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Angiopoietin-related growth factor (AGF) supports adhesion, spreading, and migration of keratinocytes, fibroblasts, and endothelial cells through interaction with RGD-binding integrins

Yueqing Zhang, Xiaobo Hu, Ruiyang Tian, Wangui Wei, Wei Hu, Xia Chen, Wei Han, Huayou Chen, Yi Gong *

Shanghai Institute for Biological Sciences, Graduate school of Chinese Academy of Sciences, 500 Cao Bao Road, Shanghai 200233, PR China

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Abstract

Angiopoietin-related growth factor (AGF) is a newly identified member of angiopoietin-related proteins (ARPs)/angiopoietin-like proteins (Angptls). AGF has been considered as a novel growth factor in accelerating cutaneous wound healing, as it is capable of stimulating keratinocytes proliferation as well as angiogenesis. But in our paper, we demonstrate that AGF stimulates keratinocytes proliferation only at high protein concentration, however, it can potently promote adhesion, spreading, and migration of keratinocytes, fibroblasts, and endothelial cells. Furthermore, we confirm that the adhesion and migration cellular events are mediated by RGD-binding integrins, most possibly the α_v-containing integrins, by *in vitro* inhibition assays using synthetic competitive peptides. Our results strongly suggest that AGF is an integrin ligand as well as a mitogenic growth factor and theoretically participates in cutaneous wound healing in a more complex mechanism.

Keywords: Angiopoietin-related growth factor; RGD-binding integrins; Keratinocyte; Fibroblast; Endothelial cell; Cell proliferation; Cell adhesion; Cell spreading; Cell migration

Angiopoietin-related proteins (ARPs)/angiopoietin-like proteins (Angptls) and angiopoietins (Angs) are structurally resemblant glycoproteins characterized by two domains, a N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain. Similar to Angs (Ang1, 2, and 4), ARPs (ARP1/Angptl1, ARP2/Angptl2, Angptl3, ARP4/Angptl4, and AGF/ARP5/Angptl6) regulate angiogenesis [1]. However, recent studies have shown that ARPs exhibit pleiotropic effects not only on vascular cells but also on other cell lineages [2–4].

Among the ARPs, angiopoietin-related growth factor (AGF/ARP5/Angptl6) was recently cloned using EST screening and RACE technology. It was a multimeric glycoprotein secreted by hepatic parenchyma cells and several hematopoietic cells [5]. Some significant biological functions of this protein have been identified based on the investigations from genetically modified mice. Most (>80%) AGF-deficient mice died in embryonic stage with apparent cardiovascular defects, and the survivors developed obesity, lipid metabolic disorders, and insulin resistance [6]. These observations indicate AGF plays an important role in regulating cardiovascular development and energy metabolism. The K14-AGF transgenic mice overexpressing this molecule in epidermal keratinocytes, however, featured thickened epidermis and hypervascularization in the skin. Consistent with the histological characteristics, rapid wound closure was observed in these transgenic mice. Further *in vitro* studies revealed that AGF stimulated keratinocytes proliferation in culture system as well as induced angiogenesis [7]. In addition, AGF was detected from infiltrated platelets and mast cells in

* Corresponding author. Fax: +86 21 64369607.
E-mail address: ygong@sibs.ac.cn (Y. Gong).

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wounded skin but not in normal skin tissue of wild-type mice [5]. Taken together, AGF is considered to be a novel growth factor capable of accelerating wound healing and has the potential to be developed as a new therapeutic agent.

Up to now, three AGF orthologs with complete sequences from humans and rodents can be searched out from NCBI database (Accession Nos: *Homo sapiens* NP_114123.2; *Mus musculus* NP_660136.1; *Rattus norvegicus* XP_216613.3). Noticeably, all the three AGF molecules contain a conservative RGD (Arg-Gly-Asp) motif in the fibrinogen-like domain, whereas none of the other ARPs encompass this motif. This tripeptide sequence extensively exists in many extracellular matrix (ECM) proteins, such as fibronectin, fibrinogen, vitronectin, von Willebrand factor, laminin, tenacin, etc., and mediates adhesion and migration of various cell lineages by binding to single or promiscuous surface integrins [8]. This prompts us to explore the possibility of AGF as an integrin ligand to promote adhesion and migration of the wound healing related cells.

In this paper, we employed HaCaT, NIH/3T3, and PIEC cells as the representative of keratinocytes, fibroblasts, and endothelial cells, respectively. Our results demonstrate that the recombinant human AGF prepared from mammalian cells is capable of promoting adhesion, spreading, and migration of all the three types of cells, while it only stimulates keratinocytes proliferation at high concentration. Furthermore, both the adhesion and migration cellular events are mediated through RGD-binding integrins, most possibly α_v-containing integrins, as the events can be markedly inhibited by the specific competitive peptides.

