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Proteoglycan from salmon nasal cartridge promotes *in vitro* wound healing of fibroblast monolayers via the CD44 receptor

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ABSTRACT

Proteoglycans (PGs) are involved in various cellular functions including cell growth, adhesion, and differentiation; however, their physiological roles are not fully understood. In this study, we examined the effect of PG purified from salmon nasal cartilage (SNC-PG) on wound closure using tissue-cultured cell monolayers, an *in vitro* wound-healing assay. The results indicated that SNC-PG significantly promoted wound closure in NIH/3T3 cell monolayers by stimulating both cell proliferation and cell migration. SNC-PG was effective in concentrations from 0.1 to 10 μg/ml, but showed much less effect at higher concentrations (100–1000 μg/ml). The effect of SNC-PG was abolished by chondroitinase ABC, indicating that chondroitin sulfates (CSs), a major component of glycosaminoglycans (GAGs) in SNC-PG, are crucial for the SNC-PG effect. Furthermore, chondroitin 6-sulfate (C-6-S), a major CS of SNC-PG GAGs, could partially reproduce the SNC-PG effect and partially inhibit the binding of SNC-PG to cells, suggesting that SNC-PG exerts its effect through an interaction between the GAGs in SNC-PG and the cell surface. Neutralization by anti-CD44 antibodies or CD44 knockdown abolished SNC-PG binding to the cells and the SNC-PG effect on wound closure. These results suggest that interactions between CS-rich GAG-chains of SNC-PG and CD44 on the cell surface are responsible for the SNC-PG effect on wound closure.

1. Introduction

Proteoglycans (PGs), one of the major components of extracellular matrices, are macromolecules comprising glycosaminoglycan (GAG) chains covalently linked to the core protein. PGs are involved in various cellular functions including cell growth, adhesion, and differentiation [1,2]. These features of PGs indicate that they have potential clinical applications as a safe pharmaceutical agent, although PG purification has been costly. Very recently a low-cost method for large-scale PG purification from salmon nasal cartilages (SNCs-PG) was established [3], which also has an ecological benefit because the raw material is salmon heads, a fishery and foodstuff waste. PGs have become a promising candidate for medical and pharmaceutical applications. Thus, research on the physiological activities of SNC-PG has advanced vigorously. SNC-PG can suppress inflammatory responses in various animal models [4–7]. Furthermore, daily oral administration of SNC-PG reduces the severity of experimental colitis [3,8,9], improves intestinal microbiota [10], promotes the recovery of photo-damaged skin after ultraviolet B irradiation [11,12], and attenuates arthritis in a mouse model [13].

Aggrecan is a primary component of SNC-PG [14]. The GAG chains in aggrecan contain a large number of chondroitin sulfates (CSs) [15]. Moreover, a distinctive feature of salmon aggrecan is its higher proportion of chondroitin 6-sulfate (C-6-S) (approximately 60%) than chondroitin 4-sulfate (C-4-S) [5,16]. Because C-6-S promotes wound healing in fibroblastic cell monolayers [17,18], we hypothesized that SNC-PG would be effective in wound healing and have potential as a wound-healing ointment. In this study, we examined the effect of SNC-PG on wound healing using a scratch assay in cultured fibroblast and keratinocyte cell monolayers, and the role of SNC-PG CS chains and their possible cell receptors.

2. Materials and methods

2.1. Materials

Highly purified SNC-PG was obtained from Biomatec Japan Co., Kushiro, Japan, and Wako Pure Chemical Industries Ltd., Osaka,

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Japan. Anti-β-actin, C-4-S, C-6-S, fibronectin, mitomycin-C, and CI-1033 (Canertinib) were purchased from Sigma–Aldrich (St. Louis, MO). The anti-CD44 antibody used for western blotting analyses was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). Alexa 568 and Alexa 660 succinimidyl esters were obtained from Molecular Probes (Eugene, OR). CD44-blocking and control antibodies were purchased from Beckman Coulter Inc. (Brea, CA).

2.2. Cell culture

The mouse fibroblast line NIH/3T3 (#JCRB0615) and human fibroblast TIG-118 (#JCRB0535) and TIG-121 (#JCRB0536) lines were obtained from JCRB Cell Bank (Osaka, Japan). Human primary cultured keratinocytes were purchased from EIDIA Co., Ltd. (Tokyo, Japan). All fibroblast cells were maintained in DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (Invitrogen), 100 U/ml penicillin-G, and 100 μg/ml streptomycin (Invitrogen). Human keratinocytes were grown in keratinocyte growth medium (KGM-2, Lonza, Walkersville, MD). Human CD44 DNA (wild-type) was obtained from the Kazusa DNA Project [19].

