The Effect of AgK114 on Wound Healing

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AgK114 is a newly isolated membrane-associated protein which is expressed on keratinocytes. Its expression is restricted to dermal sheath cells near sebaceous glands in normal skin. However, it is transiently induced by UV exposure or injury stimulation (Tatefuji T. et al., Biol. Pharm. Bull. 27, 1742—1749, 2004). Thus, the expression pattern of AgK114 suggested its potential role in wound healing response. We report here that expression of AgK114 is induced in the initial 24 h at the edge keratinocytes during keratinocyte migration, followed by disappearance once epithelialization is completed in the murine excisional wound model. We also demonstrate that exogenous recombinant mouse AgK114FL promoted wound healing process. Mouse AgK114FL up-regulated matrix-metalloproteinase-9, vascular endothelial growth factor, transforming growth factor-β, IL-6, and IL-1β production in the early stage of wound tissue. Moreover, mouse AgK114FL induced the matrix-metalloproteinase-9 activity of wound fibroblasts prepared from impaired skin in the presence of proinflammatory cytokines. These results suggest that the AgK114 participates in the wound response during the healing process, and promotes wound repair.

Key words AgK114; keratinocytes; wound healing

We have identified a novel glycosylphosphatidylinositol (GPI) anchored membrane-associated protein, AgK114, from hamster keratinocytes that is restrictedly expressed on the dermal sheath cells near the bulged area of hair follicle, and on the differentiated sebocytes in normal adult hamster skin.1) Interestingly, AgK114 is transiently expressed in the epidermis in association with tissue damage caused by selected stimuli to the skin. For instance, AgK114 is induced in basal keratinocytes after UV exposure and is also induced on edged keratinocytes adjacent to the incision area, suggesting that AgK114 may play some role(s) in the injury response in the skin.1)

The epidermis is the first line of defense in the skin and responds to damage by the production of a number of wound response proteins.2) Cutaneous wound healing is a complex process involving blood clotting, inflammation, new tissue formation and finally tissue remodeling.3) When skin is injured, the repair process is initiated immediately by the release of various growth factors, cytokines, chemokines, and low-molecular-weight compounds. Inflammatory cells such as neutrophils, monocytes and lymphocytes, produce growth factors, cytokines and chemokines, which initiate the proliferative phase of wound repair in several hours after injury. At 3—7 d after injury, migration and proliferation of keratinocytes at the wound edge commence and are followed by proliferation of dermal fibroblasts in the proximal area of the wound.4—7)

In our previous study, the expression of AgK114 was found to be induced on the edge keratinocytes at 3—5 d after inducing full-thickness wounds by immunohistochemical staining. No biological functions have been reported for any AgK114 molecules of any species except for a report on murine homologue, mouse placenta expressed transcript 1 (mPLET1, Swiss Protein AAQ72439).8) To help understanding the biological functions of AgK114 during the wound healing process and its relationship to other proteins, we have succeeded in preparing soluble recombinant mouse AgK114FL (mAgK114FL) by replacing the C-terminal hydrophobic region with a FLAG sequence and in investigating the autocrine or paracrine functions of this novel protein in the mouse excisional wound model.

In this study, we show specific expression of AgK114 during wound healing process and its activity to enhance the production of cytokines related to wound healing, suggesting a potential application of this protein as an accelerator of wounds repair in future.

MATERIALS AND METHODS

Construction of mAgK114FL Fusion Protein Expression Plasmid The information of mAgK114 cDNA suggested that AgK114, a cell membrane-associated protein, has a GPI anchoring site in a hydrophobic region near the C-terminal. To produce the soluble form of mAgK114FL, the extracellular domain of mAgK114 was fused with FLAG tag sequence. The amino acid sequence of the fusion protein of mAgK114FL and FLAG is shown in Fig. 1. The cDNA was inserted into pREF-XN, an eukaryotic expression vector,9) in investigating the autocrine or paracrine functions of this novel protein in the mouse excisional wound model.

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mAgK114FL. The selected clone (CHO-smAgK114FLBP23) cells were seeded at 1 × 10^6 cells/cm² in 40 roller bottles (1500 cm²/bottle, Becton Dickinson, Franklin Lakes, NJ, U.S.A.) and cultured for 3 d until subconfluent in 400 ml of Ham’F12 medium supplemented with 5% FBS. After changing to ASF medium (Ajinomoto, Yokohama, Japan) for protein harvest, the supernatants were harvested 3 times at 3-d intervals. The supernatants were concentrated using membrane filters (cut-off 6 kDa, Asahi Kasei, Miyazaki, Japan) and were subjected to affinity chromatography with anti-FLAG-M2-agarose (Sigma-Aldrich) according to the manufacturer’s instructions. The eluate fractions were pooled and concentrated using Ultrafree®-MC (cut-off 10 kDa, Millipore, Bedford, MA, U.S.A.). Then we performed Superdex 200 gel filtration chromatography to remove endotoxin.