Materials and methods

Expression plasmid construction. The entire human angiopoietin-related growth factor coding sequence (hAgf) was cloned by fusion of two overlapped fragments amplified from cDNA library and genomic DNA, respectively. First, primers of *cf* (5′ → 3′: CGT CAC CCA GCA GAG CAG TC) and *cr* (5′ → 3′: GAA CAC AGA GTC ACA GCT TCA G) were designed to amplify the 3′ part of hAgf (914 bp fragment) from MATCHMAKER human liver cDNA library (Clontech) by using LA *Taq* polymerase (TaKaRa) with GC I buffer. Second, another pair of primers of *gf* (5′ → 3′: ATG GGG AAG CCC TGG CTG C) and *gr* (5′ → 3′: GGC ACA GGC GCT CCA GGC) were designed to amplify the 5′ part of hAgf (556 bp fragment) from human genomic DNA by using LA *Taq* polymerase with GC II buffer due to its extremely high GC content. Finally, we got entire hAgf by PCR fusing the two fragments shared with 15 bp overlap region using Pyrobest polymerase (TaKaRa) and then inserted it into pMD18T vector (TaKaRa) by T-A cloning. Several clones were subjected to ABI 3730 sequencing (Bioasia, Shanghai), and one accurate clone, designated as pMD18-hAgf, was selected. To express recombinant human angiopoietin-related growth factor (rhAGF) in mammalian cells, an expression vector based on pcDNA3 was constructed. Three used primers were: *pf-out* (5′ → 3′: AAG CGG ATC CGC CAC CAT GAA GTG GGT AAC CTT TCT CCT CCT CCT CTT CAT CTC CG, *Bam*HI); *pf-in* (5′ → 3′: CCT CTT CAT CTC CGG TTC TGC CTT TTC TCA TCA TCA TCA TCA TCG GGC GGG CGC CC) and *pr* (5′ → 3′: ACT GAA TTC AGA GTC ACA GCT TCA G, *Eco*RI). PCR was performed using pMD18-hAgf as the template and *pf-out*, *pf-in*, and *pr* as the primers. The yielded product encodes the mature human angiopoietin-related growth factor (amino acids 21–470) and amino-terminal hexahistidine tag and a rat serum albumin leader (MKWVTFLLLLFISGSAFS) instead of native signal sequence for secretion. After digested with *Bam*HI and *Eco*RI restriction enzymes, the PCR fragment was ligated to pcDNA3 cut with the same enzymes. Accuracy of the recombinant plasmid, pcDNA3/RSAL-His-hAgf, was verified by ABI 3730 sequencing.

Expression and purification of recombinant human AGF (rhAGF). Expression and purification of rhAGF were performed as Chia-Hung Wu et al. described [9]. In brief, NIH/3T3 cells, maintained in DMEM containing 5% calf bovine serum (Invitrogen), were transfected with pcDNA3/RSAL-His-hAgf using Lipofectamine2000 (Invitrogen) according to the manufacturer's manual. The transfectants were selected in 750 μg/ml G418 (Genebase, Shanghai) and individual colonies isolated by cloning cylinders. The clone that secreted the highest amount of rhAGF detected by dot blot was expanded. After several runs of expansion, the cells were transferred into two TripleFlasks (Nunc) and allowed to grow to confluence. Then serum in the culture medium was directly decreased to 0.5%, and conditioned medium (CM) was harvested every two days. The CM (about 800 ml) was pooled and the total proteins were precipitated at 85% ammonium sulfate saturation and collected by centrifugation. The protein pellets were dissolved in 50 ml Ni^{2+} binding buffer (20 mM Tris–HCl, pH 7.9, 0.5 M NaCl, and 10% glycerol) and purified on a Ni^{2+}-NTA resin column (Qiagen). The purified rhAGF was analyzed by SDS–PAGE on 8% polyacrylamide gels under reducing (with 200 mM DTT) and non-reducing (no DTT) conditions, and the separated proteins were stained with Coomassie brilliant blue. The concentration of the purified rhAGF was determined by Bradford Protein Quantification Kit (Biocolor, Shanghai).

Cell culture. HaCaT cells (simultaneous human immortal keratinocyte cell line) were grown in DMEM:F12 medium supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (Amresco). NIH/3T3 cells (mouse fibroblast cell line) and PIEC cells (porcine iliac artery endothelial cell line) were grown in DMEM supplemented with 10% fetal bovine serum and the same antibiotics. All the cell lines were cultivated in a humidified atmosphere containing 5% CO_2 at 37 °C.

MTT assays. MTT assays were conducted to determine the cell proliferation [10]. HaCaT, NIH/3T3 or PIEC cells were trypsinized and adjusted density to 5×10^4 cells/ml by the respective culture medium. The suspended cells were seeded at 5×10^3 cells (100 μl) per well in 96-well cell culture plate (Costar) and incubated for 24 h. Then the cells were gently washed with DMEM:F12 or DMEM twice to remove the serum. Purified rhAGF and EGF (ToYoBo), FGF-10 (provided by Dr. Chen Xia) or VEGF (ProSpec) were serial diluted in the same serum free medium and added into the plate. After 5 days (HaCaT) or 3 days (NIH/3T3, PIEC) stimulation, 50 μl of 1 mg/ml MTT (Amresco) was added to each well followed by incubation for 4 h at 37 °C. The products, formazan crystals, were solubilized with 100 μl acidic isopropanol, and the optical density was determined at OD_{570} on a Bio-Rad microtiter reader.

Cell adhesion assays. Cell adhesion assays were performed as described by Leavesley et al. [11]. Briefly, rhAGF and plasma derived fibronectin (FN) (Roche) were diluted to indicated concentrations in PBS and coated on 24-well flat-bottomed non-tissue-culture-treated polystyrene plate overnight at 4 °C. Nonspecific sites were blocked with 10 mg/ml BSA (Fraction V, Sigma) for 2 h at 37 °C. After blocking, wells were rinsed with PBS for three times just before adding the cells. HaCaT, NIH/3T3 or PIEC cells were harvested when they were grown to 80–90% confluence by limited trypsin–EDTA treatment, washed three times by DMEM:F12 or DMEM containing 1 mg/ml BSA, and diluted to 2×10^5 cells/ml in the same medium. One hundred thousand cells (1×10^5 cells) in 0.5 ml medium were added into the coated wells, and the plates were incubated at 37 °C for 2 h. Nonadherent cells were removed by gently PBS washes. Remaining cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and stained by 0.5% crystal violet in 25% methanol for 30 min. The wells were rinsed with tap water carefully, stained cells were solubilized with 1% SDS, and OD_{570} values were determined. For adhesion inhibition assays, the synthetic blocking peptides, linear RGDS, RFDS (Genebase, Shanghai), and cyclic GPenGRGDSPCA (American

peptide) peptides, were preincubated with cells for 30 min at 37 °C before their addition to the wells coating with 4 μg/ml rhAGF.

