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CXCL14 Enhances Proliferation and Migration of NCI-H460 Human Lung Cancer Cells Overexpressing the Glycoproteins Containing Heparan Sulfate or Sialic Acid

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ABSTRACT

CXCL14 is a chemokine family member that is involved in various cellular responses in addition to immune cell activation. Although constitutive CXCL14 expression in normal epithelial cells may help protect against infection by activating immune systems, its expression in cancer cells has raised controversy regarding its possible role in tumorigenesis. However, the underlying mechanisms for this disparity remain unknown. Investigation of cellular CXCL14 binding properties might increase our understanding of the peptide's roles in tumorigenesis. In the present study, we found that CXCL14 binds to various cell types. Interestingly, binding to NCI-H460 cells was prevented by heparan sulfate and N-acetyl neuraminic acid. Next, we examined effect of CXCL14 binding in NCI-H460 and NCI-H23. CXCL14 enhanced proliferation and migration in NCI-H460 but had no effect on NCI-H23. A reporter gene assay with various transcription factor response elements revealed that only nuclear factor-κB (NF-κB) signaling was activated by CXCL14 in NCI-H460 cells, which was blocked by BAPTA-AM, TPCA-1, and brefeldin A. Exogenous expression of some glycoproteins such as syndecan-4, podoplanin, and CD43 in these cells enhanced CXCL14 binding and NF-κB activity. Collectively, these results demonstrate that CXCL14 binding to glycoproteins harboring heparan sulfate proteoglycans and sialic acids leads proliferation and migration of some cancer cells. J. Cell. Biochem. 114: 1084–1096, 2013.

KEY WORDS: CXCL14; HEPARAN SULFATE PROTEOGLYCAN; SIALIC ACID; GLYCOPROTEIN; MIGRATION; NF-κB

CXCL14 is a member of the CXC chemokine family that is also known as BRAK (breast- and kidney-expressed chemokine). CXCL14 is constitutively expressed in many tissues, including breast, kidney, and healthy human skin, especially in keratinocytes and dermal fibroblasts [Cao et al., 2000; Frederick et al., 2000; Sleeman et al., 2000]. Like other chemokines, CXCL14 can induce the migration of several immune cells, including macrophages, dendritic precursor cells, and natural killer cells [Cao et al., 2000; Sleeman et al., 2000]. CXCL14 was implicated in the homeostasis of epithelial tissues, especially skin; it attracts dendritic precursor cells to distinct regions of skin where they differentiate into the antigen presenting cells [Kurth et al., 2001; Schaerli et al., 2005]. However, CXCL14 is one of only two orphan chemokines whose endogenous receptor has not been identified. Although we examined CXCL14-stimulated activation of all chemokine receptors and most orphan G-protein coupled receptors (GPCRs) showing rhodopsin-like structure using several GPCR activation assay systems, none of them responded to CXCL14 (unpublished results).

The CXC chemokine family can be divided into subgroups by the presence or absence of a conserved sequence (Glu-Leu-Arg) called an ELR motif that is located next to the first cysteine residue of the CXC motif. CXCL14 belongs to the ELR(−) CXC chemokine group

Abbreviations: BRAK, breast- and kidney-expressed chemokine; HSPG, heparan sulfate proteoglycan; GPCR, G protein-coupled receptor; Neu5Ac, N-acetyl-neuraminic acid; SDC, syndecan.

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along with CXCL4, CXCL9, and CXCL10, which negatively regulate angiogenesis and endothelial cell proliferation [Angiolillo et al., 1995; Arenberg et al., 1997]. CXCL14 also inhibits angiogenesis in both in vivo and in vitro conditions by blocking endothelial chemotaxis [Shellenberger et al., 2004].

The gross structure of CXCL14 is similar to other chemokines, especially interleukin-8(CXCL8) [Peterson et al., 2006]. However, there are some peculiarities in amino acid sequences that distinguish it from others. CXCL14 has just two amino acids in front of the CXC motif, whereas others contain more than five amino acids, some of which include an ELR motif. Although no functional significance with the exception of the ELR motif has been defined in this region, the short sequence might influence regional structure or receptor interaction. In most chemokine N-loop that connects N-terminal cysteine residues to the first strand of β-sheets, there is a well-defined structure due to hydrophobic interaction with the C-terminal α-helix. However, this contact is not found in CXCL14, and its N-loop is more flexible than that of other chemokines. CXCL14 has a unique sequence (^{41}VSRYR^{45}) between its β2 and β3 strands, and this was suggested as a target site for proteosomal degradation by Peterson et al. [2006]. Because CXCL14 expression in normal tissues is easily detected by biochemical methods using specific antibodies, the degradation process is likely to occur in unusual conditions, and the sequences might confer a novel functional role to CXCL14. These distinctive features of CXCL14 seem to be the reason its receptor has not been identified yet.

To date, several research groups have explored the biological properties of CXCL14 in addition to its chemotactic activity in immune cells. Constitutive CXCL14 expression in epithelia may help protect against microorganism infection for two reasons: It has bactericidal activity and it recruits immune cells to the skin and other epithelial tissues [Schaerli et al., 2005; Maerki et al., 2009]. In terms of glucose metabolism, CXCL14 attenuates insulin-stimulated glucose uptake in myocytes. Animal studies demonstrated that CXCL14 deficiency attenuated obesity and maintained an insulin-sensitive phenotype despite a high-fat diet [Nara et al., 2007]. Furthermore, feeding behavior of CXCL14 knock-out mice is significantly suppressed in a novel environment [Tanegashima et al., 2010]. This evidence suggests that CXCL14 may be involved in both glucose metabolism and neuronal regulation of feeding behavior. During tumorigenesis, CXCL14 is decreased or even absent in many types of cancers. In head and neck cancer cells, its expression is down-regulated by epidermal growth factor, which is responsible for cell proliferation [Shurin et al., 2005]. At the genome level, the *CXCL14* gene is highly methylated, and its mRNA is not detectable in lung adenocarcinomas and lung-derived cancer cell lines [Tessema et al., 2010]. Furthermore, exogenous CXCL14 inhibits cell growth and induces cell death, suggesting that CXCL14 is anti-carcinogenic. However, CXCL14 is up-regulated in other cancers, such as prostate and pancreatic cancers and stromal cells around tumors [Allinen et al., 2004; Augsten et al., 2009]. Stimulation with CXCL14 increases the motility and invasiveness of breast and pancreatic cancer cells [Wente et al., 2008]. These discrepancies may be due to unknown functional characteristics of CXCL14.

Chemokines can interact with target cells in two ways. A canonical interaction is through GPCRs expressed in target cells, which mainly induces chemotactic cell migration. The other is through polysaccharide-containing proteins (e.g., glycosaminoglycan) and lipids [Rybak et al., 1989; Kuschert et al., 1999]. Although the physiological effects of the latter interactions are not necessarily obvious, some chemokines likely regulate target cell biological activities by interacting with glycoproteins. CXCL10 and platelet factor 4 (PF4) bind to a specific cell surface heparan sulfate and inhibit endothelial cell proliferation [Luster et al., 1995]. CXCL10 also interacts with syndecan-4, a proteoglycan on pulmonary fibroblasts, thereby inhibiting fibroblast migration and subsequent fibrosis [Jiang et al., 2010]. A previous study reported that CXCL14 can bind to the membrane surface of some cells, and this interaction is blocked by heparin [Shellenberger et al., 2004]. They suggested that CXCL14 also regulates cellular behaviors by binding to glycosaminoglycans.

The purpose of this study was to evaluate CXCL14 binding to saccharides and the cellular responses to this interaction. Using alkaline phosphatase-fusion protein, we found a specific cell surface saccharide composition related to CXCL14 binding. This interaction may provide insight into CXCL14-mediated mechanisms that favor the progression of some cancers.

