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Induction of Nucleolin Translocation by Acharan Sulfate in A549 Human Lung Adenocarcinoma

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ABSTRACT

Acharan sulfate (AS), isolated from the giant African snail *Achatina fulica*, is a novel glycosaminoglycan, consisting primarily of the repeating disaccharide structure α-D-*N*-acetylglucosaminyl (1 → 4) 2-sulfoiduronic acid. AS shows anti-tumor activity in vitro and in vivo. Despite this activity, AS is only weakly cytotoxic towards cancer cells. We examine the interactions between AS and cell-surface proteins in an effort to explain this anti-tumor activity. Using flow cytometry and affinity column chromatography, we confirm that AS has strong affinity to specific cell-surface proteins including nucleolin (NL) in A549 human lung adenocarcinomas. Surprisingly, we found the translocation of NL from nucleus to cytoplasm under the stimulation of AS (100 μg/ml) in vitro. Also, as NL exits the nucleus, the levels of growth factors such as bFGF and signaling cascade proteins, such as p38, p53, and pERK, are altered. These results suggest that the communication between AS and NL plays a critical role on signal transduction in tumor inhibition. J. Cell. Biochem. 110: 1272–1278, 2010.

KEY WORDS: ACHARAN SULFATE; NUCLEOLIN; TRANSLOCATION; A549 HUMAN LUNG ADENOCARCINOMA

Glycosaminoglycans (GAGs) are known to play essential roles of normal cell and tumor cell behavior influencing proliferation, differentiation, migration, and adhesion through their binding to a large number of ligands and receptors regulating these processes [Capila and Linhardt, 2002]. GAGs are linear polysaccharides consisting of repeating, negatively charged disaccharides of particular structures. In tumor biology, GAGs have a double-edged activity, both stimulating and inhibiting angiogenesis, tumor growth, and metastasis, depending on GAG structure and the types of cancer or animal models [Borsig et al., 2001; Sasisekharan et al., 2002]. Despite the ambiguous nature of these phenomena, GAGs such as heparin and heparan sulfate are considered a critical component of cell–cell communications at the cell membrane and in the extracellular matrix.

A novel GAG, acharan sulfate (AS), was isolated from the body of the giant African snail *Achatina fulica* by our laboratory having the primary structure →4)-2-acetamido-2-deoxy-α-D-glucopyranose(1 → 4)-2-sulfo-α-L-idopyranosyluronic acid (1 → (GlcNAc-IdoA2SO$_3^-$) [Kim et al., 1996]. While related to heparin and heparan sulfate family, it is distinctly different from the vertebrate GAGs. In previous studies, AS showed anti-angiogenic activity in inflammation models [Ghosh et al., 2002], in vivo anticoagulant activity, anti-mitogenic activity on heparin-mediated basic fibroblast growth factor (bFGF) [Wang et al., 1997], and immunomodulating action [Shim et al., 2002]. AS also showed little cytotoxicity (0–200 μg/ml) on various cancer cells, inhibiting tumor growth in vivo through an anti-angiogenic effect [Lee et al., 2003].

To explain these effects, the specific proteins interacting with AS were explored using biotinylated A549 human lung adenocarcinomas and AS affinity column. We had previously reported that cell-surface nucleolin (NL) in mouse Lewis lung carcinoma was an AS-binding protein and confirmed its inhibitory effect on tumor growth in vivo [Joo et al., 2005]. NL was first reported by Orrick et al. [1973], is known to function in the nucleus in rDNA transcription, rRNA maturation, ribosome assembly, and nucleolus formation [Chen et al., 1991; Ginisty et al., 1999; Srivastava and Pollard, 1999;

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Ma et al., 2007]. Recently, the role of NL as shuttle protein, between the nucleus and the cytoplasm, was demonstrated. Cell-surface NL has also been described as a tumor marker [Christian et al., 2003] and a receptor related to angiogenesis and cancer [Huang et al., 2006; Shi et al., 2007; Reyes-Reyes and Akiyama, 2008]. Here, we have demonstrated that AS not only binds to cell-surface NL but also contributes to signaling cascades resulting in tumor inhibition.

