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Matrix metalloproteinase and cytokine production by bone marrow adherent cells from multiple myeloma patients

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Abstract

Introduction: Cultures of bone marrow stromal cells derived from the bone marrow of multiple myeloma (MM) patients were shown to exhibit several abnormalities compared with control cultures from healthy subjects. The aim of the study was to examine whether cultures of bone marrow adherent cells, at low passage level, exhibit differences in matrix metalloproteinases (MMPs) and cytokine production compared with cultures from normal donors.
Materials and Methods: MMP production was evaluated by gel zymography and by ELISA in supernatants of serum-free cultures of bone marrow adherent cells derived from 20 MM patients and 23 healthy controls. Spontaneous and lipopolysaccharide (LPS)- or Newcastle disease virus (NDV)-induced cytokine release was assessed in the supernatants of the cultures by the ELISA method.
Results: Both cultures produced MMP-1, -2, -3, and -9 under serum-free conditions; however, the levels of MMP-1 and MMP-2 were significantly higher in cultures derived from MM patients, while MMP-3 was significantly higher in control cultures. The level of MMP-9 was comparable in the cultures derived from MM patients and controls. All cultures produced interleukin (IL)-10 and IL-11 spontaneously, but after LPS or NDV induction the levels of IL-10, IL-11, interferon α, and tumor necrosis factor α, were significantly higher in the cultures derived from MM patients than in control cultures.
Conclusions: The results indicate that both the abnormalities in MMP production and the overproduction of cytokines (in the presence of LPS or virus, which mimic inflammatory conditions) may be involved in bone destruction and tumor spread in multiple myeloma.

Key words: multiple myeloma, bone marrow adherent cells, cytokines, matrix metalloproteinases

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INTRODUCTION

Multiple myeloma (MM) is a progressive B-lineage neoplasia characterized by the accumulation of slow-proliferating malignant plasma cells in the bone marrow compartment, where the microenvironment seems to be favorable for their growth and survival [9, 14]. Long-term bone marrow cultures (LTBMCs) are characterized by the development of a stromal adherent layer with several cell types (mostly fibroblast-like cells) producing cytokines and growth factors anchored to cell membranes and/or released into the culture medium. Marrow stromal cells act on human hematopoietic cells by cell-to-cell contact and by secreting cytokines and growth factors that modulate hematopoiesis [4, 7, 27]. Some stromal abnormalities have been detected in LTBMCs from MM patients, for example an increased number of osteoclasts, a reduction in the deposition of extracellular matrix (ECM), and overall loser organization than in normal LTBMCs [11, 13]. These differences between normal and MM stroma persist after the elimination of co-cultured MM cells, suggesting intrinsic stromal abnormalities. Moreover, compared with normal bone marrow stroma, cells derived from MM patients have been described as exhibiting increased spontaneous and MM cell-induced interleukin (IL)-6 production. In some papers, increased basal levels of IL-1β and tumor necrosis factor (TNF)-α has also been reported [6, 23, 32].

Matrix metalloproteinases (MMPs) and their tissue inhibitors regulate not only the turnover of ECM, but also cell survival, proliferation, differentiation, adhesion, and migration. On the basis of their domain structure and substrate specificity, MMPs are classified into four major subgroups: collagenases, gelatinases, stromelysins, and membrane-type MMPs [20]. Recently it has been shown that MMP-2 and MMP-9 are produced in LTBMCs from normal donors [25]. Bone marrow stromal cells (BMSCs) from MM patients constitutively express MMP-1 and MMP-2. MMP-1 expression is positively regulated by IL-1β and TNF-α, but MMP-2 is not modulated by cytokines. Co-cultures of BMSCs with MM cells upregulate MMP-1 expression and convert pro-MMP-2 into its active form. MMP-9 expression is similar in normal and MM-derived cells [12, 30].