Cell migration assays. Cell migration assays were performed with 24-well Transwell plate (8.0 μm pore size, Costar) according to a modified protocol described by Leavesley et al. [12]. The under surface of the membrane was coated with rhAGF or FN, diluted to appropriate concentration in DMEM:F12 or DMEM at 4 °C overnight, and blocked with 1 mg/ml BSA at 37 °C for 2 h. HaCaT, NIH/3T3, and PIEC cells were harvested and washed as described above. After last wash step, cells were adjusted to the density of 4×10^5 cells/ml with the same medium. Forty thousand cells (4×10^4 cells) in 100 μl medium were added in the upper chamber and allowed to migrate from upper chamber to the lower chamber for 20 h. Nonmigratory cells were removed from the upper surface with cotton swabs. Cells that had migrated through the membrane were then fixed with 4% paraformaldehyde for 30 min and stained by 0.5% crystal violet for 30 min. After extensively washing, the stained cells were solubilized with 500 μl of 1% SDS and OD_{570} value was determined. For migration inhibition assays, linear RFDS and cyclic GPenGRGDSPCA peptides were premixed with the cells before their addition to the wells.

Results

RGD motif is conserved in AGF orthologs of humans and rodents

Three complete AGF sequences, human AGF (hAGF), mouse AGF (mAGF), and rat AGF (rAGF), are obtained from NCBI protein database. The hAGF, mAGF, and rAGF molecules contain 470, 457, and 387 amino acids, respectively, and share with more than 65% amino acid identities. Each AGF sequence contains a conservative RGD motif in its fibrinogen-like domain, whereas no other members of ARPs and Angs have this tripeptide sequence (Fig. 1). The RGD motif existence reminds us AGF may support adhesion and migration of the wound healing related cells as many typical RGD-containing ECM proteins do.

Preparation of recombinant human AGF (rhAGF) in mammalian cells

To study biological functions of AGF on wound healing related cells, rhAGF comprising mature hAGF and N-terminal hexahistidine tag were expressed and purified from conditioned medium of transfected NIH/3T3 cells (Fig. 2A). The rat serum albumin leader is a strong signal peptide that can efficiently lead rhAGF to secrete into the medium. The concentration of rhAGF in the conditioned medium was estimated to 2–4 mg/l by semi-quantitative dot blot assay (data not shown). After one-step purification, we obtained about 2 ml of 420 μg/ml rhAGF with more than 90% purity. SDS–PAGE revealed that rhAGF was a huge protein with >220 kDa molecular mass under nonreducing condition. While under reducing condition, the observed molecular mass of the major rhAGF band is about 66 kDa (Fig. 2B). These electrophoresis results under different conditions indicate that rhAGF is a multimeric protein in its natural state.

High concentration of AGF stimulates keratinocytes proliferation

Former study demonstrated that AGF could promote keratinocytes proliferation by comparing the colony mag-

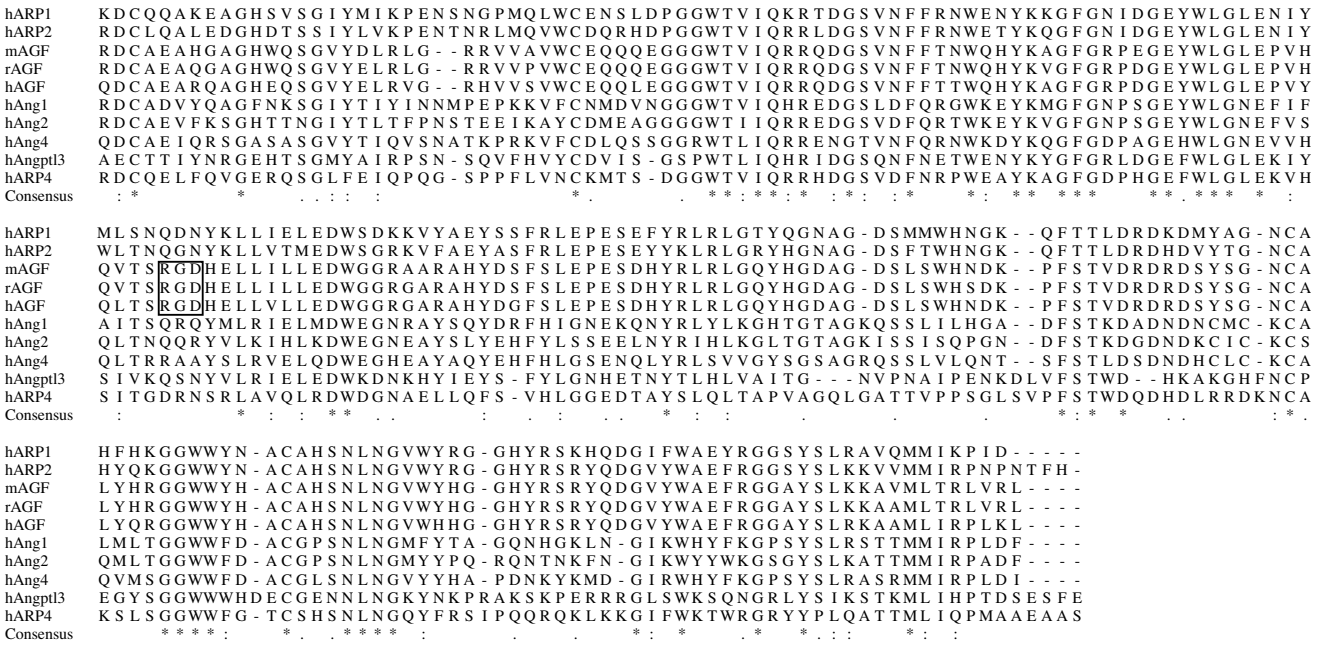

Fig. 1. Alignments of the amino acid sequences of the fibrinogen-like domain of human angiopoietin-related proteins (ARPs)/angiopoietin-like proteins (Angptls) and angiopoietins (Angs). Domain sequences from human AGF (hAGF), mouse AGF (mAGF), rat AGF (rAGF), human ARP1 (hARP1), human ARP2 (hARP2), human Angptl3 (hAngptl3), human ARP4 (hARP4), human Ang1 (hAng1), human Ang2 (hAng2), and human Ang4 (hAng4) are aligned by ClustalX 1.83. All the three AGF orthologs (hAGF, mAGF, and rAGF) contain the conservative RGD motif (in black box). In consensus line, identical residues were indicated by a "*", high similarity residues by a ":", less similarity residues by a "." and unrelated residues by a space.

