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ORIGINAL ARTICLE

Recombined humanized endostatin-induced suppression of HMGB1 expression inhibits proliferation of NSCLC cancer cells

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Abstract

Background: Recombined humanized endostatin (Rh-endostatin) exhibits a potent anti-cancer effect involving multiple molecular targets and signaling pathways. HMGB1 is a highly conserved DNA-binding protein involved in cancer development. The therapeutic effect of Rh-endostatin on HMGB1 has not been reported, thus we investigate the effect in non-small cell lung cancer (NSCLC) cells.

Methods: Quantitative real-time PCR and Western blot were used to analyze the messenger RNA and protein expression of HMGB1 in A549 cancer cells, while enzyme-linked immunosorbent assay was used to detect the release of HMGB1. Western blot was performed to evaluate HMGB1 expression in SK-MES-1 and H661 NSCLC cells.

Results: Rh-endostatin inhibited the proliferation of A549 cancer cells and distinctly downregulated the expression and release of HMGB1 in dose and time dependent manners. Rh-endostatin-induced HMGB1 downregulation was confirmed in different types of NSCLC cells.

Conclusion: These results demonstrate the general phenomenon that Rh-endostatin can induce HMGB1 suppression in a variety of NSCLC cells. Rh-endostatin may suppress HMGB1 expression and release in A549 cancer cells, thus inhibiting cell proliferation.

Introduction

Non-small cell lung cancer (NSCLC) is the most common malignancy and the leading cause of cancer-related death.[1] After recurrence, the five-year survival rate is merely 20%.[2] As standard therapeutic modalities for NSCLC, chemotherapy and radiation significantly decrease tumor recurrence and prolong patient survival. However, the use of chemotherapy and radiation in clinical practice is restricted because of toxicity. Therefore, there is an urgent need to determine a safer therapeutic approach to improve the survival of NSCLC patients.

Rh-endostatin is a new recombinant human endostatin developed by Simcere Biopharmaceutical Co. Ltd. (Nanjing, China), different to the original endostatin identified by O'Reilly *et al.*[3] Rh-endostatin is designed to halt cancer progression by depriving the tumor of oxygen and nutrients for growth. Rh-endostatin works by inhibiting angiogenesis: the proliferous formation of new blood vessels in and around the tumoral tissue. Preclinical studies

have shown that this novel agent could inhibit tumor growth and shrink existing tumor blood vessels.[4–6]

HMGB1 is a highly conserved DNA-binding protein present in most cell types. As a nuclear protein, HMGB1 modulates chromatin structure, promotes interaction of proteins, and plays a role as a transcription factor in gene expression regulation.[7] In addition to its nuclear expression, HMGB1 can also be passively released by necrotic cells.[8] It can be actively secreted into the extracellular matrix by inflammatory cells and some cancer cells, where it acts as a damage-associated molecular pattern to mediate inflammation or plays a role as a chemoattractant factor.[9] Cheng *et al.* showed that the induction of HMGB1 contributed to NSCLC tumorigenesis, while HMGB1 silencing suppressed cancer progression.[10] Tang *et al.* found that HMGB1 promoted differentiation syndrome in acute promyelocytic leukemia cells.[11] Considering its role as mediator of tumorigenesis, HMGB1 targeted therapy may be of meaningful value in anti-cancer treatment.[12]

Given the importance of HMGB1 in tumor initiation and progression, the aim of this study was to investigate whether Rh-endostatin can suppress the proliferation of A549 human NSCLC cells by inhibiting HMGB1 expression.

Methods

Materials

Ethyl pyruvate was purchased from Sigma Chemical (St. Louis, MO, USA). An HMGB1 enzyme-linked immunosorbent assay (ELISA) kit was purchased from Elabscience Biotechnology Co., Ltd. (Wuhan, China). Antibodies against HMGB1 were purchased from Wanlei Life Sciences (Shenyang, China). A Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan).

