ORIGINAL RESEARCH

CD133⁺ human pulmonary adenocarcinoma cells induce apoptosis of CD8⁺T cells by highly expressed galectin-3

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Manuscript submitted 28th October, 2009
Manuscript accepted 4th January, 2010

Clin Invest Med 2010; 33 (1): E44-E53.

Abstract

Purpose: To investigate the expression and function of Galectin-3 (Gal-3) in CD133⁺ pulmonary adenocarcinoma.

Methods: CD133⁺ pulmonary adenocarcinoma cells were separated by MACS from excised pulmonary adenocarcinoma specimens of 11 patients. The percentage of CD133⁺ cells in different cells population was determined by flow cytometry (FCM). Expression of Gal-3 in cancer cells was detected by Fluorescence Quantitation RT-PCR (FQRT-PCR) and Western blot whereas extracellular expression was detected by ELISA. CD133⁺ cells were transfected with Gal-3 specific siRNA to explore the affects of Gal-3 inhibition on cancer cell growth and induction of CD8⁺ T cell apoptosis.

Results: Cells expressing CD133 constituted 90% of the CD133⁺ subpopulation after separation by MACS whereas they made up only 1.2% of the unsorted cell population. Expression of Gal-3 was 1.24 fold, 1.5 fold and 2 fold higher in CD133⁺ cells than in CD133⁻ cells as detected by FQRT-PCR, Western blot and ELISA respectively ($p < 0.05$ for each). The supernatants of CD133⁺ cells induced apoptosis of CD8⁺ T cells to a greater degree ($27.1 \pm 2.6\%$) compared with supernatants from CD133⁻ cells ($10.1 \pm 2.2\%$), and could be down-regulated by lactose, anti-galectin-3 polyclonal antibody and Gal-3 siRNA. Downregulation of Gal-3 resulted in significant inhibition of cancer cell growth *in vitro*.

Conclusion: Gal-3 is expressed at a relatively higher level in CD133⁺ lung adenocarcinoma cells and could induce

CD8⁺ T cell apoptosis *in vitro*, both of which could be down-regulated by Gal-3 siRNA. These findings indicate that Gal-3 may play an important role during oncogenesis, implying a potential therapeutic target for pulmonary adenocarcinoma.

List of Abbreviations

MACS	magnetic activated cell sorting
FCM	Flow cytometry
FQRT-PCR	Fluorescent quantitation real time polymerase chain reaction
ELISA	enzyme linked immunosorbent assay
Sangon	Shanghai Sangon Biological Engineering Technology & Services Co., Ltd
CD133+cell	CD133+ human pulmonary adenocarcinoma cells
CD133-cell	CD133- human pulmonary adenocarcinoma
sup.	supernatant

Lung cancer is often incurable and remains the leading cause of death due to cancer worldwide. Histologically it is divided into four major subtypes: squamous cell carcinoma, adenocarcinoma, small cell carcinoma and large cell carcinoma. Among them, adenocarcinoma is the most common, and currently ac-

counts for almost half of all non-small cell lung carcinomas. Despite continuous efforts to improve the therapeutic response, the overall five-year survival rate for such tumors is lower than 15%.¹⁻³

In the last decade, accumulating evidence has been lending support to the idea that tumors contain a small population of cancer stem cells that are responsible for tumor maintenance and spread. The same phenomenon exists in lung cancer. To date, CD133 has been recognized as a stem cell marker for cancerous tissues and it is being currently used alone or in combination with other markers for identification and isolation of the putative cancer stem cell population from malignant tumors.⁴⁻⁶ In lung cancer, there is a rare population of CD133⁺ cancer stem-like cells able to self-renew and capable to generate unlimited progeny of differentiated cells that constitute the major tumor population. Molecular and functional characterization of such a tumorigenic population may provide valuable information to the development of effective therapies.

