Suppression of SERPINA1-albumin complex formation by galectin-3 overexpression leads to paracrine growth promotion of chronic myelogenous leukemia cells

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ARTICLE INFO

Article history:
Received 20 May 2013
Accepted 12 July 2013
Available online xxx

Keywords:
Galectin-3
Chronic myelogenous leukemia
SERPINA1
Bone marrow stromal cell

ABSTRACT

Galectin-3 is induced in chronic myelogenous leukemia (CML) cells by co-culture with bone marrow stromal cells, making paracrine growth promotion of CML cells in conditioned medium (CM) from galectin-3 overexpressing CML cells more potent. We used gel filtration chromatography to demonstrate that the bovine SERPINA1-fetal bovine serum albumin (BSA) complex was specifically suppressed in CM from galectin-3 overexpressing cells. The SERPINA1-BSA complex as well as human plasma SERPINA1 inhibited the growth of CML cells, while exogenous galectin-3 partly offset this effect. These findings suggest that galectin-3 overexpression promotes paracrine growth of CML cells by interfering with the action of the growth inhibitory SERPINA1-albumin complex.

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1. Introduction

The BCR-ABL1 fusion oncoprotein with constitutive tyrosine kinase (TK) activity induced by the Philadelphia (Ph) chromosome, a reciprocal t(9;22)(q34;q22) chromosomal translocation, is causative of chronic myelogenous leukemia (CML) [1,2]. While BCR-ABL1 TK stimulates various cell-intrinsic downstream signaling cascades for cell survival and deregulated cell proliferation, the bone marrow microenvironment (BMME), which consists of various growth factors or bone marrow (BM) supporting cells, also supports the proliferation and maintenance of leukemic cells and protects leukemic cells from cytotoxic stimuli in a BCR-ABL1 signaling-independent manner [3].

We recently demonstrated that galectin-3 (Gal-3), a member of the β-galactoside-binding galectin family, is specifically induced in CML cells in the presence of BM stromal cells (BMSCs). Gal-3 overexpression promotes cell proliferation, impairs apoptosis induction by inhibitors for BCR-ABL1 TK (TKIs), and promotes lodgment of CML cells in BM in a BCR-ABL1 TK signaling-independent manner. Gal-3 overexpression also promotes paracrine proliferation of leukemia cells, since the conditioned medium (CM) from Gal-3-overexpressing CML cells promoted in vitro cell proliferation of CML cells more than CM from parental cells did [4]. In this study, we analyzed CM components to investigate the underlying mechanisms of Gal-3-mediated paracrine cell proliferation, and identified Gal-3 overexpression as causative of modulation of the SERPINA1-bovine serum albumin (BSA) complex in CM from leukemic cells. SERPINA1, a 52 kDa glycoprotein, is a member of a serine protease inhibitor superfamily, which is crucially involved in various proteolytic processes involved in inflammation, turnover of extracellular matrix, blood coagulation and hematopoietic stem/progenitor cell mobilization [5–8]. SERPINA1 has also been shown to function as a tumor suppressor in breast and lung cancer cells by interfering with the release of membrane bound precursors of growth promoting factors, including transforming growth factor-α or interleukin-8 [9–12], or by interfering with transferrin iron uptake in K562 cells [13]. These findings suggest that galectin-3 overexpression promotes paracrine growth of CML cells by interfering with the action of the growth inhibitory SERPINA1-albumin complex in the tumor microenvironment.
2. Materials and methods

2.1. Cell lines, generation of subclones and reagents

The human CML-derived cell lines MYL and K562, as well as HS-5, an immortalized human BMSC-derived cell line, were utilized in this study [14,15]. Cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C and 5% CO2 in a humidified atmosphere. Gal-3 overexpressing sublines of MYL cells and K562 cells were generated by means of transfection of pEFGal3.Gal3.neo plasmid (a generous gift from Dr. Fu-Tong Liu, University of California, Davis School of Medicine, CA) [16] and designated MYL/G3 and K562/G3, respectively [4]. The synthetic human SERPIN1-albumin complex (Silk) was generated by polymerization of human plasma SERPIN1 (a1-antitrypsine) (Sigma, St Louis, MO) and human albumin by means of glutaraldehyde cross-linking.