Cell culture

Human lung cancer cells (A549, SK-MES-1, H661) were obtained from American Type Culture Collection (Manassas, VA, USA). A549 and SK-MES-1 cells were cultured in Dulbecco's modified Eagle medium, whereas H661 cells were incubated in RPMI-1640 at 37°C in 5% CO_2 (Gibco, Carlsbad, CA, USA). Both mediums were supplemented with 10% fetal bovine serum (Gibco), 100 μg/mL streptomycin, and 1% (v/v) penicillin.

Cell viability assay

Cell viability was assessed using a CCK-8 kit. To obtain single cell suspension, adherent cells that reached 80% growth density were rinsed with phosphate buffered saline and digested with 0.25% trypsin. The cells were then seeded in 96-well plates at a density of 2 × 103 cells/100μL/well. After 24 hours of incubation at concentrations of 0, 5, 10, 20, 40, 80, and 160 nmol/L, Rh-endostatin was added to the cells for 24 hours, and a fixed concentration of 20 nmol/L Rh-endostatin for 12, 24, 48, 72, and 96 hours, respectively. The other settings were treated with 20 nmol/L Rh-endostatin alone and in combination with 20 ng/mL rHMGB1 or 10 mmol/L ethyl pyruvate for 24 hours. In each well, 10 μL CCK-8 was then added and cultivated for two hours. ELISA was used to detect the absorbance of each well at 450 nm. Each experiment was conducted five times and the average values are reported.

Colony formation assay

Cells were inoculated in six-well plates with 500 cells/well. After 24 hours of incubation, various concentrations of Rh-endostatin (0, 5, 10, 20, 40 nmol/L) were added for 12 hours and the cells were then cultured in a normal condition for two weeks. The colonies were fixed with 4% paraformaldehyde solution and stained with 0.1% crystal violet. Colonies with > 50 cells were scored. Each test was undertaken in triplicate, and the average values are reported.

Real-time PCR

To validate the microarray data, we selected another eight patient samples for RT-PCR. Total RNA was isolated from thymoma and thymic cyst tissue using TRIzol Reagent (Goldenbridge Biotech, Beijing, China), according to the manufacturer's instructions. Complementary DNA was digested with DNase 1, and then synthesized via reverse transcription from RNA samples using oligo (dT) primers and TransScript RT/RI/Enzyme Mix (Takara, Beijing, China). Quantitative real-time (qRT) PCR was used to determine the relative messenger RNA (mRNA) transcription levels of HMGB1 to the control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) with SYBR green PCR master mix buffer (Takara) and specific primers. The qRT-PCR primers were designed and synthesized by Sangon Biotech Company (Shanghai, China) (Table 1). PCR amplification was performed as follows: pre-denaturation at 94°C for three minutes, followed by 35 cycles of 94°C for 30 seconds, 56–58°C for 30 seconds, 72°C for one minute, and finally, extension at 72°C for five minutes. The relative expression levels of targeted genes were normalized to GAPDH and analyzed by $2^{-\Delta\Delta Ct}$.

Western blot analysis

To extract the total protein, the harvested cells were suspended in radioimmunoprecipitation assay lysis buffer containing a protease inhibitor cocktail (Solarbio, Beijing,

Table 1 Primers for quantitative real-time PCR

Gene	Forward	Reverse
HMGB1	TGTGCAAACTTGTCGGGAG	TCTTTCATAACGGGCCTTGTC
GAPDH	ACCGAGCGCGGCTACAG	CTTAATGTCACGCACGATTTCC

GADPH, glyceraldehyde 3-phosphate dehydrogenase.

China). The protein expression level was examined via the bicinchoninic acid method. An aliquot of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Solarbio). After blocking with 5% non-fat milk for one hour at room temperature, the membranes were incubated overnight at 4 °C with primary antibodies against HMGB1 (1:500), followed by horseradish peroxidase-conjugated secondary antibodies. The data were evaluated using QuantityOne software (Hercules, CA, USA). The relative expression of the target protein content was analyzed by the gray value ratio of target and β-actin.