MATERIALS AND METHODS

MEASUREMENT OF CELL CYTOTOXICITY

Human lung adenocarcinomas A549 (Korea Cell Line Bank, Seoul, Korea) were maintained and cultured in RPMI (Hyclone, MA, USA) supplemented with 10% fetal bovine serum at 37°C under 5% CO_2. Aliquots (100 μl) of 2×10^4 cells was seeded into 96-well plates and stabilized for 24 h. The cells were treated with AS at 0–200 μg/ml for 24, 48, and 72 h. Next, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT; Sigma, MO, 100 μl of 0.5 mg/ml) was added and incubated for 3 h. The crystal of produced formazan was dissolved with DMSO and the optical density was measured at 540 nm using an Emax microplate reader (Molecular Devices, CA) for the quantification of cell viability. All assays were performed in triplicate.

CELL-BINDING ASSAY

The binding affinity of each GAG to cells was analyzed by flow cytometry. Aliquots of 2×10^5 cells were incubated for overnight

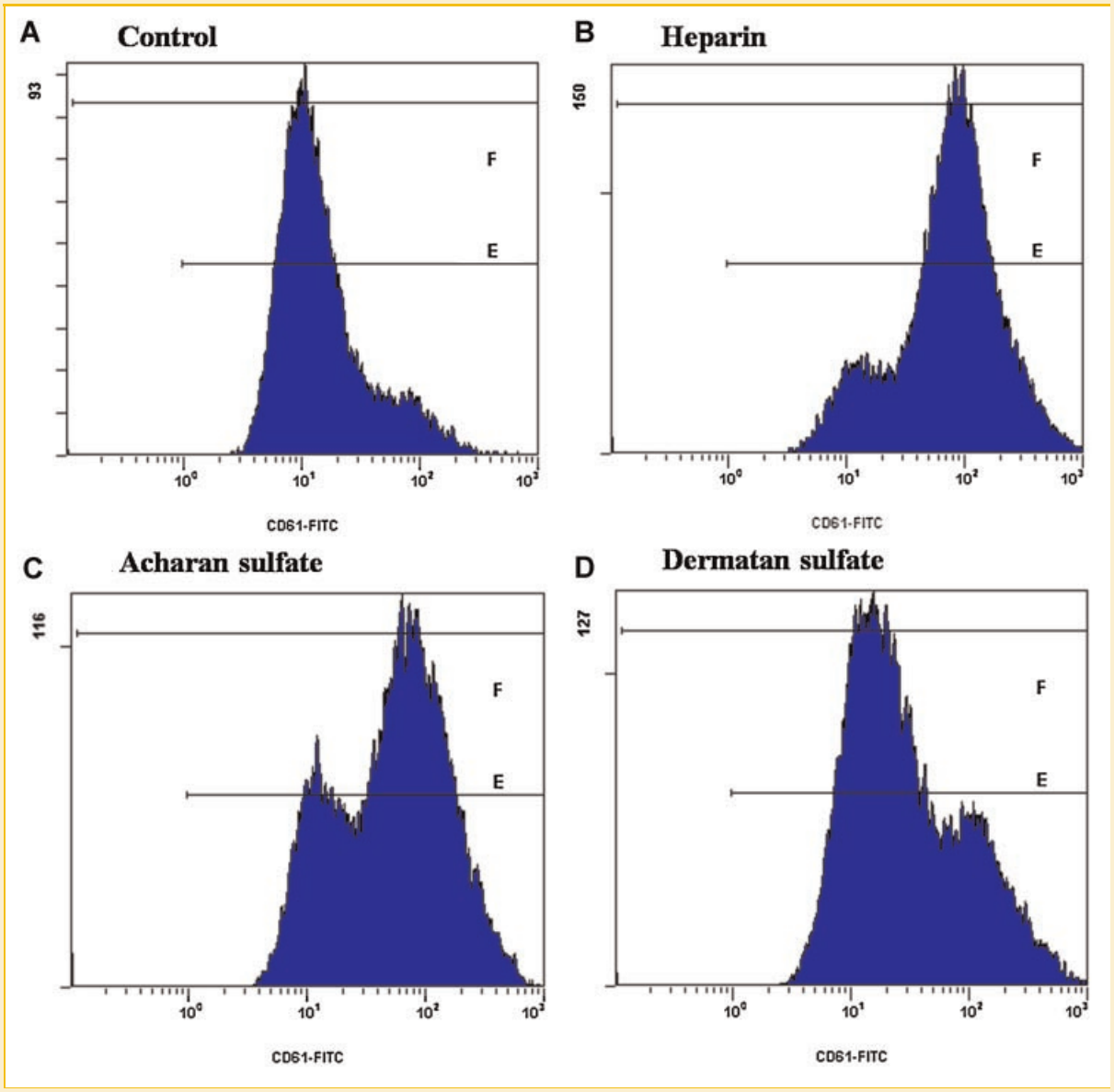

Fig. 1. The binding affinity of acharan sulfate (AS), heparin, and dermatan sulfate to A549 human lung adenocarcinoma was analyzed by flow cytometry. After incubation of AS (100 μg/ml) for 24 h, the cells were reacted with each anti-GAG antibody. Compared to control (A), heparin (B), and AS (C) showed a strong affinity to the A549 cells, while dermatan sulfate (D) bound weakly to A549 cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

with AS (100 μg/ml in 200 μl of PBA, PBS containing 1% BSA). Cells were treated with heparin or dermatan sulfate in the same manner or 200 μl of PBA as a negative control. Cells were washed with PBA to remove the unbound GAGs. After washing three times, cells were incubated with 1:2,000 dilutions of anti-AS antibody, anti-heparin antibody, and anti-dermatan sulfate antibody for 1 h at 37°C. Anti-AS antibody tagged with c-myc