Galectin-3 (Gal-3), a member of the beta-galactoside-binding protein family, is a multifunctional protein capable of acting both intracellularly and extracellularly, affecting such processes as tumor cell adhesion, proliferation, differentiation, angiogenesis, apoptosis, cancer progression, and metastasis.⁷⁻¹¹ Of note, high levels of Gal-3 proteins are found in serum from cancer patients with metastases.¹² Because the functions of Gal-3 overlap with the roles tumor stem cells play, and there are high levels expression of Gal-3 in lung cancer,^{13,14} we conjectured that Gal-3 plays a role in the activity of tumor stem cells, such as CD133⁺ lung cancer cells. Therefore, we conducted this study to investigate the expression of Gal-3 and its function in tumor cells isolated from lung adenocarcinomas, which can be separated into two subpopulations by the expression of CD133.

Methods

Patients and tissue specimens

Eleven surgically resected adenocarcinoma specimens and 10 mL of peripheral blood were obtained from patients diagnosed with pulmonary adenocarcinoma between 2006 and 2008 at Huazhong Science and Technology University Union Medical Hospital (Wuhan, China) and who had not yet received radiotherapy or chemotherapy. The patients ranged in age from 28 to 68 years with an average of 52.5 years. Six patients (54.5%) were male and 5 (45.5%) were female. All studies were performed with the patients' informed consent with the study approved by the Institutional Review Board of Tongji School of Medicine.

Magnetic cell separation (magnetic activated cell sorting, MACS)

Surgical specimens were washed 3 times and left overnight in DMEM-F12 medium supplemented with 200 IU/ml penicillin, 200 µg/ml streptomycin, and 20 µg/ml amphotericin B to avoid contamination. The tumor tissue specimens were mechanically disrupted and cells were separated by density centrifugation as described previously in detail.¹⁵⁻¹⁷ Tissue dissociation was carried out by enzymatic digestion (20mg/mL collagenase II, Gibco-Invitrogen, Carlsbad, CA) for 2 h at 37°C. Recovered cells were cultured in serum-free medium containing 50mg/mL insulin, 100mg/mL apo-transferrin, 10mg/mL putrescine, 0.03mM sodium selenite, 2mM progesterone, 0.6% glucose, 5mM HEPES, 0.1% sodium bicarbonate, 0.4% BSA, glutamine and antibiotics, dissolved in DMEM-F12 medium (Gibco-Invitrogen) and supplemented with 20mg/mL EGF (Epidermal Growth Factor) and 10mg/mL bFGF (basic Fibroblast Growth Factor). After 3 subcultures, single cells were prepared with enzymatic digestion and then were magnetically labeled with anti-CD133 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) in the dark at 4°C for 30 min and applied to

the prepared MS Column (Miltenyi Biotec). CD133⁻ cells were collected in the flow-through of the column; CD133⁺ cells bound to the beads were flushed out by applying the plunger as described by the manufacturer.

Flow cytometry

The percentage of CD133 expressing cells in the original cell populations, the flow-through, and the flushed-out fractions were analyzed by flow cytometry. Cells were washed twice with phosphate-buffered saline (PBS) and then were stained at the concentration of 1×10^6 cells per 90 μ L PBS buffer and 10 μ L control or specific antibody. The antibody used was PE-conjugated anti-CD133 purchased from Miltenyi Biotec. After 30 min incubation at 4°C, cells were washed twice with PBS and analyzed with a FACScan (Beckman Coulter, Brea, CA).

RNA Isolation and Real Time Fluorescence Quantitation RT-PCR (FQRT-PCR)

Total RNA was extracted from the cell lysates using TRIZOL (Gibco-Invitrogen). RNA was quantitated by the real-time reverse transcription polymerase chain reaction using the QuantiTect™ SYBR Green PCR Handbook Kit (QIAGEN, Valencia, CA). The samples were heated to 95°C for 10 min, followed by 30 cycles of 95°C for 10 s, 53°C for 15 s, and 72°C for 20 s. The oligonucleotide primers used were as follows: Galectin-3 forward: 5'-TACTGAAGGCCGTGGAC-3', reverse: 5'-CCTCGTGGCTCCAGTACA-3'; Beta-actin forward: 5'-TACTGAAGGCCGTGGAC-3', reverse: 5'-CCTCGTGGCTCCAGTACA-3'. Primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The relative expression of Gal-3 in each sample was normalized by comparison with that of Beta-actin.