2.3. RNAi-mediated knockdown of CD44

CD44 silencing was performed with Accell SMARTpool siRNAs (Thermo Fisher Scientific, Waltham, MA) against mouse CD44 or a non-target control according to the manufacturer's instructions. NIH/3T3 cells were transfected with 1 μM siRNA, incubated at 37 °C with 5% CO_2, and used in the wound-healing assay 72 h after siRNA transfection. The knockdown efficiency of CD44 protein was evaluated by western immunoblotting [20].

2.4. Wound-healing Assay

Wound healing was evaluated by an *in vitro* assay using the Culture-Inserts system (Ibidi, Martinsried, Germany). Thirty-five thousand cells were seeded in a culture insert on low 35-mm μ-dishes (Ibidi) precoated with fibronectin (0.5 μg/ml), and grown overnight to be confluent. After removal of the culture insert, a 500 μm cell-free gap was formed in the cell monolayer. After changing to fresh culture medium (complete growth medium noted above), cell migration toward the gap area was recorded by time-lapse photography in a heated chamber (37 °C, Tokai Hit CO., Ltd., Shizuoka, Japan) on the stage of an inverted light microscope (TE-3000, Nikon, Tokyo, Japan) equipped with a camera (Cascade II, Photometrics, Tucson, AZ), or with a Time Lapse Imaging System, BioStation IM (Nikon). In some cases, the cells were pretreated for 30 min and incubated throughout the assay with drugs or antibodies as noted.

Gap closure was quantified using phase contrast images of the same location. The perpendicular distance (L) from one side of the gap to the other was measured, and ΔL was calculated as the displacement from the initial distance (time 0) ($L_0 - L_t$). In each sample, at least ten distance readings were measured, and each experiment was repeated as noted. Wound closure rate (%) was calculated as the ratio of ΔL in the experimental versus control conditions.

2.5. Cell-proliferation

Cell proliferation was measured by determining the incorporation of 5-bromo-2′-deoxy-uridine (BrdU). Fibroblasts cultured at 10^3 cells/cm^2 on cell culture μ-slides (Ibidi) precoated with fibronectin (0.5 μg/ml) were incubated in complete growth medium supplemented with SNC-PG for 10 h. Subsequently, 10 μM BrdU (Roche) was added for 2 h before fixation. BrdU was detected according to the manufacturer's protocol (Roche, labeling and detection kit I).

2.6. Conjugation of Alexa 568 or Alexa 660 with SNC-PG

SNC-PG reacted with Alexa succinimidyl esters according to the manufacturer's instructions. We used labeled SNC-PG that had a 1.8–3.3 dye to protein ratio.

2.7. Flow cytometry

NIH/3T3 cells were incubated in complete medium supplemented with Alexa-660-labeled SNC-PG (10 μg/mL) at 4 °C for 60 min in the dark. The cells were pretreated with antibodies or chondroitin sulfates at 4 °C for 60 min, if necessary. Controls included cells treated with media containing labeled ovalbumin. The cells were washed for 3 × 5 min with ice-cold phosphate-buffered saline (PBS) to remove the excess ligand, and then harvested with 5 mM EDTA-PBS(−). The cells were resuspended in ice-cold fluorescence-activated cell-sorting (FACS) buffer and analyzed by flow cytometry (FACS Calibur, Becton Dickinson, San Jose, CA). Data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR).

2.8. Immunofluorescence

NIH/3T3 cells grown on 35-mm ibiTreat μ-dishes (Ibidi) were treated with Alexa-568-labeled SNC-PG as described above and, without harvesting or fixation, observed under a laser-scanning confocal microscope (LSM510, Carl Zeiss). Fluorescent images were processed with Adobe Photoshop software.

2.9. Statistical analysis

Significant differences between control and experimental groups were determined by Student's t test. $^{*}p < 0.05$; $^{**}p < 0.01$; NS, not significant.

