HIGHLY SENSITIVE AND SELECTIVE ANTI-CANCER EFFECT BY CONJUGATED HA-CISPLATIN IN NON-SMALL CELL LUNG CANCER OVEREXPRESSED WITH CD44

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ABSTRACT

In spite of severe side effects, chemotherapy is widely used as a major anticancer treatment in non-small cell lung cancer (NSCLC). In order to enhance the therapeutic properties and reduce side effects, enormous efforts have been devoted to direct anticancer agents specifically to tumor tissues by the use of nanoparticles, or cancer cell marker attached drugs. However, cell-specific chemotherapy is still in its infancy and is not applicable to all types of cancers due to the complexity of the cancer occurrence and progress. In this study, we demonstrate that hyaluronan (HA)-conjugated cisplatin has highly selective and sensitive anticancer effects in NSCLC cells that overexpress the trans-membrane receptor, CD44, as HA is a specific ligand for CD44. In proliferation experiments, HA-conjugated cisplatin showed dramatic cell viability decreases (from 100% to 42.31%) in H1299 cells, which overexpress CD44, whereas no such change was observed in A549 and HFL1, which have little to no expression of CD44. More importantly, conjugation with HA decreased the dosage concentration of cisplatin by 50-fold for both cytotoxic and anti-metastatic effects. In addition, HA-cisplatin conjugate treatment selectively decreased migration (from 100% to 7.8%) and invasiveness (from 100% to 21.4%, respectively) of H1299. Based on our experimental results, we strongly believe that HA-cisplatin conjugate is a potential anticancer chemotherapeutic agent, which target CD44 overexpression in NSCLC, with minimal side effects and high therapeutic properties.

KEYWORDS

CD44, hyaluronan-cisplatin conjugate, lung cancer.

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths worldwide. The majority of patients with lung cancer have non-small cell lung cancer (NSCLC). Overall prognosis of NSCLC is poor, with low 5-year survival rate, due to late discovery, disease relapse, and lack of curative systemic therapy. Recently, cancer stem cells (CSCs) came under the spotlight in the treatment of lung cancer. The theory behind CSC proposes that cancer is maintained by subpopulations of tumor cells that possess stem cell or progenitor cell characteristics [1]. Several putative surface markers for lung CSC have been identified, including CD133 and CD44 [2]. Those cells with the CSC markers can initiate tumor formation, differentiate along multi-potent pathways, and become relatively resistant to conventional chemotherapy. Thus, the development of CSC-targeted anti-lung cancer drugs has become more important.

In treating NSCLC, the most widely used anticancer agent is platinum-based chemotherapy [3]. Cisplatin (cis-diamminedichloroplatinum) is a chemotherapeutic agent used in a variety of solid
tumors like lung cancer. However, intravenously administered cisplatin can increase risks of severe side effects including leukopenia, nausea, and anemia [4–7]. The most significant downside is neurotoxicity and nephrotoxicity, both of which are strongly influenced by the peak plasma concentration of cisplatin [8]. These detrimental factors can lead to decreased patient compliance, inability to complete chemotherapy courses, or use of less effective chemotherapeutic agents [9]. Systemic therapy is rarely completely successful because only a limited dosage of chemotherapeutic drug actually reaches tumor cells. Therefore, development of a drug delivery system that can enhance cisplatin accumulation in the tumor, while reducing collateral cellular injury to other organs, such as the kidney, would provide a promising approach in achieving enhanced antitumor activity, as well as reducing adverse effects. Thus, an active tumor-targeting delivery system of chemotherapeutic of cell division and rapid matrix remodeling that occur during embryonic morphogene drug is urgently needed.