**Generation of Monoclonal Antibody (mAb) against mAgK114** To generate mAbs against the mAgK114, BN/Cj rats (Charles River Japan, Yokohama, Japan) were immunized intraperitoneally with 20 μg of purified recombinant mAgK114FL with Complete Freund Adjuvant (CFA, Sigma-Aldrich) two times at 2 weekly intervals. The immunized rats and fused with murine myeloma Sp2/0. mAbs were screened for reactivity to mAgK114FL without CFA, splenocytes were prepared from the immunized rats, and fused with murine myeloma Sp2/0. mAbs were screened for reactivity to mAgK114FL by the EIA procedure. As a negative control, FLAG peptide was used. Briefly, Maxisorp® 96 well microplates (Nalge A/S, Roskilde, Denmark) were coated with 2 μg/ml of mAgK114FL or FLAG peptide. After blocking with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS), serially diluted culture supernatant from each hybridoma was added to each well of the plates and incubated. The plates were further incubated with HRPO-conjugated anti-rat Igs antibodies (DakoCytomation A/S, Glostrup, Denmark) diluted at 1:1000 followed by visualization with the ECL western reagents (Amersham Biosciences U.K., Buckinghamshire, U.K.) according to the manufacturer’s instructions.

**Cutaneous Wound Assay** Animal use throughout this study complies with the Declaration of Helsinki and was reviewed by the Committee of Animal usage at our institute. Under anesthesia, 8-week-old male ICR mice (Charles River Japan) were shaved, and 8 mm square full thickness excisional wound was made on each dorsal lateral flank. To investigate the expression of endogenous mAgK114 during wound healing, wounded skins were collected on days 1, 2, 4, and 7 after injury, and immunohistochemical analyses were performed. To test the action of mAgK114FL on wound tissues, 5 μg of mAgK114FL was dissolved in 10 μl of PBS supplemented with 10% glycerin, and administered onto the wounds three times, immediately, 1 d, and 2 d after injury. Equal amounts of BSA were administered onto the control wounds. At the 4 and 7 d after injury, the wound dimensions were determined by measuring the length and width of the wounded area at the defined points, calculated as (length × width).

**Histochemical Studies** Wound tissue was fixed with 10% formalin, dehydrated through an ascending ethanol gradient, and was then embedded in paraffin according to the standard procedures. The tissue was then sectioned at 4 μm with a microtome, and stained with haematoxylin and eosin according to the standard procedures. For immunostaining, after blocking with Block Ace™, the samples received 2 μg/ml of affinity-purified anti-mAgK114 mAb, and were incubated at 4 °C over night. After several washes with 0.05% Tween 20 in PBS, positive staining was detected using Liquid DAB-Black substrate kit (Zymed Laboratories, South San Francisco, CA, U.S.A.) according to the manufacturer’s instructions.

**Western Blotting** Protein samples were denatured by boiling for 5 min in the presence of 2% SDS and 2% dithiothreitol, then separated in 10—20% gradient SDS-PAGE, and were transferred onto nitrocellulose membranes. To detect the immunoreactive protein, after incubating with Block Ace™ (Dainippon Pharmaceutical, Tokyo, Japan), the membranes were incubated with anti-FLAG mAb (Sigma-Aldrich) or affinity-purified anti-mAgK114 mAb, and further incubated with HRPO-conjugated anti-rat Igs diluted at 1:1000 followed by visualization with the ECL western reagents (Amersham Biosciences U.K., Buckinghamshire, U.K.) according to the manufacturer’s instructions.

**Fig. 1. Amino Acid Sequence of mAgK114**
(A) Natural, glycosylphosphatidylinositol (GPI)-anchored type mAgK114 (PLET1: AAQ72439). B: Artificial soluble form (mAgK114FL). C-terminal hydrophobic region was replaced with a FLAG sequence. *: Potential N-glycosylation site, #: predicted GPI-anchor addition site, underlined: signal sequence, double underlined: hydrophobic region, dotted line: FLAG sequence.
mAgK114FL dissolved in 10 μl of PBS mixed with 10% glycerin was administered onto the wounds four times, immediately, 6, 24, and 30 h after injury. Equal amount of BSA was administered on the control wounds. Wounded skins were excised and homogenized in protein lysis buffer (pH 7.8) consisting of 20 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 10 mM EDTA, 10% (v/v) glycerin and complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) on ice, and were centrifuged to remove insoluble tissue debris. Protein concentration of the skin homogenate was determined by the Bradford method using the Bio-Rad Protein Assay (Bio-Rad Laboratories) according to the manufacturer’s instructions.