MATERIALS AND METHODS

MATERIALS AND CELL LINES

Heparin, heparan sulfate, chondroitin sulfate A and B, N-acetyl-neuraminic acid (Neu5Ac), heparinase I, and neuraminidase were obtained from Sigma (St. Louis, MO). Human recombinant CXCL14 was purchased from PeproTech (Rocky Hill, NJ). Anti-His antibody was from Abfrontier, Inc. (Seoul, Korea), and peroxidase-labeled antibodies were from Kirkegaard & Perry Laboratory (Gaithersburg, MD). Fluorescein isothiocyanate (FITC-) conjugated anti-rabbit immunoglobulin (IgG) was purchased from Abcam (Cambridge, UK). HEK293, HEK293T, Detroit 551, NCI-H460, NCI-H23, U937, and LNCaP cell lines were obtained from American Type Culture Collection (Rockville, MD). SNU-C1 was purchased from the Korean Cell Line Bank (Seoul, Korea). Cell culture media including Dulbecco's Modified Eagle Medium (DMEM) and RPMI 1640 were obtained from WELGENE Inc. (Daegu, Korea). HEK293, HEK293T and Detroit 551 cells were maintained in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. NCI-H460, NCI-H23, U937, LNCaP, and SNU-C1 cells were maintained in RPMI1640 medium containing 10% FBS and 1% penicillin and streptomycin. TRIzol and SuperScript™ II reverse transcriptase were purchased from Invitrogen (Carlsbad, CA). All chemicals including various inhibitors were from Sigma. All glycoptrotein genes including syndecans (SDC), CD44, podoplanin (PDPN), glycophorin C (GYPC), 67LR, CD44, CD43, podocalyxin-like 2 (PODXL2), galectin 3, glypican 4, patatin-like phospholipase domain containing 2 (PNPLA2) and asialoglycoprotein receptor 2 (ASGR2) were obtained from 21C Frontier Human Gene Bank (Daejeon, Korea) or isolated by RT-PCR with specific primers. They were inserted to pcDNA3.1 vector for mammalian cell expression.

PRODUCTION OF FC FUSION PROTEIN

cDNA of the Fc domain of rabbit IgG kindly provided by Dr. Junho Chung in Seoul National University was inserted into enzyme sites *Sfi* I/*Bam*H I in a pCEP4 vector. Human CXCL14 cDNA lacking a termination codon was inserted into the plasmid in frame. pCEP4/CXCL14-Fc plasmids were transfected into HEK293T cells using the calcium phosphate method. Cells were maintained with serum-free media for 3–4 days. Culture supernatants were collected, centrifuged to remove detached cells, and stored at −70°C until they were used in binding experiments.

FLUORESCENCE ACTIVATED CELL SORTING (FACS) ANALYSIS

Adherent cells were detached with 0.25% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA). Both adherent cells and non-adherent cells were washed twice with binding buffer [phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA)]. A total of 5×10^5 cells were incubated with culture supernatants containing rabbit Fc or CXCL14-rabbit Fc on ice for 30 min. After washing four times with binding buffer, cells were incubated with 1 μg FITC-conjugated anti-rabbit IgG for 30 min on ice, washed four times, then analyzed on a FACScan with Cell Quest software (BD Biosciences, San Diego, CA).

PRODUCTION OF ALKALINE PHOSPHATASE (AP) FUSION PROTEIN

Human cDNAs of CXCL14 and CXCL10 were amplified by polymerase chain reaction (PCR) with primers lacking a termination codon, digested with *Nhe* I/*Bgl* II, and inserted into a pAPtag5 vector (GenHunter, Nashville, TN). Protein products from the plasmid contain the authentic signal sequences and entire mature proteins linked and secreted alkaline phosphatase through the four amino acid linker Arg-Ser-Ser-Gly. The CXCL14-APtag plasmids were transfected into HEK293T cells using the calcium phosphate method. The next day, culture media was removed, and cells were maintained with serum-free DMEM for 3–4 days. Culture supernatants were collected and centrifuged to remove detached cells. The fusion proteins were purified with an ÄKTAFPLC (fast protein liquid chromatography) system (GE Healthcare Life Science, Pittsburgh, PA) using an Ni-NTA column. Purified proteins were dialyzed with PBS, concentrated, and stored at -70°C.

AP FUSION PROTEIN BINDING ASSAY

Cells (1×10^5) were seeded in 24-well plates and cultured for 2 days. Cells were washed twice with 2 ml ice-cold binding buffer (HBSS containing 10 mM HEPES, 0.1% BSA) and incubated with the indicated concentrations of CXCL14-AP and competitors in a total of 500 μl ice-cold binding buffer for 2 h on ice with occasional rocking. Cells were washed six times with ice-cold binding buffer, lysed with 100 μl 10 mM Tris, pH 8.0, 1% Triton X-100, and transferred into an E-tube. Cellular APs were inactivated by heating at 65°C for 2 h. After centrifugation at 14,000×g for 10 min, 30 μl soluble lysate was mixed with 60 μl p-nitrophenyl phosphate (pNPP) solution (Thermo Scientific, Rockford, IL) in a 96-well plate at room temperature, and the optical density (OD) of the colorimetric product was measured with spectrophotometry at 405 nm. To remove saccharides from the plasma membrane, cultured cells were washed with serum-free DMEM and incubated in 500 μl serum-free DMEM containing 2 U/ml heparinase I or 2 U/ml neuraminidase at 37°C and 5% CO_2 for 1 h. After four washes with ice-cold binding buffer, a cell-bound AP activity assay was performed as described above. To examine the effect of salt on binding, cells were incubated with 1 M NaCl for 1 min after CXCL14-AP binding, washed six times with binding buffer, and lysed with lysis buffer, as described in previous report [Luster et al., 1995].

WESTERN BLOTTING

To assess protein expression, 10 μl culture supernatant from HEK293T cells expressing fusion proteins were mixed with 5× sample buffer, boiled for 5 min, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). For immunoblotting, proteins were transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with Tween 20 (TBST) washing buffer, probed with appropriate antibodies, and signal was developed using an enhanced chemiluminescence (ECL) kit (GE Healthcare Life Science). After the generation of CXCL14-expressing cells, 20 μl culture supernatant was used for SDS–PAGE and western blotting with anti-CXCL14 antibodies.

ESTABLISHMENT OF CELLS EXPRESSING CXCL14

The human *CXCL14* gene was cloned into a lentiviral FUGW vector under control of a ubiquitin promoter as previously described [Lois et al., 2002]. HEK293T cells were transfected with the expression vector and accessory plasmids using calcium phosphate precipitation. Forty-eight hours later, the culture media was harvested and concentrated on Amicon Ultra columns (Millipore, MA). An enhanced green fluorescence protein (EGFP-) expressing virus was used as a control to monitor viral titer. We employed 10 multiplicity of infection of viral titer to infect NCI-H460 and NCI-H23 cells. CXCL14 expression was confirmed by western blotting with anti-CXCL14 antibodies.

CELL GROWTH ASSAY

Growth of NCI-H460 and NCI-H23 cells in real time was measured by using an RTCA DP instrument (xCELLigence System, Roche Inc., Basel, Switzerland). Initially, 1×10^4 cells/well were grown in E-plate 96 plates with RPMI media containing 1% FBS and antibiotics. Data were collected at 1 h intervals. After 24 h, cells were treated with 100 ng/ml CXCL14 and 1 μg/ml AP fusion proteins and cultured until the indicated time.

As another method to test cell proliferation, we used a Cell Counting Kit-8 (CCK-8) from Dojindo Molecular Technologies, Inc. (Rockville, MD). Briefly, 4×10^3 cells/well were seeded in 96-well plates. After 24 h, cells were treated with CXCL14 for 72 h and then incubated with 10 μl CCK-8 solution for 2 h. The absorbance of each well was measured at 450 nm using a microplate reader. CXCL14-expressing cell proliferation was determined by same procedure without the peptide.

WOUND HEALING ASSAY

For the in vitro wound healing assay, a confluent monolayer of NCI-H460 cells was serum starved overnight, and the bottom of the well was scratched with a pipette tip. After several washes with

serum-free media, cells were incubated with 0.1% serum-containing media with or without CXCL14. Images of cell migration into the wound were captured at 24-h intervals.

TRANSWELL MIGRATION ASSAY

We obtained a suspension of cells expressing CXCL14 or not with 2-min trypsin-EDTA treatments at 37°C. Prior to the migration assay, transwell inserts with 8-μm pores (Corning Inc., Corning, NY) were coated with matrigel (Invitrogen, Carlsbad, CA) for 2 h at 37°C. Cells (5×10^4) in RPMI media containing 0.1% BSA were added to transwell inserts. RPMI media containing 10% FBS were added to the bottom wells, and the plates were incubated in a humidified incubator with 5% CO_2 at 37°C for 24 h. Transwell inserts were washed in PBS, and non-migrated cells in the inner wells were removed with a cotton swab. The membranes were fixed in 4% formalin and stained with hematoxylin, and migrated cells were counted in five high-power fields under a light microscope. To examine CXCL14 chemotactic activity, RPMI media containing various concentration of CXCL14 peptides and 0.1% BSA were added to the bottom wells, and NCI-H460 cells (5×10^4) in RPMI media containing 0.1% BSA were added to the transwell inserts.

