Blockade of interleukin-6 receptor suppresses the proliferation of H460 lung cancer stem cells

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Received December 30, 2011; Accepted March 26, 2012

DOI: 10.3892/ijo.2012.1447

Abstract. IL-6/6R signaling is closely associated with tumor growth and poor prognosis. Although there is evidence that interleukin-6 receptor (IL-6R)-mediated signaling promotes the growth and malignancy of cancer, the role of IL-6R in cancer stem cells (CSCs) is poorly defined. This study investigated the role of IL-6R in the proliferation of CSCs. Sphere-forming cells were isolated from the H460 non-small cell lung cancer (NSCLC) cell line and identified as CSCs using confocal microscopy, RT-PCR and WST-1 assay. The H460 spheres demonstrated the typical characteristics of CSCs, including CD133 expression, upregulation of Nanog, self-renewal, and drug resistance to methotrexate (MTX) and fluorouracil (5-FU). The release of IL-6R and its ligand, IL-6, were quantitatively determined and compared between CSCs and non-CSCs. The concentration of soluble IL-6R (sIL-6R) was remarkably high in CSCs compared to that in non-CSCs. Furthermore, significant upregulation of the IL-6R gene was also observed in the CSCs. The growth of CSCs was significantly inhibited by transfection with IL-6R small-interfering RNA (siRNA), as well as with the IL-6R monoclonal antibody (mAb). In addition, blocking both IL-6R and IL-6 using siRNA or mAbs intensified the inhibition of CSC proliferation. These findings indicate that IL-6R is present in CSCs and has an important role in the proliferation of CSCs in the H460 lung cancer cell line. Therefore, we suggest that IL-6R is both a viable target for the development of CSC-directed lung cancer therapeutics and a potential CSC marker in NSCLC.

Introduction

According to the cancer stem cell (CSC) theory, tumors have rare subpopulations of undifferentiated cells that possess stem cell properties, such as self-renewal, heterogeneity, and resistance to anticancer drugs (1-3). Due to these characteristics, CSCs have been regarded as the mediators of metastasis and/or cancer recurrence. Thus, new strategies for cancer treatment that target CSCs are needed to eradicate the disease. Because it has important clinical implications, many researchers have been attempting to isolate and characterize CSCs. CSCs were first isolated from leukemia and has since been isolated in many solid tumors as well, including breast, brain, prostate, pancreatic, colon, and lung cancer (4-10). The Hoechst 33342 dye has been identified as a putative functional marker for CSCs that extrude the dye through transporters (11,12). Although a general marker for CSCs has not yet been identified, many researchers documented CD133 expression in several types of CSCs (5,7,9,10,13). Recently, CSC enrichment was found *in vitro* within tumor spheres grown in a serum-free medium supplemented with appropriate growth factors (14,15). The sphere-forming cells showed cancer stem cell features, such as self-renewal, higher tumorigenicity, and resistance to anticancer drugs (16-18).

The IL-6 receptor (IL-6R) is a 80 kDa molecule that binds directly with the IL-6 ligand. When IL-6R binds with IL-6, the signal transducer gp130 is activated. Subsequently, Janus kinase (JAK) and the signal transducers and activators of transcription (STAT) are phosphorylated, which activate target genes that mediate cell growth and survival, differentiation, cell mobility and angiogenesis (19,20). The aberrant production and increased secretion of IL-6 in cancer patients is profoundly linked to tumor progression and poor prognosis in many cancer types, including lung cancer (21-23). IL-6R has been targeted for cancer treatments, resulting in cell growth inhibition or reduced angiogenesis (24,25). Recently, Wang *et al* showed that knockdown of IL-6R diminished stemness and survival of glioma stem cells (26). In our previous studies, we have isolated lung CSCs using the Hoechst 33342 exclusion assay and demonstrated that IL-6R expression is upregulated in CSCs (27,28).

