Unexpected Role Of Fibrinogen In Attenuating Primary Osteosarcoma Angiogenesis Through Inhibition Of Mmp-9 Processing Of Vegf-a

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Introduction: A specific association between cancer and abnormalities of the clotting system has been recognized for over a century. Recently, we determined that venous thrombosis in patients with osteosarcoma is laden with tumor embedded in fibrin and that treatment of mice inoculated with osteosarcoma with anticoagulants attenuates osteosarcoma growth. In addition to its role in coagulation, fibrin polymer formation is thought to enhance tumor adhesion to platelets and endothelial cells and/or to provide a matrix to support tumor growth. In support of this finding, fibrinogen-deficient mice have diminished growth of non-sarcoma in vivo malignancy (1). In an effort to elucidate the mechanism by which treatment of mice with anticoagulants reduced osteosarcoma growth we designed experiments to test the hypothesis that these effects were due to a reduction in fibrin. Specifically, we monitored osteosarcoma growth and vascularity in mice inoculated with osteosarcoma and treated with a fibrinogen antisense oligonucleotide (ASO).

Methods: In vitro

Murine osteosarcoma K-lines (K7M3) initially derived from a primary osteosarcoma in a Balb/C mouse were provided by Dr. Eugenie Kleinerman (Department of Pediatrics, The University of Texas MD Anderson Cancer Center, Houston, Tex)

Fibrin binding Assay. To determine whether fibrin clot absorbed tumor-produced MMP-9/VEGF, K7M3 cells were cultured on type I collagen gel or fibrin clot for 48h. Condition media (CM) driven from K7M3 was extracted after 48 h incubation and added to 12-well plate with type I collagen gel or fibrin clot. After a 2 hour incubation CM was extracted and tested with ELISAs for VEGF and total MMP-9.

In vivo

Single-cell suspensions (1 x 105) of murine osteosarcoma (K7M3) cells were injected into the right tibias of 6-week-old BALB/c mice as previously described (1). Fibrinogen antisense oligonucleotide (Fib-ASO) or control-ASO were injected subcutaneously once per week starting 2 weeks (at 4 weeks of age) before tumor injection. To determine whether fibrinogen treatment rescues the osteosarcoma exacerbation seen in the fibrinogen ASO mouse, we injected fibrinogen intraperitoneally (400mg/kg twice a week). We performed in vivo experiments 4 times individually (n=20 control ASO, n=20 fibrinogen ASO, n=12 fibrinogen treatment group in total) and median samples of each group were used for angiogram (n=4 for each group). Weekly radiographic evaluations were performed and assessment for enlargement of the soft tissues surrounding the injected tibia was conducted by analysis of radiographs as previously described(1). Long bones were fixed and sections were stained with hematoxylin and eosin (H&E). Immunofluorescence for VEGF, CD31, MMP-9 and Fib(rinogen) was performed using the manufacturer’s protocol and imaged with Nikon AZ100M fluorescent microscope. Angiogram with barium sulfate technique was performed and 3D characterization of the tumor angiogenesis was analyzed by uCT (Fig B).

Results: Loss of fibrinogen promotes tumor growth and tumor-induced osteolysis.

By radiographic analysis, Fib-ASO treated mice had significantly increased tumor size compared to control-ASO treated mice. Intramedullary osteolysis, as determined by bone fractional volume from μCT, was significantly increased in Fib-ASO treated mice.

Loss of fibrinogen promotes tumor angiogenesis.

We found extreme neoangiogenesis in tumor region accompanied by osteosarcoma progression. Fib-ASO mice had significantly higher volume vascularity at the tumor site than control-ASO mice (Fig A.C). We found fibrinogen treatment via intraperitoneal injection rescues the tumor growth and angiogenesis exacerbation observed in Fib-ASO mice in association with plasma VEGF level.

Plasma fibrinogen level of Fib ASO mice strongly correlates with tumor burden.

