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Kallistatin antagonizes Wnt/ β -catenin signaling and cancer cell motility via binding to low-density lipoprotein receptor-related protein 6

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Abstract

Kallistatin, a plasma protein, exerts pleiotropic effects in inhibiting angiogenesis, inflammation and tumor growth. Canonical Wnt signaling is the primary pathway for oncogenesis in the mammary gland. Here we demonstrate that kallistatin bound to the Wnt co-receptor low-density lipoprotein receptor-related protein 6 (LRP6), thus blocking Wnt/ β -catenin signaling and Wnt-mediated growth and migration in MDA-MB-231 breast cancer cells. Kallistatin inhibited Wnt3a-induced proliferation, migration and invasion of cultured breast cancer cells. Moreover, kallistatin bound to LRP6 in breast cancer cells, as identified by immunoprecipitation followed by western blot. Kallistatin suppressed Wnt3a-mediated phosphorylation of LRP6 and glycogen synthase kinase-3 β , and the elevation of cytosolic β -catenin levels. Furthermore, kallistatin antagonized Wnt3a-induced expression of c-Myc, cyclin D1 and vascular endothelial growth factor. These findings indicate a novel role of kallistatin in preventing breast tumor growth and mobility by direct interaction with LRP6, leading to blockade of the canonical Wnt signaling pathway.

Keywords

kallistatin; LRP6; Wnt; β -catenin

Introduction

The Wnt proteins are a group of secreted cysteine-rich glycoproteins that are involved in differentiation events during embryonic development, and can lead to tumor formation when aberrantly activated [1]. Activation of the canonical Wnt pathway involves stabilization of β -catenin through binding of Wnt ligands to cell surface receptors: Frizzled (Fz) family receptors and low-density lipoprotein receptor-related proteins (LRP5 or LRP6). However, in the absence of Wnt ligands, β -catenin is phosphorylated by a protein complex containing glycogen synthase kinase 3 β (GSK-3 β), leading to ubiquitination and targeting for

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degradation by the proteasome. Upon binding of certain Wnt ligands, Frizzled receptor binds to LRP5/6, resulting in the phosphorylation of LRP6 and subsequent blockade of β -catenin phosphorylation by GSK-3 β [2, 3]. The non-phosphorylated form of β -catenin accumulates in the cytoplasm and is translocated to the nucleus where it acts as a cotranscriptional activator in regulating downstream target genes to promote cell proliferation, differentiation and tissue development [4–6]. Moreover, Wnt/ β -catenin signaling has been shown to be the primary pathway for Wnt-mediated oncogenesis in the mammary gland [7].

Kallistatin, a plasma protein, has been demonstrated to exert multiple biological functions. Plasma levels of kallistatin are known to be reduced in patients with sepsis, liver disease [8], obesity [9], as well as various cancers (unpublished observations). Kallistatin's pleiotropic effects include inhibiting angiogenesis, inflammation, tumor growth and metastasis in animal models and cultured cells [10–14]. Kallistatin suppressed tumor growth and angiogenesis in nude mice via antagonizing VEGF-mediated cell proliferation, migration and invasion of cultured endothelial cells [10, 11]. Kallistatin also inhibited inflammation via competing with the binding of tumor necrosis factor (TNF)- α to its receptor, thus reducing NF- κ B activation and pro-inflammatory gene expression in endothelial cells [14]. Moreover, a previous study showed that SERPINA3K (kallistatin) blocked Wnt ligand-induced signaling by binding to LRP6 in retinal epithelial cells, leading to inhibition of retinal neovascularization [15]. The goal of the present study is to determine the effect of kallistatin on canonical Wnt signaling and cancer cell mobility through Wnt3a interaction with LRP6 in human breast cancer MDA-MB-231 cells.

Materials and methods

Cell culture and recombinant human kallistatin

The human breast cancer cell line MDA-MB-231 was chosen for this study due to their high expression of LRP6 [16]. MDA-MB-231 cells were obtained from American Type Culture Collection (ATCC) and grown in high-glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen, CA) supplemented with 2 mM L-glutamine and 10% FBS (Atlanta Biologicals, GA). Recombinant human kallistatin (KS) was expressed and secreted into the serum-free medium of cultured HEK293 cells. The cultured medium was concentrated by ammonium sulfate precipitation and then purified by nickel-affinity chromatography as previously described.

