The influence of bone marrow stromal cells on matrix metaloproteinases expression in myeloma cell line L 363

Influența celulelor stromale din maduva hematogenă asupra expresiei metaloproteinazelor matriceale în linia celulară mielomatoasă L 363

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Abstract

Multiple myeloma (MM) involves slow proliferation of malignant plasma cells and is associated with high serum levels of monoclonal antibodies, increased angiogenesis and osteolysis. The interaction of plasma cells with the bone marrow microenvironment represents a key factor in the development of osteolytic lesions. Matrix metalloproteinases (MMPs) are implicated in tumor growth and invasion, angiogenesis and bone remodeling in many cancers including MM. To assess the influence of the bone marrow microenvironment upon gene expression levels of MMPs from malignant plasma cells, we have developed an in vitro experiment involving co-culture of bone marrow stromal cells (BMSCs) with the L363 myeloma cell line. Following co-culture, microarray analysis was used to compare the expression of all 24 human MMP genes known. Our data revealed no significant change in the expression of MMP-2 in non-adherent L363 cells exposed to interaction and possibly elevated results for MMP-2 gene in the adherent population. Although our microarray data shows a 5.82 fold increase for this gene in the adherent L363 group, it is possible to attribute this to contaminating stroma because MMP-2 is known to be expressed by both BMSCs and MM cells and the purity we have achieved for L363 cells after separation from BMSCs was in the range of 90-95%. For the other MMP genes, both from adherent and non-adherent populations, our results indicate that the BMSC influence on L363 is not significant, this most likely due to the fact that L363 is a leukemic MM cell line and less dependent on stroma as opposed to other MM cell lines or primary MM samples.

Keywords: *multiple myeloma, bone marrow stromal cells, matrix metalloproteinases, microarray, gene expression, co-culture.*

Rezumat

Mielomul multiplu (MM) presupune proliferarea plasmocitelor maligne şi se asociază cu un nivel crescut de anticorpi monoclonali în ser, angiogeneză crescută şi osteoliză. Interacţiunea dintre micromediul

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medular şi plasmocite reprezintă un factor cheie în dezvoltarea leziunilor osteolitice. Metaloproteinazele matriceale (MMPs) sunt implicate în creşterea şi invazia tumorală, angiogeneza şi remodelarea osoasă din numeroase neoplazii, inclusiv MM. Pentru a evalua influenţa micromediului medular asupra expresiei genelor MMPs din plasmocitele maligne, am realizat un experiment in vitro ce presupune co-cultura liniei celulare mielomatoase L363 cu celulele stromale din măduva osoasă hematogenă (BMSCs). După co-cultură am comparat expresia tuturor celor 24 de gene MMPs folosind analiza microarray. Rezultatele noastre au arătat ca nu sunt modificări semnificative ale expresiei genei MMP-2 în populaţia neaderentă de celule L363 expusă interacţiunii şi au mai arătat un nivel posibil crescut al expresiei aceleiaşi gene în populaţia aderentă de celule L363. Deşi datele de microarray prezintă o creştere de 5.82 ori pentru MMP-2 din populaţia adherentă, este posibil ca acest lucru să se datoreze stromei contaminante deoarece MM-2 este exprimată atât de către plasmocitele mielomatoase cât şi de către BMSCs, iar nivelul purităţii L363 după separarea de BMSCs a fost de 90-95%.. Pentru celelalte gene MMPs, atât din populaţia aderentă cât şi cea neaderentă, rezutatele arată că influenţa BMSCs asupra L363 nu este semnificativă, cel mai probabil datorită faptului că L363 este o linie mielomatoasă leucemică şi este mai puţin dependentă de stromă comparativ cu celelalte linii mielomatoase sau cu plasmocitele pacienţilor cu MM.

Cuvinte cheie: *mielom multiplu, celule stromale medulare, matrix metaloproteinaze, microarray, expresie genică, co-cultură.*

Introduction

Multiple myeloma (MM) is a debilitating malignancy characterized by the accumulation and proliferation of monoclonal plasma cells within the bone marrow (BM). MM is associated with osteolytic bone disease, the production of a monoclonal immunoglobulin, increased angiogenesis and ineffective hematopoiesis (1-5). The interaction of myeloma cells with bone marrow microenvironment plays a key role in survival and proliferation of malignant plasma cells and confers protection against therapeutic drugs (6-11).