We confirmed Fib-ASO treated mice had lower fibrinogen levels than control (no tumor) and fibrinogen injection increased fibrinogen level to about 1mg/ml. Plasma fibrinogen levels of Fib-ASO treated mouse negatively correlates with tumor burden as measured by radiographic analysis.

MMP-9 produced by aggressive osteosarcoma cells releases sequestered VEGF from osteosarcoma cell itself.

ELISA measuring conditioned media without treatment for 48 hours revealed K7M3 cells produce MMP-9. We found increased VEGF secretion from K7M3 cells in an exogenous MMP-9 concentration-dependent fashion. To test the function of endogenous MMP-9 in VEGF relaxing, we treated K7M3 with a broad MMP inhibitor (GM6001) or MMP-9 selective inhibitor to for 48hours. ELISA study revealed that both GM6001 and MMP-9 selective inhibitor decrease secretion of VEGF into condition media.
Fibrin decreases VEGF secretion from osteosarcoma cells by absorbing tumor produced MMP-9.

Total MMP-9 and VEGF concentration of CM co-cultured cells with fibrin clot drastically decreased. Additionally, ELISA revealed co-culture with CM derived from K7M3 with fibrin clot for 2 hours decreased only MMP-9 but not decreased VEGF concentration. These results indicated fibrin clot decrease VEGF secretion from osteosarcoma cells by absorbing tumor produced MMP-9.

**Discussion:** Surprisingly, we found that our hypothesis was incorrect and that instead of promoting osteosarcoma growth, fibrin directly reduces osteosarcoma growth, bone destruction and tumor related angiogenesis. As such reducing fibrin resulted in extreme neo-angiogenesis in accompanied by osteosarcoma progression. Confirming our results, immunofluorescent staining revealed Fib-ASO treatment significantly reduced fibrin deposition in tumor site. Additionally, such treatment increased MMP9/VEGF expression within the tumor microenvironment. Importantly, fibrinogen treatment via intraperitoneal injection attenuated the osteosarcoma exacerbation observed in Fib-ASO mice.

Recently, evidence support plasma fibrinogen level as one of inflammatory markers may be considered a possible prognostic marker in several kinds of cancers. In contrast, physiological role of fibrinogen in tumor progression was largely unknown. We found plasma fibrinogen levels negatively correlates with tumor burden only in low level (0-1.5mg/ml) in vivo. Based on our in vivo findings, we set out to determine if these findings were secondary to enhanced osteosarcoma MMP9/VEGF production. The rationale for this works stems from the recent studies suggest that VEGF and MMP-9 might act with a synergistic effect and can positively regulate the angiogenesis in tumor cites of some malignancies. Additionally, MMP-9 has been shown to have a distinct role in tumor angiogenesis mainly regulating the bioavailability of vascular endothelial VEGF, the most potent inducer of tumor angiogenesis and a major therapeutic target (2). Despite the fact that VEGF and MMP-9 have been shown to bind to fibrin in a dose dependent manner (3), the effect fibrin clot on tumor-produced MMP-9/VEGF is unclear. Here, we found that endogenous MMP-9 continually induces VEGF secretion from aggressive osteosarcoma cells and fibrin reduces VEGF secretion from osteosarcoma cells by absorbing tumor produced MMP-9.

In conclusion, we show that the high aggressive phenotype of osteosarcoma results in tumor induced angiogenesis caused by tumor expression of MMP-9/VEGF axis. We found that at least 1.5mg/ml fibrinogen was essential to prevent osteosarcoma progression through this axis. Thus, in osteosarcoma, fibrin can inhibit tumor angiogenesis by sequestering VEGF via absorbing tumor produced MMP-9.

**Significance:** Unexpected role of fibrinogen in attenuating primary osteosarcoma angiogenesis through inhibition of MMP-9 processing of VEGF-A

**References:**

**Acknowledgments:** 0