Various targeting molecules have been developed for the delivery of anticancer drugs to tumor tissues, such as folic acid, biotin, and hyaluronic acid (HA). Among these, HA has been widely investigated due to its unique properties. The glycosaminoglycan HA is a major component in the extracellular matrix (ECM) of most mammalian tissues, is known to be involved in wound healing, and accumulates in sites of inflammation, and tumor regeneration [10–12]. Recently, HA polymers have become a topic of interest for developing sustained drug delivery systems of peptide and protein drugs in subcutaneous formulations. A number of strategies for chemical modification of HA have been developed to improve its physicochemical properties [13–19]. Indeed, HA uptake in cells is known to be mediated through endocytosis with specific receptors, such as cluster determinant 44 (CD44) and receptor for hyaluronate-mediated motility (RHAMM) [14, 16]. The adhesion/homing molecule CD44, which is implicated in cell–cell and cell–matrix adhesion, is a cell surface receptor for several extracellular matrix components, and is involved in a wide variety of biological processes, including cell migration and tumor metastasis [4, 13]. There is now ample evidence for the importance of CD44 expression in the progression of many tumor types. Enhanced CD44 expression has been reported for glioma, breast carcinoma, non-small cell lung carcinoma, colon carcinoma, ovarian carcinoma, and CSCs [20, 21]. When compared with conventional anticancer agents, HA-conjugated anticancer drugs tend to have reduced toxicity in normal cells, and demonstrate higher potency in cancer cells [5, 22].

Many researchers have demonstrated the targeting ability of HA, though human cancer cell-targeting drug delivery systems have only been studied in vitro [14, 23, 24]. Additionally, no study has been published on the minimal concentration of HA-conjugated anticancer drug in the context of CD44 expression NSCLC [25–29]. Thus, the objective of this study is to investigate the anti-proliferation, metastatic effects of HA-cisplatin conjugates in CD44 overexpression NSCLC (Figure 1).

RESULTS

Synthesis of HA-Cisplatin Conjugates

HA was bound to cisplatin to obtain HA-cisplatin conjugates, and free cisplatin concentration was confirmed using inductively coupled plasma-optical emission spectroscopy (ICP-OES). As illustrated in supplemental Table S2, the conjugation efficiency of HA-cisplatin was 27.29% and the remains percentage of cisplatin in HA-cisplatin conjugates at 24 hours and 48 hours were 61.53% and 40.23%, respectively (supplementary Figure S1). We used minimal concentrations of HA-cisplatin conjugates to test whether they have selective and sensitive anticancer effects on NSCLC.

HA-Cisplatin Conjugates Selectively Decreases Proliferation of CD44 Overexpression NSCLC

It is well known that cellular HA receptors CD44 and RHAMM are overexpressed in many types of cancer cells, demonstrating enhanced binding and internalization of HA by those cancer cells [30]. To select for a HA-binding receptor CD44 overexpression cell line, we analyzed the expression profile of surface CD44 in NSCLC (H1299, H1793, H23, and A549) and lung fibroblast cells (HFL1). The results showed that CD44 receptor is much more strongly expressed in the H1299, H1793, and H23 cell lines than in A549 and HFL1 (Figure 1 and supplementary Figure S2). These results are consistent with other studies on CD44 expression in the same cell lines [2]. Based on the above results, we also examined the selective effects of HA and cisplatin to obtain the lowest lethal concentration of these chemotherapeutic compounds. We used CD44-overexpressing H1299, H1793, and H23 cells and CD44 less or negative (A549 and HFL1) expression cells to confirm the selective anticancer effects of HA-cisplatin to NSCLC. The in vitro cytotoxicity of HA-cisplatin was investigated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. In the
Sensitive and Selective Anticancer Effects of HA-Cisplatin

FIGURE 1. The anti-proliferative effects of HA-cisplatin conjugate in CD44(+) NSCLC. Cells (5 × 10³ cells/well) were seeded in 96-well plates and cultured for 24 hours and were treated with minimal concentration cisplatin (0.2 μg/mL), free cisplatin (10 μg/mL), and HA-cisplatin conjugate (0.2 μg/mL of cisplatin). Cell viability was measured by MTT assay at 24 hours. The t-test analysis resulted in statistically significant differences; with *P < .05; **P < .01.