Enzyme-linked immunosorbent assay

Supernatants were collected and the HMGB1 level was analyzed via ELISA using commercial kits as the standard method.

Statistical analysis

SPSS version 19.0 (IBM Corp., Armonk, NY, USA) was used for data analysis and a *t*-test of independent samples for continuous variables. All data are expressed as the mean ± standard deviation. Statistical differences were assessed by Student's *t*-test or one-way analysis of variance. An alpha of $P < 0.05$ was used to determine statistical significance.

Results

Recombined humanized endostatin (Rh-endostatin) inhibits A549 human non-small cell lung cancer (NSCLC) cell proliferation

We investigated the biological function of Rh-endostatin on cell proliferation. CCK-8 and colony formation assays were performed to detect the proliferation of A549 cells treated with Rh-endostatin. As expected, Rh-endostatin significantly suppressed cell viability and colony formation in dose and time dependent manners (Fig 1), indicating that Rh-endostatin significantly inhibited A549 cell proliferation.

Rh-endostatin inhibits HMGB1 expression in A549 human NSCLC cells

To explore the effects of different concentrations and the activation time of Rh-endostatin on the expression of HMGB1 in A549 cells, qRT-PCR of A549 cells treated with different doses of Rh-endostatin at different time points was conducted. As shown in Figure 2a,c, Rh-endostatin suppressed the expression of HMGB1 mRNA in dose and time dependent manners, with significant downregulation noted as early as three hours. To analyze the profile of HMGB1 expression, we further investigated whether Rh-endostatin-induced HMGB1 suppression could be identified at a translational level. Western blot analyses revealed

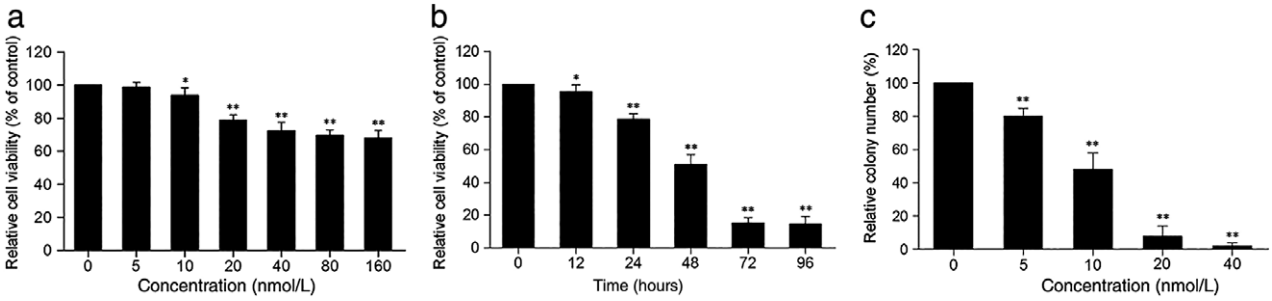

Figure 1 Recombined humanized endostatin (Rh-endostatin) inhibits A549 cancer cell proliferation. A549 cells were treated with (**a**) the indicated concentrations of Rh-endostatin for 24 hours (*$P < 0.05$ and **$P < 0.01$ vs. control group ([0 nmol/L]); and (**b**) 20 nmol/L of Rh-endostatin for the indicated durations (*$P < 0.05$ and **$P < 0.01$ vs. control [0 hours]). Cell viability was measured using Cell Counting Kit-8 assay. Data are expressed as mean ± standard deviation (SD) of five independent experiments. (**c**) A549 cells were pretreated with the indicated concentrations of Rh-endostatin for 12 hours and then cultured at the normal condition for two weeks. Cell colony formation was measured using plate colony formation assay (**$P < 0.01$ vs. control [0 nmol/L]). Data are expressed as (mean ± SD) of three independent experiments.