Although there is evidence that IL-6/6R signaling acts as a critical factor for the growth and malignancy of cancer cells, the role of IL-6R in cancer stem cells (CSCs) is not well defined. Cancer stem cell researches have achieved considerable progress in solid tumors, such as breast, brain, and colon cancers. However, there has been minimal progress in lung

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Key words: cancer stem cell, interleukin-6 receptor, H460, lung cancer, sphere

CSCs because of a deficient understanding of lung epithelial stem cell hierarchy (29). Thus, in this study, we isolated CSCs from H460 lung cancer cells and investigated the functional expression of IL-6R in CSCs to assess the potential for targeting IL-6R in CSC-targeted lung cancer therapy.

Materials and methods

Cell culture. The H460 non-small cell lung cancer (NSCLC) cell line was obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were grown in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco) at 37˚C in a humidified incubator (5% CO_2) as recommended by the KLCB.

Serum-free sphere culture. H460 cells were plated at a density of 60,000 cells per well in an uncoated cell culture dish (Corning Inc., New York, NY, USA) in BEBM cell medium (Lonza Group Ltd., Basel, Switzerland), supplemented with BEGM SingleQuots (Lonza Group Ltd.) containing retinoic acid, bovine pituitary extract, insulin, hydrocortisone, transferrin, triiodotyronine, epinephrine, human epidermal growth factor, gentamicin, and amphotericin B, plus human EGF (10 ng/ml; Peprotech Inc., Rocky Hill, NJ, USA) and human bFGF (10 ng/ml; Peprotech Inc.). Cells were incubated in a humidified atmosphere at 37˚C with 5% CO_2. Fresh aliquots of EGF and bFGF were added twice a week. After 72 h of culture, spheres were visible by inverted phase-contrast microscopy. After the first-generation of spheres (Sphere #1) were grown for 8 days, the spheres were collected by centrifugation at 1,200 rpm for 5 min and dissociated into single-cell suspensions that can generate second-generation spheres (Sphere #2). Third-generation spheres (Sphere #3) were derived from second spheres (Sphere #2) in the same way.

Anticancer drug treatment. The anticancer drugs, fluorouracil (5-FU) and methotrexate (MTX), were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA) and dissolved in phosphate-buffered saline (PBS) (pH 7.4). Adherent cells, first-generation (Sphere #1) and third-generation spheres (Sphere #3) were seeded in a 96-well cell culture plate (Corning Inc.) and incubated overnight in a humidified atmosphere at 37˚C with 5% CO_2. After MTX (50 μg/ml) or 5-FU (25 μg/ml) were treated for 48 h, WST-1 solution (Dojindo Laboratory, Kumamoto, Japan) was added, and after another 4 h incubation, optical density was recorded using an ELISA plate reader (Magellan TECAN, Austria) at reference wavelengths of 450 and 620 nm.

RNA extraction and semi-quantitative RT-PCR. We performed semi-quantitative RT-PCR to identify the gene expression level of IL-6R in CSCs. The RNA was quantified by its absorption at 260 nm and stored at -80˚C before use. Using a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an internal control, the mRNA expression of different molecular markers was analyzed by semiquantitative RT-PCR. Briefly, first-strand cDNAs were synthesized from 5 μg of total RNA with Superscript III transcriptase (Invitrogen, Carlsbad, CA, USA). PCR amplification was performed with specific primer pairs designed from published human gene sequences (Table I) for different markers using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). Image J software (NIH, http://rsb.info.nih.gov/nih_image/index.html) was used to analyze the PCR product.

Confocal microscopy. Adherent cells were grown on a slide glass for confocal microscopy examination. Spheres were collected by gentle centrifugation at 1,200 rpm for 5 min. After washing with phosphate buffer solution, spheres and adherent cells were incubated with FITC-IL-6R (Abcam, Cambridge, MA, USA) and PE-CD133 (Miltentyi Biotec, Bergisch Gladbach, Germany) antibodies for 30 min. The cells and spheres were mounted in aqueous mounting medium before coated on slide glass. Immunofluorescence was observed with a confocal laser scanning microscope (Olympus FV-1000 spectral, Tokyo, Japan) at x200 magnification.