3. Results

3.1. The effect of SNC-PG on wound healing in a cell-scratch assay

To examine whether SNC-PG has an effect on wound healing, we performed an *in vitro* scratch assay to mimic cell behavior during wound healing *in vivo*. Initially, we used cultured murine fibroblasts (NIH/3T3 cells). When a 500 μm wide cell-free gap was created in a NIH/3T3 cell monolayer, more than 30 h incubation was needed to close the gap. When SNC-PG was applied exogenously at a concentration of 10 μg/ml, the cells migrated toward the gap faster than that in the control (Fig. 1A and B). SNC-PG-treated cells were able to close the gap in 24 h (Fig. 1B). Our quantitative analysis showed that SNC-PG significantly accelerated the wound closure rate by a factor of 1.3 at lower concentrations (0.1–10 μg/ml) but did not affect wound closure at higher concentrations (100–1000 μg/ml; Fig. 1C). The wound-healing effect of SNC-PG at lower concentrations was also observed in experiments using human fibroblast and primary cultured keratinocyte (Fig. 1D and E), suggesting that SNC-PG could exert a positive effect on wound healing in a wide range of cell types.

3.2. SNC-PG stimulates both cell proliferation and migration

The wound-healing process generally involves both cell migration and proliferation. To examine if SNC-PG affects either or both of these, we performed time-lapse imaging of migrating cells and immunofluorescent staining of proliferative cells with BrdU in

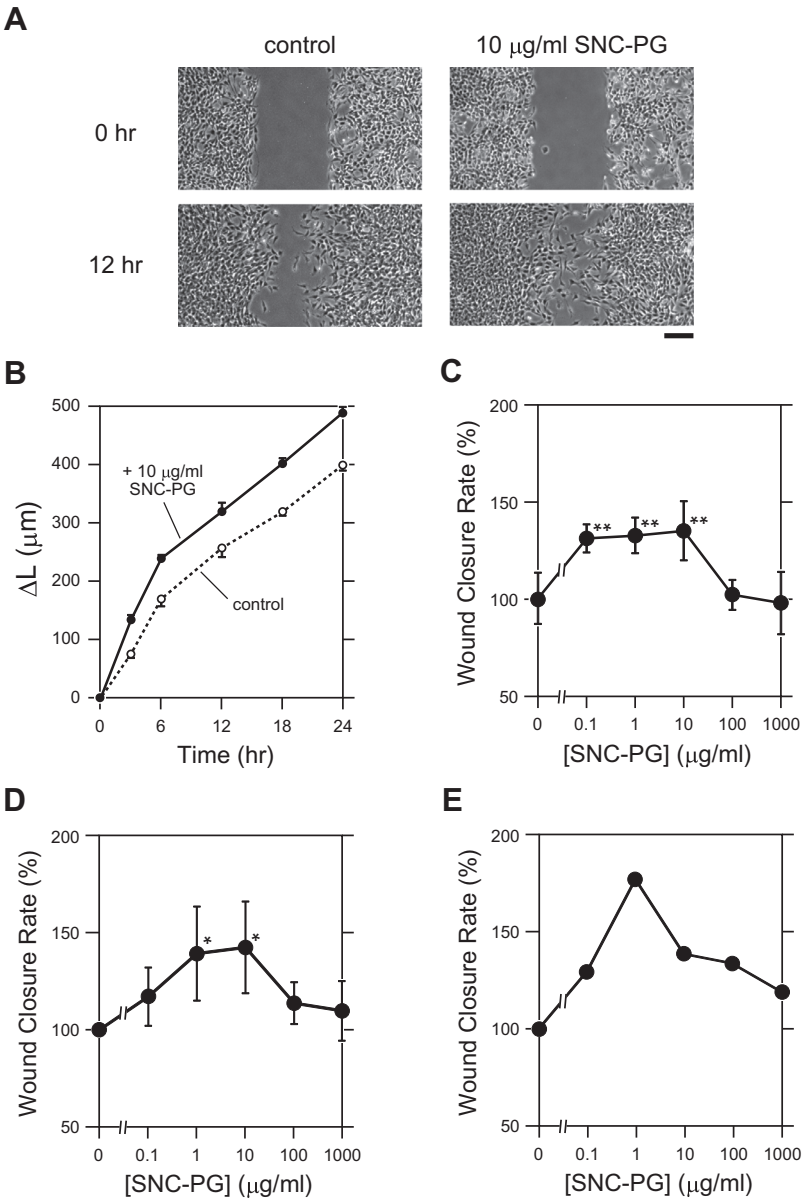

Fig. 1. SNC-PG accelerates wound closure in an *in vitro* wound-healing assay. (A) Analysis of NIH/3T3 fibroblast migration with SNC-PG (10 μg/ml) (right) and vehicle control (left) in the *in vitro* wound-healing assay. Phase contrast images were acquired at 0 and 12 h after the wound occurred. Bar = 100 μm. (B) Time course of wound closure in the presence of SNC-PG (10 μg/ml) and vehicle control. Dose-dependency of the acceleration of wound closure by SNC-PG in (C) NIH/3T3 fibroblasts at 12 h after the onset of closing, (D) human fibroblasts at 18 h, and (E) human primary cultured keratinocytes at 42 h. (C and D) Data from four separate experiments are expressed as mean ± S.D. (E) Representative data from one of two experiments.