REPORTER GENE ASSAY

The luciferase activity assays with transcription factor-response elements were performed as previously described [Kim et al., 2010]. In brief, NCI-H460 or NCI-H23 cells (8×10^4/well) were cultured overnight in 24-well plates and subsequently transfected with liposome complexes containing reporter genes (200 ng) and pCMV/β-galactosidase plasmid (20 ng). After a 48-h, incubation with CXCL14 (20 ng/ml) or CXCL14-AP (200 ng/ml) for 6 h, cells were washed with PBS and solubilized with lysis buffer. We used ten times higher amount of AP-tag proteins compared with intact peptides considering molarities equivalent. Luciferase activities of cell extracts were determined using a standard luciferase assay system from BioTek Instrument, Inc. (Winooski, VT). NCI-H460 cells containing the nuclear factor-κB (NF-κB) reporter gene were incubated with 500 μl serum-free DMEM containing 2 U/ml heparinase I or 2 U/ml neuraminidase at 37°C and 5% CO_2 for 1 h to remove cell surface saccharides. After four washes with serum-free media, cells were treated with CXCL14 (20 ng/ml) for 6 h. To investigate the effect of the inhibitors on NF-κB activation, cells transfected with the reporter gene were treated with various inhibitors for 20 min prior to CXCL14 stimulation. Transfection efficiency was determined and normalized to β-galactosidase activity. All data were collected from at least three independent experiments and normalized to untreated groups.

RT-PCR

Total RNA was extracted from NCI-H460 and NCI-H23 cells using Trizol (Invitrogen) and a reverse transcription reaction was performed with 1 μg of RNA and superscript™ II Reverse Transcriptase, according to the instructions of the manufacturer. PCR amplification with specific primers for each glycoprotein was performed (35 cycles) and PCR products were loaded on a 1.5% agarose gel.

DATA ANALYSIS

All data were obtained from at least three independent experiments and presented as means ± standard error. Group means were compared using Student's *t*-test or one-way analysis of variance (ANOVA). $P < 0.05$ was accepted to indicate statistical significance.

RESULTS

CXCL14 SPECIFICALLY BOUND TO THE PLASMA MEMBRANE OF MANY CELL TYPES

Because radiolabeled chemokines produced in *E. coli* easily aggregate in solution and on the cell surface, it is hard to evaluate a specific interaction between the peptide and membrane molecules [Lodi et al., 1994; Luster et al., 1995]. To overcome this problem, CXCL14 was expressed as a fusion form with either the Fc region of rabbit immunoglobulin IgG or alkaline phosphatase, which resulted in the secretion of non-aggregating monomeric proteins. We used the Fc gene but substituted the codon for cysteine with that for serine because wild-type Fc contains a cysteine responsible for disulfide bond dimerization in the hinge region. The fusion proteins were secreted to the culture media and were stably expressed in HEK293T cells (Fig. 1A). To screen for cells that bound to the fusion protein, culture supernatants were incubated with many types of cells, and binding properties were determined by FACS using FITC-conjugated anti-rabbit IgG antibodies. As shown in Figure 1B, NCI-H460, SNU-C1, Detroit, and NCI-H23 cells bound to CXCL14-Fc with relatively high affinity. Trypsin was not likely to inhibit CXCL14-Fc binding to these cells because they bound to the peptides when they were detached from the dish by trypsin prior to the assay. U937 and HEK293 cells did not show any binding to the fusion protein. When NCI-H460 and SNU-C1 cells were incubated with serially diluted supernatants, they bound to CXCL14-Fc in a dose-dependent manner, implying that CXCL14 attached to certain cell surface molecules with specificity (Fig. 1C).

We used CXCL14-AP for the next set of binding studies because the enzymatic activity of the fusion protein was sensitive enough for quantitative analysis of the peptide binding properties. AP-tagged proteins were not cleaved in the culture media and even after several freezing and thawing cycles (Fig. 2A). Endogenous APs should be inactivated by heating because they interfere with the measurement of the specific activity of AP fusion protein by competing for the substrate. However, we found that heat inactivation method recommended by the supplier was not enough to eliminate endogenous AP activity in SNU-C1 cells (data not shown). For this reason, we used NCI-H460 cells for the next experiments. CXCL14-AP bound to the cells with a K_d of 26 nM (Fig. 2B). When the cells were treated with 200 nM CXCL14-Fc and 400 nM CXCL14 peptide prior to 30 nM CXCL14-AP incubation, the AP activities were markedly decreased (Fig. 2C), suggesting that CXCL14 specifically binds to NCI-H460 cells with relatively high affinity.

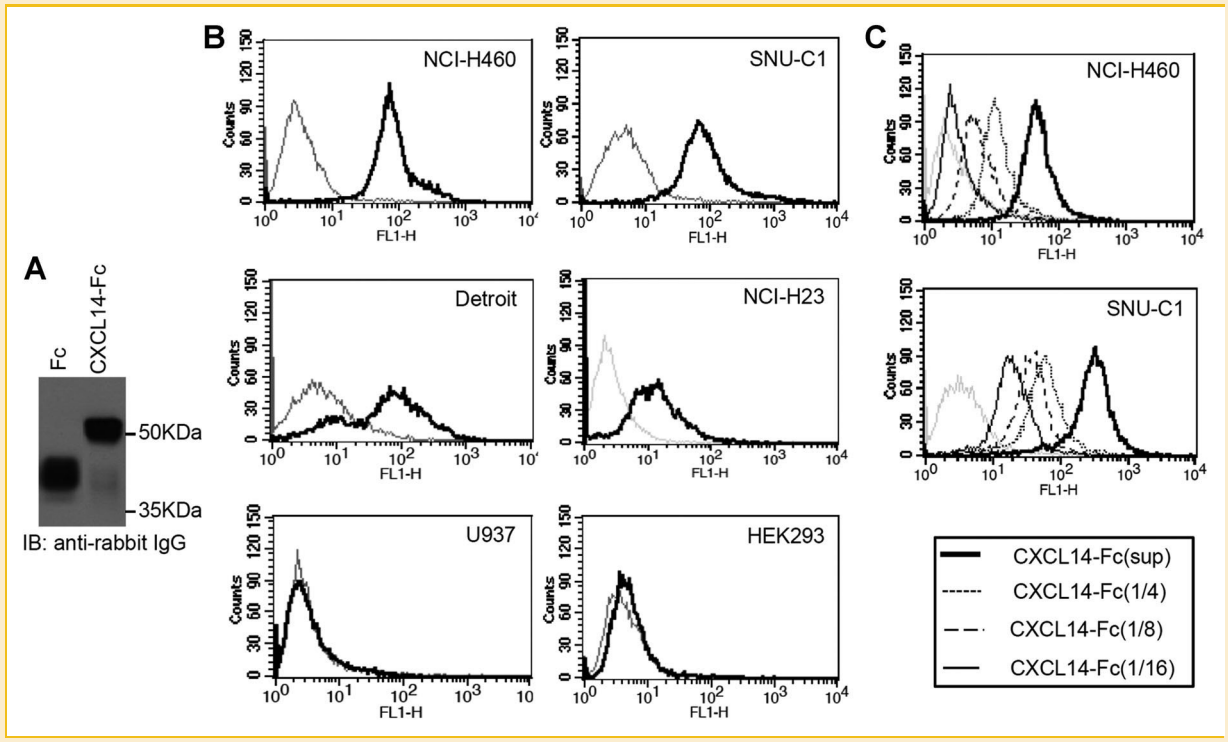

Fig. 1. CXCL14-Fc binds to several types of cells. A: Expression of CXCL14-Fc. Human CXCL14 gene was fused with the gene for the Fc region of rabbit IgG and expressed in HEK293T cells. The culture supernatants were subjected to western blotting with anti-rabbit IgG antibodies. B: FACS analysis. Cells (5×10^5) were incubated with culture supernatants containing Fc or CXCL14-Fc followed by FITC-conjugated anti-rabbit IgG. Binding was analyzed by FACS. C: Dose-dependent binding of CXCL14-Fc. NCI-H460 and SNU-C1 cells were incubated with serially diluted culture supernatants, and their binding levels were analyzed by FACS.