Detection of IL-6R by ELISA assay. The amount of released IL-6 or IL-6R in culture supernatants of CSCs and non-CSCs was determined by human IL-6 ELISA Ready-SET-Go kit (Ebioscience, San Diego, CA, USA) and human IL-6R ELISA kit (Abcam). The supernatants were harvested daily, starting the day after seeding until six days after seeding. The assays were performed according to the instruction manuals for IL-6 and IL-6R.

Small interfering RNA transfections. Small-interfering RNA (siRNA) for IL-6 and IL-6R were ordered from Genolution pharmaceuticals Inc., (Seoul, Korea). The following siRNA sequences were used: IL-6, sense 5'-GGACAUGACAACUCAUCUCtt-3', antisense 5'-GAGAUGAGUUGUCAUGUCCtg-3'; IL-6Rα, sense 5'-CGACUCUGGAAACUAUUCAtt-3', antisense 5'-UGAAUAGUUUCCAGAGUCGtg-3'. Scrambled siRNA was used as a negative control. We cultured H460 cells (non-CSCs) and its spheres (CSCs) on 96-well plates. A final concentration of 5 nM siRNA was transfected into 50-60% confluent cells using Lipofectamine RNAimax (Invitrogen) according to the manufacturer's instructions. Briefly, the siRNA was diluted in a serum-free media followed by the addition of Lipofectamine RNAimax. After a 20 min incubation, 20 μl of the solution was added to the cells in antibiotic-free optimal media in 96 well-plates. After a 48 h incubation of the transfected cells, WST-1 solution (Dojindo Laboratory, Kumamoto, Japan) was added, and after another 4 h incubation, optical density was recorded using an ELISA plate reader (Magellan, TECAN, Austria) at reference wavelengths of 450 and 620 nm.

Effect of the IL-6R mAb on cell proliferation. Mouse monoclonal antibodies for IL-6 and IL-6R (Abcam) were treated according to the manufacturer's guide. Ten microliters of mAb was added to the 96-well plates containing 1,000 cells per well in 100 μl of media. The final concentration of both IL-6 and IL-6R were 100 μg/ml. After a 48 h incubation, WST-1 solution was added for cell proliferation as described above.

Statistical analysis. Results are expressed as the means ± SD. Student's t-test was used to compare the differences between groups. $P<0.05$ was considered significant. Statistical analyses

Table I. Human primer sequences used for semi-quantitative RT-PCR.

Gene	Sense primer (5'-3')	Antisense primer (5'-3')	Product (bp)
IL-6	ATGAACTCCTTCTCCACAAGCG	TGCTACATTTGCCGAAGAGC	641
IL-6R	ACGCCTTGGACAGAATCCAG	TGGCTCGAGGTATTGTCAGA	398
Nanog	CTGTGATTTGTGGGCCTGAAG	GTAGGTGCTGAGGCCTTCTG	442
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	498

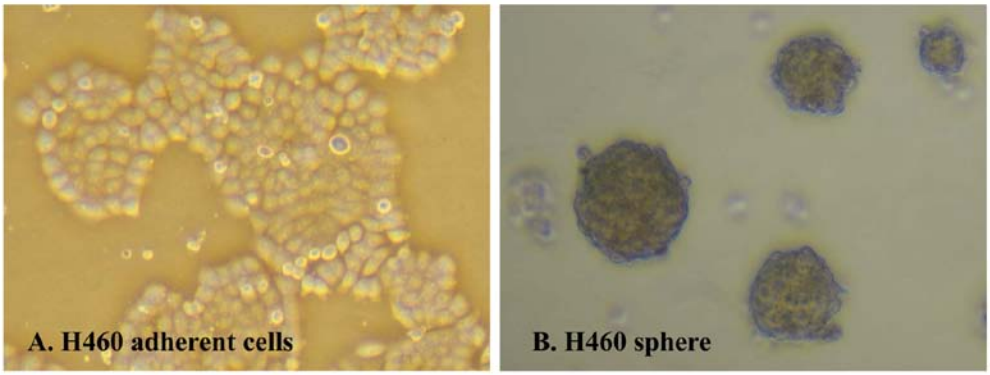

Figure 1. Lung cancer spheres were isolated from H460 NSCLC cells. (A) H460 lung cancer cells, cultivated in RPMI media with 10% FBS, revealed adherent and flat shapes. (B) H460 cells, cultivated in serum-free BEGM media supplemented with EGF and bFGF, formed floating spheres (x100).