the presence or absence of SNC-PG. SNC-PG facilitated both the proliferation of cells and the migration speed of lead cells in a similar concentration-dependent manner (Fig. 2A and B). These results indicate that SNC-PG has the potential to upregulate both cell proliferation and migration. However, cell proliferation and migration speed may be interrelated. To precisely evaluate the SNC-PG effect on cell migration speed separately from that on cell proliferation, experiments were performed in the presence of mitomycin C (10 μg/ml), which inhibits cell proliferation. Mitotic inactivation by the drug reduced the wound closure rate regardless of the presence of SNC-PG, but the facilitating effect of SNC-PG on cell migration speed was preserved (Fig. 2C). These results indicate that SNC-PG facilitates both cell proliferation and migration independently, and that cell proliferation contributes significantly to the wound closure rate.

3.3. Chondroitin-6-sulfates in SNC-PG GAGs are crucial for the SNC-PG effect

Next, we examined which components of SNC-PG are responsible for the effect on wound closure. SNC-PG has an EGF-like motif sequence in its core polypeptide and this motif is considered to function as an EGF ligand to stimulate cellular proliferation and migration. However, the pan-ErbB receptor tyrosine kinase inhibitor, CI-1033, did not inhibit the SNC-PG effect on wound closure (Fig. 3A), indicating that the activation of the EGF receptor family is dispensable for the SNC-PG effect. However, digestion by chondroitinase ABC completely abolished the SNC-PG effect on wound closure (Fig. 3B), indicating that the effect requires the GAG side chains containing CSs, and that these interact with cells to produce the SNC-PG effect. Consistent with this, our FACS analysis showed

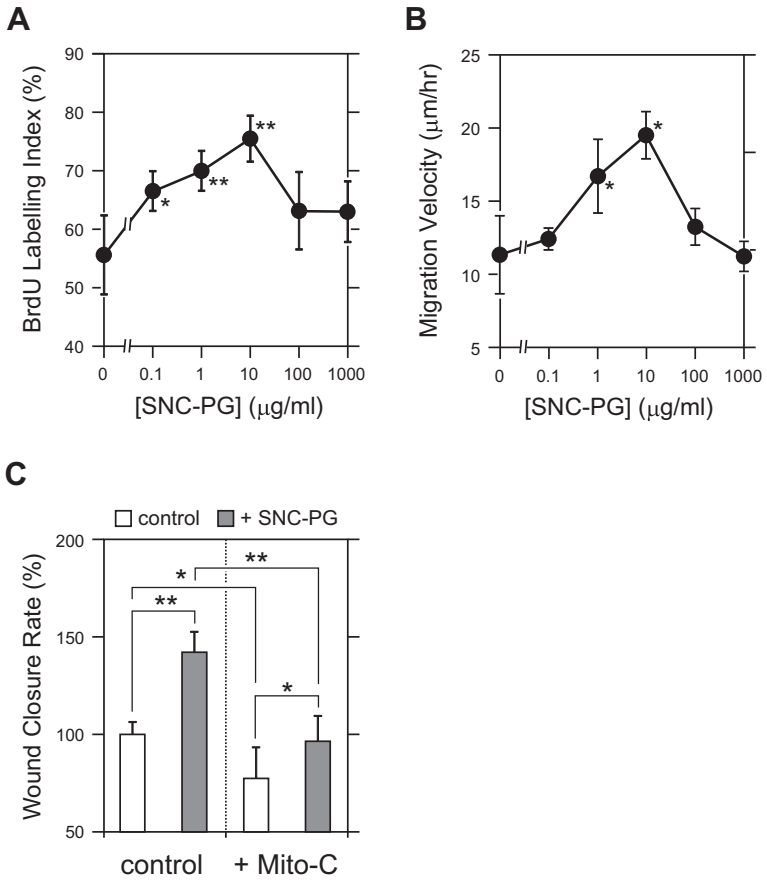

Fig. 2. SNC-PG stimulates both cell proliferation and cell migration. (A) Upregulated cell proliferation in NIH/3T3 cells in the presence of SNC-PG, measured by BrdU labeling. Data from six experiments are expressed as mean ± S.D. (B) Cell migration speed was accelerated by SNC-PG with a peak at 10 μg/ml concentration. Migration speed was measured by quantifying the total migratory distance that the cells at the edge of the wound (>10 cells) moved in 3 h. Data from four experiments are expressed as mean ± S.D. (C) Wound closure in the presence of mitomycin C. Data from six independent experiments are expressed as mean ± S.D.