was provided by Dr. Gerdy ten Dan from Netherlands [ten Dam et al., 2004]. After washes, cells were incubated with goat anti-mouse IgG Alexa488 (Abcam, Cambridge, UK) for 2 h at 4°C. After three additional washes, cells were re-suspended in PBA and analyzed using Coulter EPICS™ XL™ (Brea, CA).

BIOTYNLATION OF CELL-SURFACE PROTEINS AND ISOLATION OF ACHARAN SULFATE-BINDING PROTEINS

The identity of AS-binding proteins on the cell surface was next explored. Cells were biotinylated with membrane-impermeable Sulfo-NHS-LC-biotin (Pierce, IL) and whole biotinylated lysates were applied to the AS affinity column as previously described [Joo et al., 2005].

CONFIRMATION OF ACHARAN SULFATE-BINDING PROTEINS

The proteins eluted from the AS affinity column were separated by SDS–PAGE and transferred to nitrocellulose membrane (Pall Sciences, FL). The membranes were incubated in blocking solution containing 5% non-fat dried milk for 1 h to inhibit non-specific binding. The membranes were incubated with streptavidin conjugated with horseradish peroxidase (HRP; Pierce) for 1 h. After several washes, the blots were developed using *o*-phenylenediamine (OPD; Sigma) in phosphate-citrate buffer.

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A549 cells were seeded in 24-well plates or glass chamber slides (NUNC, NY). The cells were incubated with AS 0, 10, 100 μg/ml for 0–24 h. The cells were then washed gently two times with ice-cold PBS. After fixation using 2% paraformaldehyde solution, the cells were incubated with anti-NL antibody for overnight at 4 or 37°C for 1 h. After several washes, the cells were incubated with goat anti-mouse IgG Alexa488 or goat anti-rabbit IgG Alexa610 for 1 h at room temperature (Abcam). The nucleus was stained by 4′-6-diamidino-2-phenylindole (DAPI; Sigma). The stained cells were