Immunoassay The amounts of pro-matrix-metalloproteinase-9 (MMP-9), vascular endothelial growth factor (VEGF), IL-1β, IL-6 and transforming growth factor-β (TGF-β) in the homogenates prepared from wounded skin were determined using Quantikine® series (R&D SYSTEMS, MN, U.S.A.) according to the manufacturer’s instructions.

Preparation of Wound Fibroblasts from Wounded Skin The mice with wounds were sacrificed, and the wounded skin was excised 1 d after injury. The tissues were minced and digested with 0.25% collagenase in DMEM (Nissui Seiyaku, Tokyo, Japan) with 3 mM CaCl₂ for 4 h at 37°C. The resultant cell suspensions were passed through 100 μm Nylon mesh and washed with DMEM twice, and the wound fibroblasts were grown for 2 d until subconfluent on collagen type I-coated dishes in DMEM supplemented with 20% FBS and 5 ng/ml basic fibroblast growth factor (bFGF). Then, 2×10⁴ cells/well of wound fibroblasts were seeded onto collagen I-coated 96 well microplates (Asahi Techno Glass, Tokyo, Japan) in the same medium and cultured for 2 d. After changing the medium to serum-free DMEM, wound fibroblasts were treated with various agents.

Zymographic Analysis Gelatinase activity in the culture supernatant of wound fibroblasts was analyzed by gelatin zymography. Gelatinolytic activities were assessed under non-reducing conditions using modified SDS-PAGE. Fifteen microliters of the culture supernatant was mixed with 5 μl of loading buffer (160 mM Tris–HCl, 50% glycerin, 8% SDS, and 0.08% bromphenol blue), and the mixture was applied onto 10% polyacrylamide gels containing 1 mg/ml gelatin. After electrophoresis, the gels were washed 3 times with 2.5%Triton X-100 to remove the SDS and to allow the enzymes to renature. Then the gels were incubated inzymography buffer (5 mM CaCl₂ and 50 mM Tris–HCl, pH 7.5) for 6 h at 37°C and stained with Coomassie brilliant blue G-250 (Sigma-Aldrich). Gelatinolytic activity was identified as a clear band on a blue background.

Statistical Analysis Data were considered to have a parametric distribution and a Student’s t test (two-tail) assuming unequal variance was performed for comparisons between in vivo data points. A Student’s t test assuming equal variance was used for in vitro data points.

RESULTS

Generation of mAb Raised against Mouse AgK114 and mAgK114FL Preparation We established hybridoma clones that produce mAb against mAgK114FL. We here show that mAb #K33-1 (IgG1) recognized mAgK114FL by Western blotting. The molecular range of mAgK114FL that was recognized by both anti-FLAG mAb and anti-mAgK114 mAb was comparable, and this protein appeared as 50—110 kDa smear band (Fig. 2A). There was a discrepancy in the molecular weight of AgK114FL between the value estimated from amino acid sequence (27 kDa) and that from SDS-PAGE. We previously reported that hamster AgK114 would be modified by diverse glycosylation. Another anti-mAgK114 mAb #T21-11G (IgG2a) restrictedly recognized outer root sheath cells and differentiated sebocytes in the hair follicle in normal adult mouse skin tissue by immunohistochemistry (Fig. 2B). The immunoreactivity of mAb #T21-11G in the immunohistochemistry was the same as that we described before with anti-hamster AgK114 mAb. Characterization of mAb #T21-11G by Western blotting was previously described.

Induction of mAgK114 Expression in Wounded Skin In the normal skin, the expression of mAgK114 was restricted in the hair follicle of outer root sheath cells and in the differentiated sebaceous gland cells as shown in Fig. 2B. Although mAgK114 was not expressed in basal keratinocytes, edge of elongating keratinocytes in the basal layer of the epidermis expressed the antigen on the first day after injury (Fig. 3A). Expression increased as keratinocytes elongated and reached a maximum on day 2, and high expression levels were maintained until day 4 after injury (Figs. 3B, 3C). When elongating keratinocytes came in contact with keratinocytes migrating from the opposite side of the wound, mAgK114 expression was extinguished (Fig. 3D).