that the binding of fluorescently labeled SNC-PG onto NIH/3T3 cells was partially inhibited by C-6-S and C-4-S (Fig. 3C). Furthermore, C-6-S, but not C-4-S, was found to have a similar effect on wound closure to SNC-PG (Fig. 3D), which is consistent with an earlier report [17]. Taken together, we conclude that C-6-S in the GAG side chains of SNC-PG may be crucial for the SNC-PG effect on wound healing.

3.4. SNC-PG binding to CD44 is a crucial step in the elevated wound healing rate

Lastly, we focused on the mechanism by which C-6-Ss of SNC-PG act on cells. CSs can interact with several types of membrane receptors to stimulate cellular signaling [21]. CD44 is expressed ubiquitously in a broad range of cell types, including fibroblasts and keratinocytes [22], and binds to aggrecan [23]; therefore, we examined if CD44 is involved in the SNC-PG effect on wound closure. NIH/3T3 cells overexpressing human CD44 exhibited an elevated binding of fluorescently labeled SNC-PG (Fig. 4A). Pretreatment neutralization of the cells with an anti-CD44 antibody decreased the amount of fluorescently labeled SNC-PG bound to the cells, indicating that CD44 is involved in the binding of SNC-PG to the cells (Fig. 4B). We observed that SNC-PG molecules not only adhered to the cell surface but were also incorporated into intracellular vesicles 30 min after the SNC-PG application (Fig. 4C). However, in the presence of neutralizing anti-CD44 antibodies, SNC-PG remained on the cell surface (Fig. 4C). We scarcely observed labeled SNC-PG on the bottom surface of the dish coated with fibronectin (data not shown), implying that SNC-PG exerts its effect mainly through binding to the cell surface of fibroblasts. In the *in vitro* wound-healing assay, neutralizing anti-CD44 antibodies inhibited the positive effect of SNC-PG on wound closure (Fig. 4D). Furthermore, CD44 depletion by siRNA treatment (Fig. 4E) significantly inhibited the SNC-PG facilitation of wound closure (Fig. 4F). These results suggest that the binding of SNC-PG to CD44 is the primary mechanism for the effect on *in vitro* wound closure.

4. Discussion

We demonstrate that SNC-PG accelerates wound closure at relatively low concentrations (0.1–10 μg/ml) in an *in vitro* wound-healing assay that uses fibroblast cell monolayers. SNC-PG produces this effect by stimulating both cell proliferation and cell migration, and requires an interaction between the constituent CS-units and CD44 on the cell surface. SNC-PG has various physiological effects including anti-inflammatory action [3–13]. However, to the best of our knowledge, there has been no prior report describing a positive effect of SNC-PG on wound healing.

4.1. Possible modes of SNC-PG action

There are several potential mechanisms by which the SNC-PG effect may occur. First, EGF-like motifs in the SNC-PG core polypeptide may function as an EGF ligand to stimulate cell proliferation and migration [14] in a similar manner to versican, a member of