The presence of HA-cisplatin conjugate, proliferation of CD44 overexpressing H1299, H1793, and H23 cell lines significantly decreased (57.69%, Figure 1 and supplementary Figure S4), while CD44 less or negative cell lines A549 and HFL1 did not exhibit any significant differences (A549 and HFL1 decreased by 15.64% and 0.85%, respectively). The LD₅₀ of free cisplatin in all three cell lines was 10 μg/mL, but that of HA-cisplatin conjugate was 20 μg/mL (containing 0.2 μg cisplatin) in H1299. In the CD44 overexpressing NSCLC cell lines, the concentration for HA-conjugated cisplatin was 1/50th of that for free cisplatin (Figure 1). These results may be caused by the conjugation of cisplatin to HA, which increased in vitro uptake by CD44-dependent endocytosis of cells.

To further confirm that HA-cisplatin has selective and sensitive apoptotic effects on CD44 overexpression cell lines, we investigated effects of HA-cisplatin on the expressions of two pro-apoptotic (Bax and caspase-3) proteins in a time-dependent manner. The results showed that HA-cisplatin significantly increased caspase-3 and Bax protein expressions in the H1299 cell line. At 3 hours, the intensity of caspase-3 and Bax/GAPDH increased by factors of 1.6 and 2.6, respectively. However, HA-cisplatin had no effects on A549 and HFL1 (Figure 2). Furthermore, there were no differences in the caspase-3 expression of free cisplatin (0.2 μg/mL) treated cells (supplementary Figure S3). These results provided evidence that HA-cisplatin has stronger influence than free cisplatin on the apoptosis of CD44 overexpressing NSCLC.

HA-Cisplatin Conjugates Suppress Migration and Invasion of CD44 Overexpressing Lung Cell Lines

In cancer cells, CD44 interacts with the HA-rich microenvironment, which affects cell-signaling pathways that trigger the ability of malignant cells to migrate, invade basement membranes, and lodge at distant sites from the tumor [2, 31–33]. One attempt to elucidate the role of HA binding to CD44 in tumor progression was made with the BSp73AS pancreatic carcinoma cells transfected with the CD44v4–v7 isofoms, which conferred the metastatic potential [34]. To confirm, once again, whether HA-cisplatin has selective anti-metastatic effects, we investigated cell motility and invasion using migration and invasion assays. The results show that HA-cisplatin (contained 0.2 μg/mL cisplatin) significantly decreased motility and invasiveness of lung cancer H1299 cells but not those of A549 and HFL1 cells (Figure 3, 4). However, free cisplatin
FIGURE 2. The effects of HA-cisplatin conjugate on apoptosis-related proteins in lung cell lines. Cells (2.5 × 10^5 cells/well) were treated with HA-cisplatin conjugate (0.2 μg/mL of cisplatin) and harvested at 1, 2, 3, and 5 hours. The expressions of the caspase-3 and Bax proteins were investigated by western blotting. The t-test analysis resulted in statistically significant differences; with *P < .05; **P < .01.

HA-Cisplatin Conjugate’s Anticancer Effects Inhibited CD44 mAb Treatment in CD44 Overexpressing Lung Cell Line

Presence of CD44 receptors on cell surface is a fundamental requirement for the internalization of HA-cisplatin. Thus, to provide evidence for the HA-cisplatin anticancer effects through binding with CD44 receptor, we used CD44 monoclonal antibody pretreatment. When CD44-HA interaction was blocked by CD44 monoclonal antibodies (10, 20 μg/mL), the CD44 expression decreased in a dose-dependent manner, but there were no significant differences in proliferation, migration, and invasiveness (Figure 5a and b). The anti-proliferative and anti-metastatic effects of HA-cisplatin were also decreased by CD44 Ab treatment (Figure 5b). These results indicate that anticancer effects of HA-cisplatin are dependent on the CD44 receptor.

DISCUSSION

The overall prognosis of lung cancer is poor with low 5-year survival rates due to late presentation, disease relapse, and lack of curative systemic therapy. Cisplatin is one of the most common chemotherapeutic agents for solid tumors such as lung cancer [35]. However, its toxicity and chemotherapeutic tumor resistance severely limits its use in many patients [6, 36]. Developing anticancer drug delivery system to selectively kill cancer cell could reduce systemic toxicity. Therefore, many researchers have focused on cancer cell targeted anticancer drug delivery system.