As no papers are available concerning the production of some metalloproteinases, such as MMP-3, and some cytokines, such as interferon (IFN)-α, by bone marrow adherent cells of MM patients, in the current study we compared MMP-1, MMP-2, MMP-3, and MMP-9 production as well as IFN-α, TNF-α, IL-10, and IL-11 production, both spontaneous and under inflammatory conditions mimicked by bacterial lipopolysaccharide (LPS) or virus presence, in bone marrow adherent cell cultures derived from MM patients and normal donors.

MATERIALS AND METHODS

Patients

Bone marrow samples were obtained from 20 freshly diagnosed MM patients after receiving informed consent according to the requirements of the local ethics committee. The clinical characteristics of the patients are presented in Table 1. Bone marrow samples were also obtained from 23 healthy volunteers (median age: 42.9±12.6 years, 9 men and 14 women). The healthy volunteers were either donors for allogenic bone marrow transplantation or were undergoing orthopedic surgery, and the samples were collected during the operation.

Table 1. Clinical characteristics of multiple myeloma (MM) patients

	MM n=20
Age (mean ±SD)	61.3±11.9
Men/woman (n)	11/9
Disease stage (Durie and Salmon [10])	
I/II/III; A/B	2/1/17; 15/5
% of plasma cells in bone marrow (mean ±SD)	42.7±24.1
IgG mg/dl (mean ±SD)	3683.7±2804.8
IgA mg/dl	297.2±610.6
IgM mg/dl	76.5±30.7
β_2 microglobulin mg/dl	7.68±7.62

Bone marrow adherent cell cultures

Anti-coagulated bone marrow samples were diluted 1:2 with Iscove's Modified Dulbecco's Medium (IMDM; Gibco, BRL, UK) containing 0.2% methylcellulose (Sigma, St. Louis, MO, USA). After sedimentation of erythrocytes (30–40 min) at room temperature, supernatants were collected, washed two times with IMDM supplemented with 2% fetal bovine serum (FBS; Gibco), and resuspended in IMDM supplemented with 10% FBS, 10% horse serum (Gibco), 1 μM hydrocortisone (Sigma), and 1% antibiotic-antimycotic solution (Gibco). Cells from the supernatants were seeded in T25 cell culture flasks (Nunc, Roskilde, Denmark) at a density of 2×10^6 cells/ml. The cultures were incubated for 4 weeks at 37°C in a humidified atmosphere containing 5% CO_2. After 1, 2, and 3 weeks of incubation, the cultures were demi-populated and non-adherent cells were aspirated together with half of the medium volume. After 4 weeks of incubation, when a continuous network of adherent cells occupying the entire bottom of each flask was obtained, the whole medium with non-adherent cells was removed and replaced by a new one.

Into half of the flasks with an adherent cell layer, the medium without FBS was added. The cultures were incubated for one week and the MMP concentration was estimated in the supernatants. Half of the flasks with an adherent cell layer were treated with 0.25% trypsin (Sigma). The cells were subcultured in 24-well plates (3×10^5 of adherent cells/ml/well) and used for cytokine induction.

Induction of cytokines

Twenty-four hours after seeding, the culture medium was replaced by fresh IMDM medium supplemented with 2% FBS. Cytokine inducers, such as LPS from *E. coli* 0111:B4 (Sigma) at 10 μg/ml or Newcastle disease virus (NDV) at 10 MOI (multiplicity of infection, i.e. the number of infectious particles per cell), were added. After 24 h of incubation, the supernatants were collected and stored at –80°C until cytokine assay.

Gelatin substrate gel zymography

Supernatants from the cultures of bone marrow adherent cells were diluted 1:30 in SDS sample buffer without a reducing agent and subjected to SDS-polyacrylamide gel electrophoresis in 7.5% polyacrylamide gel containing gelatin at 0.5 mg/ml, as previously described by Heussen and Dowdle [15]. Molecular-weight standards of 97.4, 66, 45, and 31 kDa were included in each gel. After electrophoresis, SDS was removed from the gel by incubation in 2.5% Triton X100 for 1 h at room temperature, and after that it was incubated in 0.05M Tris-HCl buffer, pH 7.4, supplemented with 0.01M $CaCl_2$ and 0.02% NaN_3 for 20 h at 37^0C. The gels were stained with Coomassie blue R250 (0.025%) in 40% methanol and 10% acetic acid. After staining, the

gels were destained in 40% methanol and 10% acetic acid for 30 min. at room temperature. Proteolytic activities were observed as clear bands against the blue background of the stained gel.