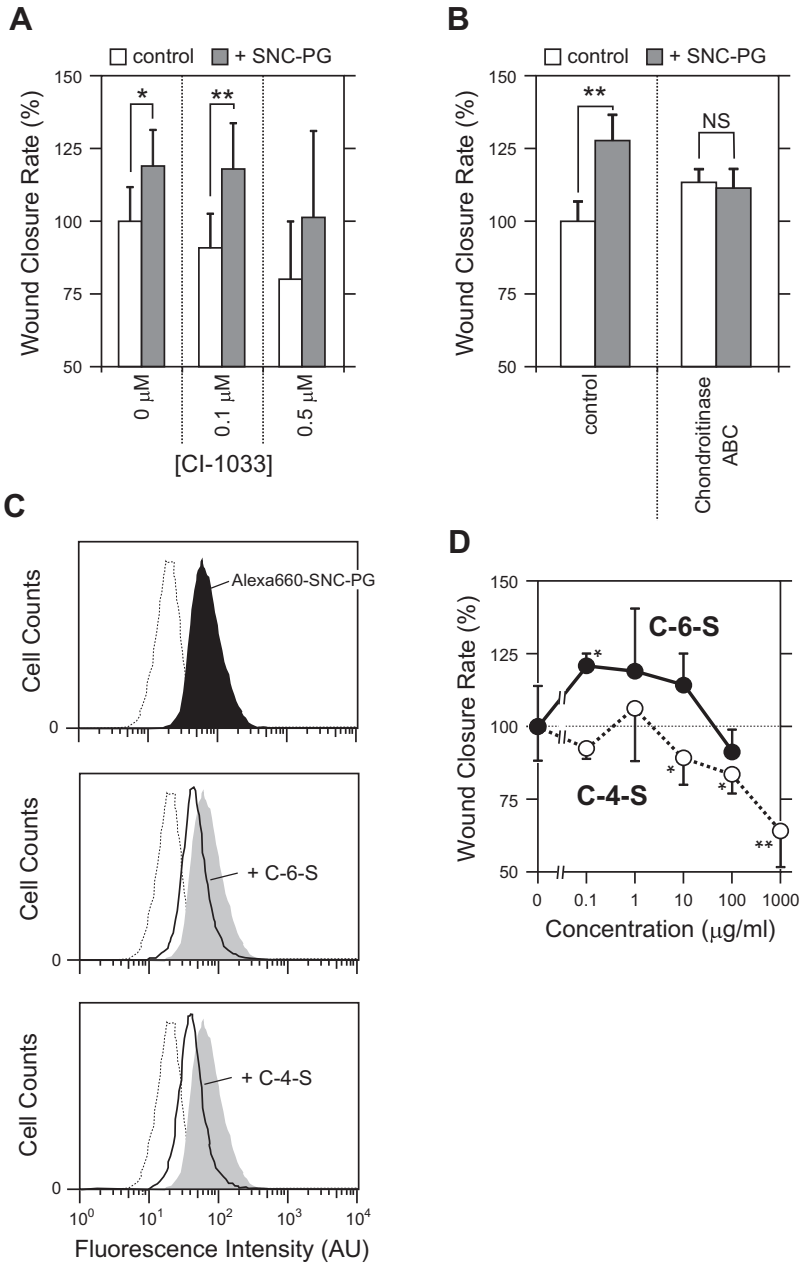

Fig. 3. SNC-PG wound-healing effect requires chondroitin-6-sufate. (A) EGFR kinase inhibitor CI-1033 did not alter the SNC-PG effect. Each bar represents the mean ± S.D. from six independent experiments. (B) Chondroitinase ABC impaired the SNC-PG effect. Each bar represents the mean ± S.D. from four independent experiments. (C) Flowcytometry of the amount of fluorescently labeled SNC-PG bound to NIH/3T3 cells. Top, harvested NIH/3T3 cells were incubated with Alexa-660-labeled SNC-PG (50 μg/ml, closed histogram) or ovalbumin (dotted line). Middle and bottom, co-incubation with C-6-S or C-4-S (50 μg/ml) partially reduced the binding of Alexa-660-labeled SNC-PG to the cells. (D) C-6-S (0.1 μg/ml) accelerated *in vitro* wound healing in NIH/3T3 cells but C-4-S did not. Data from six experiments are expressed as mean ± S.D.

the CS proteoglycan family that can enhance cell proliferation [24]. However, pharmacological inhibition of EGF receptor tyrosine kinase did not change the effect of SNC-PG on wound closure. Second, it is possible that SNC-PG in the medium could adhere to the bottom surface of the culture dish and form adhesive sites that enable cell proliferation and migration. However, we could scarcely detect fluorescently labeled SNC-PG bound to the fibronectin-coated bottom surface of the culture dish, including the cell-free gap (data not shown), suggesting that SNC-PG does not function as a substrate for migrating and proliferating cells. Third, it is conceivable that SNC-PG could bind to a particular receptor on the cell surface and activate intracellular signaling pathways leading to a promotion of cell proliferation and migration. This was strongly supported by our observation of intense signals from fluorescently labeled SNC-PG on the surface of and inside cells (Fig. 4C).