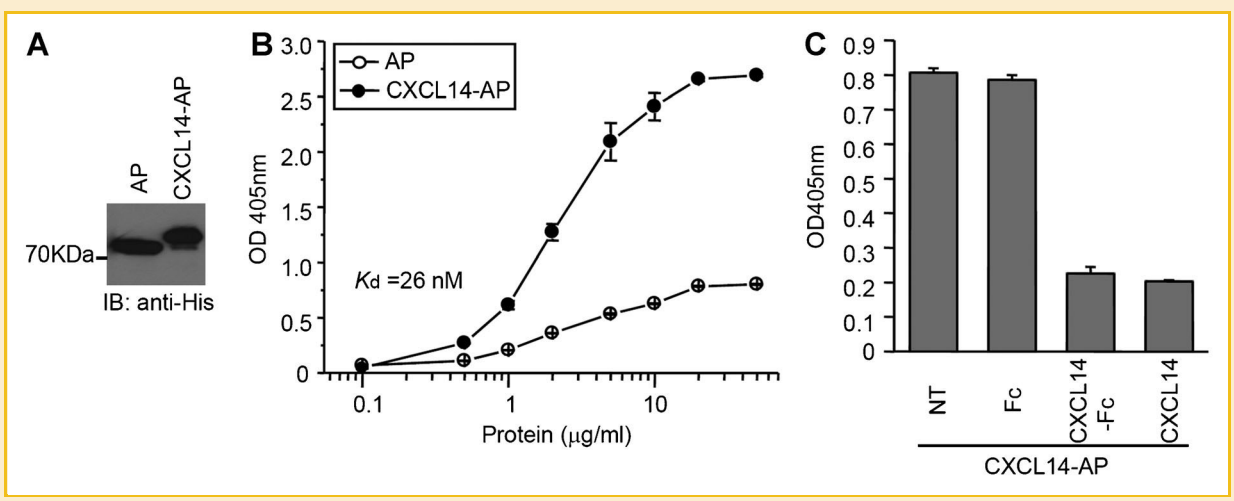

Fig. 2. CXCL14-AP binding to NCI-H460 cells is specific and saturable. A: Expression of CXCL14-AP. The human *CXCL14* gene was inserted into a pAPtag5 vector and expressed in HEK293T cells. Culture supernatants were harvested, and AP proteins were purified with Ni-NTA. Western blotting was done with anti-His antibodies. B: CXCL14-AP binding to NCI-H460 cells. NCI-H460 cells grown in 24-well plates were incubated with increasing concentrations of CXCL14-AP. Binding efficiency ($K_d = 26$ nM) was calculated from specific binding (Specific binding = CXCL14-AP binding − AP binding). Nonspecific binding of CXCL14-AP determined in the presence of 10 μM unlabeled CXCL14 was quite similar to the AP binding value ($P < 0.02$ differences). C: NCI-H460 cells were incubated with 2 μg/ml CXCL14-AP in the presence of competitors (20 μg/ml). NT, non-treatment.

CXCL14 BOUND TO NCI-H460 CELLS THROUGH SURFACE SACCHARIDES

Several chemokines including CXCL10 and PF4 have been shown to bind to cell surface heparan sulfate proteoglycans (HSPG) [Luster et al., 1995] and may block the receptor binding of some growth factors that require HSPGs as a part of their ligand-receptor complexes. They are likely to bind membrane surface HSPGs with different affinities and specificities. To explore CXCL14 binding properties, we incubated cells with various saccharides and CXCL14-AP. Heparin and heparan sulfate blocked CXCL14-AP binding to cells in a dose-dependent manner (Fig. 3A), but even high concentrations did not completely block binding. Chondroitin sulfates A and B, which are also glycosaminoglycans, had no effect on CXCL14-AP binding even at 200 μg/ml. This binding property was somewhat different from that of other chemokines, including CXCL10 and PF4, which were blocked from binding by heparin and heparan sulfate [Luster et al., 1995]. Interestingly, neuraminic acid (Neu5Ac) inhibited CXCL14-AP binding more potently than heparin and heparan sulfate. Moreover, 100 μg/ml completely blocked binding. To compare the binding properties of CXCL14 and CXCL10, cells were incubated with AP-fusion proteins and 10 μg/ml of each saccharide. Heparin and heparan sulfate inhibited CXCL10-AP binding to cells, which was in an agreement with a previous report [Luster et al., 1995], but Neu5Ac did not inhibit binding. Conversely, CXCL14-AP binding was potently blocked by heparin, heparan sulfate, and Neu5Ac (Fig. 3B), implying that CXCL14 is able to bind HSPGs and sialic acid-containing glycoproteins with higher affinity than CXCL10.

Next, we explored whether CXCL14 interacts with cells via saccharides. Figure 3C revealed that heparinase I dramatically decreased the binding sites of both CXCL14-AP and CXCL10-AP on NCI-H460 cells. Neuraminidase also removed CXCL14-AP binding

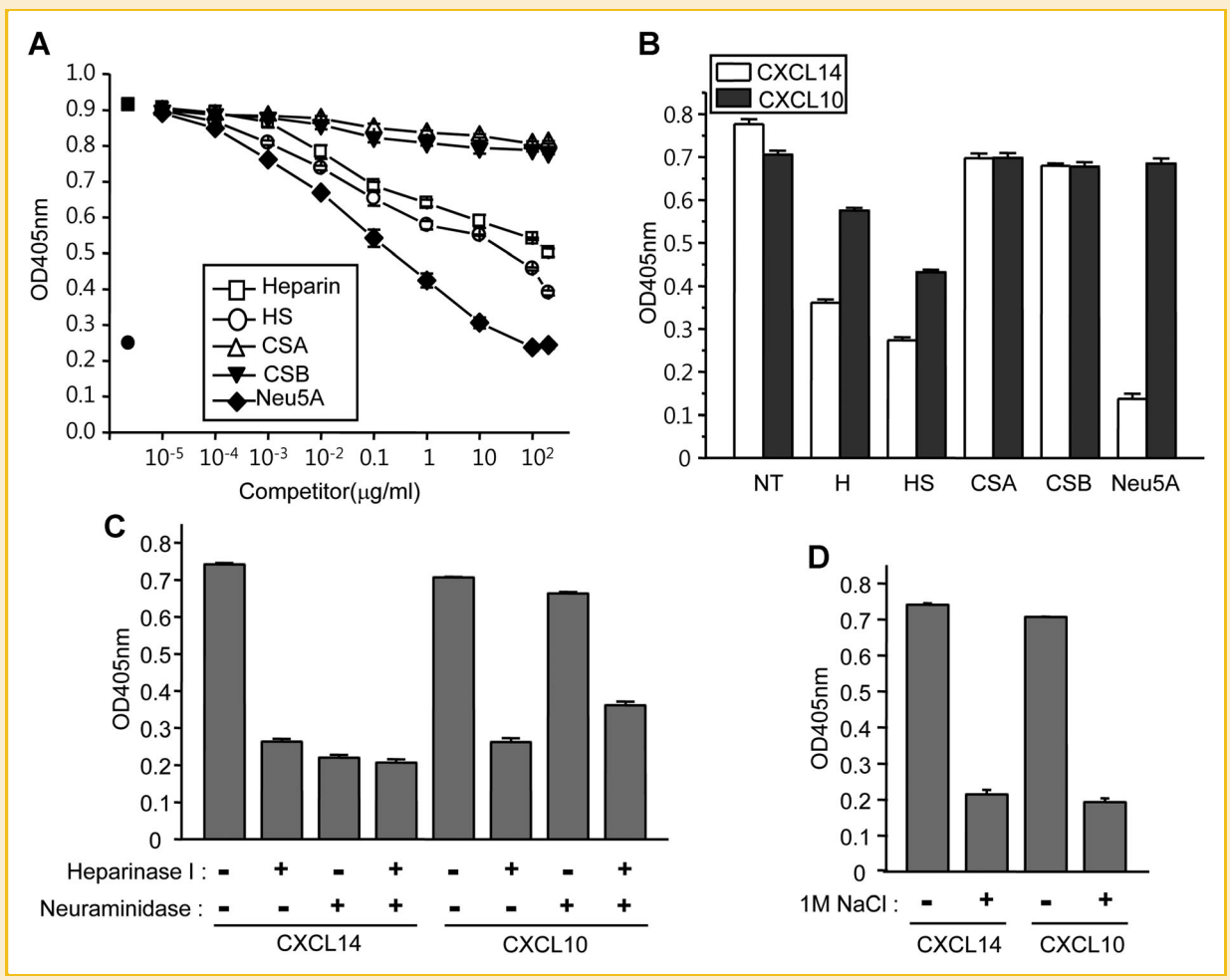

Fig. 3. CXCL14-AP binding was inhibited by specific saccharides. A: Concentration dependent inhibition. NCI-H460 cells were incubated with CXCL14-AP in the presence of increasing concentrations of saccharides. HS, heparan sulfate; CSA and CSB, chondroitin sulfate A and B; Neu5Ac, N-acetylneuraminic acid. ■, 2 μg/ml CXCL14-AP binding without competitor; ●, 2 μg/ml AP binding. B: Comparison of CXLC14 and CXCL10 in saccharide binding. NCI-H460 cells were incubated with 2 μg/ml CXCL14-AP or CXCL10-AP in the presence of 10 μg/ml saccharides. All values were specific binding (AP bindings were subtracted.) NT, non-treatment; H, heparin. C: The effect of saccharide-degrading enzymes. NCI-H460 cells were treated with 2 U/ml heparinase I or neuraminidase prior to incubation with CXCL14-AP or CXCL10-AP. All values were specific binding. D: The effect of salt. After AP-fusion protein binding, cells were washed with 1 M NaCl. All values were specific binding.

sites from the cells but had no effect on CXCL10-AP binding, suggesting that only CXCL14 is able to bind cells through Neu5Ac. Because the interaction between proteins and saccharides is primarily mediated by ionic interactions, we considered that chemokine binding might be salt sensitive. As shown in Figure 3D, washing with 1 M NaCl removed almost all of the AP-fusion chemokines from NCI-H460 cell surfaces, which is consistent with previous results [Luster et al., 1995].

