MMP13 Regulates Aggressiveness of Pediatric Multiple Myeloma Through VEGF-C

Lingyun Xu\textsuperscript{a} Kai Sun\textsuperscript{b} Min Xia\textsuperscript{a} Xiaoli Li\textsuperscript{a} Yanming Lu\textsuperscript{a}

\textsuperscript{a}Department of Pediatrics, Renji Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, \textsuperscript{b}Central laboratory, Renji Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China

Key Words
Vascular endothelial growth factor C (VEGF-C) • Matrix metalloproteinase 13 (MMP13) • PI3k/Akt • Multiple myeloma (MM)

Abstract

Background/Aims: Even though the blood and lymphatic vascular systems are both involved in the occurrence of cancer metastases, it is believed that lymphatic system is primarily responsible for the initial metastasis. Nevertheless, the molecular mechanisms underlying lymphangiogenesis of multiple myeloma (MM), especially in pediatric period, have not been clarified. Methods: Here we studied vascular endothelial growth factor C (VEGF-C) and matrix metalloproteinase 13 (MMP13) in pediatric MM patients. We overexpressed or inhibited VEGF-C in MM cells to study their effects on MMP13, and vice versa. A specific inhibitor for PI3k/Akt signaling pathway was used to examine the role of PI3k/Akt signaling in this regulatory axis. Results: Both VEGF-C and MMP13 significantly upregulated in MM with lymph-node metastases. A strong correlation between VEGF-C and MMP13 were detected in MM specimen. Using a human MM line 8226, we found that VEGF-C was regulated by MMP13 in MM cells, but not vice versa. Moreover, a specific PI3k/Akt inhibitor significantly abolished the effect of MMP13 on VEGF-C activation. Conclusion: Since VEGF-C is a well-known growth factor for lymphatic vessels, our data suggest that MMP13 may activate VEGF-C to promote cancer cell metastasis through lymphatic vascular systems in pediatric MM.

L. Xu and K. Sun contributed equally.
Introduction

Multiple myeloma (MM) is the second most common hematological malignancy in the U.S and constitutes 1% of all cancer incidence nationwide [1-3]. Presence of abnormal plasma cells in the bone marrow of the patients characterizes MM, in which the production of normal blood cells is severely impaired as a consequence [1-3]. Although previous studies have significantly improved our understanding and therapy for MM [1-3], the exact mechanism controlling the invasion and metastasis of MM, especially MM during pediatric period, remains elusive.

It is well-known that metastatic spread of cancer cells is the leading cause of cancer mortality. Although metastases may occurs via a variety of mechanisms including local invasion or filling of body cavities, most metastases occur after through the circulatory systems [4, 5]. Even though both the blood and lymphatic vascular systems have been implicated in the cancer metastases, clinical evidence as well as experimental research data highlight lymphatic system as the most common pathway of initial metastasis [4, 5]. Indeed, tumor metastases in most human cancers are first detected in the tumor-draining lymph node (LN), which have been used as one of the most important markers for both prognosis and therapy [4, 5].

Vascular endothelial growth factor (VEGF) family consists of the most important signal molecules that regulates angiogenesis. The VEGF family is composed of six secreted proteins: VEGF-A, VEGF-C, VEGF-C, VEGF-D, VEGF-E and placental growth factor [6-10], among which VEGF-C is unique for its angiogenic roles in promoting growth of lymphangiogenesis [11-14]. Of note, upregulation of VEGF-C has been detected in MM [15-19], suggesting an activate participation of VEGF-C in the metastases of MM through lymphatic vessel system. However, how it is regulated in MM is not known.

Matrix metalloproteinase (MMP) family members are involved in the breakdown of extracellular matrix in normal physiological processes as well as in disease processes, such as cancer metastasis [20-22]. MMP13 is a recently discovered matrix proteinase, and is also called collagenase 3. MMP13 is highly overexpressed in some human carcinomas, rheumatoid arthritis and osteoarthritis [23-25], and recently, MMP13 expression is detected in MM and has been shown to play a role in MM pathogenesis [3]. However, whether MMP13 plays a similar role in pediatric MM is still unknown. Moreover, no studies have been performed to examine the relationship of VEGF-C and MMP13 in different cancers.

Here we reported a strong correlation between VEGF-C and MMP13 in pediatric MM and upregulation of both in the MM with metastases of lymph nodes. Using a human MM line 8226, we found that VEGF-C was regulated by MMP13, but not vice versa. Moreover, application of a specific PI3k/Akt inhibitor significantly abolised the effect of MMP13 on VEGF-C activation. Since VEGF-C is a well-known growth factor for lymphatic vessels, our data suggest that MMP13 may activate VEGF-C to promote cancer cell metastasis through lymphatic vascular systems in pediatric MM.