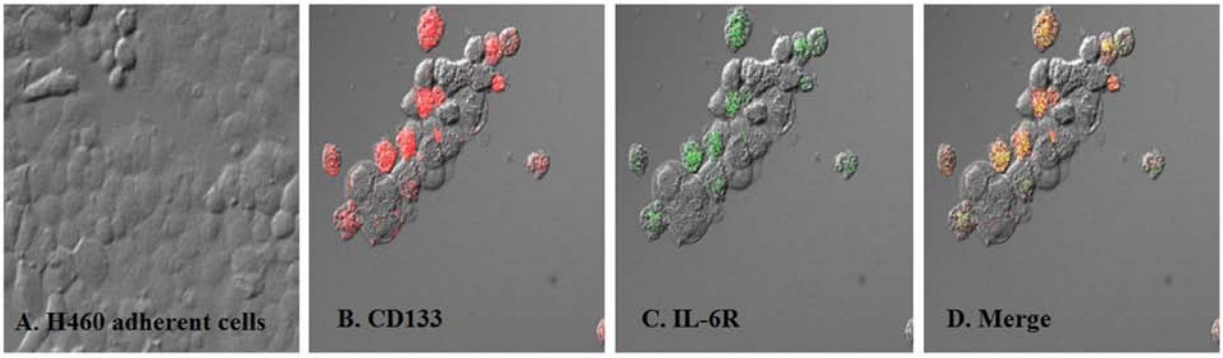

Figure 2. Expression of CD133 and mIL-6R on the cell surfaces of spheres (A-D). (A) The adherent cells and (B-D) floating spheres were incubated with CD133-PE antibodies (red) and FITC-IL-6R antibodies (green) for confocal microscopy observation (x200).

were performed with Statistical Analysis Systems software (SAS/STAT version 8.1, Cary, NC, USA).

Results

Isolation of H460 lung CSCs. Lung CSCs were isolated from H460 NSCLC cells under stem cell-selective conditions as previously described by Tirino *et al* (17). Adherent cancer cells formed floating spheres within a few days in serum-free BEGM media supplemented with EGF and bFGF (Fig. 1). The first-generation spheres showed self-renewal capacity, forming second-generation spheres. When the tumor spheres were plated onto adherent plates in an FBS-supplemented culture medium, they generated differentiated progeny with the phenotypic features of H460 lung cancer cells. The spheres expressed the lung CSC marker CD133 on their cell surface, while adherent cells sparsely expressed CD133 (Fig. 2A and B). In the mRNA expression study, Nanog, an embryonic stem cell marker, was highly expressed in the spheres as compared to adherent cells (Fig. 3). As shown in Fig. 4, the H460 spheres also appeared to be resistant to the anticancer drugs MTX and 5-FU. The growth of adherent cells was significantly inhibited by 63% with MTX treatment, while the growth inhibition of the spheres was only 12% in the first generation and negative in the third generation. A similar pattern of growth inhibition was also observed during treatment with 5-FU. Based on the presence of CSC properties, represented by CD133 expression, Nanog expression, and drug resistance, the H460 spheres and