Western blotting

Two million cells were lysed with 500 μ L SDS-PAGE 1x loading buffer. After boiling for 5 min, samples were separated by 12.5% SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. Skim milk powder solution (10%, w/v) was used for blocking. Monoclonal mouse anti-Gal-3 antibody (Vector laboratories, Peterborough, UK) was used at a dilution of 1:2,000 at room temperature in TBST buffer (50mM Tris-HCl, pH 7.6, 137mM NaCl, 0.1% Tween-20) with 3% fetal bovine serum (Hyclone, Logan, UT). The membrane was incubated with primary antibody for 1 h at room temperature and washed three times and then incubated with HRP conjugated Goat anti-mouse IgG diluted 1:3000. Signals were visualized by using enhanced chemiluminescence (ECL).

Separation and preparation of CD3⁺CD8⁺ T cells

PBMCs were isolated from 10 mL of whole blood using Ficoll-paque density gradient separation solution (Sigma-Aldrich, St. Louis, MO). In brief, whole blood was collected into sodium heparin tubes, diluted with an equal volume of PBS, and then layered over Ficoll-paque. After centrifugation at 300 x g for 20 min at room temperature, PBMCs were collected from the interphase layer and washed three times with RPMI 1640. CD3⁺CD8⁺ cells were then isolated from PBMCs by magnetic activated cell sorting (BD Bioscience, San Diego, CA) following the manufacturer's instructions. CD3⁺CD8⁺ cells were resuspended in RPMI 1640 supplemented with 10% FBS and 2 mM glutamine at a concentration of 1×10^6 cells/mL.

Stable Transfection

As previously reported, wild-type Gal-3 siRNA and mutant Gal-3 siRNA used in our studies were as follows: siRNA forward: 5'-CUUUCUUCCTAA AUCUGCUGCUGGA-3', siRNA reverse: 5'-UCCA GCAGCAGAUUUGGGAAGAAAG-3'. siRNA_{mut} forward: 5'-CUUUCUUCCTAA AUCUGCUGCUG

GA-3', siRNAmut reverse: 5'-UCCAGCAGCAGAU AAGGGAAGAAAG-3'.¹⁸ siRNA were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. Transfection of siRNAs were performed with LipofectamineTM 2000 (Gibco-Invitrogen) at a final concentration of 100 nmol/L as described previously.¹⁹ Cells were cultured for 48 h in complete medium and transferred to complete medium containing 300 µg/mL zeocin (Gibco-Invitrogen).

Cell proliferation assay

A 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) proliferation assay was used to determine the anti-growth effects of Gal-3 siRNA in CD133⁺ cells. The cells were resuspended in fresh medium, diluted to 1.25x10⁵ cells/mL, and seeded into four 96-well plates at 100 µL per well. After transfection of siRNA, cells were grown for up for 4 days and one plate was used for the MTT assay each day for a total of 4 days. Twenty microliters of MTT (5 mg/mL; Sigma, St. Louis, MO) was added to each well, and the cells were incubated at 37°C for another 4 h. The culture medium was then replaced with 150 µL dimethylsulfoxide (Sigma). After shaking for 10 min, the plate was read for the absorbance at 490 nm on a plate reader. The proliferation rate was determined as follow: absorbance of transfected cells/absorbance of untransfected cells.

ELISA (enzyme linked immunosorbent assay)

At 72h post-transfection, the medium of transfected and non-transfected cells was collected separately by centrifugation, of which the Gal-3 content was then determined using a commercially available ELISA (Merck-Calbiochem, Whitehouse Station, NJ) as described by the manufacturer.

Apoptosis Assay

A total of 1x10⁶ CD3⁺CD8⁺T cells were treated with the supernatant of CD133⁺ cells or CD133⁺ cells 48h post-transfection. Meanwhile, Gal-3 recombinant protein (1 µg/mL), lactose (1mM) or Gal-3 polyclonal antibody (1:500; Vector laboratories) were co-cultured with CD8⁺ T cell separately as controls. After 48h, apoptotic cells were identified by using annexin V and propidium iodide (PI) according to the manufacturer's instructions (R & D Systems, Minneapolis, MN). Samples were analyzed on a FACScan flow cytometer.