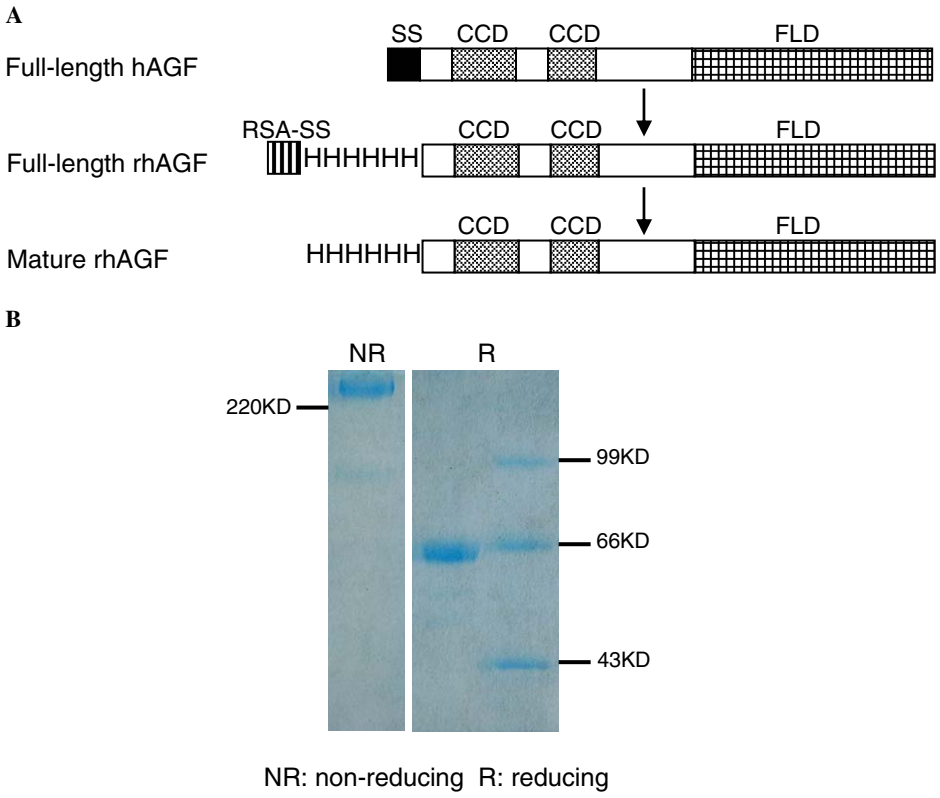

Fig. 2. Recombinant human AGF (rhAGF) preparation. (A) Schematic diagram of the protein structure of recombinant human AGF. SS, putative native signal sequence (amino acids: 1–20); RSA-SS, rat serum albumin signal sequence; CCD, coiled-coil domain; FLD, fibrinogen-like domain. Hexahistidine tag is fused at the N-terminal of mature AGF sequence (amino acids: 21–470) to facilitate protein purification. (B) Coomassie-stained SDS–polyacrylamide gel of purified rhAGF under reducing and non-reducing conditions.

nitudes of stably transfected and primary keratinocytes [5]. Nevertheless, such mitogenic activity is still lack of confirmation on the protein level. In our experiment, we verified that rhAGF do stimulate HaCaT proliferation, but only at high concentration (>1 μg/ml). To NIH/3T3 and PIEC cells, rhAGF did not show mitogenic effect (Fig. 3), which is consistent with the result from HUVEC cells [7].

AGF supports adhesion and spreading of keratinocytes, fibroblasts, and endothelial cells

To test whether or not AGF can serve as a substrate to support adhesion and spreading of keratinocytes, fibroblasts, and endothelial cells, rhAGF were coated on non-tissue-culture-treated 24-well plates. As shown in Fig. 4A, these three types of cells were all capable of adhering to immobilized rhAGF in a dose-dependent manner. The adhesive curves were quite similar, all the cells achieved maximal adhesion at the coating concentration of 2–3 μg/ml within 2 h. When compared with positive control of fibronectin, rhAGF showed almost equivalent adhesive potency for NIH/3T3 and PIEC cells, but less effectiveness for HaCaT cells in the coating range of 1–20 μg/ml. On the other hand, cell morphology was also examined under the phase-contrast microscope after the cells were added into the coated wells. We observed that all the HaCaT, NIH/3T3, and PIEC cells began to spread on immobilized rhAGF and fibronectin about 20 min later, and achieved complete spreading within 2 h, whereas the cells in BSA-coated wells (negative control) still kept round shape and floating (Fig. 5). These experimental results suggest that AGF can act as a potent substrate supporting adhesion and spreading of keratinocytes, fibroblasts, and endothelial cells.