Nanoparticles modified with HA have previously been designed for tumor targeted delivery of many drugs, such as paclitaxel, doxorubicin, and cisplatin [37–39]. Generally, HA links to the CD44 surface by covalent modification. Cancer delivery of the HA-cisplatin conjugate may be useful in the treatment of solid tumor by reducing systemic toxicities and increasing cisplatin deposition and retention within tumors. The present report aims to understand the interaction between HA-conjugated cisplatin and CD44 receptors, and confirm whether
this conjugate compound has selective and sensitive anticancer effects on NSCLC.

Overexpression of CD44 receptors on malignant cell membranes is associated with poor prognosis and tumor control [40–42]. Indeed, CD44 up-regulation tends to generate more aggressive and rapidly progressive cancer. Recently, there are many researcher highlighted the importance of CD44 molecules in the onset of malignant transformation, and there is evidence that a small population of tumor cells, such as CSCs or cancer-initiating cells for the formation of new tumors at metastatic states [7, 43, 44]. Hence, there are many studies on CD44-targeted chemical groups of HA, such as the carboxylate on glucuronic acid, the N-acetylg glucosamine hydroxyl, and the reducing end, which can potentially be used to conjugate a drug. In fact, it has been reported that the anti-proliferative activity of targeted anticancer drug is several folds higher than that of the free drug. And also in some cases similar to and rarely higher than that of the parent drug [45]. Our results show that the concentration of HA-cisplatin required 20 μg/mL (0.2 μg of cisplatin) to selectively inhibit CD44 overexpression H1299 cells’ growth, which was almost 50-folds more effective than free cisplatin (0.2 μg/mL). The sensitive cytotoxicity may have occurred because HA was specifically recognized by CD44 on the surface of cancer cells and enhanced the intracellular uptake of cisplatin. Furthermore, HA-cisplatin affected apoptotic pathway by increasing the expression of pro-apoptotic Bax and
FIGURE 5. The anticancer effects of HA-cisplatin conjugate were inhibited by CD44 monoclonal antibody in CD44 overexpressing cell lines. (a) CD44 expression inhibited by CD44 monoclonal antibody treatment in H1299 cells on Confocal Laser scanning; (b) cell proliferation, motility, invasiveness were measured by MTT assay, migration assay, matrigel invasion assay, respectively. The t-test analyses resulted in statistically significant differences; with *P < .05; **P < .01.

caspase-3 proteins. HA have biopharmaceutical, chemical, pharmaceutical, and toxicological benefits, which make it a unique molecule to be explored in targeted drug delivery systems.

Another interesting observation of the HA-cisplatin conjugate was cancer metastasis regulation. CD44 has been identified as an important marker of CSCs in breast, pancreatic, and colorectal cancers [46–48]. This protein is known to regulate growth, survival, differentiation, and migration, and is thereby involved in tumor progression and metastasis. Together, these findings suggest that CD44 plays an important role in the initiation and or maintenance of CSCs in some tumors. HA–CD44 interactions are known to promote tumor invasion by modulating the motility of cancer cells. In this study, we showed that motility and invasiveness of CD44 over-expression cells are selectively and sensitively inhibited by the HA-cisplatin conjugate.

The results of CD44 monoclonal antibody experiment also confirmed that the anti-proliferation and anti-metastasis effects of HA-cisplatin conjugate depended on the CD44 receptor. The targeting capacity of HA-cisplatin conjugates or HA ligand particulate formulations can be assessed by chemosensitivity assay using CD44 overexpression cell lines. Enhancement of chemosensitivity can result from other factors as well cytotoxicity of excipients of formulation, and additional cell line studies may be necessary to make a conclusive statement. Another important finding in our study was that conjugation of HA and cisplatin is able to significantly inhibit CD44-activated NSCLC metastatic signaling pathway with a minimal concentration of the conjugated compound.
We clearly demonstrate that HA–CD44 interaction contributes to selective and sensitive anticancer effects of HA-cisplatin conjugate in CD44 overexpression NSCLC cells. Despite the in vitro nature of this investigation, the results demonstrated interesting anti-proliferative, pro-apoptotic, and anti-metastatic activities that suggest HA-cisplatin as a promising candidate in the treatment of NSCLC.