Statistical analysis

The statistical software was adopted to deal with all the results and the significance of differences among groups was determined by the Student's *t* test.

Results

MACS efficiency detected by flow cytometry (FCM)

The single cell suspensions were separated into CD133⁺ and CD133⁻ cells by magnetic cell separation and then analyzed by flow cytometry for CD133 expression. We confirmed that CD133⁺ cells were infrequent but consistently detectable in all the different patient samples analyzed (Fig. 1), which were in accordance with a prior report.²⁰ However, there is a good degree of enrichment of the CD133⁺ subpopulation sorted by magnetic cell separation (Fig. 1).

FIGURE 1 NEAR HERE

Expression of Gal-3 is increased in CD133⁺ cells

In order to determine the expression of Gal-3 in different subpopulations of lung cancer cells, FQRT-PCR and Western blotting were performed as described above. These two separate assays, gave rise to similar results indicating that Gal-3 expression was significantly higher in CD133⁺ cells than CD133⁻ cells. The FQRT-PCR and Western blot showed the

FIGURE 1. The percentage of CD133 expression in pulmonary adenocarcinoma cells analyzed by flow cytometry. On left, CD133 was expressed in 1.2% of the unsorted pulmonary adenocarcinoma cells; in the middle, cells expressing CD133 constituted 90% of the CD133⁺ subpopulation after sorting. On right, cells expressing CD133 constituted 0.2% of the CD133⁻ subpopulation after sorting.

expression of galectin-3 in CD133⁺ cells was 1.24 fold and 1.5 fold higher than in CD133⁻ cells respectively (Fig. 2a, c). Secreted Gal-3 was also detected by ELISA. The concentration of Gal-3 in the supernatant of CD133⁺ cells cultured for 48h was two fold higher than that from CD133⁻ cells (Fig. 3). These results indicate that Gal-3 was highly expressed in CD133⁺ cells compared with CD133⁻ cells.

Gal-3 siRNA down-regulates the expression of Gal-3 in CD133⁺ cells in vitro

As previously reported, wild-type Gal-3 siRNA and mutant Gal-3 siRNA have been synthesized.¹⁹ It remains unknown whether the siRNA could knockdown the expression of Gal-3 in CD133⁺ cells. Here we confirm the interference efficiency by FQRT-PCR, Western blot and ELISA. The results of FQRT-PCR show that siRNA was able to interfere with Gal-3 with an interference efficiency of 80.3%, slightly higher than 73.8% reported previously¹⁹ (Fig. 3a). The Western blot and ELISA results show that siRNA could efficiently decrease the Gal-3 protein production (Fig. 3b, c). The mutant siRNA had no effect on Gal-3 as shown in Fig. 3. The siRNA specific for Gal-3 could

efficiently knockdown the expression of Gal-3 in CD133⁺ cells *in vitro*.

CD133⁺ cells induce apoptosis of CD8⁺ T cells by Gal-3

It could be expected that highly expressed Gal-3 in CD133⁺ cell would have biological significance. As inducing CD8⁺ T cell apoptosis is an important path by which cancer cells escape from attack by immune system, we investigated the activity of galectin-3 secreted by lung cancer cell on CD8⁺ T cells. Annexin V and PI double staining showed that the supernatant of CD133⁺ cells cultured for 48h can induce apoptosis of CD8⁺ T cells (18.5±3.5% apoptotic cells)(Fig.4) with recombinant Gal-3 protein as a positive control. The supernatant of CD133⁻ cells induced apoptosis in just 10.1±2.2% of the CD8⁺ T cells (Fig. 4b). To determine if apoptosis of CD8⁺ T cells induced by culture medium was due to the high concentration of Gal-3, lactose (an inhibitor of Gal-3) or antibodies to Gal-3 were added to the cultures. As expected, the induction of apoptosis was decreased by either lactose or Gal-3 polyclonal antibody (Fig.4). Furthermore, the supernatant of CD133⁺ cells 48h after transfection with siRNA, but not siRNA mutant, were not able to in-