AGF supported adhesion is mediated by the RGD-binding integrins

Many RGD-containing ECM proteins promote cell adhesion and migration via specific members of integrins at this tripeptide sequence [8]. These integrins, named RGD-binding integrins, include $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_{IIb}\beta_3$, and α_v-containing integrins ($\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, and $\alpha_v\beta_8$) [13]. It is natural to ask whether rhAGF supported adhesion and migration are also mediated by the RGD-binding integrins. To answer this question, linear synthetic peptide of RGDS that could competitively bind to these integrins as well as the non-competitive control of RFDS was employed to perform adhesion inhibition assays [14]. RGDS peptide showed dose-dependent inhibition of all the three types of cells adhering to the immobilized rhAGF, whereas RFDS did not exhibit marked inhibition even when the concentration increased up to 1000 μM. To

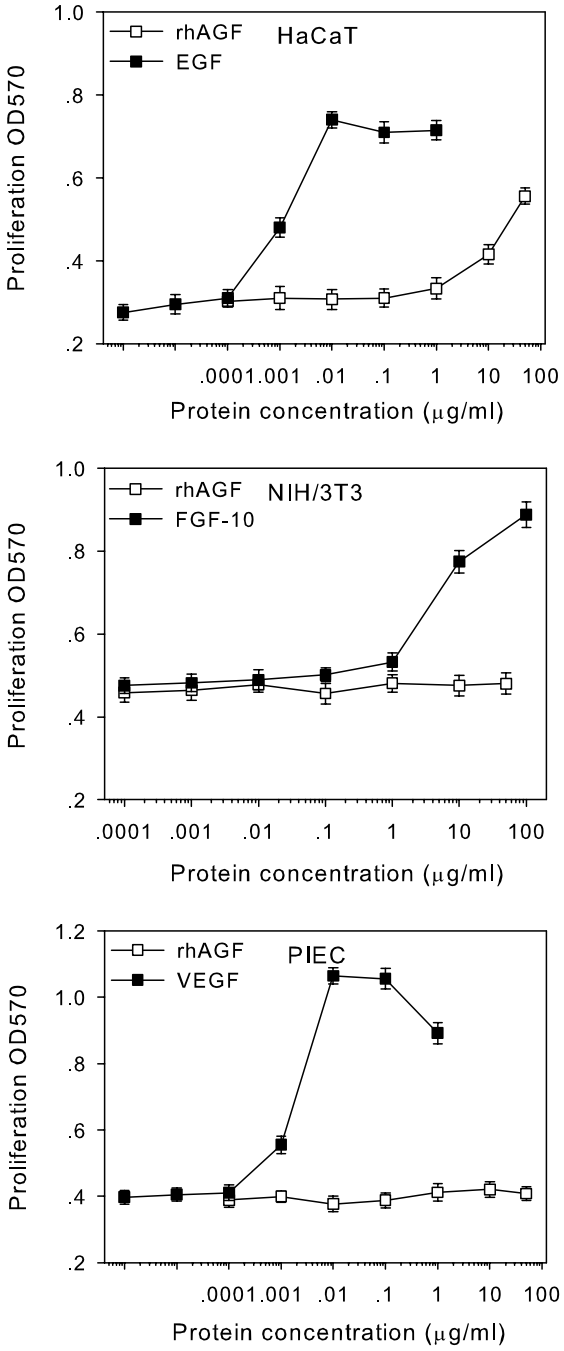

Fig. 3. AGF stimulates keratinocytes, but not fibroblasts and endothelial cells, proliferation. MTT assays were employed to evaluate the mitogenic activity of rhAGF on HaCaT, NIH/3T3, and PIEC cells. EGF, FGF-10, and VEGF growth factors were used as the positive controls. The results demonstrate that rhAGF does stimulate HaCaT proliferation, but only at high protein concentration. Values represent means ± SD from triplicate samples.

HaCaT, NIH/3T3, and PIEC cells, RGDS achieved 50% inhibition at the concentration of about 200 μM, 250 μM, and 600 μM, respectively. Competitive inhibition assays were further conducted with a cyclic peptide containing the penicillamine motif, GPenGRGDSPCA, which has been shown to have some specificity for the integrins containing the α_v subunit. This cyclic peptide, as reported, displayed almost 10 times more effective activity in inhibiting α_v-containing integrins mediated cell adhesion than the linear RGDS did [15]. It achieved 50% inhibition of HaCaT, NIH/3T3, and PIEC cells at about 8 μM, 25 μM, and 100 μM, almost 25-fold, 10-fold, and 6-fold lower than the linear RGDS peptide, respectively. These inhibition results strongly suggest that the adhesion of keratinocytes, fibroblasts, and endothelial cells on AGF is mediated by RGD-binding integrins, most possibly α_v-containing integrins.

AGF promotes migration of keratinocytes, fibroblasts, and endothelial cells

To test whether AGF could promote keratinocytes, fibroblasts, and endothelial cells migration, we applied the migration assays using transwell chambers. In our experiments, all the HaCaT, NIH/3T3, and PIEC cells could migrate through the filter within 20 h after seeding whereas little migration was observed on the filter coated with BSA. Numbers of migrated cells, measured by crystal violet staining, were related with the coating concentration (Fig. 6A). Fibronectin at the concentration of 4 μg/ml was also chemotactic for all the cells we tested, in agreement with earlier reports [16–18].

AGF promoted migration is also mediated by RGD-binding integrins

Many studies indicate that integrins participate in not only cell adhesion but also cell migration [19]. In our experiments, the cyclic GPenGRGDSPCA peptide was directly employed to carry out migration inhibition assays. As shown in Fig. 6B, this peptide effectively inhibited approximately 62%, 68%, and 52% of the migration of HaCaT, NIH/3T3, and PIEC cells to the lower chamber rhAGF, respectively. Our results suggest that RGD-binding integrins, most possibly α_v-containing integrins, are involved in the AGF promoted migration of keratinocytes, fibroblasts, and endothelial cells.