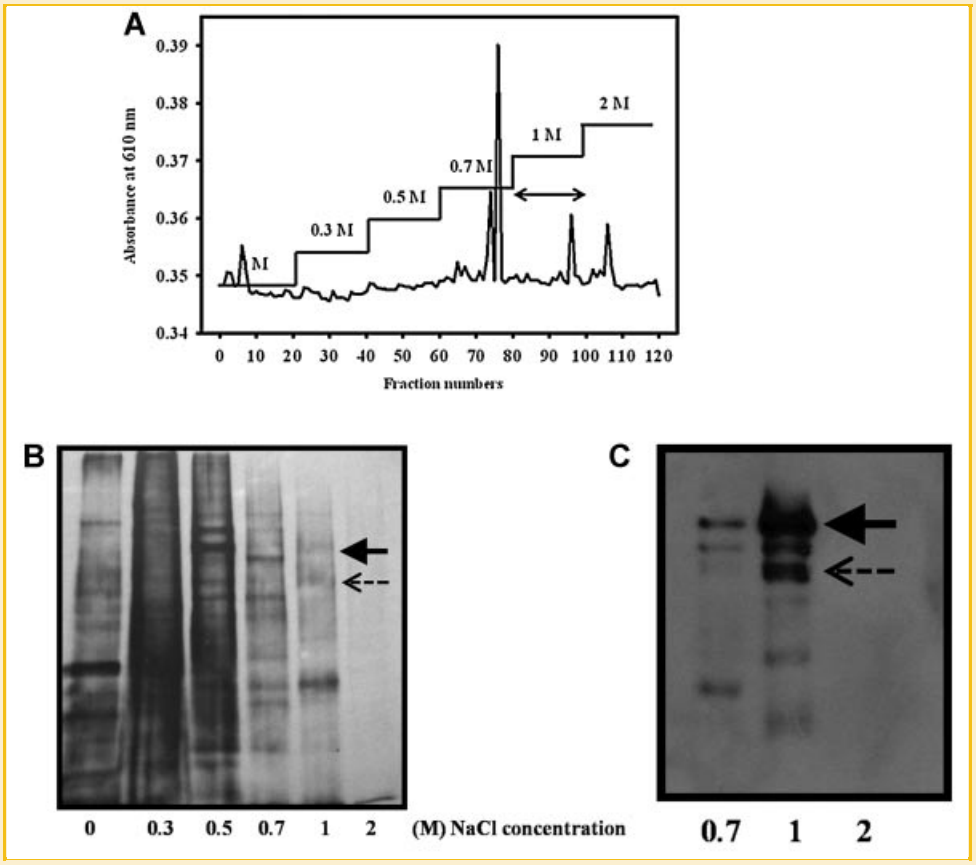

Fig. 2. After biotinylation of cell-surface proteins, the cells were lysed and the whole lysates were applied to the AS affinity column. The whole proteins were fractionated by a stepwise salt gradient and collected (A). After SDS–PAGE and Western blotting, the blots were reacted with streptavidin conjugated with HRP (B) and anti-nucleolin antibody (C). Because biotinylated proteins were reacted to streptavidin, the bands in (B) revealed only cell membrane proteins. The bold arrow indicates for full-size nucleolin (100–110 kDa) and the dotted arrow for fragmented nucleolin (80 kDa). M, molecular markers; W, whole lysates.

imaged under CKX41 fluorescence microscope (Olympus, Tokyo, Japan).

WESTERN BLOT ANALYSIS

A549 cells were seeded and incubated with AS 100 μg/ml for 0–24 h. For total extract, the cells were washed twice with ice-cold PBS and lysed in lysis buffer (20 mM HEPES, pH 8.0, 150 mM NaCl, 1% Nonidet P-40) containing a protease inhibitor cocktail (Sigma). For cytoplasmic and nuclear (Nu) fractionation, AS treated cells were washed with ice-cold PBS twice. And the cells were suspended in ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail) and incubated for 15 min on ice. After 10% Nonidet P-40 (Sigma) was added, the suspension was mixed for 10 s and centrifuged for 1 min at 16,500*g*. The residual pellet was re-suspended in ice-cold buffer C (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail) for 15 min and centrifuged for 5 min at 16,500*g*. Each lysate (total, cytoplasmic, Nu fraction) was analyzed by 8% or 12% SDS–PAGE. The proteins were transferred to nitrocellulose membranes and probed with primary antibodies such as NL, pAkt, pJNK, pERK, p38, pp38, p53, pp53, bFGF, caspase3, and survivin, followed by species-specific secondary antibodies conjugated with HRP (Santa Cruz Biotechnology, CA). The signals were detected by LAS-1000 Image Analyzer (Fujifilm, Tokyo, Japan).