FIGURE 2. Gal-3 expression in CD133+ or CD133- pulmonary adenocarcinoma cells. FQRT-PCR (a) showed that Galectin-3 expression was significantly higher in CD133+ cells than CD133- cells ($p < 0.05$), with an average of 1.536 fold vs. 1.247 fold based on the expression of Beta-actin, which was confirmed by Western blotting (b, data from five patients are shown), with the expression of Gal-3 in 11 patients normalized based on the expression of Beta-actin by gray scale scanning (c).

FIGURE 3. Gal-3 siRNA efficiently down-regulated the expression of Gal-3 in CD133+ cells in vitro. (a) The expression of Gal-3 in CD133+ cell treated with siRNA, siRNA mutant or untreated 48h after transfection was detected by FQRT-PCR. (b) Western blotting was used to evaluate the expression of Gal-3 in CD133+ cells treated with siRNA, siRNA mutant or untreated 72h after transfection. (c) Secreted Gal-3 from CD133- cell, CD133+ cell, and CD133+ cell treated with indicated siRNA were measured by ELISA. The data shown were calculated as mean $\pm$ s.e.m. ($*p < 0.05$), and data are from three independent experiments (a, c).

FIGURE 4. Apoptosis of CD8+T cells from peripheral blood mononuclear cell detected by flow cytometry 48h after co-culture with different supernatants. (a)The percentage of apoptotic cells in each sample was measured by annexin V binding and PI permeability using flow cytometric analysis. (b)The percentage of Annexin V positive CD8+ T cells. The data shown were calculated as mean $\pm$ s.e.m. (* p <0.05, compared with supernatants from CD133+ cells) Data are from three independent experiments.

FIGURE 5. The proliferation rate of CD133+ cell treated with indicated siRNA after transfection was detected by MTT assay. The proliferation rate was determined as follows: absorbance of transfected CD133+cells/absorbance of untransfected CD133+cells. The data shown were calculated as mean $\pm$ s.e.m. (* p <0.05). Data are from three independent experiments.

duce apoptosis of CD8+ T cells. All of these results confirm that CD133+ cells induce apoptosis of CD8+ T cells by the expression of galectin-3.

Down-regulation of Gal-3 in CD133+ cells inhibit proliferation in vitro

To determine whether the inhibition of Gal-3 by siRNA affects the growth of CD133+ cells post-transfection, the proliferation of CD133+ cell post-transfection was evaluated by MTT test as described above. The MTT assay showed that the proliferation rate of CD133+ cell treated with siRNA was decreased with a greater decrease occurring after a greater time after transfection (Fig. 5). At the point 96h after transfection, the proliferation rate of CD133+ cell treated with siRNA was significantly lower than that of CD133+ cell treated with siRNA mutant (p <0.05), whereas there was no significant difference between the proliferation rate with siRNA or siRNA mutant at the point 48h after transfection (Fig.5). These data indicated that specific siRNA for Gal-3 influence the growth of CD133+ cell by down-regulating expression of Gal-3 and therefore

it appears that Gal-3 is not only important for CD133⁺ cells to escape immune attack but also for proliferation.

Discussion

In conventional theory, tumors were viewed as simple monoclonal expansions of transformed cells. However, the growing understanding of cancer biology has led to the clear concept that tumors are hierarchically organized and are composed of a heterogeneous population of cells in which a rare population referred to as Tumor Stem Cells (TSC) sits at the top, and these cells play a critical role in the development of cancer. Therefore, the identification and characterization of such cell populations responsible for lung cancer may contribute significantly to develop new therapeutic strategies and improve the prognosis of this malignancy.