Discussion

Cutaneous wound healing is an orchestrated process involving three overlapping phases: inflammation, tissue repair, and matrix remodeling [20]. Tissue repair, comprising reepithelialization, and granulation tissue formation, is the central part of this process. In reepithelialization, epidermal keratinocytes from wound margins migrate over the clot provisional matrix and proliferate to cover the denuded wound surface. Meanwhile, granulation tissue forms and expands under the clot after macrophages, dermal fibroblasts, and capillary vessels move in the wound bed. The macrophages provide a continuing source of growth factors necessary to stimulate fibroplasia and angiogenesis; The immigrated fibroblasts proliferate and synthesize new ECMs provided as structural scaffold for

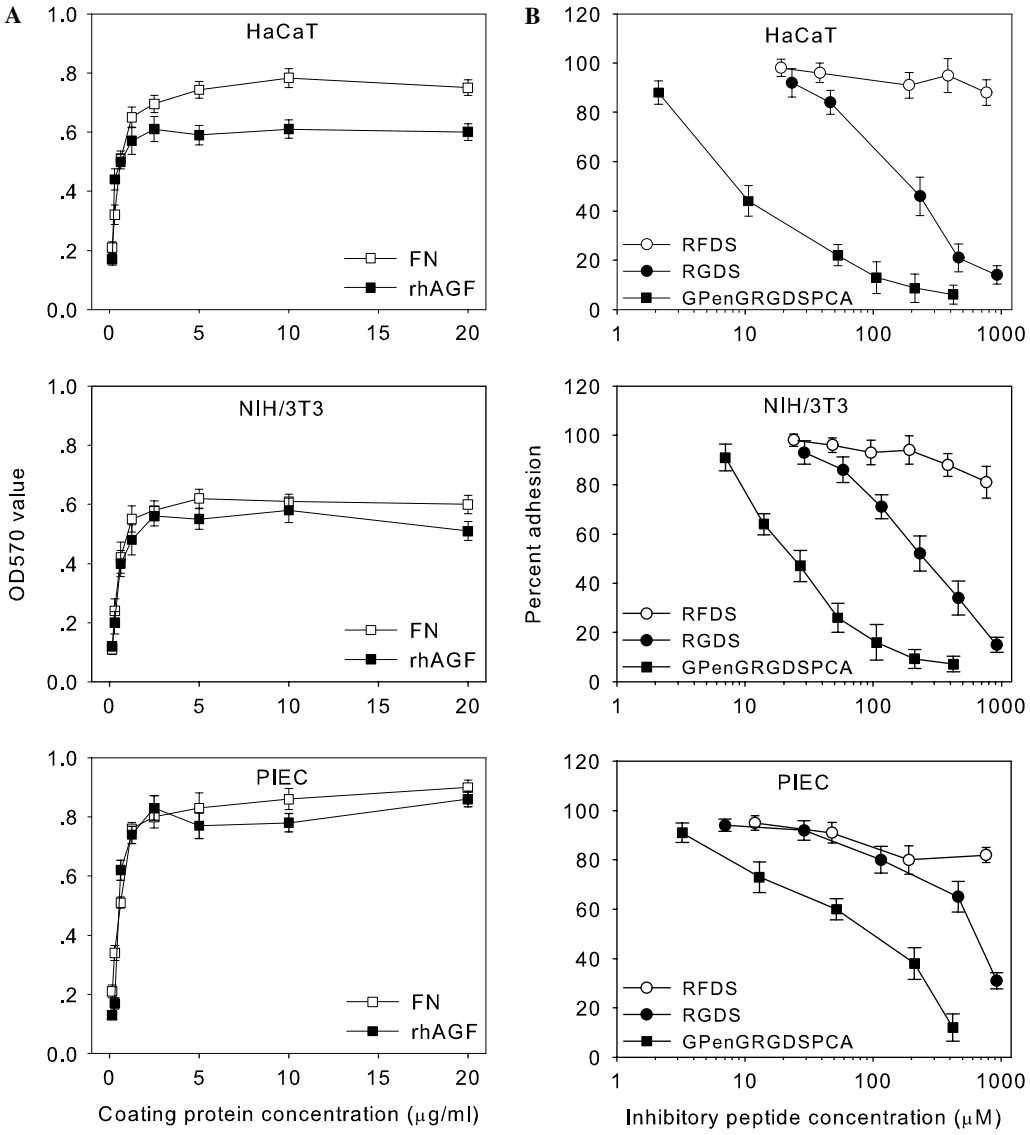

Fig. 4. AGF supports adhesion of keratinocytes, fibroblasts, and endothelial cells, and this activity is mediated by the RGD-binding integrins. (A) Dose-dependent adhesion of HaCaT, NIH/3T3, and PIEC cells on the coated rhAGF. All the cells achieved maximal adhesion on rhAGF at the coating concentration of 2–3 μg/ml within 2 h. In the negative control wells, which only blocked with 10 mg/ml BSA, the OD_{570} values are less than 0.1. (B) Adhesion of the HaCaT, NIH/3T3, and PIEC cells on rhAGF (4 μg/ml) was competitively inhibited by the increasing concentrations of synthetic linear (RGDS) and cyclic (GPenGRGDSPCA) RGD-containing peptides. GPenGRGDSPCA was much more effective than RGDS. This cyclic peptide inhibited more than 80% adhesion of the HaCaT, NIH/3T3, and PIEC cells. There was less than 20% inhibition even the concentration increased up to 1000 μM. All the values represent means ± SD from triplicate samples.

more cells to grow in; and numerous capillary vessels supply the necessary oxygen and nutrients to maintain cell metabolism [21]. Many factors including various growth factors, cytokines, and ECMs that are able to promote proliferation and/or migration of the wounded cells, especially keratinocytes, fibroblasts, and vascular endothelial cells, have been reported effective in accelerating reepithelialization and granulation tissue formation, and consequently ameliorating wound healing [22].

The researches created K14-AGF transgenic mice demonstrated that AGF promoted proliferation of keratinocytes as well as angiogenesis and thereby played a role in the wound healing process [5,7].

