early stage and to accelerate the destruction of the wounded tissues. On the other hand, it also stimulated the development of blood vessels to regenerate cutaneous tissues. Overall, AgK114 promoted the wound healing of the impaired skin.

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by pruritic, rubor, edema, ulcer formation, dry skin and cutaneous hypertrophy (4). The incidence of AD has been gradually increasing recently, and it is suggested that environmental and social factors may be involved (4, 5). AD is an intractable disease and its etiology
nor treatment are fully understood, mainly because of a lack of suitable animal models. Recently, it was reported that itching dermatitis, which is very similar to human AD, develops spontaneously in NC/Nga mice, fed under conventional conditions (6). Furthermore, it has been reported that repeated application of picryl chloride (PiCl) to NC/Nga, mice under specific pathogen-free (SPF) conditions, causes the development of AD-like skin lesions (7). Several articles mentioned that PiCl-treated NC/Nga mice revealed skin lesions macroscopically characterized by erythema, edema and excoriation (7, 8). Histologically, hypertrophy of the epidermis and dermis and massive infiltration of inflammatory cells, which resemble typical human AD symptoms, are observed.

Since mAgK114 enhances inflammatory responses and promotes wound healing in impaired skin, the effects of mAgK114 on AD skin lesions were examined using the NC/Nga mouse model.

Materials and Methods

Mice. All animal procedures were in accordance with the Declaration of Helsinki, and were approved by the committee of animal rights in our institute. Female 5-week-old NC/Nga mice were purchased from Charles River Japan (Yokohama, Japan) and were maintained under SPF conditions. The mice had solid feed (CRF-1, Oriental Yeast, Tokyo, Japan) and ultrafiltration (UF) membrane-filtrated water ad libitum.

Preparation of recombinant mAgK114-1 protein. The total RNA was prepared from primarily cultured mouse ear keratinocytes. The cDNA fragment encoding the presumptive soluble form of mAgK114 (NCBI Nucleotide Accession No. AK002767, NCBI Protein Accession No. BAB22342, 194 amino acid residues (a.a.)) was cloned by reverse transcription-polymerase chain reaction (RT-PCR). After confirmation of the nucleotide sequence, the cDNA was inserted into pREF-XN, an eukaryotic expression vector (9), in which the expression of the gene is under the control of the cytomegalovirus enhancer and the EF-1 promoter. The resulting expression vector was transfected into CHO-K1 cells, maintained in Ham’s F12 medium supplemented with 10% fetal calf serum (FCS), by electroporation with a Gene Pulser (Bio Rad Laboratories, Hercules, CA, USA). After selecting the transformed cells in the presence of 400 μg/ml G-418 (Geneticin, Invitrogen, Carlsbad, CA, USA) and screening, the clone with the highest production level, CHO-mAgK114b 16-38, was chosen. The soluble mAgK114 protein was purified from the culture supernatants by a series of column chromatography on WGL-sepharose 6B (Amersham Bioscience, Piscataway, NJ, USA), chelating-sepharose (Amersham Bioscience) and Superdex 200 (Amersham Bioscience).

Reagents. Picryl chloride (PiCl, 1-chloro-2,4,6-trinitrobenzene, Katayama Chemical Industries, Osaka, Japan) was used after recrystallization with ethanol. Fifty μg of mAgK114 was dissolved in 0.1 g of a hydrophilic ointment (Merck Hoei, Osaka, Japan). Prednisolone (Wako Pure Chemical Industries, Osaka, Japan) (0.5% w/w) was dissolved in 0.1 g of the hydrophilic ointment.

Development and treatment of dermatitis. The dermatitis was induced by PiCl in NC/Nga mice according to the standard instructions provided by Charles River Japan. Briefly, the abdomen and foot pad of the mice were sensitized epicutaneously with 150 μl of 5% PiCl dissolved in a mixture of ethanol and acetone (4:1). Five days after the sensitization, the dorsal skin of the mice was challenged with 150 μl of 1% PiCl dissolved in olive oil under anesthesia with pentobarbital sodium (Takeda Schering-Plough Animal Health, Tokyo, Japan). After the first challenge, 1% PiCl solution was repeatedly applied to the dorsal skin of the mice 8 more times at intervals of 1 week. The AgK114-1 ointment (2.5 mg/kg) was applied to the dorsal skin, 3 times a week for 5.5 weeks starting from the 17th day after sensitization with 5% PiCl. As a positive control for the treatment of dermatitis, 0.1 g of 0.5% prednisolone ointment (25 mg/kg) was applied. As a negative control, 0.1 g of the hydrophilic ointment was applied to the dorsal skin 3 times a week. The body weights of the NC/Nga mice were monitored once a week during the experimental period.