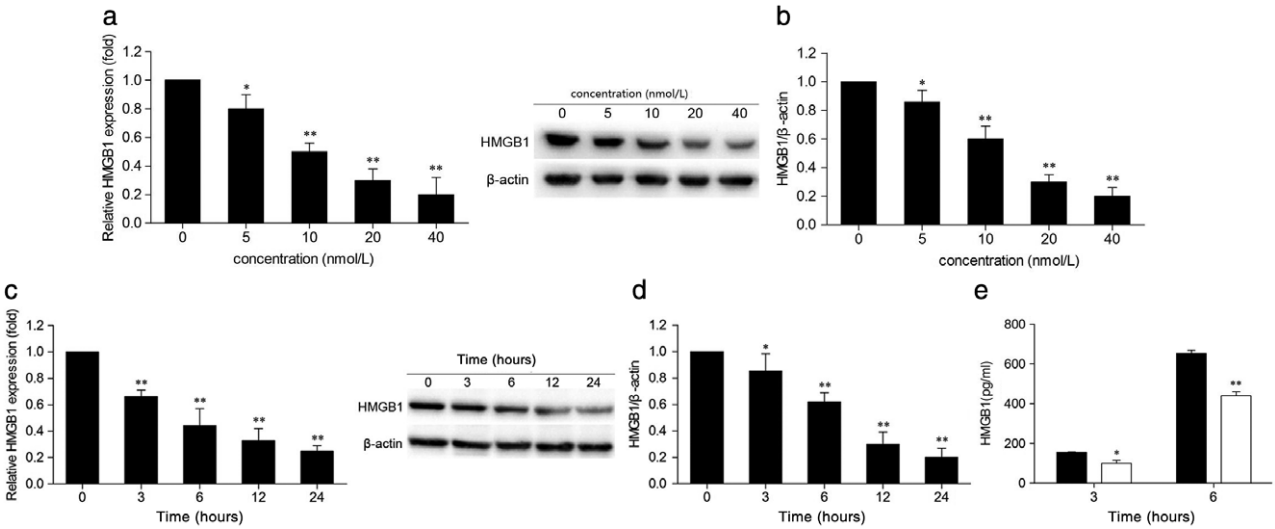

Figure 2 Recombined humanized endostatin (Rh-endostatin) suppresses the expression and release of HMGB1 in A549 cancer cells. A549 cells were treated with the indicated concentrations of Rh-endostatin for 24 hours, and (**a**) messenger RNA (mRNA) and (**b**) protein levels of HMGB1 were subsequently determined by quantitative real-time (qRT) PCR and Western blot, respectively (*P < 0.05 and **P < 0.01 vs. control [0 nmol/L]). A549 cells were treated with 20 nmol/L of Rh-endostatin for the indicated durations, and then the (**c**) mRNA and (**d**) protein levels of HMGB1 were analyzed by qRT-PCR and Western blot, respectively (*P < 0.05 and **P < 0.01 vs. control [0 hours]); and (**e**) the supernatants of HMGB1 release were measured by ELISA (*P < 0.05 and **P < 0.01 vs. control (0 nmol/L). Data are expressed as the mean ± standard deviation of three independent experiments. (■) 0 nmol/L and (□) 20 nmol/L

that Rh-endostatin induced HMGB1 protein suppression in dose and time dependent manners, with significant downregulation noted as early as three hours (Fig 2b,d).

Furthermore, we conducted ELISA analysis of supernatants to examine HMGB1 release after Rh-endostatin treatment. Consistently, supernatant levels of HMGB1 were distinctly reduced as early as three hours after Rh-endostatin treatment (Fig 2e). These results imply that Rh-endostatin can potently inhibit HMGB1 expression and extracellular secretion.

Rh-endostatin downregulates HMGB1 expression in various human NSCLC cells

To investigate whether Rh-endostatin-induced suppression of HMGB1 occurred in other NSCLC cells, Western blot analyses were conducted with SK-MES-1 and H661 NSCLC cells treated with Rh-endostatin for 24 hours. Interestingly, Rh-endostatin suppressed the expression of HMGB1 in all cancer cells tested (Fig 3). These data demonstrate that Rh-endostatin could widely downregulate the expression of HMGB1 in various human NSCLC cells.