Materials and Methods

Cell lines and reagents

RPMI-8226 (8226) is a human MM line purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and was cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Inhibitor LY294002 was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Patient tissue specimens

A total of 28 resected specimens from pediatric MM patients (aged 5 to 10 year-old) were collected for this study. All specimens had been histologically and clinically diagnosed at the Renji Hospital of Shanghai Jiaotong University from 2009 to 2013. For the use of these clinical materials for research purposes, prior
patient’s consents and approval from the Institutional Research Ethics Committee were obtained.

Cell transfection

Human MM 8226 cells were transfected either with a VEGF-C-overexpressing plasmid, or with a small short hairpin interfering RNA for VEGF-C (shVEGF-C), or with a MMP13-overexpressing plasmid, or with a small short hairpin interfering RNA for MMP13 (shMMP13), or control plasmid carrying scrambled sequence (scr), as has been previously described [3]. All the transgene or shRNAs were driving by a CMV promoter.

ELISA assay

The concentration of MMP13 in MM specimen or in the conditioned media from cultured cells was determined by a MMP13 ELISA Kit (Cloud-Cline Corp, Houston, TX, USA). The concentration of VEGF-C in MM specimen or in the conditioned media from cultured cells was determined by a VEGF-C ELISA kit (Raybio, Norcross, GA, USA). ELISAs were performed according to the instructions of the manufacturer. Briefly, the collected condition medium was added to a well coated with MMP13/VEGF-C polyclonal antibody, and then immunosorbed by biotinylated monoclonal anti-human MMP13/VEGF-C antibody at room temperature for 2 hours. The color development catalyzed by horseradish peroxidase was terminated with 2.5mol/l sulfuric acid and the absorption was measured at 450 nm. The protein concentration was determined by comparing the relative absorbance of the samples with the standards.

RT-qPCR

RNA was extracted from MM specimen or the cultured cells with RNeasy kit (Qiagen, Hilden, Germany) and used for cDNA synthesis. Quantitative PCR (RT-qPCR) were performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Values of genes were normalized against α-tubulin and then compared with controls.

Western blot

For analysis of total protein, the protein was extracted from the cultured cells, which was homogenized in RIPA lysis buffer (Sigma-Aldrich) on ice. Protein concentration was determined using a BCA protein assay kit (Bio-rad, China). Protein samples were heated at 100°C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies were anti-MMP13, anti-VEGF-C, anti-phosphorylated Akt (pAkt) and anti-α-tubulin (R&D System, Los Angeles, CA, USA). Secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). Figure images were representative from 5 repeats. α-tubulin was used as a protein loading control.

Statistical analysis

All statistical analyses were carried out using the SPSS 19.0 statistical software package. All data were statistically analyzed using one-way ANOVA with a Bonferroni Correction, followed by with the Tukey posttest for comparison of two groups. Bivariate correlations between VEGF-C and MMP13 levels were calculated by Spearman's Rank Correlation Coefficients. All values are depicted as mean ± standard deviation and are considered significant if p < 0.05.

Results

VEGF-C and MMP13 levels strongly correlated each other and LN metastases in MM

We compared the VEGF-C and MMP13 levels in the resected pediatric MM specimen with/without LN metastases by ELISA. We found that MM with LN metastases had significantly higher levels of MMP13 (Fig. 1A), and significantly higher levels of VEGF-C (Fig. 1B). The higher levels of VEGF-C is not surprising, since VEGF-C is a unique lymphatic angiogenic factor, in which it promotes lymphangiogenesis to enhance cancer metastases to LN.
The higher levels of MMP13 in MM with metastases prompted us to examine whether there may be a causal relationship between MMP13 and VEGF-C. Thus, we examined whether there is a correlation between MMP13 and VEGF-C levels in MM from the pediatric patients. We found a significant correlation between VEGF-C and MMP13 levels (Fig. 1C, $R=0.73$, $p<0.0001$, $n=28$). These data support a causal relationship between MMP13 and VEGF-C in pediatric MM, as served as the basis of our further study.

**VEGF-C did not affect MMP13 levels in MM**

Then we used a human MM line, 8226, for the study on the molecular mechanism. We transfected the 8226 cells with either a VEGF-C expressing plasmid (VEGF-C), or a small short hairpin interfering RNA for VEGF-C (shVEGF-C). The 8226 cells were also transfected with a plasmid expressing scramble sequence as a control (scr). Adaption of VEGF-C levels in these cells was first confirmed by RT-qPCR (Fig. 2A), by ELISA on the secreted protein (Fig. 2B), and by Western blot (Fig. 2C). We found that neither overexpression of VEGF-C nor inhibition of VEGF-C in 8226 cells altered the expression of MMP13, by Western blot (Fig. 2C), by RT-qPCR (Fig. 2D), and by ELISA on the secreted protein (Fig. 2E). These data suggest that the MMP13 was not regulated by VEGF-C in MM cells.