2.2. Cell proliferation and apoptosis assays

Cells were seeded at 2 x 10³ cells/ml. MYL cells were cultured with CM from parental MYL cells (CM/MYL) or CM from MYL/G3 cells (CM/MYL/G3), while K562 cells were cultured with CM from parental K562 cells (CM/K562) or CM from K562/G3 cells (CM/K562/G3) for 72 h. In other experiments, cells were incubated for 48 h with various concentrations of human plasma SERPIN1, which has been shown to contain the SERPIN1-albumin complex [17], with or without recombinant human Gal-3 (R&D Systems, Minneapolis, MN). Cell proliferation was determined with a modified methyl-thiazol-diphenyl-tetrazolium (MTT) assay using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). For the examination of apoptosis, cells were counterstained with Annexin V-FITC and propidium iodide and subjected to flow cytometric analysis according to the manufacturer's instructions (BD Pharmingen, San Jose, CA).

2.3. Gel filtration chromatography (GFC), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and amino-acid sequencing

Conventional GFC was performed on CMs from leukemia cells [18]. Briefly, CM was filtered through a Cosmofine filter (0.45 μm; Nacalai Tesque, Kyoto, Japan), and 200 μl of the filtrate was injected at 0.5 ml/mininto Superdex 75 or Superdex 200 (GE Healthcare Japan, Tokyo, Japan) equilibrated with 20 mM sodium phosphate buffer, pH 7.2 containing 150 mM NaCl. Fractions were collected every 1 ml. The medium was also fractionated by means of Superdex 75 or Superdex 200 equilibrated with the cell culture medium. Aliquots of the medium, mixed with the same volume of 8 M urea, 0.01% bromophenol blue and 2.0% SDS, were used for cell culture. Twenty μl of the samples was resolved by means of SDS-PAGE using 7.5% gel with Laemmli's buffer system in a vertical electrophoresis apparatus (AE-6450; Atto, Tokyo, Japan). The proteins were developed with a Silver Stain Kit (Nacalai Tesque). In addition, proteins in the gel were electrophotographed transferred to a PVDF membrane (Clear blot Membrane-P, ATTO) using a semi-dry blotting apparatus (AE-6550, ATTO) with a transfer buffer consisting of glycine (373.3 mM), Tris (49.5 mM), and SDS (3.5 mM) at 100 mA per gel. Proteins on the membrane were developed with 0.04% Coomassie Brilliant Blue R-250 in 10% acetic acid and 25% isopropanol, followed by destaining with 10% acetic acid. The protein band of interest was then removed and subjected to sequence analysis by Edman degradation using a PPSQ-21 sequencer (Shimadzu, Kyoto, Japan).

2.4. Human cytokine ELISA assay

Leukemic cells were seeded at 2 x 10³/ml in fresh complete media and grown for 72 h, after which CMs from MYL, MYL/G3, K562 and K562/G3 cells were obtained by means of conventional centrifuge. For wide-range screening, CMs were first analyzed with the Human Cytokine ELISA Plate Array (Signosis, Sunnyvale, CA) to determine relative concentrations of 31 cytokines of two samples: CM/MYL/G3 was compared with CM/MYL and CM/K562/G3 with CM/K562. The results of the screening for 31 cytokines were then used for further analysis of the concentrations of cytokine (C-X-C motif) ligand 10 (CXCL10), interleukin 10 (IL10) and transforming growth factor-β (TGF-β) in CMs were with the Human CXCL10 (IP-10) ELISA Kit (MyBioSource, San Diego, CA), Human IL-10 ELISA (Thermo Fisher Scientific, Rockford, IL) and Human TGF-beta 1 Instant ELISA (eElcience Austria, Vienna, Austria), respectively. Chemiluminescence detection was performed with the PowerScan HT (DS Pharma Biomedical, Osaka, Japan).

2.5. Statistical analysis

Data are expressed as mean ± standard error (SE). Cell proliferation assays were performed in triplicate, and the significance of the difference was determined by Student's t-test.