CXCL14 also bound to NCI-H23 and Detroit cells (Fig. 1B). Thus, we investigated whether the binding properties of NCI-H460 cells could be applicable to interactions with other cells. Washing with 1 M NaCl removed the CXCL14-AP from the surface of all cells. CXCL14-AP binding to LNCaP cells was also inhibited by heparinase and neuraminidase. However, neither of the enzymes inhibited CXCL14-AP binding to NCI-H23 or Detroit cells (Fig. 4). This result suggests that some cells including NCI-H23 and Detroit cells are able to bind chemokines through cell surface molecules other than HSPGs and Neu5Ac.

CXCL14 STIMULATED NCI-H460 CELL PROLIFERATION AND MIGRATION

CXCL14 has demonstrated dichotomous functions that induce cell growth and death, depending on experimental conditions and cancer cell types. Here, we used an xCELLigence system to test the effect of CXCL14 on the growth of NCI-H460 and NCI-H23 cells, both of which originate from non-small cell lung cancers. CXCL14 and CXCL14-AP enhanced the growth rate of NCI-H460 cells compared with the control treatment (Fig. 5A). This result indicates that the functional activity of CXCL14-AP is equivalent to CXCL14 itself. In contrast, the curve of NCI-H23 cells was lowered by CXCL14 (Fig. 5B). To confirm the activity of CXCL14 on cell growth, we performed another proliferation assay using CCK-8. NCI-H460 cells grew rapidly following CXCL14 and CXCL14-AP treatment compared with control, which is consistent with the results of the xCELLigence System assays. However, CXCL14 had no effect on NCI-H23 cell growth (Fig. 5C). This result implies that the effect of CXCL14 on NCI-H23 cells observed in the xCELLigence System (Fig. 5B) seems to be related to cellular behaviors other than proliferation. Different effects of the peptide on the cell growth are likely dependent on cell surface glycoproteins because CXCL14 binding properties of both cell types were different.

CXCL14 also has positive and negative roles on cell migration. The effect of the peptide on NCI-H460 cell migration was determined in the wound healing assay. The wound in the CXCL14-treated cell monolayer was nearly closed after 48 h, whereas control cells were only 50% closed, demonstrating that CXCL14 stimulates NCI-H460 cell migration (Fig. 5D).

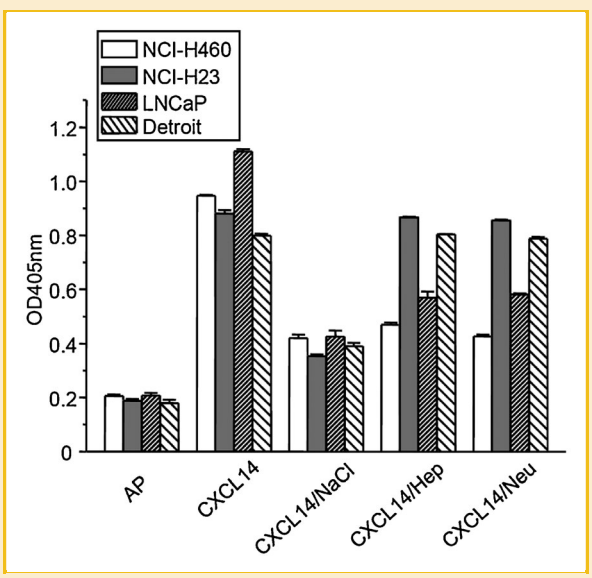

Fig. 4. CXCL14-AP binding properties in different cells. To test the effect of salt, cells grown in 24-well plates were incubated CXCL14-AP followed by 1 M NaCl. After they were washed with binding buffer, cells were lysed and used in AP activity assays. To eliminate cell surface saccharides from surface, cells were treated with 2 U/ml heparinase I or 2 U/ml neuraminidase prior to CXCL14-AP binding. Hep, heparinase; Neu, neuraminidase.

EXOGENOUS EXPRESSION OF CXCL14 ENHANCES NCI-H460 CELL PROLIFERATION AND MIGRATION

The expression pattern and biological activity of CXCL14 in cancer cells are controversial [Wente et al., 2008; Tessema et al., 2010]. CXCL14 in NCI-H460 and NCI-H23 cells was hardly detected by RT-PCR (data not shown). To explore the effect of exogenous CXCL14 expression, we established CXCL14-expressing cells using a lentiviral vector (Fig. 6A). CXCL14-expressing NCI-H460 cells grew slightly faster than control cells, whereas the growth of NCI-H23 cells was not changed in the presence of CXCL14 (Fig. 6B). In wound healing assay, CXCL14 enhanced closing the wounded area compared with control (Fig. 5D). This would provoke the idea that wound healing may be due to growth stimulation of the peptide to NCI-H460 cells. To exclude the possibility, we examined transwell migration of NCI-H460 cells toward serum or CXCL14 peptide. As shown in Figure 6C, NCI-H460 cells expressing CXCL14 were better able to migrate toward serum. However, CXCL14 was dispensable for the migration of NCI-H23 cells toward serum (Fig. 6D). To examine whether CXCL14 itself stimulates cellular migration, we added various concentrations of CXCL14 to the bottom wells of transwell migration assay plates. Some migrated cells were observed at 4 μg/ml CXCL14, but no migrated cells were detected at lower CXCL14 concentrations (Fig. 6E). The results imply that CXCL14 is likely to have mitogenic activity and enhance cellular mobility, but it does not stimulate strong chemotaxis of NCI-H460 cells by itself.

CXCL14 INDUCES NF-κB ACTIVATION THROUGH CALCIUM-DEPENDENT SIGNALING AND ENDOCYTOSIS

Because cellular proliferation is generally accomplished through the activation of transcription factors, we examined the effect of CXCL14 on transcription factors in NCI-H460 cells with reporter gene assays. As shown in Figure 7A, CXCL14 activated NF- κB but not the other analyzed transcription factors, suggesting that NF-κB signaling is an important pathway responsible for the cellular responses to CXCL14. We found that CXCL14-AP stimulated NF-κB activity with similar potency to CXCL14, implying that the fusion protein is functionally equivalent to the intact chemokine (Fig. 7B).