RESULTS AND DISCUSSION

CELL CYTOTOXICITY OF ACHARAN SULFATE

To evaluate the cytotoxicity of AS, various cancer cell lines, including A549 human lung adenocarcinomas, were treated with AS and cell viability measured by MTT assay. AS at 500 μg/ml showed only ~30% inhibitory effects after a 72 h incubation (data not shown). We concluded that anti-tumor activity of AS is not the result of immediate cytotoxicity but is attributable to the induction of a slower response from their interactions with one or more cell-surface proteins.

BINDING AFFINITY OF ACHARAN SULFATE TO A549 CELLS

Various GAGs are understood to exhibit their biological activities by binding to cell-surface proteins. We measured the binding of the purified AS, heparin, and dermatan sulfate to A549 cells by flow

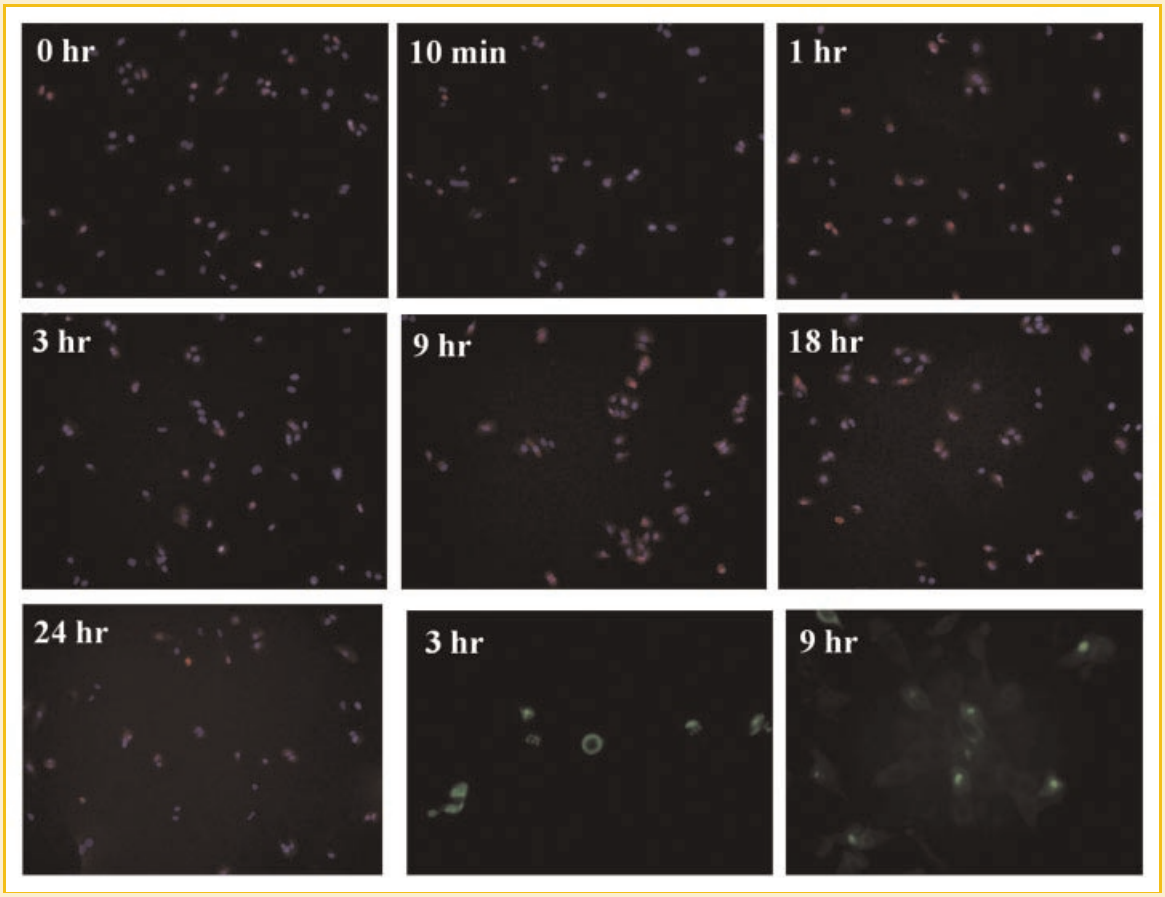

Fig. 3. For the observation of nucleolin translocation by AS, the cells were incubated with AS (100 μg/ml) in a time-dependent manner. The cells were fixed at each time point, reacted with anti-nucleolin antibody and observed under fluorescence microscopy. The stained nucleolin is indicated by red or green and every nucleus is stained as blue using DAPI. After 3-h incubation time, nucleolin in the nucleus gradually decreases. After 9 h of incubation, cytoplasmic nucleolin is clearly detected. Cells are observed at the magnification of 10 × 10 in CKX41 fluorescence microscope. The bold boxes (3 and 9 h) show nucleolin in green at higher magnification (40 × 10). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cytometry. Compared with the control peak, heparin showed the highest binding to A549 cells followed by dermatan sulfate, which only showed weak binding affinity (Fig. 1). The moderate affinity of AS for cancer cells represents one line of evidence explaining its anti-cancer activity.