In this study, we separated the rare population of CD133⁺ cells from excised pulmonary adenocarcinoma specimens of 11 patients for further analysis. Subsequent study found Gal-3 to be differentially expressed in lung cancer cells and predominantly in CD133⁺ cells. Our data indicate that the distribution of Gal-3 in pulmonary adenocarcinoma tissue was asymmetrical, although Gal-3 was highly expressed throughout non-small lung cancer tissues confirmed by immunohistochemistry.¹³ We speculated that Gal-3 might correlate closely with the biological properties of tumor stem cells. As the induction of T-cell apoptosis by secreted Gal-3 may play a role in the immune escape mechanism during tumor progression through the induction of apoptosis to cancer-infiltrating T cells,²¹ we investigated the activity of Gal-3 secreted by CD133⁺ cells on CD8⁺ T cells. Results indicated that the supernatants of CD133⁺ cells induced more apoptosis of CD8⁺ T cells than supernatants from CD133⁻ cells, and could be down-regulated by lactose and anti-Gal-3 polyclonal antibodies. Furthermore, the CD133⁺ cancer cell transfection with siRNA targeting Gal-3 could efficiently inhibit the extracellular

expression of Gal-3. More importantly, down-regulation of Gal-3 resulted in significant inhibition of cancer cell growth *in vitro* as well as a reduction of CD133⁺ cell capacity to induce CD8⁺ T cell apoptosis.

Specific siRNA for galectin-3 may non-specifically suppress the proliferation of CD133⁺ cells, so it is possible that the low concentration of Gal-3 in the supernatants of CD133⁺ cell transfected with siRNA could be due to the decreased number of viable CD133⁺ cells rather than decreased expression induced by siRNA (Fig.3C). Surprisingly, there was no significant difference in the proliferation rate between the CD133⁺ cells treated with siRNA and siRNA mutant 48h post-transfection (Fig. 5), although the expression of Gal-3 in two groups was significantly different (Fig.3C). These data indicate that the decreased expression of Gal-3 was caused by the inhibitory effect of siRNA, not by the reducing number of CD133⁺ cells 48h post-transfection.

Previous research has shown that Gal-3 has the ability to act in a dual manner. It can protect cells from apoptosis or stimulate cell death depending on whether the protein is functioning intracellularly or extracellularly.^{7,21} Extracellular Gal-3 can induce apoptosis in activated T cells as treatment of T cells with extracellular recombinant Gal-3 induces apoptosis.^{7,11} These findings are consistent with our results. Likewise, we demonstrate that Gal-3 is expressed intracellularly (detection by Western blot) and extracellularly (detection by ELISA) and that Gal-3 recombinant protein can efficiently induce apoptosis of CD8⁺ T cells isolated from peripheral blood mononuclear cells. It has also been shown that Gal-3 directly initiates thymocyte and T cell death, and therefore is potentially immunosuppressive.^{7,11} In view of these recent advances, it was both reasonable and logical to speculate that Gal-3 may possibly play an important role in the oncogenesis and maintenance of lung adenocarcinoma.

Although recent evidence suggests the likely presence of cancer stem cells in pulmonary tumors, the phenotype of lung cancer stem cells remains contro-

versial. According to a recent report by A Eramo et al., only CD133⁺ cells serve as a tumorigenic population in lung cancer tissue, being able to reproduce the original tumor in immunocompromised mice, while the CD133⁻ cancer cell population was completely devoid of tumor-initiating activity. In contrast, Xiang-jiao Meng et al. reported that both CD133⁺ and CD133⁻ subpopulations of A549 and H446 cells contained cancer-initiating cells.^{22,23} The latter authors pointed out that a possible explanation is that there are differences between lung cancer tissue and cell lines. Thus, further studies are still necessary to provide more definitive evidence.

In summary, our work provides concrete experimental evidence that Gal-3 is highly expressed in CD133⁺ cells from lung adenocarcinoma tissue and can induce apoptosis of CD8⁺ T cells, which can be efficiently inhibited by Gal-3 siRNA. Therefore, understanding the role of Gal-3 in the CD133⁺ cell will yield important insights into such malignancies and delineate novel strategies for disease intervention.

Acknowledgments

None of the authors have any commercial or other associations that might pose a conflict of interest. This work was supported by grants to Jian-jun Wang from the Ph.D.Programs Foundation of ministry of Education of China (20060487046).

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