Cell proliferation, migration and invasion assays

MDA-MB-231 cells were serum-deprived, pretreated with kallistatin (0.5 μ M) for 30 min, and then cultured with Wnt3a (100 ng/ml) for 24 h prior to the addition of 5-bromo-2'-deoxyuridine (BrdU) for an additional 2 h before performing the Cell Proliferation ELISA, BrdU colorimetric assay (Roche Applied Science, IN). Modified Boyden's chambers (Corning Incorporated, GA) were used to quantify cell migration and invasion as previously described [18].

Immunoprecipitation and western blot

Immunoprecipitation was performed as described previously [19]. Anti-kallistatin polyclonal antibody [8] or anti-LRP6 antibody (5 µg) (Cell Signaling, MA) was used to precipitate 1 mg of whole cell lysate. The precipitates and input samples were then immunoblotted with antibodies for LRP6 and monoclonal antibody against kallistatin (G4C10), respectively [8]. For western blot, cultured MDA-MB-231 cells were serum-deprived, pretreated with kallistatin at different concentrations (0.5, 1 µM or 2 µM) for 30 min or left untreated, and then stimulated with Wnt3a (100 ng/ml) (R&D Systems, MN) for 2 or 24 h for different experiments. Cell extracts were subjected to western blot analysis for detection of phosphorylated LRP6 and GSK-3β, non-phosphorylated β-catenin, as well as total LRP6, GSK-3β, β-catenin, cyclin D1 (Cell Signaling, MA) and c-Myc (Santa Cruz, CA). GAPDH was used as a loading control (BioAbchem, SC).

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted with TRIzol reagent (Invitrogen, CA). RNA was then reverse transcribed with a cDNA archive kit (Applied Biosystems, CA). Quantitative real-time PCR was carried out on a 7300 real-time PCR system (Applied Biosystems, CA) and normalized against 18S RNA (Hs99999901_ml). The following primers were used: c-Myc (Hs00905030_ml), cyclin D1 (Hs00765553_ml), and VEGF (Hs00173626_ml).

Enzyme-linked immunosorbent assay (ELISA)

Levels of VEGF in the cell culture media were measured by DuoSet ELISA development system (R&D Systems, MN) according to the manufacturer's protocol.

Statistical analysis

Data are presented as mean ± SEM and compared among experimental groups with the use of a one-way ANOVA followed by Newman-Keuls multiple comparison test. A difference of $P < 0.05$ was considered statistically significant.

Results

Kallistatin inhibits Wnt3a-induced proliferation, migration and invasion of breast cancer cells

The effect of human kallistatin on the proliferation of tumor cells in response to Wnt pathway activation was investigated. We measured BrdU uptake by MDA-MB-231 breast cancer cells treated with or without Wnt3a (100 ng/ml) in the presence or absence of kallistatin (0.5 µM). As shown in Fig. 1a, Wnt3a markedly increased MDA-MB-231 cell proliferation about 1.6-fold compared with non-stimulated cells, but the effect was abolished by kallistatin. Kallistatin, in the absence of Wnt3a, had no effect on cell proliferation in MDA-MB-231 cells, as determined by both BrdU uptake and ³H-thymidine incorporation (unpublished results). Moreover, the migration and invasion of Wnt3a-treated cells significantly increased 1.5-fold and 1.3-fold, respectively, compared with control cells, and kallistatin attenuated these effects (Fig. 1b, c).

Kallistatin binds to Wnt-co-receptor LRP6

To determine whether kallistatin specifically binds to Wnt-co-receptor LRP6, MDA-MB-231 cells were placed in serum-free medium with and without human kallistatin (0.2 μ M) for 2 h. Western blot analysis using anti-kallistatin antibody showed that LRP6 was present in both control and kallistatin-treated input samples, but was only co-precipitated in kallistatin-treated cell extracts (Fig. 2a). To further confirm the interaction between LRP6 and kallistatin, LRP6 antibody was used for immunoprecipitation followed by western blot analysis, thus showing that LRP6 co-precipitated with kallistatin (Fig. 2b). These results indicate that kallistatin specifically bound to the Wnt co-receptor LRP6 in MDA-MB-231 cells.