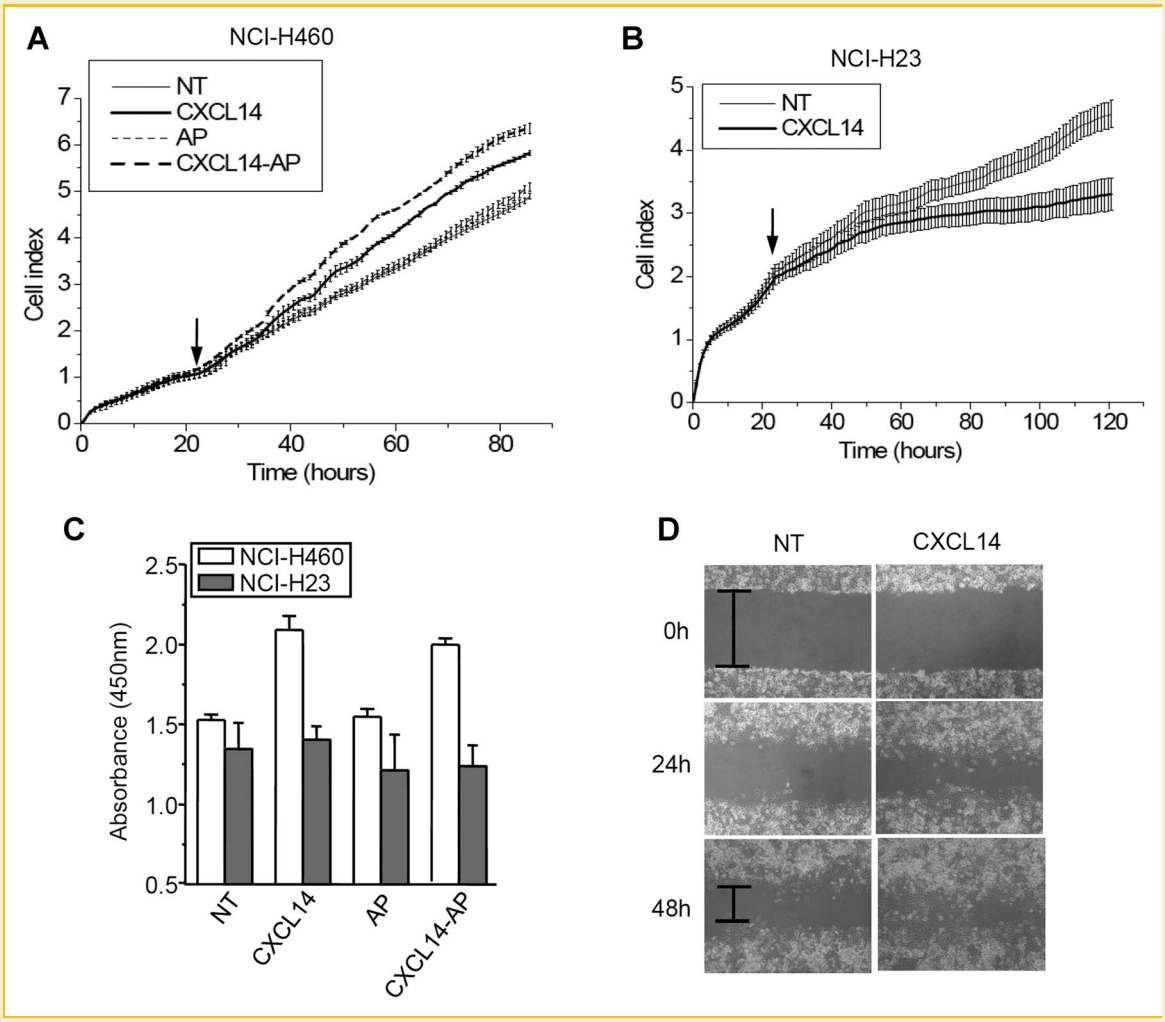

Fig. 5. The effect of CXCL14 on cell proliferation. A, B: Cells (1×10^5/well) were seeded in E-plates (96-well) from RTCA DP instruments, and data were collected every 1 h. Twenty-four hours later, cells were treated with 100 ng/ml CXCL14 and 1 μg/ml AP fusion proteins and cultured until the indicated time. Arrows indicate protein treatment points. NT, non-treatment. C: Cells (4×10^4/well) were seeded in 96-well plates. The next day, cells were treated with 1% FBS media containing 100 ng/ml CXCL14 and 1 μg/ml AP fusion proteins. After 72 h, cells were incubated with CCK-8 solution, and a microplate reader was used to measure absorbance at 450 nm. D: Wound healing assay. NCI-H460 cells were cultured in six-well plates until they reached monolayer confluency and starved overnight. The monolayer was scratched with a pipette tip, and cells were washed several times and incubated with 0.1% FBS media with containing 100 ng/ml CXCL14. The images of cell migration into the wound were captured at 24 h intervals. All experiments were performed more than three times. The data in the figures are the representative of the results.

However, NF-κB activation was not observed in NCI-H23 cells (data not shown). Because cellular CXCL14 binding was dependent on surface HSPGs and sialic acids, we treated cells with heparinase I and neuraminidase prior to CXCL14 stimulation to remove cell surface saccharides. These enzymes markedly decreased CXCL14-mediated NF-κB activation (Fig. 7C).

Many intracellular signaling events induced by exogenous stimuli converge on NF-κB [Rothwarf and Karin, 1999; Perkins, 2012]. To identify signaling pathways that transduce NF-κB activation, cells were treated with various signaling inhibitors prior to CXCL14 stimulation. TPCA-1 and IKK-16 blocked NF-κB activation, suggesting that IκB kinase mediates CXCL14 stimulation of NF-κB. Interestingly, BAPTA-AM, an intracellular calcium chelator, completely inhibited NF-κB activation, implying that CXCL14 is likely to increase intracellular calcium and trigger NF-κB activation. Unfortunately, a CXCL14-mediated calcium increase was not observed with Fura2-AM (data not shown). This indicates that NCI-H460 cells do not express GPCRs that respond to CXCL14, and peptide binding is likely to slowly and steadily increase intracellular calcium just enough to induce NF-κB signaling. Because cell surface molecular interactions are dependent on endocytosis, we treated NCI-H460 cells with brefeldin A, a protein transport inhibitor. Interestingly, CXCL14-stimulated NF-κB activation was eliminated in the presence of brefeldin A, suggesting that CXCL14 binding-mediated endocytosis is a prerequisite of NF-κB activation (Fig. 7D). Taken together, these results demonstrate that CXCL14 stimulated NCI-H460 cell proliferation by triggering calcium-dependent NF-κB activation.

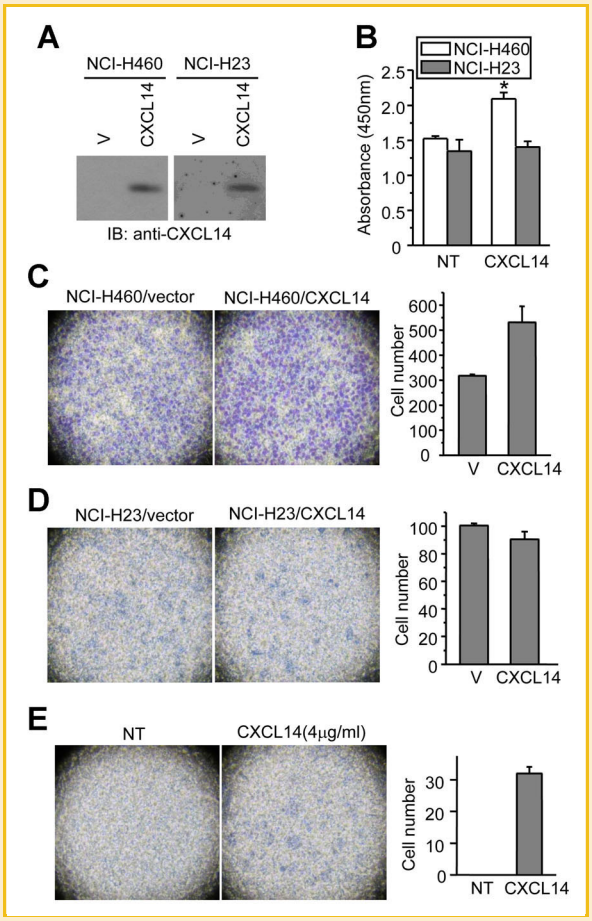

Fig. 6. Exogenous expression of CXCL14 enhances NCI-H460 cell proliferation and migration. A: After lentiviral infection, CXCL14 expression and secretion was examined by western blotting with anti-CXCL14 antibodies. B: CXCL14-expressing NCI-H460 cells and NCI-H23 cells were seeded in 96-well plates (4,000/well). After 72 h, cells were incubated with CCK-8 solution, and a microplate reader was used to measure absorbance at 450 nm. *The growth rate of CXCL14-expressing cells was significantly higher than empty vector-infected cells (V) ($P < 0.05$). C, D: Cells (5×10^4) were used in a migratory assay toward 10% FBS. The cells that migrated through transwell inserts were counted in five high-power microscope fields, and these values were averaged. E: NCI-H460 cells (5×10^4) were used for CXCL14-dependent transwell migration assay. A few cells migrated in the 4 μg/ml.

CXCL14 STIMULATES NF-κB SIGNALING THROUGH THE INTERACTION WITH SEVERAL GLYCOPROTEINS IN NCI-H460 CELLS

Many kinds of glycoproteins are expressed as conjugating forms with various saccharides on cell surface and conduct their biological functions. As described previously, they act as binding partners of extracellular matrix and other membrane proteins or receptors of some ligands. Syndecan-1, a typical HSPG is involved in osteoprotegerin-induced chemotaxis of human monocytes [Mosheimer et al., 2005]. Some glycoproteins are highly expressed in cancer cells and somehow related with deterioration of the tumors. We collected some glycoproteins which seem to be related with cancers or cell migration, and then investigated their binding activities on CXCL14. As shown in Figure 8A, syndecan-2, syndecan-4, PDPN, GYPC, and CD43 enhanced CXCL14 binding when they were exogenously expressed in NCI-H460 cells. In the reporter gene assay, SDC-2, SDC-4, PDPN, and CD43 slightly enhanced NF-κB activity (Fig. 8B). This result suggests that several glycoproteins are likely involved in CXCL14-stimulated cellular responses by binding to the peptide. However, GYPC had no effect on CXCL14-stimulated NF-κB activation, although it attended CXCL14 binding. Next, we examined transcriptional expression of the glycoproteins in NCI-H460 and NCI-H23 cells. SDC-2, SDC-4, PDPN, and GYPC were detected in RT-PCR with NCI-H460 cells but not with NCI-H23 cells, implying that these proteins may be involved in CXCL14 binding (Fig. 8C).