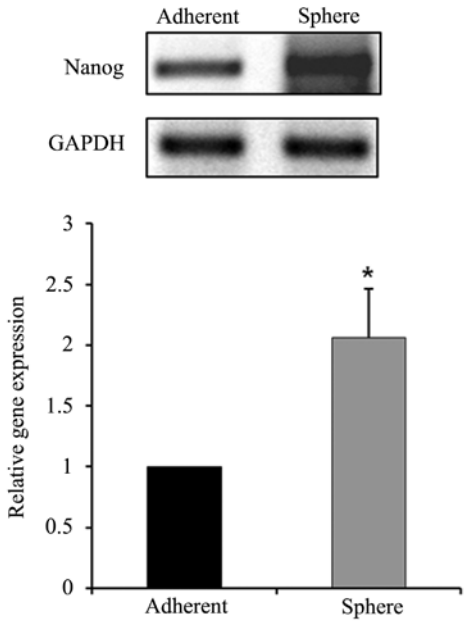

Figure 3. Nanog mRNA expression in H460 adherent cells and spheres. The spheres were collected by centrifugation at 1,200 rpm for 5 min. H460 adherent cells and spheres were used to determine the mRNA levels by RT-PCR. (n>3; *P<0.05).

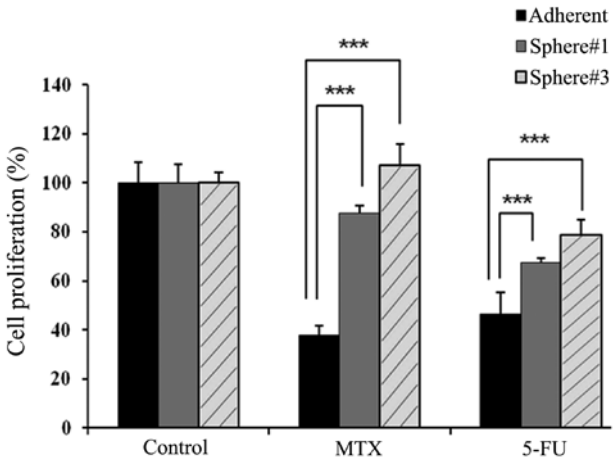

Figure 4. Effect of MTX and 5-FU on the proliferation of H460 lung cancer spheres. After the first generation of spheres (sphere #1) were grown for 8 days, the spheres were collected by centrifugation at 1,200 rpm for 5 min and dissociated into single-cell suspensions. Adherent cells and spheres (Sphere #1 and Sphere #3) were cultured overnight followed by MTX (50 μg/ml) or 5-FU (25 μg/ml) treatment for 48 h and subjected to the cell proliferation study (WST-1). (n>3; ***P<0.001).