Kallistatin suppresses Wnt/ β -catenin signaling

Given the direct interaction of kallistatin with LRP6, we next investigated whether kallistatin could modulate Wnt/ β -catenin signaling. MDA-MB-231 breast cancer cells were treated with Wnt3a (100 ng/ml) for different time periods. Western blot analysis of cell lysates showed that Wnt3a began to induce phosphorylation of LRP6 at 1 h, peaked at 2 h, and then returned to basal levels at 24 h (Fig. 3a). The change in phosphorylated GSK-3 β and non-phosphorylated β -catenin levels paralleled that of phosphorylated LRP6 during the same time course. However, cytosolic β -catenin levels did not return to the basal level at 24 h. Based on the time course results, we selected 2 h as the time point for kallistatin treatment. Cells were pre-treated, with or without kallistatin, at different concentrations (1 μ M or 2 μ M) for 30 min, followed by incubation with Wnt3a (100 ng/ml) for 2 h. Western blot analysis revealed that Wnt3a treatment increased LRP6 phosphorylation, and the effect was suppressed by both doses of kallistatin (Fig. 3b). The 2 μ M dose of kallistatin reduced Wnt3a-induced phosphorylated GSK-3 β levels. In addition, non-phosphorylated β -catenin levels in the cytosol were significantly decreased by the 2 μ M dose of kallistatin. The differences in the effect of kallistatin on phosphorylated LRP6, phosphorylated GSK-3 β , and non-phosphorylated β -catenin might lie in the time point selected. Since GSK-3 β and β -catenin are the downstream components of Wnt signaling, 2 h may not be the best time point to elicit the same inhibitory effect of lower dose of kallistatin for phosphorylated LRP6. Nevertheless, these results collectively indicate that kallistatin acts as an antagonist of canonical Wnt signaling by direct interaction with LRP6 in MDA-MB-231 cells.

Kallistatin attenuates Wnt/ β -catenin target gene expression in breast cancer cells

In order to elucidate the mechanism by which kallistatin suppresses Wnt3a-mediated tumor cell growth and motility in MDA-MB-231 breast cancer cells, we examined the Wnt/ β -catenin target genes that have documented roles in cell proliferation, migration and invasion – namely c-Myc, cyclin D1 and VEGF [20–23]. After treatment of MDA-MB-231 cells with Wnt3a (100 ng/ml) for 24 h, the protein levels of c-Myc, cyclin D1 and VEGF were increased, but were attenuated by kallistatin pre-treatment at 0.5 μ M (Fig. 4a, c, e). Similarly, kallistatin significantly blocked Wnt3a-mediated increases of c-Myc, cyclin D1 and VEGF mRNA levels (Fig. 4b, d, f).

Discussion

This is the first study to demonstrate that kallistatin inhibits the Wnt/ β -catenin signaling cascade in breast cancer cells. Our results showed that kallistatin forms a complex with the Wnt co-receptor LRP6, thereby antagonizing Wnt3a-induced phosphorylation of LRP6 and GSK-3 β , and suppressing cytosolic β -catenin levels. Kallistatin treatment also retarded the growth and motility of MDA-MB-231 breast cancer cells in association with the down-regulated expression of c-Myc, cyclin D1 and VEGF induced by Wnt3a. A schematic depicting the effect of kallistatin on Wnt/ β -catenin signaling is shown in Fig. 5.