CHARACTERIZATION OF ACHARAN SULFATE-BINDING PROTEINS USING AS AFFINITY COLUMN CHROMATOGRAPHY

In our experiments, we used water-soluble and membrane-impermeable Sulfo-NHS-LC-biotin as a reagent to label A549 cell-surface proteins. The biotinylated cells were lysed and applied to the AS affinity column. The bound proteins were eluted with a stepwise salt gradient (Fig. 2A). The eluted fractions were collected, concentrated, and analyzed using 8% or 12% SDS–PAGE. Although the silver-stained gel showed all proteins from total lysate (data not shown), only the biotinylated cell membrane proteins were visualized using streptavidin (Fig. 2B). A few of prominent bands of molecular weight 100–110 kDa (bold arrow) were observed in the high affinity fraction eluted with 1.0 M NaCl, which showed specific binding to anti-NL antibody (Fig. 2C). Bands corresponding to smaller size proteins (80 kDa, dotted arrow) were also detected and were attributed to fragmented forms of NL.

INDUCTION OF NUCLEOLIN TRANSLOCATION BY THE STIMULATION OF ACHARAN SULFATE

Once it was confirmed that NL is a strong candidate for AS-binding protein among innumerable cell-surface proteins, we investigated the influence of AS on NL. We treated A549 with AS (100 μg/ml) in a time-dependent manner (Fig. 3). NL was stained by anti-NL antibody and secondary antibody tagged with red or green (within bold box) fluorophore. At early incubation time (within 1 h), NL was localized within nucleus. Unexpectedly, ~3 h after incubation with AS most of nuclear NL started to translocate to cytoplasm. Cells were observed at the magnification of 10 × 10 or 10 × 40 under CKX41 fluorescence microscope. There were no noticeable changes in cell morphology and cell number. Using different concentrations of AS (10 and 200 μg/ml), we observed a similar pattern of NL translocation (data not shown). These results demonstrate that AS