Discussion

Various factors involved in different signal pathways take part in the development of cancer. HMGB1 is a conserved nuclear protein and plays a critical role in nucleosome stabilization and gene transcription. Moreover, HMGB1 upregulation has recently been proven to act as the link between tumor-associated inflammation and tumorigenesis.[13] HMGB1 is stably expressed in the nucleus of

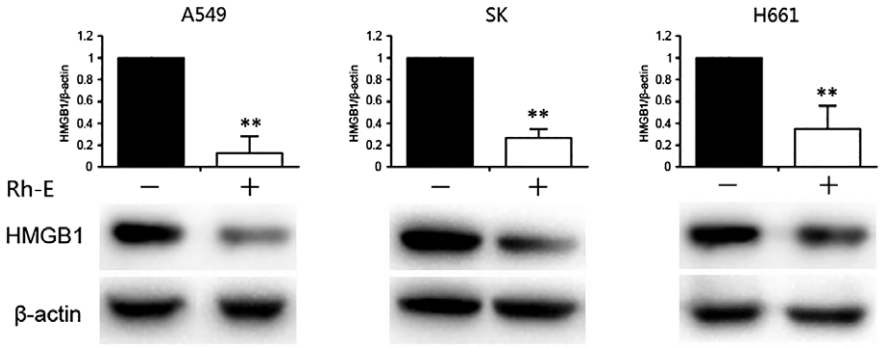

Figure 3 Recombined humanized endostatin (Rh-endostatin)-induced HMGB1 suppression occurs in various human cancer cells. Various NSCLC cells were treated with Rh-endostatin (20 nmol/L) for 24 hours. The level of HMGB1 expression was assayed by Western blot (**P < 0.01 vs. control [0 nmol/L]). Data are expressed as the mean ± standard deviation of three independent experiments.

quiescent cells and HMGB1 secretion has been confirmed as its translocation from the nucleus to the cytoplasm.[9] Recently, many reports have shown that overexpression of extracellular HMGB1 contributes to cancer carcinogenesis and metastasis by promoting apoptotic evasion; mediating tumor-associated inflammation; and increasing tumor cell proliferation, migration, and angiogenesis.[14] Enhanced HMGB1 expression has been observed in patients with various cancers, including breast cancer,[12] cervical carcinoma,[15] metastatic prostate cancer,[16] and hepatocellular carcinoma.[17]

This research explored the effect of Rh-endostatin on HMGB1 expression and the possible mechanisms of proliferation in A549 cells. We confirmed that Rh-endostatin inhibits A549 cell proliferation (Fig 1). Our findings also suggest that the expression and release of HMGB1 are significantly suppressed by Rh-endostatin treatment in A549 cells (Fig 2). Downregulation of HMGB1 by Rh-endostatin may be a general phenomenon in other types of cancer cells (Fig 3). Additionally, NF-κB is of crucial importance in the synthesis of mediators involved in tumor development and progression. Binding to TLR4, HMGB1 activates the NF-kB signaling pathway, which promotes the expression of various genes taking part in tumor cell proliferation, migration, and invasion.[18,19] Recent research has shown that the macrophage migration inhibitory factor promotes cancer metastasis by activating the HMGB1/TLR4/NF-κB signaling pathway.[20] Therefore, we recommend that future research should be directed toward a better understanding of the relationship between the key gene and NSCLC and the mechanism and role of the HMGB1/TLR4/NF-κB pathway in NSCLC occurrence and development.

In summary, we have shown that Rh-endostatin significantly inhibits the proliferation of A549 NSCLC cells, and this therapeutic effect is a result of the downregulation of HMGB1 expression and release. Our results revealed an interesting mechanism of NSCLC and identified a novel therapeutic drug for NSCLC treatment.

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Disclosure

No authors report any conflict of interest.

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