Assay for cytokines and MMPs

The concentrations of MMP-1, MMP-2, MMP-3, and MMP-9 (total) were measured by ELISA using commercially available kits from Amersham Pharmacia Biotech, Piscataway NJ, USA, or R&D System, USA. The detection limit was 0.023 ng/ml for MMP-1 and 0.1 ng/ml for MMP-2, MMP-3, and MMP-9. The concentrations of IFN-α, TNF-α, and IL-10 were also measured by ELISA using commercially available kits (Endogen, Woburn, MA, USA). IL-11 concentration was measured using a kit from R&D System (Minneapolis, USA). The detection limit was 3 pg/ml for IFN-α and IL-10, 5 pg/ml for TNF-α, and 8 pg/ml for IL-11.

Cell phenotype

The morphology of adherent bone marrow cells growing in the cultures was studied on May-Grünwald-Giemsa-stained coverslips. The cells were also stained for tartrate-resistant acid phosphatase (TRAP) in order to detect osteoclasts.

After trypsinization and washing in PBS, the cells from the flasks were resuspended in IMDM supplemented with 1% FBS and incubated at 37^0C (5% CO_2) for 3 h. Then the cells were washed with PBS. Two-color immunofluorescence studies were performed using a combination of phycoerythrin and fluorescein isothyocyanate conjugated mouse monoclonal antibodies: CD45/CD14, CD3/CD19, CD19/CD38, CD45/CD9, CD14/CD9, and CD14/CD34. All antibodies were supplied by Dako (Copenhagen, Denmark) or by Serotec (Oxford, UK). One hundred µl of cells (1×10^6) was added to a flow cytometry tube and stained with 10 µl of the appropriate antibodies. After 20 min. of incubation at room temperature, the cells were washed twice in PBS, centrifuged, resuspended in 500 µl of PBS, and analyzed by flow cytometry (Ortho Cytoron Absolute, Becton-Dickinson, CA, USA) directly after preparation.

Statistics

Results were reported as the mean ±SD. The Mann-Whitney U-test was used to compare the differences between MMP and cytokine concentrations in the MM and control groups. The concentrations of MMPs and cytokines below the detection level were considered as 0 for the purpose of analysis. Correlation between different variables were assessed by the Kendall τ test.

RESULTS

General properties of cultured bone marrow adherent cells from MM patients and normal donors

In all the examined MM samples, the culture system allowed bone marrow adherent cells to grow. The ability of MM samples to form a confluent adherent layer was higher than in controls. After 4 weeks of culture, a confluent layer of fibroblast-like cells interspersed with macrophages and TRAP-positive osteoclasts formed (Fig. 1). Apart from fibroblasts ($CD9^+/CD45^-$), cytometric analysis revealed (Table 2) macrophages ($CD14^+/CD45^+$), endothelial cells ($CD9^+/CD45^-$, $CD34^+$), and small amounts of T and B lymphocytes. There were no significant differences in the percentages of the different cells except for the presence of plasma cells ($CD38^+/CD45^+$, $CD34^-$) and a higher number of $TRAP^+$ mononuclear cells in MM cultures (Fig. 1).