The severity of dermatitis was assessed macroscopically in a blind fashion twice a week starting from the third challenge, and was expressed as the sum of the individual score grades from 0 (no symptoms), 1 (mild), 2 (moderate) to 3 (severe) for each of the following 4 signs and symptoms: (i) erythema/hemorrhage, (ii) excoriation/erosion, (iii) scaling/dryness, and (iv) the extent of excoration, as described previously (10).

Serum IgE levels. Blood was collected from the postcaval vein 56 days after the sensitization. Serum samples were obtained by centrifugation and stored at –80°C until assay. Total serum IgE levels were determined by ELISA using the capture anti-mouse IgE monoclonal antibody (mAb; #R35-72) and detecting biotinylated anti-mouse IgE mAb (#R35-118) pairs according to the procedure suggested by BD Bioscience PharMingen (San Jose, CA, USA). Purified mouse IgE (#03121D, PharMingen) was used as a standard. The lower detection limit of this system was 7.8 ng/ml.

Histological analysis. The mice were sacrificed on day 56 after the sensitization. The dorsal skin was removed, fixed in 10% buffered formalin, embedded into paraffin, stained with hematoxylin-eosin and examined by light microscopy. From the histopathological findings, the severity of dermatitis was assessed in a blind fashion on the epidermis (hypertrophy, hyperkeratosis, parakeratosis), erosion and inflammatory cell infiltration and on the corium (inflammatory cell infiltration), and was expressed as the sum of the individual score grades from 0 (no symptoms), 1 (mild), 2 (moderate) to 3 (severe), as described previously (11).

Statistical analysis. The data were expressed as the mean±S.D. The statistical significance of differences in the severity of dermatitis, observed macroscopically and histopathologically, was assessed by the Mann-Whitney U-non-parametric test. The statistical significance of differences in serum IgE levels was assessed by the Student’s t-test. P-values less than 0.05 were considered to be statistically significant.

Results

Effect of AgK114 ointment on the clinical skin scores of AD-like skin lesions in PiCl-treated NC/Nga mice. It is known that the NC/Nga mice develop AD-like skin lesions by repeated application of PiCl under SPF conditions (7). Consistent with
this finding, the macroscopical skin severity scores in the control NC/Nga mice increased gradually depending on the times of PiCl challenge (Figure 1). All the mice in this group exhibited AD-like skin lesions, erythema, hemorrhage, excoriation, scaling and dryness. The application of mAgK114 had no obvious effects on the development of AD-like skin lesion and no significant differences in the clinical skin severity scores were observed between the control group and the mAgK114-treated group throughout the experimental period (Figure 1). In contrast, the application of the prednisolone ointment significantly suppressed the development of the skin symptoms on days 48 and 55 after sensitization. However, significant weight loss was observed in the prednisolone-treated group of mice as a side-effect (Figure 2), while the application of mAgK114 did not cause any significant weight loss compared with the control mice throughout the experimental period.

**Effects of AgK114 on serum IgE levels.** On day 56 after the sensitization, sera were obtained and examined for the serum IgE levels. Total serum IgE levels increased in the control group (IgE, 40.5±19.4 µg/ml, mean±S.D., n=10) compared with the values (0.12±0.15 µg/ml) reported in normal NC/Nga mice maintained under SPF conditions (8). Application of the mAgK114 ointment had no significant effects on the serum IgE levels (52.4±25.6 µg/ml, mean±S.D., n=10). However, application of the
prednisolone ointment suppressed the IgE levels significantly (7.3±3.4 μg/ml, mean±S.D., n=10, p<0.01) (Figure 3).

Histological analysis. When the severity of the dermatitis was expressed as the sum of score grades assessed based on the histological findings, the total score of the control NC/Nga mice reached 11.6±2.07 (mean±S.D., n=10). The application of mAgK114 resulted in a histological score comparable to that of the control group (total score 11.7±2.83). Although it is not statistically significant, the application of the prednisolone ointment (total score: 7.4±4.62) reduced the histological score compared with that observed in the control group of mice (Table I).