DISCUSSION

Cell surface and extracellular matrix glycoproteins are able to interact with low-molecular weight humoral mediators, including chemokines. They are thought to protect small molecules from degradation, present them to specific receptors on the cell surface, and help them trigger various cellular responses [Rot, 1992; Tanaka et al., 1993]. According to recent reports, some chemokines seem to regulate cellular responses by interacting with glycoproteins, especially proteoglycans, in a GPCR-independent manner. CXCL14 is regarded as a chemokine because it has typical residues, including CXC sequences for two disulfide bonds, and it stimulates the migration of some types of immune cells, even though its receptor remains unknown. However, many studies have demonstrated that CXCL14 triggers various pathophysiological cellular behaviors. As described above, CXCL14 has paradoxical effects on cancer progression. These are likely due to CXCL14's interactions with other molecules besides GPCRs. Because molecular interaction on the cell surface is a prerequisite for CXCL14-induced cellular responses, we screened cells that bind to CXCL14 using FACS. Many types of cells specifically interacted with CXCL14. Although trypsin prevented CXCL10 from binding to cell surface by digesting membrane proteins [Luster et al., 1995], CXCL14 binding was not likely to be inhibited by the protease because the cells were detached from the culture dish with trypsin prior to the assay. Furthermore, trypsin-treated SNU-C1 cells still interacted with CXCL14. We did not detect any evidence of HEK293 cell binding to CXCL14 in the FACS analysis, but they showed strong binding activity in the AP fusion protein binding assay (data not shown), indicating that CXCL14 interacted with HEK293 cells through trypsin-sensitive proteins. In contrast, trypsin-independent binding in many cell types is likely to stem from interactions with either trypsin-insensitive proteins or heavily glycosylated proteins with various glycans that protect surface proteins from attacking by the proteases.

Using AP fusion proteins, we demonstrated that CXCL14 binds to NCI-H460 cell surfaces with a K_d of 26 nM, which is similar to the binding affinity of IP-10 to A20 B cells [Luster et al., 1995]. The data suggest that CXCL14 seems to bind to cell surface saccharides like many other chemokines. Because other chemokines bind to HSPGs, it is reasonable to assess HSPG binding of CXCL14. We found that cellular CXCL14 binding was inhibited by heparin and heparan

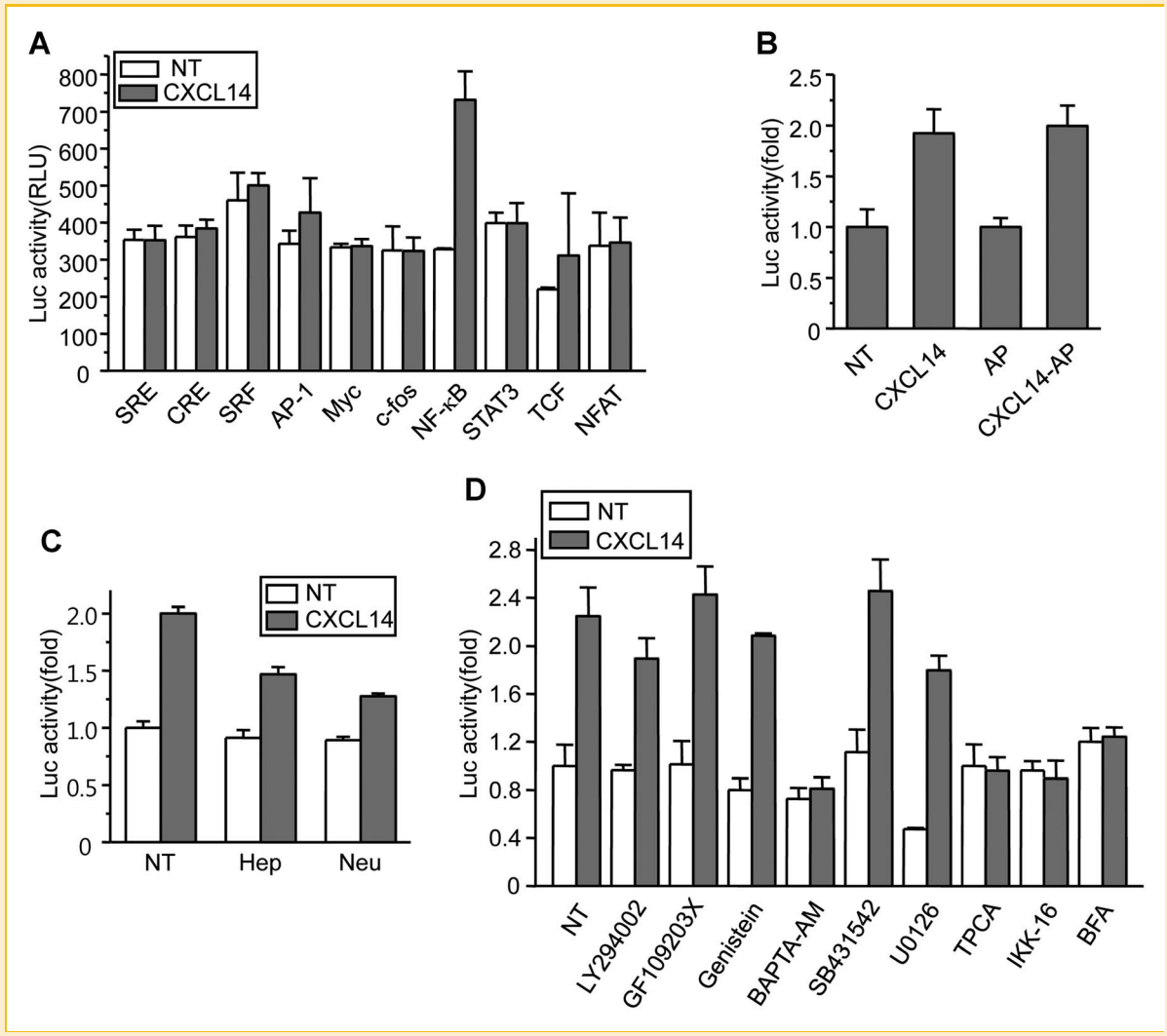

Fig. 7. CXCL14 activates NF-κB (A) NCI-H460 cells were transfected with various reporter genes and treated with 20 ng/ml CXCL14 for 6 h. Cells were lysed and used in luciferase activity assays. B: NCI-H460 cells transfected with an NF-κB reporter gene were treated with 20 ng/ml CXCL14 or 200 ng/ml AP-fusion proteins for 6 h. C: NCI-H460 cells transfected with an NF-κB reporter gene were incubated with 2 U/ml heparinase I and 2 U/ml neuraminidase for 1 h. They were washed four times with serum-free media and treated with 20 ng/ml CXCL14 for 6 h. D: Effect of the inhibitors on CXCL14-stimulated NF-κB activity. NCI-H460 cells were transfected with an NF-κB reporter gene and treated with inhibitors for 20 min prior to CXCL14 stimulation. NT, non-treatment. Concentration of inhibitors; 10 μM LY294002, 5 μM GF109203X, 25 μM Genistein, 20 μM BAPTA-AM, 10 μM SB431542, 25 μM U-0126, 2 μM TPCA-1, 2 μM IKK-16, 1 μg/ml brefeldin A.

sulfate, and the blocking efficiencies were higher than those for CXCL10, suggesting that CXCL14 binds to these HSPGs with higher affinity than CXCL10. Sialic acid is generally located in the terminal sites of polysaccharide branches in glycoconjugates, such as glycoproteins and glycolipids. Sialic acid-rich glycoproteins are often overexpressed on the cell surface in late-stage cancers [Tanaka et al., 2000; Almaraz et al., 2012]. Because the effect of CXCL14 on cancer cell behavior may be related to these proteins, Neu5Ac, a type of sialic acid, was used for the competition assay. Interestingly, compared to heparin, Neu5Ac potently blocked cellular binding to CXCL14 but not to CXCL10. Binding was also eliminated by heparinase I and neuraminidase treatments. These data demonstrate that CXCL14 can bind cells through specific HSPGs and sialic acid-rich glycoproteins and also suggest that the interaction between sialic acid-rich glycoproteins and CXCL14 may explain the role of CXCL14 on cancer progression.