In the present study, kallistatin specifically bound to Wnt co-receptor LRP6 in human breast cancer cells, as demonstrated by the method of immunoprecipitation followed by immunoblot. Consequently, kallistatin pre-treatment reduced Wnt3a-induced LRP6 and GSK-3 β phosphorylation and cytosolic non-phosphorylated β -catenin levels in MDA-MB-231 cells. These findings are consistent with the report that kallistatin (SERPINA3K) bound to LRP6 in retinal epithelial cells [15]. Specific binding of kallistatin to LRP6 on MDA-MB-231 cells indicated that the inhibitory effect of kallistatin on Wnt-mediated actions, such as cell proliferation, migration, invasion and target gene expression, is at the Wnt receptor level. Dimerization between LRP6 and Fz is the first step in the activation of the Wnt pathway [24]. By specifically binding to LRP6, kallistatin may prevent LRP6 and Fz dimerization induced by Wnt ligand. We previously showed that kallistatin exerts its pleiotropic actions through two important structural elements: the active site and a heparin-binding domain [25–27]. Kallistatin via its active site inhibits tissue kallikrein activity. Moreover, through its heparin-binding domain, kallistatin has been shown to compete with TNF- α binding to endothelial cells and thus inhibit TNF- α -induced NF- κ B activation and pro-inflammatory gene expression [14]. Kallistatin, but not its heparin mutant, was also demonstrated to suppress VEGF-induced proliferation, migration and permeability in endothelial cells [11]. Studies have indicated that LRP binds to a synthetic heparin-mimicking compound on the surface of vascular smooth muscle cells [28] and functions as a heparin-dependent adhesion receptor for connective tissue growth factor in hepatic cells [29]. Therefore, it is likely that kallistatin binds to LRP6 via its heparin-binding site. The mechanism by which kallistatin interacts with LRP6 awaits further investigation.

Both c-Myc and cyclin D1 have been identified as target genes of the Wnt/ β -catenin signaling pathway [23, 30]. Aberrant expression of cyclin D1 and c-Myc is mainly dependent on alterations in the Wnt signaling pathway in breast cancer [31]. Our studies here showed that activation of Wnt/ β -catenin signaling enhanced c-Myc expression, which was paralleled by an increase in expression of the c-Myc-dependent gene cyclin D1. Cyclin D1 is one of the cell cycle-regulating oncoproteins, playing a pivotal role not only in breast carcinogenesis, but also development of the mammary gland [32]. We found that kallistatin treatment significantly diminished Wnt3a-induced expression of c-Myc and cyclin D1 in breast cancer cells. This finding is consistent with the report that expression of c-Myc and cyclin D1 is markedly decreased in LRP6-knockout MDA-MB-231 cells [16]. In addition, we showed that the binding of kallistatin to LRP6, which would lower the phosphorylation of LRP6 induced by Wnt, led to an expected decrease in cytosolic β -catenin levels as well as reduced c-Myc and cyclin D1 transcriptional activity. Moreover, we demonstrated that

kallistatin significantly inhibited Wnt3a-induced MDA-MB-231 cell proliferation. The anti-proliferative effects of kallistatin is most likely due to the inhibition of cyclin D1 expression, as cyclin D1 is required for DNA synthesis and cell cycle progression [33].

Our studies also showed that kallistatin inhibited Wnt3a-induced migration and invasion of human breast cancer cells, possibly through reduction of VEGF expression. VEGF is a major inflammatory and angiogenic factor, as well as a target gene of the Wnt pathway [6]. A previous study showed that the migration and invasion of human cancer cells are attributed to expression of VEGF and its receptors [34]. VEGF is expressed by many epithelial cancer cells, including human breast carcinomas. Recently it has been shown that VEGF receptors are also present the surface of breast cancer cells [35]. VEGF secreted by epithelial cells stimulates the PI3-kinase/Akt signaling pathway, leading to cellular migration and invasion of breast cancer cells [35, 36]. Thus, by reducing VEGF expression, kallistatin may potentially prevent breast cancer metastasis.