Matrix metalloproteinases (MMPs) represent a family of related enzymes involved in denaturation of most components of the extracellular matrix (12, 13). MMPs play important roles in physiological processes, while their overexpression is important in pathological processes as multiple sclerosis, arthritis, bone diseases, inflammatory disorders and vascular alterations such as atherosclerosis, aortic aneurysm and myocardial infarction but mainly in cancer and metastasis (14-18).

Previous studies on MMPs in MM are controversial regarding MMP-2. Some authors have shown that human myeloma cells secrete constitutively MMP-9 but not MMP-2 and MMP-1 while at the same time, cells of bone marrow environment (BMSC) secrete MMP-2 and MMP-1 but not MMP-9 (19). Other authors have later demonstrated that the MMP2 gene is also expressed by plasma cells from MM patients (20, 21) and MM cell lines (22). Only a few studies have evaluated the effect of stroma on the expression of MMPs in myeloma cells and the number of assessed MMP genes was limited. In those studies, to simulate the stroma, myeloma plasma cells were cultured together with bone marrow endothelial cells (23, 24), osteoblasts (21), osteoclasts (25) or bone marrow stromal cells (19, 26). Our goal in this study was to determine whether there are significant variations in gene expression levels of MMPs in the L363 MM cell line following co-culture with BMSCs using an in vitro experimental model. The expression of all 24 human MMPs has been assessed: MMP 1-3, MMP 7-17, MMP 19-21, MMP 23-28.

Material and methods

Cell culture

Six consecutive experiments were performed, based upon the premise that stroma provides the same type of support for MM cells, regardless of its origin. Each experiment was performed as follows: MM cells from L363 MM cell line were cultured in the absence (control MM

cells) or presence of BMSCs obtained from one healthy donor (three distinct experiments) or BMSCs obtained from 3 different MM patients (other three distinct experiments). Therefor each experiment has generated three different types of samples: one control (L363 cells cultivated in the absence of stroma); one L363 non-adherent cell fraction; one L363 adherent cell fraction.

BMSCs. BM aspirates from MM patients and healthy volunteers obtained according to institutionally approved protocols were cultured in 75 cm2 flasks (Nunc, Denmark) with Myelocult H5100 medium (StemCell Technologies, Vancouver, British Columbia, Canada). Flasks were incubated at 37°C in a 5% CO2 humidified atmosphere. Medium was changed every 3 or 4 days and confluent layers of BMSCs were obtained within 4 to 5 weeks.

MM cells. L363 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; Gibco) at 37°C and 5% CO2. Prior to co-culture L363 cells were labeled with 10μM CFSE (carboxyfluorescein succinimidyl ester), a green fluorescent protein, in order to allow proper identification for cell sorting of adherent L363 cells.

Co-culture. Confluent layers of BMSCs were co-cultured with CFSE-labeled L363 MM cell line. After 72hrs of culture adherent and non-adherent MM cells were collected. Non-adherent L363 cells were harvested from the culture supernatant. For collecting the adherent L363 cell fraction, BMSCs (containing adherent MM cells) were detached with Trypsin EDTA. Subsequently, CFSE-labeled MM cells were sorted using a MoFlo™ High-Performance Cell Sorter (Dako-Cytomation) to a purity of 90-95%. Cell pellets were stored at -70°C.

Sample processing for gene expression analysis

RNA from frozen cells was isolated with Qiagen RNeasy Mini and Micro kits. RNA was analyzed on an Agilent 2100 Bioanalyzer and RNAs with a RIN > 7 were used for further processing.

The Ambion Message Amp II-Enhanced kit was used to generate the Biotin-labeled target cRNA according to the manufacturer's protocol; 950 ng of total RNA was reverse-transcribed with a T7-oligo-dT primer into first-strand cDNA and subsequently second strand cDNAs were generated; after cDNA purification, In-vitro-Transcription (IVT) was performed overnight; during this reaction, biotin was incorporated in the resulting cRNA; after cRNA cleanup and fragmentation, the labeled target was hybridized for 16h at 45°C on Affymetrix HG-U133 Plus 2.0 arrays; using GCOS software on an Affymetrix GeneChip system, arrays were washed, stained, and then scanned.

Statistical data analysis

The expression of all 24 human MMP genes known to the present was compared as follows: control MM cells versus adherent MM cell fraction; control MM cells versus non-adherent MM cells fraction.