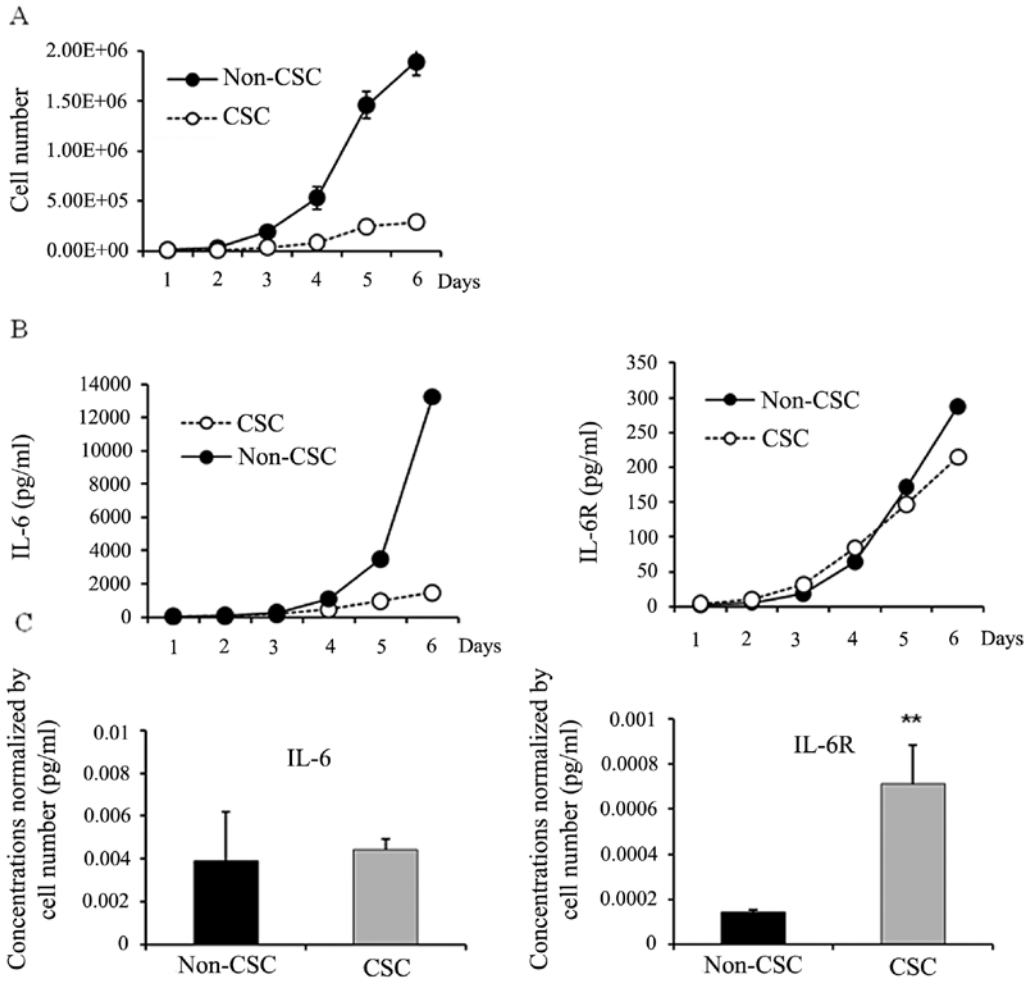

Figure 5. The concentrations of IL-6 and sIL-6R released from CSCs and non-CSCs. (A) Cells were counted for CSCs and non-CSCs for 6 days after cell seeding. (B) IL-6 and sIL-6R concentrations were measured in the cell-growth media using IL-6 and IL-6R ELISA kits. (C) IL-6 and sIL-6R concentrations were normalized to the cell numbers at cellular logarithmic growth phase, 4-6 days after seeding. (n>3; **P<0.01).

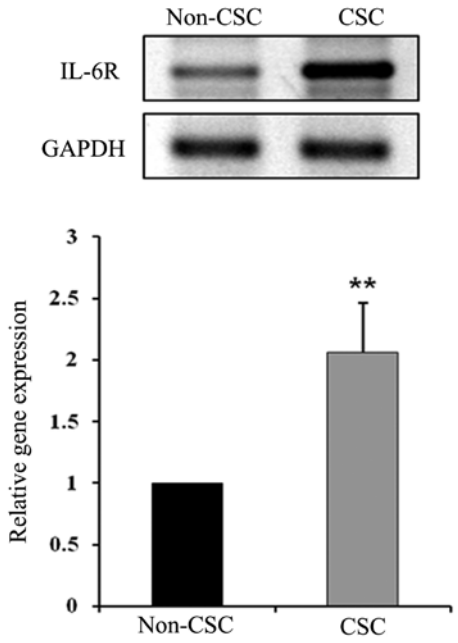

Figure 6. Gene expression of IL-6R in CSCs and non-CSCs. (A) Total RNA was extracted from CSCs and non-CSCs, and mRNA levels were analyzed by RT-PCR. PCR amplification was performed with specific primer pairs designed from published human gene sequences. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was selected as an internal control. The intensity of electrophoresis bands was quantified by Image J Software (NIH, http://rsb.info.nih.gov/nih_image/index.html). (n>3; **P<0.01).