Effect of mAgK114FL on Wound Healing Based on the results described above, we examined the effects of exogenous mAgK114 on wound healing process. The dimensions of mAgK114FL-treated wounded skins were significantly (p<0.05) smaller than those of control (FLAG peptide-treated) group on day 4 after injury (Fig. 4A). On the
Fig. 3. Induction of mAgK114 Expression in Excised Skin

(A–D) Immunohistochemical staining of mouse wound skin for mAgK114 using affinity purified anti-mAgk114 mAb (T21-11G), is shown at defined time points. Arrows indicate wound edge basal keratinoytes. (A) 1 d, (B) 2 d, (C) 4 d, (D) 7 d after injury. Original magnification: ×90. SG: sebaceous gland.

Fig. 4. mAgK114 Promoted the Wound Healing Response

(A, B) Impaired skin areas treated or untreated with mAgK114FL (day 4, n=16; day 7, n=8, circle shows the value for each mouse; *p<0.05). (C) Blood vessel counts at 7 d after wounding (n=8, circle shows the value for each mouse; *p<0.05). (D, E) Tissue sections of wounded skin on 7 d after the application of control FLAG peptide (D) and mAgK114 (E) are shown. Arrows indicate blood vessels. Original magnification: ×90.
other hand, there was no significant difference on day 7 (Fig. 4B). Histological studies indicated that the rate of re-epithelialization on day 4 and the progression of granulation on both days 4 and 7 were up-regulated. Moreover, the mAgK114FL-treated wounded skin sections showed significant increase in the formation of small blood vessels in the granulation tissue at 7 d after injury (Figs. 4C, D, E).

**Induction of Wound Response Proteins during Wound Healing**

Next, we investigated the effect of mAgK114FL on the production of wound response proteins during wound healing process. Although in the control skin homogenates the amounts of proMMP-9 and VEGF increased on the second day after injury, their increase was recognized on the first day by administration of mAgK114FL. Their production was significantly up-regulated on both 1 and 4 d after injury (Figs. 5A, 5C). Production of TGF-β1 was significantly up-regulated in the mAgK114FL-treated skin on 1 d after injury (Fig. 5B). Furthermore, the amounts of IL-6 and IL-1β in mAgK114FL-treated skin homogenates were higher than those of control at each point. Their increase was significant on 2 and 4 d after injury for IL-6 and for IL-1β, respectively (Figs. 5D, 5E).

**Induction of MMP-9 Production by Wound Fibroblasts**

To further study the paracrine action of AgK114 that is produced on edge keratinocytes during wound healing, we attempted to clarify the physiological functions of AgK114 in ex vivo. We prepared wound fibroblasts from the impaired mouse skin and treated the cells with mAgK114FL alone or combined with proinflammatory cytokines to simulate conditions elicited at wound inflammatory sites. The isolated cells showed heterogeneous phenotype, but they showed alkaline phosphatase (ALP) activity-positive and smooth muscle (SM) α-actin-positive characteristics (Figs. 6A, B). Fig. 6C shows the results of zymographic analysis of MMP-9 in 24 h-cultured supernatant in the presence or absence of 10 μg/ml of mAgK114FL alone or combined with 10 ng/ml TNF-α, IL-1β and IL-6. MMP-9 activity was weakly induced from the wound fibroblasts treated with 10 ng/ml of TNF-α or IL-1β alone. Although mAgK114FL alone did not induce MMP-9 activity at 10 μg/ml, mAgK114FL combined with either TNF-α, IL-1β or IL-6 strongly induced MMP-9 activity.

**DISCUSSION**

The mAgK114 expression during cutaneous wound healing and the effect of exogenously administered mAgK114FL in the mouse cutaneous wound model were investigated here. Although endogenous mAgK114 was constitutively but restrictedly expressed in the hair sheath keratinocytes near sebaceous glands (Fig. 2B), it was induced during the early phase of wound healing (Fig. 3A and C). When elongating keratinocytes covered as a neoepidermis, mAgK114 expression became undetectable in the basal and suprabasal epidermis (Fig. 3D). These results suggest that AgK114 is expressed at onset of keratinocyte extention and proliferation, and has important roles in the process of wound healing.