In order to do so, we used the Genedata Expressionist software (version 6.1) for further statistical data analysis. CEL files were imported into the Refiner module 6.1 of Expressionist where quantile normalization and probe summarization was performed using the Bioconductor RMA condensing algorithm (27). To identify differentially expressed genes, the data were imported into the Analyst 2.2 module. We used the paired Bayes T-test (CyberT) (28) with the Bayes Confidence Estimate Value set to 10 and a window size of 101 genes. To control the false discovery rate, the Benjamini-Hochberg q-value was calculated (29). We then used the "2 groups" activity of Analyst to calculate the Paired Effect size between the groups. Significance thresholds were set to 0.05 FDR (False discovery rate, accepting 5 % false-positives) and a Paired Effect size of 2-fold change between the paired medians of the groups.

Results and Discussions

The interaction of a confluent layer of BMSCs (*Figure 1*) with L363 has led to interesting results because the L363 cells grew into an adherent fraction onto BMSCs and into a non-adherent cell fraction (*Figure 2*).

These two populations of L363 cells gave different results in terms of gene expression. All 24 human MMP genes have been tested and we noticed significant changes only in the expression of MMP-2. The microarray chip has 3 probe sets for the MMP-2 gene: 201069_at, 1566678_at and 1566677_at.

We took into consideration the results generated from the 201069_at probe set because they passed our threshold and this probe set targets almost the entire MMP-2 gene which has 13 exons. The other two probe sets 1566678_at and 1566677_at target both sense and antisense strand of only one MMP-2 exon and moreover their results did not pass threshold criteria (*Figure 3* and *Table 1*).

Considering the 201069_at probe set, the expression level of MMP-2 from adherent L363 cells was 5.82-fold higher than in the negative control. For non-adherent L363 cells and the same gene, the increase in expression level was only 1.1 fold (*Table 1*).

There are other co-culture experiments on MMPs expression in MM where osteoblasts (21), osteoclasts (25), or bone marrow endothelial cells (23, 24) were used to simulate the micro-medium, but despite the fact that BMSCs are intense used in experimental co-culture models for MM disease there are limited BMSCs-plasma cells co-culture studies focused on MMPs expression in malignant plasma cells (19, 26).

Of great importance in MM pathology is the interaction of tumor cells with the microenvironment, which is represented by bone marrow with its components: BMSCs, endothelial cells, adipocytes, fibroblasts, osteoblasts and osteoclasts (30). The interaction between tumor cells and microenvironment is mediated via adhesion and cytokines (31). MM cells secrets MMP-7 which activates the latent form of MMP-2 secreted by BMSCs (26). MMP-7 has proteolytic activity itself and together with MMP-2 activation raises tumor invasiveness.

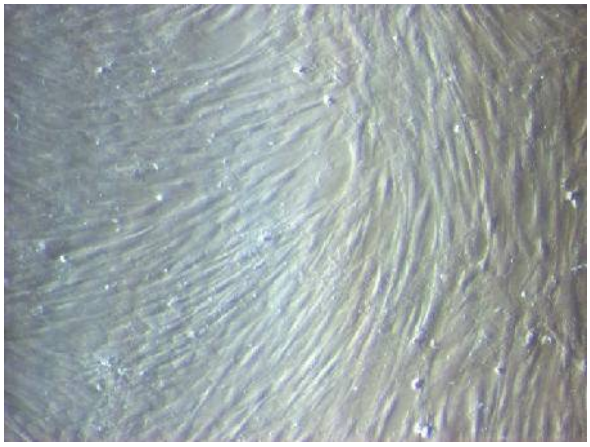

Figure 1. Confluent layers of bone marrow stromal cells in culture (objective x10, phase contrast)