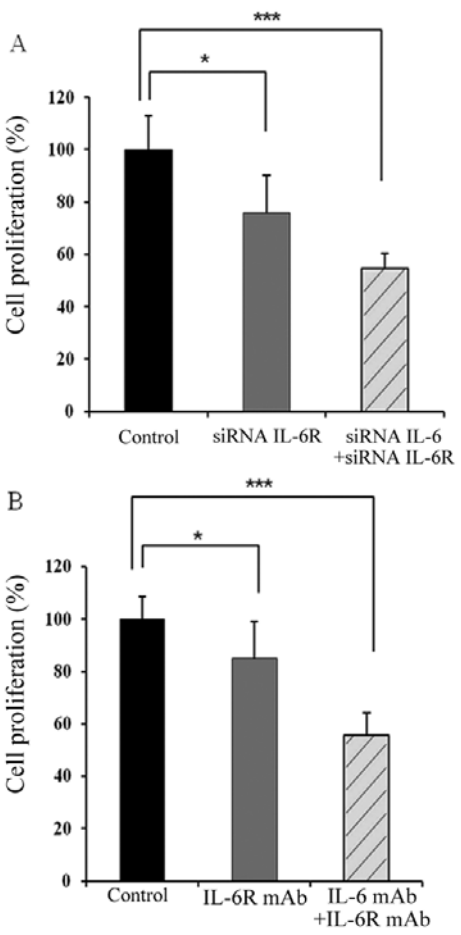

Figure 7. Effects of IL-6R siRNA or monoclonal antibodies on the proliferation of the H460 CSCs. (A) Lung CSC tumor spheres were grown in the presence/absence of IL-6R siRNA (5 nM) and IL-6 siRNA (5 nM) for 48 h. (B) Lung CSC tumor spheres were grown in the presence/absence of anti-IL-6R monoclonal antibody (mAb) (10 μg/ml) and anti-IL-6 monoclonal antibody (10 μg/ml) for 48 h. Cell proliferation was measured by WST-1. (n>3; *P<0.05, **P<0.01, ***P<0.001).

adherent cells will be termed CSCs and non-CSCs, respectively.

Expression of the IL-6R in H460 lung CSCs. To identify the contribution of IL-6R to CSC biology, we measured the amounts of soluble IL-6R (sIL-6R) and its ligand IL-6 that were released into the growing medium of CSCs and non-CSCs. Compared to non-CSCs, CSC proliferation was very slow (Fig. 5A). The concentrations of sIL-6R and IL-6 were increased in both CSCs and non-CSCs in a time-dependent manner (Fig. 5B). Because the growth rates were substantially different between CSCs and non-CSCs (Fig. 5A), we normalized the concentrations of sIL-6R based on the cell numbers at logarithmic growth phase from day 4 to day 6 after cell seeding. The concentrations of sIL-6R were remarkably higher in CSCs than in non-CSCs. On the other hand, the normalized concentrations of IL-6 were not significantly different between CSCs and non-CSCs (Fig. 5C). Direct immunofluorescence staining showed that CSCs markedly expressed membranous IL-6R (mIL-6R) on the cell surface, while non-CSCs had minimal expression of mIL-6R. Interestingly, mIL-6R colocalized with CD133 on the cell surface of spheres (Fig. 1B-D). RT-PCR results also demonstrated that the IL-6R was highly expressed in the CSCs (>2 times) compared to non-CSCs (Fig. 6).

Targeting IL-6R signaling suppresses CSC proliferation. To evaluate the effect of IL-6R on the proliferation of CSCs, the expression of IL-6R was blocked by siRNA. IL-6R knockdown significantly inhibited the growth of CSCs (Fig. 7A). Additionally, neutralization of IL-6R by mAbs inhibited CSC proliferation (Fig. 7B). Moreover, blocking both IL-6R and IL-6 using siRNA or mAbs intensified the inhibition of CSC proliferation.

Discussion

We isolated CSCs from H460 non-small cell lung cancer cells. Adherent cancer cells were transformed into spheres in serum-free stem cell selection media. CD133 has been widely proposed as a CSC marker in various cancer types, including lung cancers (9,30,31). Nanog, normally found in embryonic stem cells, has been known to play a crucial role in tumor initiation and promotes metastasis of lung adenocarcinoma (32). CSCs are known to express drug resistance proteins that result in immunity to chemotherapeutic drugs (33). This work demonstrates that, in contrast to the adherent cells, the spheres have the ability for self-renewal, show expression of CD133 and Nanog, have diminished proliferation rates, and are resistant to anticancer drugs such as MTX and 5-FU. These findings demonstrate that spheres isolated from H460 cells have the typical characteristics of CSCs, which agree with a previous report described by Levina *et al* (16). Therefore,

the current isolation method is a valuable tool for targeting specific biomolecules found in CSCs for malignant lung cancer therapy.