4.2. Which component of SNC-PG is responsible for cell binding?

Aggrecan is a major component of SNC-PG that contains a very large number of CSs in GAG chains [14,15]. CSs exert a positive effect on wound healing [17,18,25]. We hypothesized that GAG-chain CSs could underlie SNC-PG-cell binding and the effect on wound closure. The SNC-PG effect was abolished by digestion with chondroitinase ABC (Fig. 3B right) strongly supporting this hypothesis. The exogenous application of C-6-S promotes wound closure in a cultured fibroblastic cell monolayer [17,18], which we reproduced here in NIH/3T3 cell monolayers (Fig. 3D). Although C-4-S

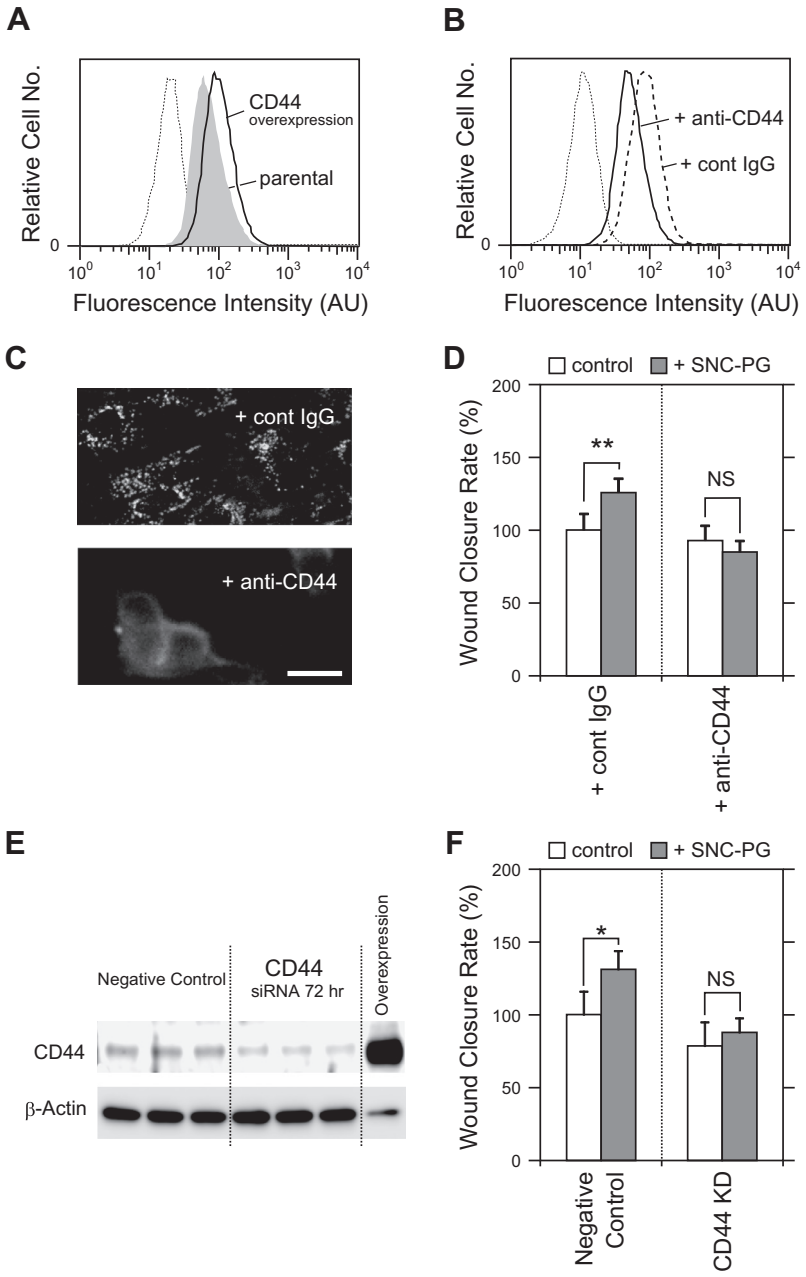

Fig. 4. CD44 is crucial for the binding of SNC-PG to the cell and for the SNC-PG effect on wound closure. (A) Overexpression of CD44 elevated the binding of SNC-PG to the cells. The amount of SNC-PG bound to the cells overexpressing CD44 (solid line) was elevated more than the parental cells (gray histogram). Dotted line indicates the negative control. (B) Preincubation with anti-CD44 neutralizing antibodies decreased the binding of SNC-PG to the cells (solid line) compared with the control IgG (broken line). Dotted line indicates the negative control. (C) Confocal microscopy images showing that fluorescently labeled SNC-PG was incorporated into intracellular vesicles in 30 min and that anti-CD44 completely blocked this SNC-PG trafficking into the cells. Bar = 30 μm. (D) Anti-CD44 antibodies neutralized the SNC-PG effect on wound closure. Data from five independent experiments are expressed as mean ± S.D. (E) Western immunoblotting of CD44 depletion by siRNA. Expression of CD44 in cells treated for 72 h with siRNA against mouse CD44 or a nontarget control (or the cells overexpressing CD44). (F) CD44 depletion blocked the SNC-PG facilitatory effect on *in vitro* wound healing. Data from three independent experiments are expressed as mean ± S.D.