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Please cite this article in press as: Nakayama R, et al. Suppression of SERPIN1-albumin complex formation by galectin-3 overexpression leads to paracrine growth promotion of chronic myelogenous leukemia cells. Leuk Res (2013), http://dx.doi.org/10.1016/j.leukres.2013.07.026
2-mercaptoethanol, indicated that this complex was disulfide-bound (Fig. 2C).

3.3. CM fractions containing SERPINA1-BSA complex are less potent for cell proliferation of CML cells

We next investigated whether Frs18-20 of CM from Gal-3-overexpressing cells and those from parental cells differ in their potency for proliferation of CML cells and found that Frs18-20/CMs/MYL containing the bovine-derived SERPINA1-BSA complex was significantly less potent for cell proliferation than Frs18-20/CMs/MYL/G3, while other fractions did not show any significant differences. The same difference in cell proliferation potency was observed in Frs18-20/CMs/K562 containing the bovine-derived SERPINA1-BSA complex compared with Frs18-20/CMs/K562/G3 (Fig. 3).

3.4. Human plasma SERPINA1 inhibits proliferation of CML cells and this inhibition is partly prevented by exogenous Gal-3

We next investigated whether commercially available human-derived SERPINA1, which contains the SERPINA1-albumin complex [17] (Fig. 4A), has a growth inhibitory effect on CML cells. As shown in Fig. 4B and C, a 48-h treatment with human plasma SERPINA1 inhibited the proliferation of MYL cells and K562 cells in a dose-dependent manner. An additional important finding was that the addition of recombinant human Gal-3 at least partly shielded...
leukemic cells from the growth inhibitory effect of human plasma SERPINA1 in MYL cells (Fig. 4D).

3.5. Human plasma SERPINA1 induces apoptosis in MYL cells

To clarify the underlying mechanism for the growth inhibitory effect of SERPINA1, we investigated whether SERPINA1 treatment affects the viability of leukemic cells. As shown in Fig. 5, SERPINA1 induced apoptosis in MYL cells in a dose- and time-dependent manner. Compared with untreated control, the viable cell ratio (fraction I) was reduced from 87.8% to 65.9% after a 48-h treatment with 20 mg/ml of SERPINA1. In contrast, the number of viable cells after 48 h of treatment with 20 mg/ml of SERPINA1 accounted for less than 30% of untreated MYL cells (Fig. 4B), suggesting that apoptosis induction was only partly responsible for the growth inhibitory effect of SERPINA1, and that other mechanisms, such as the inhibition of DNA synthesis [10,13], also contributed to the growth inhibitory effect of SERPINA1 in MYL cells.

3.6. Effects of Gal-3 overexpression on other cytokines in CM

We also examined, by means of ELISA assays, the effects of Gal-3 overexpression on the levels of other cytokines in CMs. Of the 31 cytokines screened with the Human Cytokine ELISA Plate Array, the relative concentrations of CXCL10 and IL-10 in CMs from Gal-3 overexpressing leukemic cells were estimated to be less than half of the concentrations in CMs from parental cells in both MYL and K562. In contrast, the relative concentration of TGF-β in Gal-3 overexpressing cells was estimated to be more than twice as high as that of parental cells in both cell lines. No consistently significant differences were found in the other 28 cytokines between MYL and K562 cells when analyzed with the same kit (Fig. 6). However, when the concentrations of the three cytokines were analyzed more precisely with ELISA kits, the concentrations of CXCL10, IL-10 and TGF-β in CM/MYL, CM/MYL/G3, CM/K562 and CM/K562/G3 were found to be below detectable levels, so that it was not possible to determine the effect of Gal-3 overexpression on concentrations of CXCL10, IL-10 and TGF-β in CM (data not shown).
4. Discussion

Various soluble factors may become associated with either the promotion or the inhibition of autocrine and paracrine cell proliferation of cancer cells in the tumor microenvironment, but the exact mechanisms underlying these associations may differ among cancer types and have not yet been fully identified. We previously determined that Gal-3 is one of the critical and specific regulators of leukemia microenvironment-mediated autocrine and paracrine cell proliferation in CML [4], but the effector molecules located downstream of Gal-3 have remained unidentified.