In this paper, we first prove on the protein level that AGF does stimulate keratinocytes proliferation, but only at high protein concentration. However, the most remarkable finding of our paper is that the AGF can promote adhesion, spreading, and migration of the keratinocytes, fibroblasts, and endothelial cells. Unlike its mildness as a mitogen, AGF is a potent substrate in mediating adhesion, spreading, and migration. Furthermore, we confirm that both the cellular events are mediated by RGD-binding integrins, most possibly the α_v-containing integrins, by *in vitro* inhibition assays using synthetic competitive peptides. These experiments strongly suggest that AGF is an integrin ligand as well as a mitogenic growth factor and

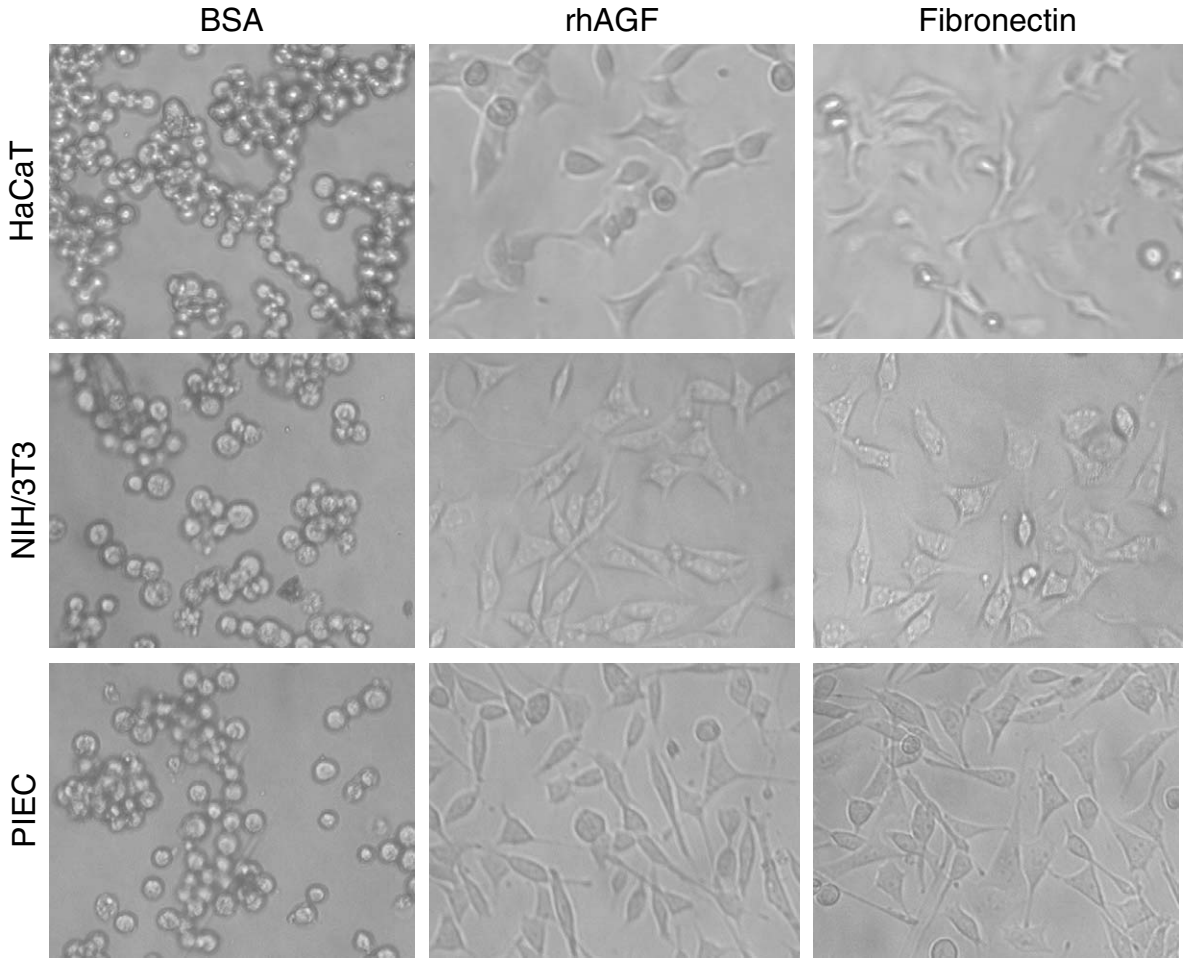

Fig. 5. Keratinocytes, fibroblasts, and endothelial cells spread on the immobilized AGF. The rhAGF (4 μg/ml) was coated on the non-tissue-culture-treated polystyrene plate and blocked with 10 mg/ml BSA. After 2 h, HaCaT, NIH/3T3, and PIEC cells adhered and spread on the immobilized rhAGF as well as on the immobilized serum-derived fibronectin (4 μg/ml). However, the cells in the wells only coated with BSA remained round and floating.

may participate in cutaneous wound healing in a more complex mechanism.

ECMs, indispensable molecules in wound healing process, are most common integrin ligands. We consider that AGF can act as an ECM protein in accelerating wound healing based on several lines of evidence: (1) AGF is a multimer with huge molecular mass. (2) AGF contains RGD motif and mediates adhesion and migration via the RGD-binding integrins. (3) The nature of AGF-mediated migration is chemotactic, not chemokinetic (data not shown). (4) AGF does not show stringent target cell selectivity, in concert with the relatively broad distribution of integrin receptors. (5) The fibrinogen-like domain of AGF shares high sequence identity with the C-terminal of the γ-chain of human fibrinogen, a well-known ECM protein. (6) AGF can possibly bind to other ECMs, as Angs, which share similar protein structure with AGF, have been proved to be able to bind to ECMs such as vitronectin, fibrinogen, and heparan sulfate proteoglycans [23,24]. In addition, AGF levels in circulation of K14-AGF transgenic mice do not increase compared with controls, which clearly indicates the secreted AGF molecules from keratinocytes are immobilized to the connective tissues around the cells [7].