exerts a similar positive effect on wound closure [18,25], we did not observe this in our *in vitro* wound-healing assay. It remains unclear how C-6-S exerts a positive effect on wound closure distinct from C-4-S (Fig. 3D), despite C-4-S having a similar cell-binding ability (Fig. 3C).

4.3. Which receptor is responsible for CS binding to cells?

Which receptor in the cell is responsible for binding SNC-PG and the subsequent effect on wound closure? Several kinds of plasma membrane receptors can interact with CSs, and some CS receptors, including CD44 and TSG6 (Tumor necrosis factor-stimulated gene 6), are involved in GAG-induced cell-mediated processes including inflammation, cell-mediated apoptosis, and cell rolling [21]. Because CD44, a receptor for hyaluronic acid, can bind to aggrecan as well as CSs [23], it seemed likely that CD44 would be involved. As shown in the results, anti-CD44 antibodies and depletion of CD44 expression could both completely eliminate the SNC-PG effect on wound closure (Fig. 4D). However, a substantial fraction of SNC-PG molecules could bind to the cells even after incubation with anti-CD44 antibodies (Fig. 4B). This result indicates that there are SNC-PG binding sites other than CD44 on the cell surface, which presently remain unclear. We speculate that when SNC-PG is present at higher concentrations

(10–1000 μg/ml), SNC-PG bound to those putative binding sites might activate signal cascades that negatively modulate the SNC-PG–CD44-dependent signaling. This might account for the bell-shaped SNC-PG dose–response curves that peak at 10 μg/ml for wound closure (Fig. 1C–E) and proliferation and migration speeds (Fig. 2A and B).

4.4. Possible downstream signaling leading to cell proliferation and migration

We find here that SNC-PG binds to CD44 on the cell surface, which is consistent with an earlier report [23]. SNC-PG interaction with CD44 may activate intracellular signaling leading to cell proliferation and migration. CD44 regulates signaling by establishing signaling platforms with other membrane receptors or by becoming associated with the actin cytoskeleton [22,26]. Our *in vitro* assay was performed in a complete growth medium containing serum and many extracellular stimulants, including growth factors or cytokines, must have activated receptor tyrosine kinases on the cell membrane. CD44 may act as a co-receptor to modulate the activation of other receptors and their signaling for cell growth and migration [22,26]. However, inhibition of the EGF receptor tyrosine kinase family did not change the SNC-PG effect on wound closure (Fig. 3A), indicating that SNC-PG acts on receptors other than ErbB via CD44. A cytoplasmic region of CD44 is associated with ERM (ezrin, radixin and moesin) family proteins [22]; therefore, the binding of SNC-PG to CD44 may induce reorganization of the actin cytoskeleton through ERM proteins [22]. Further studies are required to clarify which intracellular signaling events occur upon SNC-PG binding to CD44. Intramembranous proteolytic cleavage of CD44 molecules upon stimulation and release of the intracellular domain have been implicated in CD44-dependent signaling events [26], but we could not detect the presence of CD44 intracellular-domain fragments after treatment with SNC-PG (data not shown).

Although SNC-PG has various physiological actions [4–13], the molecular mechanism of SNC-PG-mediated cellular events remains unclear. Our results that SNC-PG stimulates cells through CD44 would help understand the molecular mechanism of intracellular signal transduction induced by PGs or CSs. Moreover, CD44, an adhesion/homing molecule, has attracted much attention from researchers across a broad range of basic science and clinical medicine because CD44 is involved in many cellular processes including cell growth, survival, differentiation, and motility and contributes to many pathological conditions including tumorization. Therefore, it is essential to characterize the molecular events involved in the cellular responses following the interaction of SNC-PG (or CSs) with CD44. In summary, we report for the first time that SNC-PG promoted wound healing using an *in vitro* assay of fibroblast and keratinocyte monolayers. Our results raise the possibility that SNC-PG could be a useful pharmaceutical agent, including ointment, to facilitate wound healing. Testing whether SNC-PG shows a similar effect *in vivo* would be an interesting future study.

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