Our in vitro study reported here demonstrated that Gal-3 overexpression causes suppression of formation of the SERPINA1-BSA complex in CM from two CML cell lines, and that human plasma SERPINA1 inhibits the growth of CML cell lines. It has also been demonstrated that Gal-3 overexpression promotes cell proliferation, cell migration, cell adhesion and the production of inflammatory cytokines of normal neutrophils as well as some of cancer cells, including CML cells [4, 19–21], and that SERPINA1 inhibits those events [5, 6, 9], indicating that Gal-3 and SERPINA1 induce opposite functional interactions. It has remained unclear, however, how Gal-3 overexpression diminishes the SERPINA1-BSA complex in CM. Because the SERPINA1-BSA complex which was diminished in CM from Gal-3 overexpressing cells in our experiments was of bovine, not human origin, the loss of the SERPINA1-BSA complex from the CM of Gal-3 overexpressing leukemic cells was presumed to be a post-translational and extracellular event. Since SERPINA1 has been shown to be one of the binding partners of Gal-3 [22], a possible mechanism for this phenomenon could be that Gal-3 secreted from human leukemic cells binds to bovine SERPINA1 through β-galactoside-mediated binding, and thereby prevents the formation or the stabilization of disulfide-bound SERPINA1-BSA complex in CM.

Indeed, our results demonstrated that the addition of human recombinant Gal-3 partly shielded leukemic cells from the growth inhibitory effect of human plasma SERPINA1 containing the SERPINA1-albumin complex. While SERPINA1 is mainly present in plasma and in the extravascular compartments, it is also found in BM, especially in the osteoblastic stem cell niche [23]. Combining these findings with ours thus leads us to speculate that BMME-mediated Gal-3 overexpression may contribute to the defense of CML cells against the cytotoxic effects of SERPINA1-albumin in BM.

Needless to say, the results of our study do not completely eliminate the potential involvement of other soluble factors in Gal-3-mediated autocrine and paracrine cell proliferation of CML cells. Indeed, results of analyses using the Human Cytokine ELISA Plate Array have suggested the possible involvement of other cytokines, namely, CXCL10, IL10 and TGF-β, in this cell proliferation, but their concentrations in the CMs used in our study were too low for validation of the results of the additional analyses. However, we cannot completely rule out the involvement of those cytokines, considering that both CXCL10 and IL10 have been found to block the stimulatory effects of growth promoting soluble factors and inhibit the growth of myeloid leukemic cells [24–28]. In addition, our result was consistent with the previous finding that Gal-3 negatively regulates IL10 expression [29, 30], while the association between CXCL10 and Gal-3 remains unknown. In spite of their extremely low concentrations, the simultaneous decrease in CXCL10 and IL10 may also be responsible to some extent for cell proliferation. The possible increase of TGF-β in CM Gal-3 overexpressing cells is also of interest. It has been demonstrated that TGF-β inhibits the growth of cycling leukemic cells in the context of BCR-ABL1 signaling [31], while TGF-β pathways promote the maintenance of leukemia-initiating cells in CML [32, 33]. It has further been shown that Gal-3 expression is required for the expression of TGF-β in a liver fibrosis model [34], while functional TGF-β signaling was found to be the prerequisite for Gal-3 expression in macrophages [35], all of which suggest an interactive association between TGF-β and Gal-3. The interaction between Gal-3 and TGF-β in the maintenance of leukemic cells in BMME would thus be a possible topic for future research.

In conclusion, Gal-3 overexpression in CML cells may promote the proliferation of adjacent CML cells through the prevention of SERPINA1-albumin complex formation observed in CM from parental cells. In addition, the interactions between Gal-3 and the
three cytokines, CXCL10, IL10 and TGF-β, although their expression levels were extremely low, would be suitable subjects for future research, especially in vivo studies.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was partly supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MT, JK and MY), and by grants from the Mochida Memorial Foundation for Medical and Pharmaceutical Research and the Hoansha Foundation (JK).

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