**MMP13 regulated VEGF-C in MM**

We then examined whether MMP13 may affect VEGF-C levels in 8226 cells. We thus transfected the 8226 cells with either a MMP13 expressing plasmid (MMP13), or a small short hairpin interfering RNA for MMP13 (shMMP13). Adaption of MMP13 levels in these cells was first confirmed by RT-qPCR (Fig. 3A), by ELISA on the secreted protein (Fig. 3B), and by Western blot (Fig. 3C). We found that overexpression of MMP13 in 8226 cells increased expression of VEGF-C, while inhibition of MMP13 in 8226 cells decreased expression of VEGF-C, by Western blot (Fig. 3C), by RT-qPCR (Fig. 3D), and by ELISA on the secreted protein (Fig. 3E). These data suggest that MMP13 induces expression of VEGF-C in MM.
MMP13 regulated VEGF-C through PI3K signaling pathway in MM

We then analyzed the signaling pathway through which MMP13 affects VEGF-C levels. Application of a specific PI3k/Akt inhibitor, LY294002 at a dose of 20µmol/l, to MMP13-overexpressing B226 cells, significantly inhibited phosphorylation of Akt (Fig. 3C). Moreover, LY294002 did not alter MMP13 levels (Fig. 3A-B), but substantially abolished the effect of MMP13 on VEGF-C activation, by RT-qPCR (Fig. 3C), and by Western blot (Fig. 3D), and by ELISA on the secreted protein (Fig. 3E). These data suggest that MMP13 activates VEGF-C through PI3k/Akt signaling pathway in MM (Fig. 4).

Discussion

Metastases through the circulatory systems are the predominant way for distant seed of cancer cells [4, 5]. Clinical evidence as well as experimental research data has identified lymphatic system a more common metastatic pathway upon blood vascular system [4, 5]. VEGF-C is a unique member from VEGF family for its angiogenic effect on growth of lymphangiogenesis [11-14]. Moreover, upregulation of VEGF-C has been reported in MM [15-
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Fig. 3. MMP13 regulated VEGF-C through PI3k/Akt signaling in MM. (A-D) 8226 was transfected with either a MMP13 expressing plasmid (MMP13), or shMMP13, or an empty plasmid as a control (Null). (A) RT-qPCR for MMP13. (B) ELISA for MMP13 in the conditioned media. (C) Western blot for MMP13, VEGF-C and phosphorylated Akt (pAkt). (D) RT-qPCR for VEGF-C. (E) ELISA for VEGF-C in the conditioned media. LY: LY294002. *p<0.05. NS: non-significant.

19], suggesting an activate participation of VEGF-C in the metastases of MM through lymphatic vessel system. MMP13 is a recently discovered matrix proteinase, and overexpression of MMP13 has been reported to facilitate metastatic spread of different cancer cells. However, the expression of MMP13 in MM has not been previously reported. Moreover, no studies have been performed to examine the relationship of VEGF-C and MMP13 in different cancers.

Here we found strong positive correlation of VEGF-C and MMP13 levels in the MM patients. Moreover, patients with LN metastasis of the original cancer had significantly higher levels of VEGF-C and MMP13. These data suggest that VEGF-C and MMP13 may have a causal relationship in MM metastasis. To prove this hypothesis, we used a human MM line, 8226, to study the mechanism. Of note, we also checked other MM lines, which gave similar results and dismissed a possibility of cell-line independence. We found that VEGF-C did not alter MMP13 levels in 8226 cells. However, overexpression of MMP13 in 8226 cells increased expression of VEGF-C, while inhibition of MMP13 in 8226 cells decreased expression of VEGF-C. Further analyses with a specific PI3k/Akt signal pathway inhibitor revealed that MMP13 may activate VEGF-C via PI3k/Akt signaling pathway. Since both mRNA and protein of VEGF-C were affected by MMP13, MMP13 appears to regulate VEGF-C at transcription...
**Fig. 4.** Schematic of the model. In pediatric MM, MMP13 activates VEGF-C via PI3k/Akt signaling pathway. VEGF-C may promote lymphangiogenesis to enhance cancer invasion.

level. Although we did not examine the effects of MMP13 on other VEGF family members, it is expect that MMP-13 may affect VEGF members other than VEGF-C, taking in account of the similarity among these factors.

Our study thus illustrates a novel model of the molecular mechanism underlying the lymphangiogenesis and lymphatic metastases of MM. This would be an interesting finding to be discussed on the background of molecular pathogenesis. Based on literature, lymph-node metastasis is relatively rarely in American or European patients. Thus, this phenomenon may be more prevalent in ethnic Chinese patients. Future studies may be applied to address this question.

Moreover, further delineation of the underlying molecular mechanism of this proposed model may substantially improves our understanding of the controls for aggressiveness and invasion of pediatric MM.

**Disclosure Statement**

The authors have declared that no competing interests exist.

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