In this study, we identified a novel signaling pathway mediated by kallistatin in inhibiting the proliferation, migration and invasion of mesenchymal-like MDA-MB-231 breast cancer cells. Our results showed that kallistatin blocked Wnt3a-mediated canonical Wnt signaling in breast cancer cells by direct interaction with the Wnt co-receptor LRP6. Wnt/ β -catenin signaling plays a crucial role in carcinogenesis [37]. Moreover, LRP6 overexpression has been characterized in a breast cancer subtype, and therefore is a potential therapeutic target in breast cancer treatment [16]. These findings indicate a potential new role for kallistatin in preventing oncogenesis in the mammary gland. Our previous studies demonstrated that kallistatin is an inhibitor of angiogenesis, inflammation, tumor growth and metastasis in animal models and in cultured cells by antagonizing VEGF- and TNF- α -mediated signaling pathways [10–14]. The current study reveals a novel mechanism mediated by kallistatin via antagonizing canonical Wnt signaling, leading to retardation of tumor progression. Our findings provide essential insights for the biological role of kallistatin in tumor growth and progression. Kallistatin administration and kallistatin-deficiency approaches would further establish the effect of kallistatin on Wnt/ β -catenin signaling and LRP6 interaction in carcinogenesis.

In summary, our present studies indicate that kallistatin is a modulator of the Wnt pathway. Importantly, we have identified a new action of kallistatin in inhibiting tumor growth and motility through direct interaction with Wnt co-receptor and subsequent blockade of Wnt/ β -catenin signaling. The inhibition of cell proliferation, migration and invasion of breast cancer cells via kallistatin administration provides evidence for its potential for cancer treatment. Indeed, kallistatin gene delivery was shown to inhibit experimental lung metastasis in mice [12]. Taken together, our findings suggest that interference of canonical Wnt signaling at the ligand-receptor level, in combination with other targeted therapies, may improve the efficacy of breast cancer treatment.

Acknowledgments

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Fig. 1.

Kallistatin inhibits Wnt3a-induced proliferation, migration and invasion of MDA-MB-231 breast cancer cells. (A) BrdU assays were performed using ELISA Kit. * $P < 0.001$ vs. Control, ** $P < 0.001$ vs. Wnt3a, $n = 6$. (B) Migration assay was performed by modified Boyden chambers method. * $P < 0.001$ vs. Control, ** $P < 0.001$ vs. Wnt3a, $n = 3$. (C) Invasion assay was performed in the same manner as the migration assay, except that upper surface of insert filter was coated with 100 μ l of Matrigel. * $P < 0.01$ vs. Control, ** $P < 0.01$ vs. Wnt3a, $n = 3$.

Fig. 2.

Kallistatin binds specifically to LRP6. MDA-MB-231 cells were serum-deprived and incubated with kallistatin (0.2 μ M) for 2 h. (A) Immunoprecipitation of cell lysate with anti-kallistatin antibody followed by immunoblot against LRP6 and kallistatin. (B) Immunoprecipitation of cell lysate with anti-LRP6 antibody followed by immunoblot against LRP6 and kallistatin.

Fig. 3.

Kallistatin inhibits LRP6-mediated Wnt signaling. Western blots were performed using 200 μ g total protein to detect phosphorylated LRP6, total LRP6, phosphorylated GSK-3 β , total GSK-3 β , non-phosphorylated β -catenin, and total β -catenin. Western blots were normalized with GAPDH. (A) MDA-MB-231 cells were serum-deprived and then stimulated with Wnt3a (100 ng/ml) at different time points. (B) Cells were serum-deprived, pretreated with kallistatin at different concentrations (1 μ M or 2 μ M) for 30 min or left untreated, and then stimulated with Wnt3a (100 ng/ml) for 2 h.

Fig. 4.

Kallistatin inhibits Wnt3a-induced expression of c-Myc, cyclin D1 and VEGF in MDA-MB-231 cells. Cells were serum-deprived, pretreated with kallistatin (0.5 μM) for 30 min, and then stimulated with Wnt3a (100 ng/ml) for 24 h. Protein levels were examined by western blot (A, C) and ELISA (E). mRNA levels were quantified by real-time PCR and normalized to control RNA (B, D, F). * $P < 0.05$ vs. Control, ** $P < 0.05$ vs. Wnt3a, $n = 3$.

Fig. 5.

Proposed mechanism by which kallistatin inhibits Wnt-induced cancer cell proliferation, migration, and invasion. DVL: Dishevelled; TCF: T-cell factor