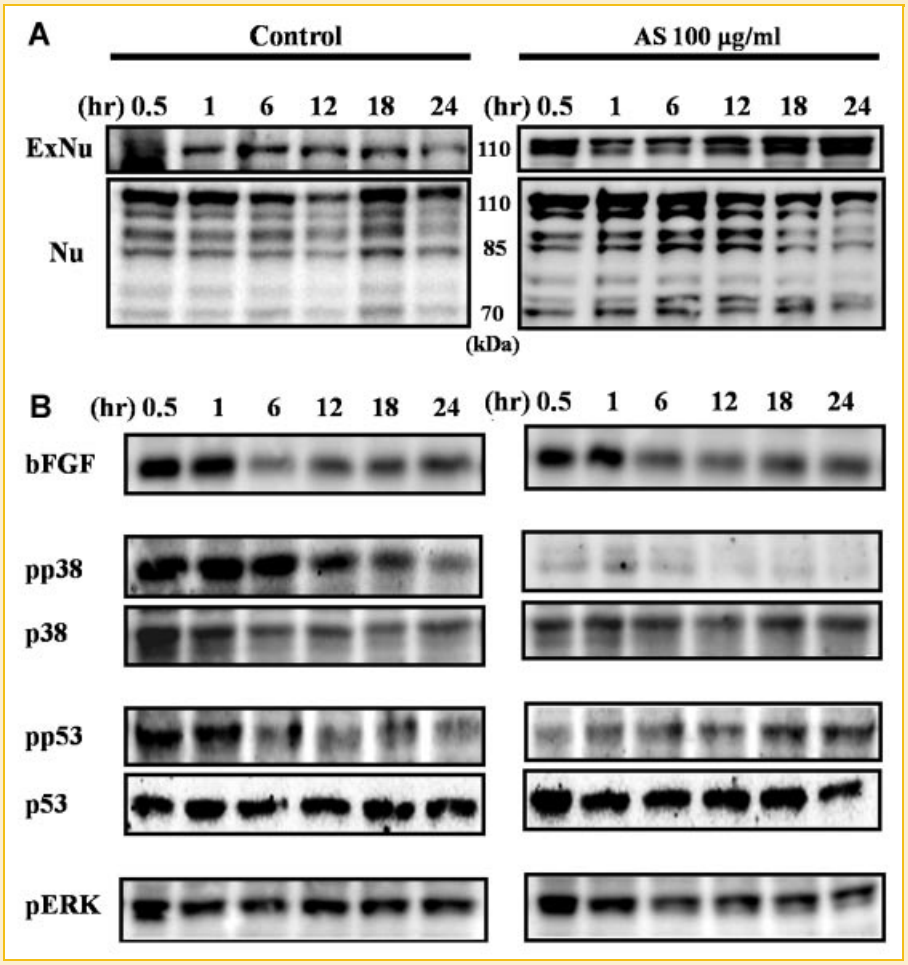

Fig. 4. To investigate of the actual movement of nucleolin, the cells treated with AS (100 μg/ml) were lysed to obtain extranuclear (ExNu) and nuclear (Nu) fractions (A). As nucleolin in the ExNu fraction increased, the amounts of full size and fragmented nucleolin in the Nu fraction decreased in a time-dependent manner. Also, Western blot analysis examines specific proteins affected by the interaction between AS and nucleolin (B). Compared with control, the amounts of bFGF, pp38, and pERK have decreased on exposure to AS and pp53 (the active form of p53) increased on exposure to AS.

stimulates the translocation of nuclear NL over a range of concentrations.