Combining earlier investigations and our findings in this paper, we propose a more complex mechanism of AGF in promoting cutaneous wound healing. First, AGF serves as a substrate to support adhesion and spreading of keratinocytes, fibroblasts, and endothelial cells via the cell surface RGD-binding integrins. Such integrin mediated adhesion and spreading have been proved crucial to several significant cellular events such as cell proliferation, differentiation, migration, and survival [25]. Second, AGF accelerates reepithelialization by stimulating epidermal keratinocytes migration and proliferation. The reepithelialization is initiated within 24 h when keratinocytes from marginal nondamaged tissue begin moving into the wound site. Keratinocytes migration, not proliferation, dominates the early reepithelialization stage to restore the surface integrity, whereas proliferation contributes to the later stage to seed more cells into the wound [26]. Third, AGF promotes granulation tissue formation by enhancing fibroblasts migration and angiogenesis. In addition, AGF is possible to promote migration of macrophages, another

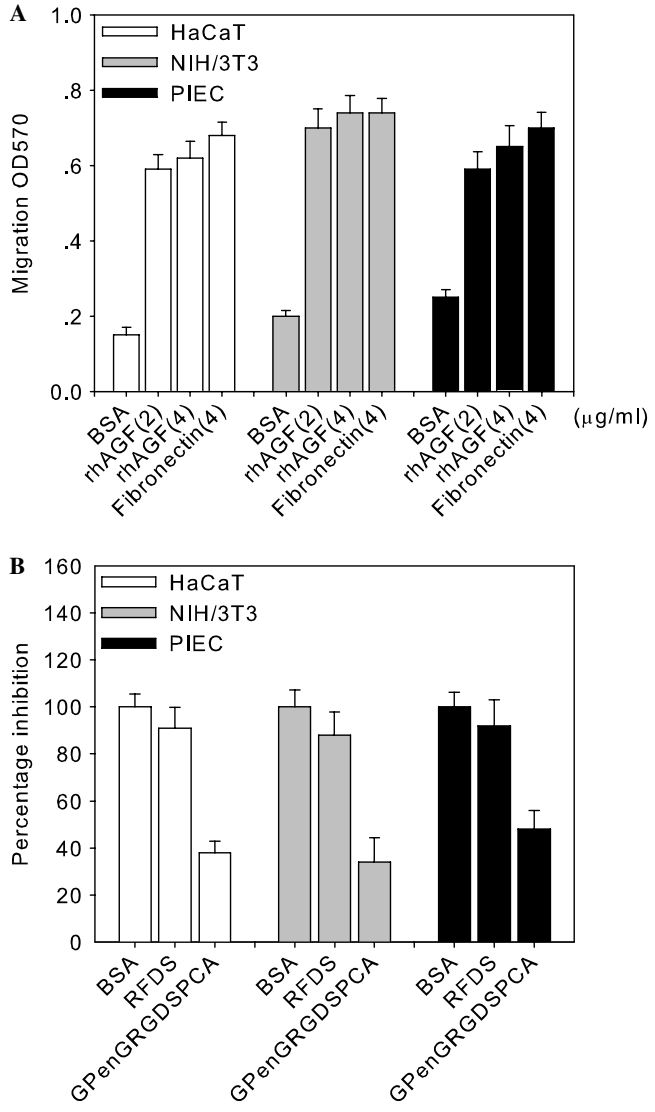

Fig. 6. AGF promotes migration of keratinocytes, fibroblasts, and endothelial cells, and this activity is also mediated by the RGD-binding integrins. (A) Dose-dependent migration of HaCaT, NIH/3T3, and PIEC cells to the rhAGF. Two concentrations (4 μg/ml and 2 μg/ml) of rhAGF and positive control FN (4 μg/ml) were added into the lower chamber of the 24-well transwell plates, whereas the cells (4×10^4 cells) were added in the upper chamber to carry out the migration assays. (B) Migration of HaCaT, NIH/3T3, and PIEC cells to rhAGF (4 μg/ml) was also mediated by the RGD-binding integrins. GPenGRGDSPCA (200 μM) inhibited about 62%, 68%, and 52% of the migration of HaCaT, NIH/3T3, and PIEC cells, respectively. There was less than 20% inhibition in the presence of equimolar RFDS control peptide. All the values represent means ± SD from triplicate samples.

vital constituent part of granulation tissue, as they also express α_v-containing integrins ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) [27].

Although we have testified that AGF mediates adhesion and migration through interaction with RGD-binding integrins and RGD motif is conservative in AGF orthologs from humans and rodents, we still cannot ensure this tripeptide sequence is the binding site of these integrins. Several studies have shown that RGD motif is not the unique sequence that the so-called RGD-binding integrins can recognize [28–31]. In ARPs and Angs protein families, recently some other members have been reported as ligands of α_v-containing integrins. For example, ANGPTL3 stimulates endothelial cell adhesion and migration via $\alpha_v\beta_3$ [32]. Ang1 and Ang2 support fibroblasts adhesion via β_1 and $\alpha_v\beta_5$ integrins [33]. These facts explicitly suggest that there exists non-RGD sequence site in the ARPs and Angs interacting with α_v-containing integrins. Another reason is that the inner RGD motif is not always exposed and accessible to the integrins. Proverbially, collagens (I, V, and VI) contain RGD motifs, yet in native conformation they mediate cell adhesion through $\alpha_2\beta_1$ and $\alpha_1\beta_1$, not RGD-binding integrins as the RGD-containing competitive peptides fail to inhibit the adhesion [34]. Therefore, further experiments especially site-directed mutations are needed to investigate the authentic sequence site in AGF binding to the integrin receptors.

Nowadays, several types of reagents, such as synthetic competitive peptides, function-blocking monoclonal antibodies and purified integrin molecules, have been extensively used in determining the integrin receptor involved in adhesion and migration mediated by a given ligand. In this paper, synthetic linear and cyclic RGD-containing peptides are employed to investigate the integrin receptors interacting with AGF, as they can competitively bind to the RGD-binding integrins [14,15]. Compared with the linear peptide (RGDS), the cyclic peptide (GPenGRGDSPCA) binds to α_v-containing integrins with apparently higher affinity, and therefore has been considered as a specific inhibitor to these integrins. But in recent studies, this specificity has been doubted based on several reliable evidences [35,36]. We suggest function-blocking monoclonal antibodies which have the best specificity should be employed in determining the genuine integrin receptors of AGF in the future studies.

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