We also investigated the effects of exogenously administered mAgK114FL on mouse cutaneous wound healing here. Since mouse AgK114FL has potential N-glycosylation sites, it is possible that recombinant mAgK114FL is modified with diverse sugar-chains and it appeared as a 50—100 kDa (Fig. 2A) smear band whereas the molecular size of the deduced mouse AgK114 protein is 27 kDa. When we applied the pro-
From the expression pattern of AgK114 (Fig. 3), it is considered that AgK114 is involved in wound healing process by interacting with these proteins at the elongating keratinocytes. Therefore, we have investigated the production of wound response proteins, such as keratin 16, bFGF, heparin-binding epidermal growth factor (HB-EGF), and transforming growth factor-α (TGF-α), are produced by wound edge keratinocytes after injury, and these proteins promote either keratinocytes migration or proliferation during the wound healing process in skin.\(^2,5,11–15\) Moreover, integrins, and matrix metalloproteinases (MMPs) that are matrix-degrading and -processing enzymes are suggested to be involved in keratinocyte migration and granulation tissue remodeling, and are induced following the inflammatory phase.\(^16–18\) From the expression pattern of AgK114 (Fig. 3), wound edge keratinocytes would be considered to be one of the producer cells of AgK114. Furthermore, it is considered that AgK114 is involved in wound healing process by interacting with these proteins at the elongating keratinocytes.

Then, we have investigated the production of wound response proteins in the impaired skin after treatment with mAgK114FL. The production of proMMP-9, VEGF, TGF-β, IL-6 and IL-1β was increased during the wound healing process during 1—4 d after injury. MMP-9 may be involved in keratinocytes detachment from the basement membrane before migration\(^19,20\) and may also facilitate matrix digestion by neutrophils and macrophages during removal of necrotic tissue.\(^21\) MMP-9 is expressed on extending edge keratinocytes on the initial 1st day after injury and is expressed in granulation tissues followed by disappearance of the basal keratinocytes.\(^18\) This expression pattern is similar to that of mAgK114. Taken together, it is considered possible that up-regulation of proMMP-9 production caused by mAgK114FL administration accelerates wound closure. VEGF is a pleiotropic cytokine that stimulates fibroblast mitosis and migration, and blood vessel formation in the presence of bFGF and TGF-β at the wound skin.\(^6,7\) TGF-β plays also important roles during wound healing and it has been shown to stimulate angiogenesis, fibroblast proliferation, myofibroblast differentiation, and matrix deposition.\(^22,23\) Increase of blood vessel formation and granulation in mAgK114FL-treated mouse skins could be explained by the effects of the enhanced VEGF and TGF-β expression. Both IL-6 and IL-1β are well known proinflammatory cytokines that also play important roles in wound healing. They likely influence various processes at the wound sites, including stimulation of keratinocyte and fibroblast proliferation, synthesis and breakdown of extracellular matrix proteins, fibroblast chemotaxis, and regulation of local the immune response.\(^3\) These cytokines are coordinately and transiently expressed at the inflammation stage of normal wound repair, but the prolonged expression may exacerbate inflammation and impair healing, such as in the case of diabetes. Although exogenous mAgK114FL up-regulated IL-6 and IL-1β production in the early stages of wound healing in our study, continuing mAgK114FL administration or high dose administration of mAgK114 may impede the following healing. These considerations suggest an important role of endogenous mAgK114 in wound healing as a trigger of the process.

We also attempted to identify the cells responding to
mAgK114 and targeted wound fibroblasts as a producer of MMP-9 activity as determined by zymographic analysis in an ex vivo experiment. Wound fibroblasts used in our experiment had ALP activity and expressed SM-α actin, and were therefore considered to be activated myofibroblast-like cells (Figs. 6A, B).\textsuperscript{24} mAgK114FL induced MMP-9 activity by the wound fibroblasts in the presence of the proinflammatory cytokines TNF-α, IL-1β and IL-6 in a synergistic manner, although mAgK114FL alone could not induce MMP-9 activity. These results suggest that at least a part of the proMMP-9 induced by mAgK114FL during wound healing (Fig. 5A) is derived from wound fibroblasts, and that proinflammatory cytokines are also involved in the proMMP-9 induction. From these results we can propose one of the considerable mechanisms of the physiological function of AgK114 in the impaired skin. AgK114 that is produced in edge keratinocytes acts synergistically with proinflammatory cytokines to increase MMP-9 activity by interacting with wound fibroblasts. Furthermore, since activated myofibroblastic cells which are known to be involved in tissue regeneration were identified as the responder cells to mAgK114 under inflammatory conditions, mAgK114 may also be involved in not only wound healing but also in organogenesis.\textsuperscript{25}

In summary, we propose a role of mAgK114 in the promotion of wound healing, as an upregulator of wound response proteins and as an inducer of MMP-9 activity from wound fibroblasts. Wounding induces endogenous mAgK114 expression in edge keratinocytes. These findings suggest the physiological significance of mAgK114 in wound healing and that mAgK114 may have a role as “ideal candidate signals” in the orchestration of the epithelial responses to wounding in the early phase.

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