**MATERIALS AND METHODS**

**Materials**

HA sodium salt (from Streptococcus equi sp.) was purchased from Fluka Chemie GmbH, Switzerland. All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA), and were of ACS grade or better. Human lung fibroblast cells (HFL1) were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were maintained according to ATCC recommendations. Human NSCLC cell lines (A549, H1299) were obtained from Korean cell line bank (KCLB) and were maintained according to KCLB recommendations.

**Synthesis of HA-Cisplatin Conjugates**

Cisplatin was conjugated to HA using silver nitrate as an activating reagent (12). HA (50 mg) (Fluka Chemie GmbH, Switzerland), cisplatin (23 mg) were dissolved in H2O (10 mL) and stirred in the dark for 3 days at ambient temperature (ca. 25°C). The reaction mixture was filtered (0.2 μm nylon membrane) and dialyzed against H2O (3500 MWCO, Pierce, Rockford, IL) for 48 hours at 4°C. Following dialysis, the HA-cisplatin was lyophilized and the degree of cisplatin substitution was determined by inductively coupled plasma optical emission spectrometry (ICP-MS) (Thermo Scientific iCAP 6300).

**Expression of CD44 in Lung Cell Lines**

To evaluate the expression of CD44 in HFL1, A549 and H1299 cell lines (2 × 10^5 cells/well) were seeded in round cover slide in 12-well plates and cultured for 24 hours. The cover slides were fixed by 4% paraformaldehyde, blocked with 3% bovine serum albumin in phosphate buffered saline (PBS) for 2 hours, and incubated with the antibody against CD44 (Thermo, Rockford, USA) in blocking solution overnight. The slides were treated with the corresponding FITC-conjugated anti-mouse IgG antibody (Santa Cruz, CA, USA) for 1 hour at 4°C, and the expression of CD44 was investigated by Zeiss observer z1 Confocal Laser scanning (ZEISS, Oberkochen, Germany). Results were obtained from at least three independent experiments, and representative results were shown.

**MTT Assay for Cell Proliferation**

The HFL1, A549, and H1299 cells were seeded into 96-well plates (5000 cells/well). The minimal concentration cisplatin (0.2 μg/mL), free cisplatin (10 μg/mL), HA-cisplatin conjugate (0.2 μg/mL of cisplatin) was applied for 24 hours. And then, the number of living cells was determined by MTT assay with 3-(4,5-dimethyl-thiazole-2-yl)-2,5-phenyltetrazolium bromide. After cells were incubated with 50 μL of MTT (1 mg/mL) for 4 hours at 37°C under a light-blocking condition, the medium was removed and 150 μL of dimethyl sulfoxide (DMSO; CALBIOCHEM, Darmstadt, Germany) was added into each well. Absorbance was measured at 595 nm using the SPECTRAMAX plus 384 (Molecular Devices, California, USA) and cell viability was calculated by

\[
\text{Cell viability} (%) = \frac{\text{OD}_{595(\text{test})} - \text{OD}_{595(\text{blank})}}{\text{OD}_{595(\text{control})} - \text{OD}_{595(\text{blank})}} \times 100\%
\]

Results were obtained from at least three independent experiments, and representative results were shown.

**Pro-Apoptotic Protein Expression**

The HFL1, A549, and H1299 cells (2.5×10^5 cells/well) were seeded in cell culture dish and cultured for 24 hours. After removing from the medium, HA-cisplatin (0.2 μg/mL of cisplatin) was added to the three cell lines and harvested at 1, 2, 3, and 5 hours. Protein expressions of caspase-3, Bax, and GAPDH were investigated by western blotting assay. Results were obtained from at least three independent experiments, and representative results were shown.

**Transwell Migration Assay**

The HFL1, A549, and H1299 cells (2×10^5 cells/well) were seeded in 12-well plates and serum-starved for 12 hours. The monolayer cells were manually scratched with a pipette tip to create extended and definite gap in the center of the well with a bright and clear field. Detached cells were removed by washing with PBS, and the rest of cells were treated...
with minimal concentration cisplatin (0.2 μg/mL), free cisplatin (10 μg/mL), HA-cisplatin conjugate (0.2 μg/mL of cisplatin), and incubated for 24 hours. The migration of cells across the scratched gap was monitored with a light microscope. Results were obtained from at least three independent experiments, and representative results were shown.