Interestingly, in the mAgK114-treated group, the score of inflammatory cell infiltration into the epidermis was significantly lower than that of the control group (Mann-Whitney, p<0.05) (Figure 4). The control NC/Nga mice showed remarkable AD-like skin lesions, including hypertrophy and severe scab of the epidermis. Severe infiltration of inflammatory cells was also observed in the epidermis and corium (Figure 5); in fact, lymphocytes were infiltrated diffusely and strongly throughout the whole epidermis. Severe lymphocyte infiltration into the epidermis caused extracellular edema of the epidermis and liquefaction degeneration of the basal layer. In marked contrast, in the mAgK114-treated group, no severe lymphocyte infiltration into the epidermis was observed (Figure 6). Furthermore, only a trace of lymphocyte infiltration was observed in the stratum basale, although hyperkeratosis and accelerated parakeratosis were observed in this group.

Discussion

AgK114 expression was transiently enhanced in the impaired epithelial keratinocytes by UV exposure and excised injury. In the UV exposure experiment, AgK114 expression preceded the appearance of regenerated keratinocytes (1). In the physical wound experiment, the expression of AgK114

Table I. Histopathological findings of the PiCl-induced AD in NC/Nga mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Endothelium</th>
<th>Epithelium</th>
<th>Inflammatory cell infiltration</th>
<th>Extracellular edema</th>
<th>Liquefaction</th>
<th>Total score</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>14</td>
<td>11.6</td>
</tr>
<tr>
<td>mAgK114</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>14</td>
<td>11.7</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>14</td>
<td>7.4</td>
</tr>
</tbody>
</table>

Grade of histopathological findings: 0: No abnormality detected. 1: Mild. 2: Moderate. 3: Marked.

Both in the mAgK114-treated group and prednisolone-treated group, inflammatory cell infiltration (*) into the epidermis was significantly decreased compared to the control (Mann-Whitney, p<0.05).
was detected on the reconstituting epidermal keratinocytes, which were elongating to the edge of the cutaneous wounding in the healing of the excised wound (3). Thus, we consider that AgK114 is an injury-responsive molecule and that it enhances the inflammatory responses in the early recovery phase of damaged tissue.

To assess the effects of exogeneously applied mAgK114 on AD-like skin lesions, NC/Nga mice were used (7, 8). The PiCl-treated NC/Nga mice in the control group revealed skin lesions, characterized macroscopically by erythema, edema, excoriation and scaling, and histologically by hypertrophy of the epidermis and severe infiltration of the inflammatory cells. These pathophysiological changes of the skin in NC/Nga mice are very similar to those in human AD patients. In a previous study, we reported that AgK114 enhanced the inflammatory responses and might accelerate destruction in damaged tissue. Therefore, there was a possibility that the AD-like symptoms might deteriorate by applying AgK114.

However, application of mAgK114-1 did not deteriorate the development of AD-like skin lesions, and no significant differences in either total clinical or histological scores were obtained compared with those of the control group. Unexpectedly, a significant difference was observed in one of the histological findings, the infiltration of inflammatory cells, in the mAgK114-treated group. In the control group, severe lymphocyte infiltration into the epidermis was observed in almost all the mice. A large number of lymphocytes were diffusely infiltrated into the epidermis, which caused extracellular edema of the epidermis and liquefaction degeneration of the basal layer. Severe lymphocytic invasion...
into the epidermis would induce breakdown of the epidermis and promote skin erosion. In contrast, in the mAgK114-treated group, only a trace of lymphocyte infiltration was observed in the stratum basale, and the epidermal structure was close to that of normal mice, although the clinical skin scores were comparable with those in the control group. The reason for these findings remains unknown. However, it is possible that epidermal turn over would easily take place in the mAgK114-treated group, resulting in a rapid recovery from the AD-like skin lesions.

AD has been reported to be induced by the activation of inflammatory cells such as T cells, mast cells and eosinophils, and the cytokine network has been suggested to play extremely important roles in the infiltration of inflammatory cells (12). As mentioned above, AgK114 enhanced the expression of several inflammation-related cytokines in skin (3). We expected that the resulting cytokines would affect the course of an experimental model of AD; however, AgK114 showed neither a deteriorating nor ameliorating effect on the experimental AD, except for the suppression of infiltrating cells.