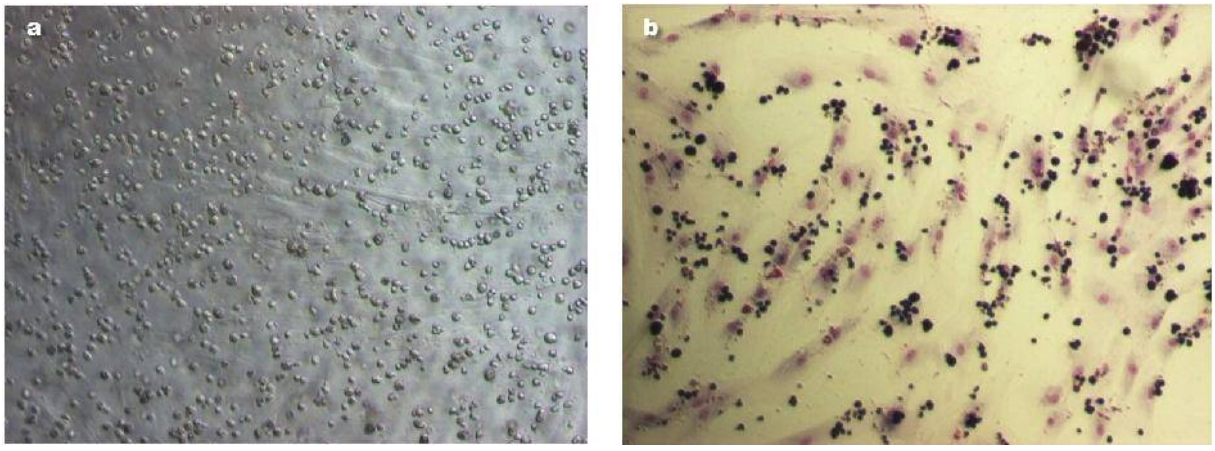

Figure 2. Co-culture of L363 MM cells with bone marrow stromal cells: a. the entire population of L363 and BMSCs (objective x10, phase contrast); b. BMSCs and adherent population of L363 (objective x10, Dia Panoptic staining)

Table 1. Expression changes for MMP-2 gene in L363 cells and FDR (false discovery rate).
Negative control is represented by L363 cultured alone.

L363 populations	probe set	paired Bayes T-Test (CyberT) FDR	Fold change
Adherent *vs* Control	201069_at	0.0004	5.82
	1566678_at	0.8502	1.08
	1566677_at	0.9470	1.03
Nonadherent *vs* Control	201069_at	0.6137	1.10
	1566678_at	0.6999	1.08
	1566677_at	0.9154	1.03

It is acknowledged from co-culture experiments that bone marrow endothelial cells increase the expression of MMP-9 in human MM cells (24) and in murine 5T33 MM cells (23). From osteoblasts-plasma cells co-culture experiments it has been suggested that osteoblasts increase the expression of MMP-2 and induce the expression of MMP-1 in MM cells while MM cells in turn induce overexpression of MMP-1 in osteoblasts; these effects enhance the invasion and migration potential of tumor cells (21). The same authors have later shown how important osteoclast-MM cell interaction is: osteoclasts activated the p44/p42 MAPK, STAT3 and PI3K/Akt pathways in MM cells and MM cells induced p38 MAPK and NF-kappaB signaling in osteoclasts; myeloma-osteoclast interactions stimulated the production of TRAP, cathepsin K, matrix metalloproteinase (MMP)-1, -9, and urokinase plasminogen activator (uPA) (25).

Several experiments on MM and MMPs were not based on co-culture models, they were concentrated instead on cytokines which are normally present in MM microenvironment; those studies illustrate how diverse soluble factors from stroma could affect MMPs expression. Thus, an experiment where RPMI 8226 myeloma cells had been cultured with interleukin-6, phorbol-12-myristate-13-acetate and tumor necrosis factor alpha has shown an increased expression for MMP-8 and MMP-13 in plasma cells, two important proteins which participate in matrix destruction and bone lesions (32). Interleukin-6 and tumor necrosis factor alpha are important cytokines present in MM micro-medium and BMSCs are a known source for interleukin-6 (33). Another experiment on four MM cell lines were I-TAC, Mig and IP10 cytokines were used has demonstrated that these soluble factors increased the MMP-2 and MMP-9 gelatinolytic activity in the cell conditioned medium, an effect mediated via CXCR3 receptor (22).