Although various chemokines interact with many cells through saccharides, the binding affinities and specificities might be different depending on cell types [Luster et al., 1995]. The binding studies with the cells showing high affinity for CXCL14 support this idea. LNCaP, a prostate cancer cell, interacted with CXCL14 in a heparinase I- and neuraminidase-sensitive manner, which was quite similar to NCI-H460 cell binding. However, NCI-H23 and Detroit cells binding were not prevented by the enzymes, even though they may be mediated by ionic interactions. These cells are likely to bind to CXCL14 through other saccharides or unknown mediators. Considering the controversial effects of CXCL14 on cancer development, the different cellular responses to the peptide may

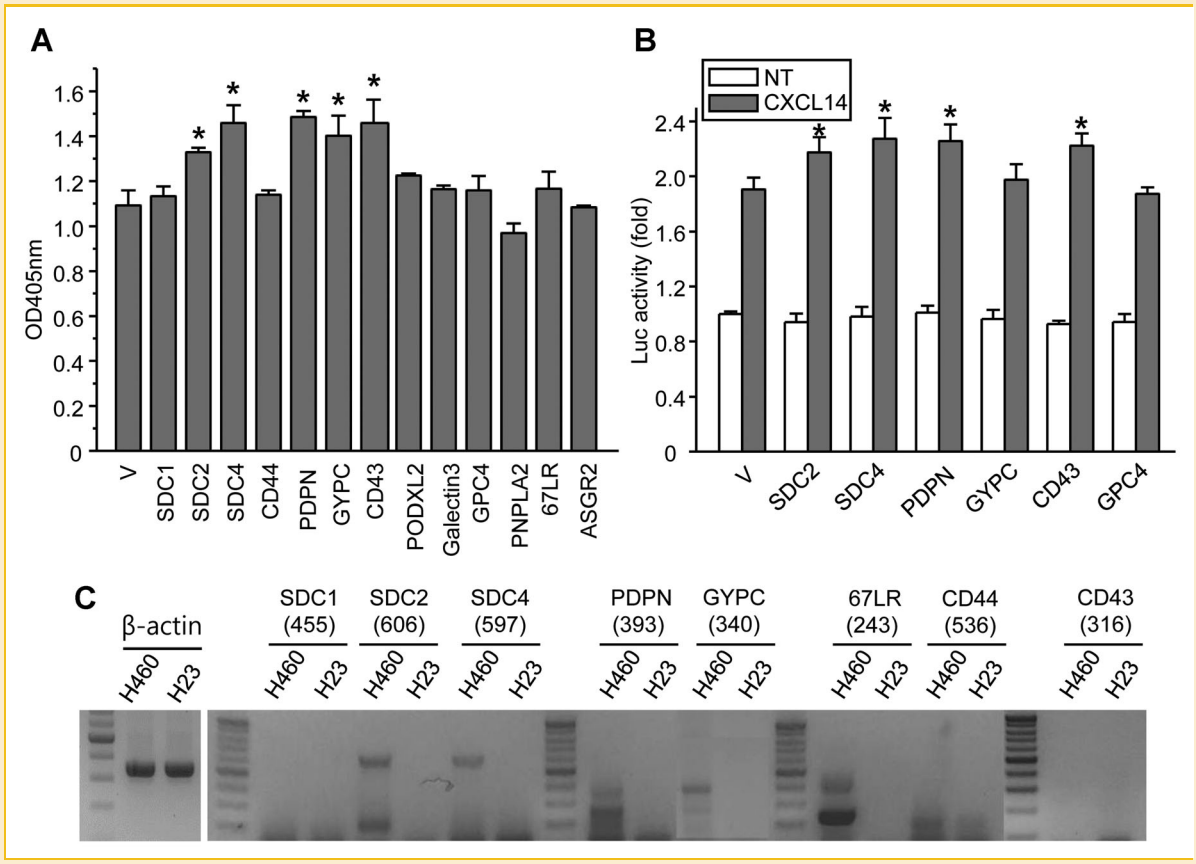

Fig. 8. CXCL14 stimulates NF-κB activation by binding some glycoproteins. A: NCI-H460 cells were transfected with glycoproteins and subjected to CXCL14-AP binding. B: NCI-H460 cells transfected with an NF-κB reporter gene and each of glycoprotein genes were incubated with 20 ng/ml CXCL14 for 6 h. Then cells were lysed and subjected to luciferase assay. V: vector (*$P < 0.05$) (C) Using RNAs from NCI-H460 and NCI-H23 cells, RT-PCR was performed with each glycoprotein-specific primer pair. Numbers in the blanks indicate estimated size of PCR products. Size markers are 1 Kb plus from Invitrogen.

be due to its different binding properties among cells. With regard to proliferation, CXCL14 contributed to the enhanced growth rate of NCI-H460 but not NCI-H23 cells, which lends further support to the idea.

As a chemokine, CXCL14 stimulates the migration of certain types of cancer and immune cells, but it inhibits angiogenesis in endothelial cells and the migration of other cancer cells. Our migration studies suggest that CXCL14 may not be a direct chemotactic factor, but it did help NCI-H460 cells migrate toward serum. The results showing that the canonical pathway involved in chemokine-mediated migration was not activated imply that NCI-H460 cells might not express the GPCR for CXCL14.

Because CXCL14 induced NCI-H460 cell proliferation and migration, it is reasonable to think that the cell surface binding of the peptide provokes intracellular signaling events. According to the reporter gene assay using various transcription factor target sequences, only the NF-κB pathway was activated by CXCL14. Interestingly, NF-κB activation was dramatically diminished by pretreatment with heparinase I and neuraminidase. The data indicate that these enzymes removed CXCL14 binding sites from cell surfaces and prevented CXCL14 from triggering intracellular signaling events. The studies using signaling inhibitors revealed that CXCL14 could stimulate NF-κB activation by increasing intracellular calcium concentration and IκB kinase activation. Although brefeldin A is thought to induce NF-κB activation by increasing endoplasmic reticulum stress [Lin et al., 1998], it blocked NF-κB signaling in NCI-H460 cells. On the basis of previous reports that brefeldin A inhibited membrane-associated proteoglycan endocytosis [Uhlin-Hansen and Yanagishita, 1995], the data imply that brefeldin A is likely to inhibit CXCL14-binding glycoprotein endocytosis, which precedes NF-κB pathway activation. In pancreatic cancer, CXCL14 is thought to play a role in cell invasion via NF-κB pathway activation [Wente et al., 2008], although the underlying mechanisms have not been verified. Our results revealed that CXCL14 triggers intracellular signaling cascades that converge on NF- κB activation by interacting with sialic acid sites of membrane glycoproteins and HSPGs.

Membrane glycoproteins including CD44 and CD168 are able to activate signaling pathways involved in cell motility and growth by interacting with their extracellular binding partners [Turley et al., 2002]. Main signalings mediating the cellular responses are the mitogen-activated protein kinase pathway and phosphtidylinositol 3-kinae-AKT pathway [Misra et al., 2003; Fuster and Esko, 2005]. According to our phosphorylation assays, CXCL14 slightly induced ERK phosphorylation but had no effect on AKT phosphorylation in NCI-H460 cells (data not shown). Along with reporter gene assay

this result indicates that CXCL14 might trigger cellular responses through interaction with different glycoproteins from previously defined ones.

Although some cancer cells consistently express CXCL14 with high level, the peptide is decreased or even absent in most malignant cells. Nevertheless, stromal cells including fibroblasts around malignant cells still express CXCL14, which is likely to influence cancer cell behavior [Augsten et al., 2009]. The different effects of CXCL14 on various malignancies might be related to functional expression levels of the unknown receptors for the peptide.

In the binding assay with NCI-H460 cells, SDC-2, SDC-4, PDPN, GYPC, and CD43 among many glycoproteins enhanced cellular CXCL14 binding, which indicate several glycoproteins act as CXCL14 receptors and are involved in the activation of NF-κB pathway. However, molecular interaction between glycoproteins and CXCL14 is not likely to be prerequisite to the transcription factor activation. GYPC was not involved in CXCL14-stimulated NF-κB activation regardless of their interaction. Compared with NCI-H23, NCI-H460 cells expressed some glycoproteins such as SDC-2, SDC-4, PDPN, and GYPC. Endogenous expression of these in NCI-H460 cells may be responsible for CXCL14-stimulated cellular responses.

In the present study, we demonstrate that CXCL14 can stimulate NCI-H460 cell proliferation and migration by interacting with sialic acid and HSPGs, thereby triggering NF-κB signaling. Because CXCL10 can exert anti-oncogenic functions by binding to HSPGs but not sialic acid, the pro-oncogenic effects of CXCL14 might be ascribed to the interaction with sialic acid-rich glycoproteins and/or HSPGs, which can elicit intracellular signaling events. In this experiment, we found several glycoproteins which are involved in CXCL14 binding and NF-κB activation. Even though other glycoproteins might interact with CXCL14, the glycoproteins we identified are likely to participate in CXCL14-stimulated cellular responses.

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