Up-regulation of ICAM-1 expression in keratinocytes has been reported in several inflammatory dermatoses such as AD. In inflammation, CD4+ T cells are recruited in the dermis and induced to produce IFN-γ. IFN-γ, in turn, up-regulates the ICAM-1 expression in keratinocytes (13, 14) and induces adhesion of T cells to keratinocytes (15, 16). Thus, T cells infiltrate into the epidermis. Although AD had been previously shown to be a Th2-type disease, recent studies have revealed that Th1-type cytokines, such as IFN-γ, especially in chronic skin lesions, play important roles in the pathogenesis of AD (17, 18). Masuda et al. showed that IFN-γ-producing cells infiltrated into the skin lesions of NC/Nga mice (6). Thus, IFN-γ may play key roles in the infiltration of inflammatory cells in the AD lesions. On the other hand, the increased IFN-γ mRNA, but not the increased interleukin-4 mRNA expression, was significantly down-regulated after successful therapy of AD (19). These results suggest that the suppression of IFN-γ production is effective for the treatment of AD. In our study, mAgK114 suppressed severe lymphocyte infiltration into the epidermis, while the extent of inflammatory cell infiltration into the dermis was similar to that observed in the control group. From our results, together with the literature data, it is tempting to speculate that mAgK114 would inhibit the production of IFN-γ by T cells, resulting in the down-regulation of ICAM-1 expression in keratinocytes and a decrease of T cell infiltration into the epidermis. In our preliminary study, IFN-γ production by LPS-stimulated splenocytes from normal mice was decreased when mAgK114 was injected into the mice intravenously (data not shown). This suggests that mAgK114 has the ability to down-regulate IFN-γ production in vivo. Further studies are required to clarify this possibility.

Previously, we observed that mAgK114 promoted the production of TGF-β1-impaired skin (3). Recently, Sumiyoshi et al. demonstrated that systemic administration of recombinant TGF-β1 to NC/Nga mice with AD-like lesions resulted in suppression of eczematous skin lesions and a down-regulation of IFN-γ production from the splenocytes (20). Furthermore, treatment with the anti-IFN-γ antibody partially ameliorated the skin lesions in NC/Nga mice. Thus, as an alternative mechanism for the mAgK114-mediated suppression of lymphocyte infiltration into the epidermis, mAgK114 may down-regulate IFN-γ production through inducing the production of TGF-β1.

mAgK114 did not suppress the total IgE production in NC/Nga mice. Recently, it has been suggested that increased IgE production and the subsequent IgE-mediated hypersensitivity reaction may not correlate with the development of AD-like skin lesions observed in some patients (21, 22). In this regard, Yagi et al. reported that STAT6-deficient NC/Nga mice, which fail to produce IgE, still suffered from AD-like skin lesions (23). The IgE-independent improvement of dermatitis in NC/Nga mice by the oral administration of persimmon leaf extract was also reported by Matsumoto et al. (24). Furthermore, oral administration of royal jelly suppressed AD-like skin lesions in NC/Nga mice without inhibiting IgE production (8). These results suggest a therapeutic pathway for AD-like skin lesions without inhibiting IgE production.

mAgK114 treatment did not cause the body weight loss and skin atrophy which was observed with prednisolone treatment. Skin atrophy by treatment with steroid ointments has been reported in animals and humans (25, 26). These results suggest that treatment of AD with mAgK114 would not cause any side-effects.

Other points to consider are the presence and the effect of intrinsic mAgK114. In a previous study, we reported that the expression of AgK114 was transiently enhanced after UV exposure and excised injury of the skin (1). However, we did not observe enhancement in the case of AD (data not shown). Although AgK114 was primarily found as a GPI-anchored membrane protein, there also exists a presumptive soluble variant. We might have failed to detect the soluble form of mAgK114 protein and its enhanced expression in the tissue sections. Furthermore, if the expression of mAgK114 were enhanced (probably as a soluble form) in AD, the expressed protein would exert its biological effects in vivo in addition to the exogenously-administered protein. The establishment of tools to detect soluble AgK114 in tissue and AgK114-knockout mice to abolish the influence of intrinsic AgK114 protein are required in order to clarify the situation.

In summary, topical mAgK114 treatment suppressed the severe infiltration of lymphocytes into the epidermis without obvious clinical effects on the skin lesions induced by...
repeated application of PiCl in NC/Nga mice. The application of mAgK114 caused no side-effects, as observed with prednisolone. Thus, mAgK114 treatment would be beneficial in the treatment of AD, at least partially, by inhibiting lymphocyte infiltration into the epidermis.

References


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