論 文 の 内 容 の 要 旨

論文題目 Multiple myeloma in a murine syngeneic model: fibrinolytic factors during disease progression

（多発性骨髄腫の疾患モデルの作製と血液線維素溶解系因子群の動態解析）

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Multiple Myeloma (MM) is a plasma cell malignancy characterized by uncontrolled proliferation of abnormal plasma cells in the bone marrow (BM). This disease is treatable but still incurable. The expected patient's survival is approximately 3-5 years depending on disease state and treatment. Novel drugs, for example, the immunomodulatory drug thalidomide and proteasome inhibitor bortezomib or cellular treatment strategies like BM transplantation have been explored in the treatment of MM. However, they are not suitable for all patients due to age limitation. Despite various options of treatments, recurrence of the disease is common. Therefore, more effective treatment is still desirable. The main clinical features of MM disease are the accumulation of myeloma cells within the BM, the overproduction of monoclonal immunoglobulin (Ig) from the malignant myeloma cell clone, anemia, hypercalcemia and bone lesions. In the late stage of tumor progression, extramedullar tumor cell spreading also occurs.

Components of the plasminogen-plasmin system, which are involved in blood clot dissolution, participate in tumor growth, invasion and metastasis. The urokinase-type and tissue-type plasminogen activator (uPA and tPA) are proteinases that convert plasminogen into plasmin, a key factor in fibrinolytic system, and are balanced by their endogenous inhibitors, the plasminogen activator inhibitor 1 or 2 (PAI-1, or PAI-2). Plasmin can render other proteolytic enzymes, like matrix metalloproteinase (MMP) -2 and -9, from its inactive into its active state. In MM patients, the increased production of MMP-2 and -9 was associated with tumor progression and MM cell homing to the BM. MMP inhibitors have been proposed for the treatment of MM, but severe side effects occurred in MMP inhibitor-treated patients. Various studies suggested that the fibrinolytic system might contribute to MM pathogenesis: Plasmin could activate

cytokine/cytokine receptor like vascular endothelial growth factor, which could promote angiogenesis and support MM growth. Moreover, the increased level of receptor for uPA (uPAR or CD87), which is expressed on primary human MM cells, and its soluble form (suPAR) were suggested as a predictor for poor prognosis in MM patients. Recent studies also reported that primary malignant human myeloma cells express uPA. Here, we hypothesize that *fibrinolytic factors play a role in MM cell growth* as they either can control the activation of disease-associated proteases like MMPs or can directly modulate other molecular targets (cytokine, receptors or extracellular matrix molecules) involved in MM.

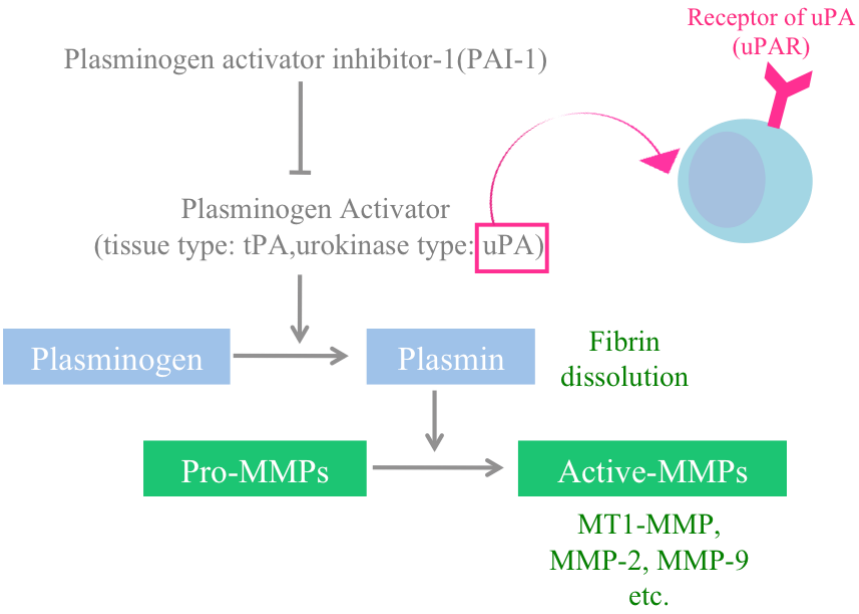

Figure 1: The fibrinolytic system

First the expression of fibrinolytic factors on human and murine MM cell lines was examined using qPCR. Whereas human MM cell lines showed low or no expression of fibrinolytic factors, the murine plasmacytoma cell lines sp2/0-Ag14 and B53 expressed uPA, uPAR, and/or PAI-1. Because the proliferation and survival of MM cell require not only autocrine, but also e.g. BM stromal cells paracrine, stromal cell lines were also included in the analysis. Both human and mouse stromal cell lines expressed uPA, tPA, uPAR, and PAI-1. Because B53 cells resembled most closely the fibrinolytic factor expression in human primary MM cells according to recent reports, we chose the murine B53 cell line for further studies. B53 cells secrete IgE allowing us to evaluate the tumor burden *in vivo* using ELISA assays and responded to treatment with common MM drugs like bortezomib and doxorubicin. To study the role of the fibrinolytic pathway for MM progression *in vivo*, we established a murine syngeneic MM model by injecting B53 cells intravenously at a