Matrigel Invasion Assay

Matrigel invasion assay was prepared in matrigel-coated invasion chamber. Briefly, Matrigel (1 mg/mL) (BD Biosciences, Beit-Ha’Emek, Israel) was added to the upper chamber of 24-well transwell plate and incubated at 37°C for 30 minutes for gelling. HFL1, A549, and H1299 cells (2 × 10⁵ cells/well) were cultured in the upper well with 200 μL medium, while 500 μL medium containing minimal concentration cisplatin (0.2 μg/mL), free cisplatin (10 μg/mL), HA-cisplatin conjugate (0.2 μg/mL of cisplatin) was added to the lower well. Plates were incubated at 37°C for 36 hours, and then non-invaded cells on top of the transwell were scraped off with a cotton swab. The amount of invading cells in the lower well as a percent of total seeded cells was evaluated with Diff-Quick staining kit and microscopy. The percent of invaded cells were calculated under microscopy. Invasion was expressed as Invasion Index (Percent of control). Results were obtained from at least three independent experiments, and representative results were shown.

Real-time PCR

Total RNA was isolated from NSCLC cells using Trizol (Invitrogen) and 1μl total RNA was reverse-transcribed using Accupower RT premix kit (Bioneer), PCR was performed in triplicate using SYBR green mix (Applied Biosystems), and a 7300 Fast Real-Time PCR System (Applied Biosystems) under the supplement Table 1 condition.

Western Blotting Analysis

Cells were harvested and washed with 1 × PBS and lysed on ice with RIPA Lysis buffer [10 mM Tris, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% Triton X-100, 0.5% NP40, pH 7.4, freshly added 0.2 mM PMSF in isopropanol, 1:50 Phosphatase Inhibitor Cocktail 2 (Sigma), 1:50 Protease Inhibitor Cocktail (Sigma)] for 1 hour. The cell lysate was then centrifuged at 13,000 rpm for 20 minutes at 4°C to remove cell debris. The protein amount in the lysate was quantified with the Protein Assay (Bio-Rad). For each lysate, 30 mg protein was loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto Nitrocellulose Membrane (Amersham, Buckinghamshire, UK). The membranes were blocked by incubation with shaking in 1% bovine serum albumin (BSA) blocking buffer at room temperature for 1 hour. Primary antibodies of caspase-3, Bax, and GAPDH (cell signaling) were diluted to 1:1000 in tris-buffered saline (TBS)/Tween 20 with 5% BSA. Secondary antibody was diluted in 1% BSA blocking buffer. Target proteins on the membrane were visualized on X-ray films by using the ECL Plus Western Blotting Detection Reagents (Amersham, Buckinghamshire, UK). Results were obtained from at least three independent experiments, and representative results were shown.

CD44 mAb Treatment

H1299 cells at a density of 1 × 10⁴ cells were pre-treated with an anti-CD44 neutralizing antibody, Hermes-1 (0, 10, and 20 mg/mL) for 2 hours at room temperature. After washes to remove free antibody, cells were subsequently incubated with HA-cisplatin (0.2 mg/mL) for 24 hours at 37°C. After washing with PBS, cells were observed using Zeiss observer z1 Confocal Laser scanning (ZEISS, Oberkochen, Germany).

Statistics

Significance of triplicates or of individual values was calculated by Student’s t-test. Null hypotheses of no difference were rejected if P-values were less than .05. Statistical software (SPSS for Windows, version 12.0; SPSS, Chicago, Illinois, USA) was used for statistical analysis.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

REFERENCES


**Supplementary Material Available Online**

Supplemental Table S1, Table S2, Figure S1, Figure S2, Figure S3, Figure S4.

Supplementary material can be viewed and downloaded at http://informahealthcare.com/elu

**Notice of correction:** Following initial online publication, the spelling of one author’s name has been corrected.