IDENTIFICATION OF SPECIFIC PROTEINS RELATED TO NUCLEOLIN REDISTRIBUTION

We next used cellular fractionation to verify the actual movement of NL following treatment with AS. After the cells were incubated with AS (100 μg/ml) for the proper time, they were detached using a scraper, lysed, and the proteins were loaded by SDS–PAGE and transferred to nitrocellulose membrane. We have analyzed the particular proteins altered by AS stimulation. First, the membranes were incubated with anti-caspase3 and anti-survivin antibodies to examine whether AS induces apoptosis. As expected, the amounts of those proteins showed no changes (data not shown). Next, the blots were visualized with anti-NL antibody. During AS incubation, the amount of NL fractionated from the total lysate remained constant (data not shown). Fragmented bands of NL were clearly observed in Nu fraction compared to control. Also, the full length of NL (~110 kDa) increased in the extranuclear fraction (ExNu) and in contrast, the bands of full size NL as well as fragmented NL (approximately 85 and 70 kDa) decreased in the Nu fraction in a time-dependent manner (Fig. 4A). These results suggest that AS drives A549 cells into a stressed condition [Daniely et al., 2002; Kito et al., 2005], resulting in the degradation and translocation of NL [Chen et al., 1991; Fang and Yeh, 1993; Mi et al., 2003; Ugrinova et al., 2007]. NL has been recently reported to have a role as a receptor [Sinclair and O'Brien, 2002; Christian et al., 2003; Chen et al., 2008; Di Segni et al., 2008; Reyes-Reyes and Akiyama, 2008] or binding protein for GFs [Take et al., 1994; Tate et al., 2006; Dai, 2009] on the cell surface. We investigated some of the specific protein ligands for NL (Fig. 4B). The level of bFGF, a GF related to angiogenesis and cell proliferation, also was reduced gradually after 6-h incubation of AS. Since, AS does not directly induce cell death, but rather inhibits tumor growth, the effects of AS on the signaling cascades related to mitogen-activated protein kinases (MAPKs) were studied. AS did not affect pAkt and pJNK but resulted in a slight decrease in the level of pERK. In cytoplasmic fraction following AS stimulation, the activated form of p38 (pp38) was hardly detected and the phosphorylated p53 (pp53) increased compared to control. Recently, several groups demonstrated that under particular circumstances NL and its mobilization are associated with p53 [Daniely et al., 2002; Ugrinova et al., 2007] and p38MAPK/PI3K [Reyes-Reyes and Akiyama, 2008]. There is another report that the binding of specific ligands to surface NL can trigger Ca^{2+} entry into cells [Losfeld et al., 2009]. Based on the activation of MAPK by Ca^{2+}, we speculate that AS might interrupt the binding of NL and Ca^{2+}, followed by blocking MAPK signaling cascades. We illustrate a proposed mechanism on anti-tumor activity of AS (Fig. 5). When cell-surface NL acts as a receptor of GF, it can induce the signals such as p38 and p53 or ERK. Activation of p38 or ERK permits cancer cells to proliferate or survive. Nuclear NL also regulates cell cycle and ribosomal biogenesis [Srivastava and Pollard, 1999]. The exogenous AS can block the binding of GFs to NL, preventing the subsequent signaling cascades from acting normally. Our data also demonstrates that AS influences the degradation of NL in nucleus. It is still unclear how AS accomplishes the interruption of NL's role

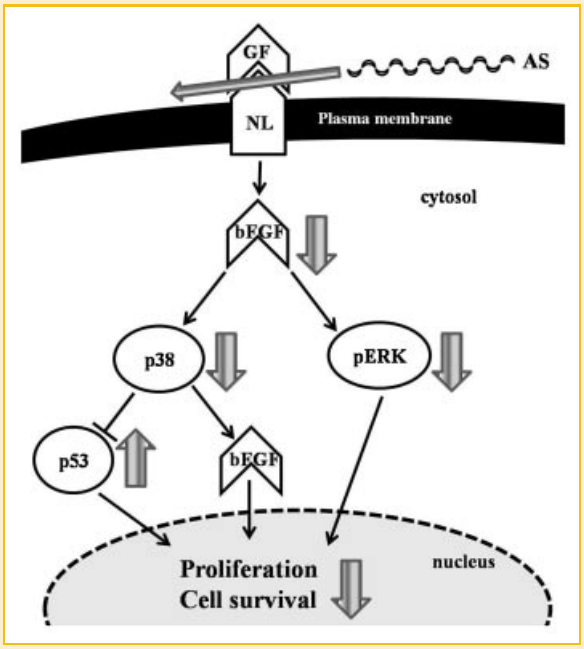

Fig. 5. The proposed mechanism of anti-tumor activity of AS. Nucleolin regulates signaling cascades and ribosomal biogenesis by activating MAPK pathways such as p38 and ERK, making cancer cells survive and avoid apoptosis. If exogenous AS binds to cell surface nucleolin (NL) instead of specific growth factors (GF) or influences on the degradation of nuclear nucleolin by some unknown mechanism, the signaling cascades as well as ribogenesis by "normal" nucleolin might be disrupted. (The striped and grey-colored arrows stand for the states of signals activated or inhibited by AS.) We speculate that AS binding to cell surface nucleolin blocks the roles of nucleolin both cell surface and nucleus resulting in tumor inhibition.

both on the cell surface and in the nucleus. Further studies will be necessary to understand the dual role of AS. AS clearly binds to cell-surface NL in vitro, allowing NL to redistribute gradually from nucleus to cytoplasm, causing the degradation of nuclear NL. It is clear that the interaction between exogenous AS and NL results in tumor inhibition and induces intracellular signaling for cell death.

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