IL-6/6R signaling has been well documented to have a role in the growth and malignancy of cancer. IL-6R is not only a membranous protein localized on the cell membrane (mIL-6R) but is also a soluble form secreted from the cells to the extracellular area (sIL-6R) (34). The soluble receptor binds IL-6 with an affinity similar to that of the membranous receptor (35). In our study, the expression of IL-6R in the CSCs was markedly upregulated at both the gene and protein levels. The prominent distribution of mIL-6R was confirmed on the cell surface of CSCs, but not on non-CSCs. The release of sIL-6R in CSCs was also greater than that in non-CSCs. These results explain that the CSCs actively show IL-6R compared to the non-CSCs. On the other hand, we found that our sphere-forming CSCs also produced IL-6, but there was no quantitative difference in the release of the ligand between CSCs and non-CSCs. The IL-6R receptor may be of greater importance as a target molecule than the IL-6 ligand in cancer therapies employing stem cell strategies. Levina *et al* (10) reported that the release of IL-6 was elevated in CSCs of H460 lung cancer cells compared to non-CSCs, though they did not monitor the level of IL-6R. Although we do not know the reason for this disparity (10), it may be due to differences in isolation methods of CSCs, where we employed the sphere-culturing method and Levina *et al* used the drug-selected method (10). However, Wang *et al* demonstrated that the mRNA level of IL-6 was not greater in CSCs than non-CSCs that were separated from glioma cells, despite the fact that IL-6R was much greater in the CSCs (26). Taken together, it is clear that IL-6R occurs predominantly in the CSCs of H460. Interestingly, IL-6R was coexpressed with CD133, a typical CSC marker, on the cell surface of CSCs. We have also demonstrated upregulation of the IL-6R gene in A549 lung CSCs isolated by Hoechst 33342. Therefore, we suggest IL-6R as a possible stem cell marker for non-small cell lung cancer cells.

Because various studies have revealed that elevated IL-6 levels are associated with cancer development and poor prognosis (36-38), IL-6R has been targeted to suppress angiogenesis, invasiveness, and malignancy of several cancers (25,39). It has also been observed that the growth of cancer cells can be inhibited by blocking IL-6R (24,40). The role of IL-6R is poorly understood in CSCs, though IL-6/6R signaling was reported to contribute to glioma malignancy through the promotion of CSC growth and survival (26). Moreover, to our knowledge, IL-6R has never been identified and characterized in lung CSCs. In this study, blocking of IL-6R by siRNA or mAbs resulted in a significant inhibition of CSC proliferation. This demonstrates that IL-6R has an important role in the proliferation of CSCs from H460 cells. In addition, simultaneous blocking of IL-6R with IL-6 intensified the inhibition of CSC proliferation, indicating that IL-6 also participates in the growth of CSCs as a ligand of IL-6R. Although we have no direct evidence for the mechanism by which IL-6R has a role in CSC proliferation, it may be assumed based on the importance of IL-6/6R signaling pathways in cancer growth. The stimulation of the Ras-dependent MAP kinase cascade has been suggested to perform an important function in IL-6-mediated proliferation of cancer (41), and the STAT3 pathway is also involved in anti-apoptotic activity (42).

Conclusively, these findings provide evidence that IL-6R is found in CSCs and promotes the proliferation of CSCs in H460 lung cancer cells. Therefore, we think that targeting IL-6R would be very beneficial for the development of CSC-targeted lung cancer therapeutics and suggest that IL-6R can be a potential CSC marker in NSCLC.

Acknowledgements

This work was supported by Konkuk University in 2012.

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