We can summarize that previous studies have demonstrated that MM cell lines and plasma cells from MM patients secrete MMP-1, MMP-2,

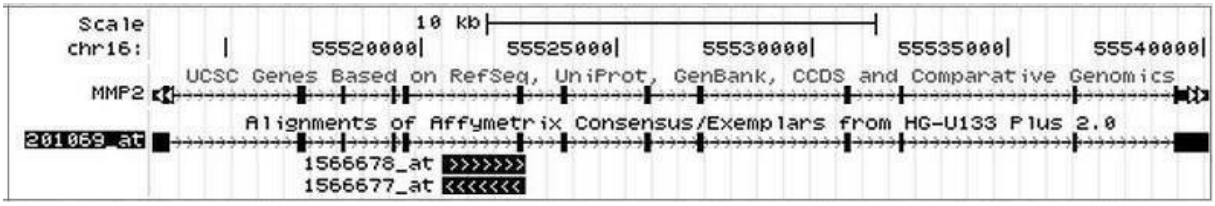

Figure 3. Alignments for 201069_at, 1566678_at and 1566677_at probe sets with MMP-2 gene. MMP-2 genomic DNA is represented here with thicker boxes representing the coding regions. It can be seen that 201069_at targets the entire gene while 1566678_at and 1566677_at probe sets overlap with only one exon. That means the 201069_at probe set gives more accurate information (USCS Genome Browser-http://genome.ucsc.edu/)

Table 2. Expression changes for BMSCs markers (fold change and false discovery rate)

Gene	probe set	Adherent vs Control		Nonadherent vs Control	
		FDR	Fold change	FDR	Fold change
ITGA4	205884_at	0.0179	3.55	0.6154	1.15
CXCL12	209687_at	0.0413	2.42	0.7682	1.06
ICAM1	202638_s_at	0.0335	2.40	0.1592	1.41
THY1	213869_x_at	0.0081	3.41	0.8329	1.05

MMP-7, MMP-8, MMP-9 and MMP-13 (19-21, 26, 32) while BMSCs secrete MMP-2 (19, 34). Studies on murine models have confirmed the expression of MMP-2, MMP-8, MMP-9 and MMP-13 in bone marrow cells isolated from 572MM mouse model (35) with MMP-9 also confirmed in plasma cells from mouse model 5T33 (23).

We have already mentioned that the expression of MMP-2 gene was controversial. In our experiment, MM-2 gene was upregulated in adherent L363 cell fraction after co-culture with BMSCs. This result is in contradiction with some studies (19) but in agreement with other experiments (20-22)

Taking into account the purity of adherent L363 and the variation of BMSCs markers (*Table 2*) we cannot safely assume that the increased expression of MMP-2 for adherent L363 is the effect of interaction with BMSCs taken alone and not the effect of contamination with BMSCs. Indeed, other studies have attributed the expression of MMP-2 to contaminating BMSCs (35).

So far, the experiments in this field have been limited to several members of MMP protein family; our work instead has investigated all 24 human MMPs: MMP 1-3, MMP 7-17, MMP 19-21, MMP 23-28.

Based on our analysis settings, MMP genes except MMP-2 had no significant change in expression after co-culture with BMSCs because they did not pass the fold change threshold. One explanation could be the fact that L363 is a leukemic MM cell line which is not too much dependent on stroma unlike other MM cell lines or primary MM plasma cells.

Conclusions

The results of our experiment revealed no significant change in expression for MMPs in the non-adherent L363 population exposed to their interaction with BMSCs and a possible up-regulation of MMP-2 gene in the adherent L363 cell population.

In the present report we show how L363 cells grew into two subsets: one adherent to BMSCs and the other to a non-adherent cell fraction. Both groups where evaluated for gene expression differences. Of note was that it is difficult to obtain a distinct and highly pure separation of L363 from BMSCs (close to 100%), because some of these cells are highly adherent. The variation in the MMP-2 gene that we observed might have been caused by BMSCs' influence in co-culture or could be the effect of contaminating BMSCs since the MMP-2 gene is highly expressed by BMSCs (34). One solution in order to discriminate the expression signal of L363's MMP-2 from the one coming from stroma could be the use of immunohistochemistry in co-culture.

We consider our results of importance since the evaluation of MMPs production in MM is valuable as a therapeutic approach involving MMPs inhibitors that target several noticeable effects in osteolysis, angiogenesis, tumor growth and invasion. Inhibitors for MMPs already exist on the market and knowing exactly which cells and genes are targeted should improve therapeutic options and might lead to a better outcome of MM patients in the very future.

Abbreviations

BM = bone marrow
BMSCs = bone marrow stromal cells
FCS = fetal calf serum
FDR = false discovery rate
MM = multiple myeloma
MMP = matrix metalloproteinases

Acknowledgements

This study was possible with financial support from the Romanian Ministry of Education and Research, The National University Research Council (Consiliul National al Cercetarii Stiintifice din Invatamantul Superior), research grant PN-II-ID-PCE-2007, code 1181/2007.

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