concentration of 10^6 cells/mouse into 6-week-old female CB6F1/Slc (CBF1) mice. The number of BM mononucleated cells (BMMNC) was decreased in MM-bearing mice over time. Infiltrating B53 cells were detected on hematoxylin & eosin stained BM, spleen and liver tissue sections and were quantified by FACS using the antigen expression pattern CD138+H2kd+/H2kb- to identify MM B53 cells. B53 organ infiltration varied significantly between mice, making this method unsuitable to monitor disease progression. Osteolytic lesions could not be observed by X-ray imaging. An increase in the expression of tPA, uPAR, and PAI-1 was found on BMMNC isolated from MM-bearing when compared to non-MM bearing mice as determined by qPCR. In accordance with reports in humans, suPAR levels rose during disease progression in MM-bearing mice. Interestingly, active plasmin plasma levels increased over time in MM-bearing mice, indicating that the fibrinolytic system is activated during disease progression in the murine MM model *in vivo*. These data prompted us to evaluate whether plasmin inhibition could be a novel treatment modality for MM by using the selective plasmin inhibitor named YO-2.

The plasmin inhibitor YO-2 has been reported a) to selectively inhibit active plasmin by binding at the catalytic site of plasmin and b) to induce apoptosis in solid cancer cell lines. Next, we examined whether the plasmin inhibitor YO-2 showed anti-MM activity *in vitro.* YO-2 treatment (IC_{50} = 20 μM) inhibited proliferation and induced apoptosis in murine B53 and human RPMI8226 MM cells as determined by cell counting (tryphan blue dye extinction) and Annexin V-PI assay, respectively. We are currently investigating which caspases are activated after YO-2 treatment. Given that YO-2 blocked MM growth *in vitro*, we next studied its effect on MM cell growth *in vivo*. The RPMI8226 xenograft murine MM model was established by injecting $4x10^6$ luciferase labeled RPMI8226 cells subcutaneously into immunodeficient NOD/SCID mice. Mice were injected daily with/without YO-2 intraperitoneally at a concentration of 3.75 mg/kg of body weight (BW). Tumor growth was similar for YO-2-treated and carrier-treated RPMI8226 xenografted mice as determined by size measurement and luciferase signal detection suggesting that in YO-2 treatment could not prevent MM cell progression *in vivo*.

We next determined the YO-2 responsiveness in B53-injected CBF1 mice. Survival and IgE plasma levels had been identified as reliable parameters to monitor disease progression in this model. Although plasmin activation was suppressed during disease progression in YO-2-treated MM-bearing mice, no difference in the survival rate, the tumor load and the tissue infiltration were found for YO-2 treated compared to control group. YO-2 treatment did not alter the expression of fibrinolytic factors (tPA, uPA, PAI-1) in BMMNCs as determined by qPCR. Similar plasma levels for PAI-1, total uPA, soluble-uPAR were

found in YO-2 or carrier-treated groups. To rule out that an insufficient concentration of YO-2 caused the failure of YO-2 treatment in controlling MM growth *in vivo*, we tested higher concentrations of YO-2 in the B53 murine MM model (7.5mg/kg of BW = 2x, or 15mg/kg of BW = 4x). Although active plasmin was almost completely blocked in treated mice, the 2x YO-2 treatment could not prevent disease progression. And at the highest 4x YO-2 concentration, it even accelerated the death of MM carrying mice. The reason for the increased mortality is currently unclear, but thromboembolic events might have occurred. Similar to data obtained using YO-2, treatment with the plasmin inhibitor tranexamic acid could not prevent MM progression. Next, we tested whether YO-2 in combination with MM-active drugs can control tumor growth. Bortezomib was chosen because bortezomib-treated MM patients showed a lower incidence of venous thromboembolism suggesting that bortezomib might stimulate fibrinolysis. When B53-bearing MM mice were treated with a combination of bortezomib and YO-2 or bortezomib alone, the addition of YO-2 significantly impaired survival in MM mice. In summary, our data suggest that although fibrinolytic factors are elevated in murine models of MM, pharmacological inhibition of plasmin was not able to prevent the progression of this aggressive disease.