MMP secretion by MM and normal bone marrow adherent cells

After monolayer formation, the cultures of adherent bone marrow cells were incubated for one week in serum-free IMDM. The supernatants were analyzed by gelatin zymography and showed gelatinolytic activity at 92 and 72 kDa, corresponding to the molecular weights of MMP-9 (gelatinase B) and MMP-2 (gelatinase A) in their zymogen forms, respectively (Fig. 2). A compari-

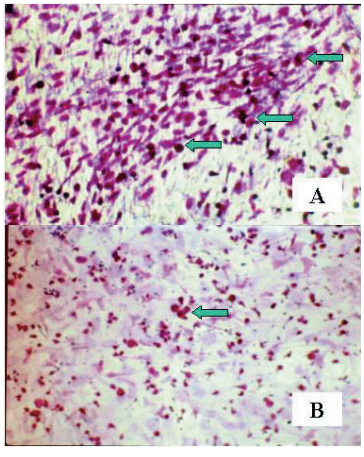

Fig. 1. Tartrate-resistant acid phosphatase-stained ($TRAP^+$) cells in cultures of bone marrow adherent cells. **A** – cultures derived from MM patients, **B** – cultures of controls. Arrows indicate $TRAP^+$ cells. In cultures derived from MM patients it was 22±27% and in controls 8±7.5% of $TRAP^+$ cells (the values are expressed as the percentage of the total cell number).

Table 2. Cytometric analysis of bone marrow adherent cells (% of cells)

Cultures	T cells ($CD3^+$)	B cells ($CD19^+$)	Plasma cells ($CD38^+$)	Fibroblasts ($CD9^+$)	Endothelium ($CD34^+$)	Macrophages ($CD14^+$)
MM	1–2	2.5–7	2–14	25–35	5–18	17–40
Control	3–5	3.0–6	0–1	27–30	12–24	17–40

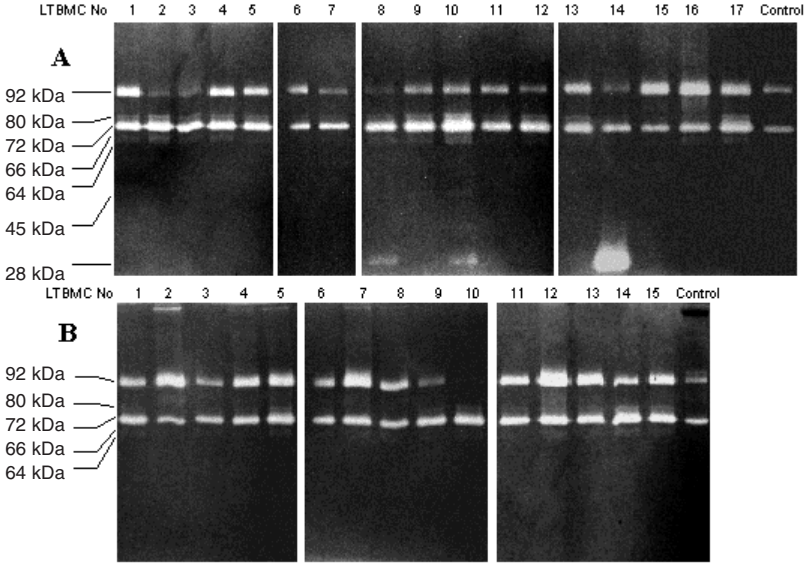

Fig. 2. Detection of MMPs in MM (**A**) and in control (**B**) bone marrow adherent cell supernatants. Zymogram of media obtained from cultures of MM patients or controls incubated for one week without serum and diluted 30-fold. As controls, 20 ng/ml of rhpro-MMP-2 and rhpro-MMP-9 were run alongside the samples.

son with the controls revealed the presence in some of the MM samples of additional 80-kDa gelatinolytic activity, corresponding to the active form of MMP-9 (94% of MM cultures and 73% of control cultures) and 28-kDa activity corresponding to the latent form of MMP-7 (present only in 18% of the MM cultures). The concentrations of MMP-1, MMP-2, MMP-3, and MMP-9 in the culture supernatants were also measured by ELISA. This assay measured both the latent and the active forms of the enzymes. All bone marrow adherent cells secreted a detectable level of MMP-1, but cells from MM patients secreted significantly more enzyme. The assay also confirmed the presence of MMP-2 in both examined groups, with the bone marrow cells of MM patients producing more MMP-2 than the controls. No difference was observed in MMP-9 secretion, while MMP-3 was mainly produced by control bone marrow cells (Fig. 3).

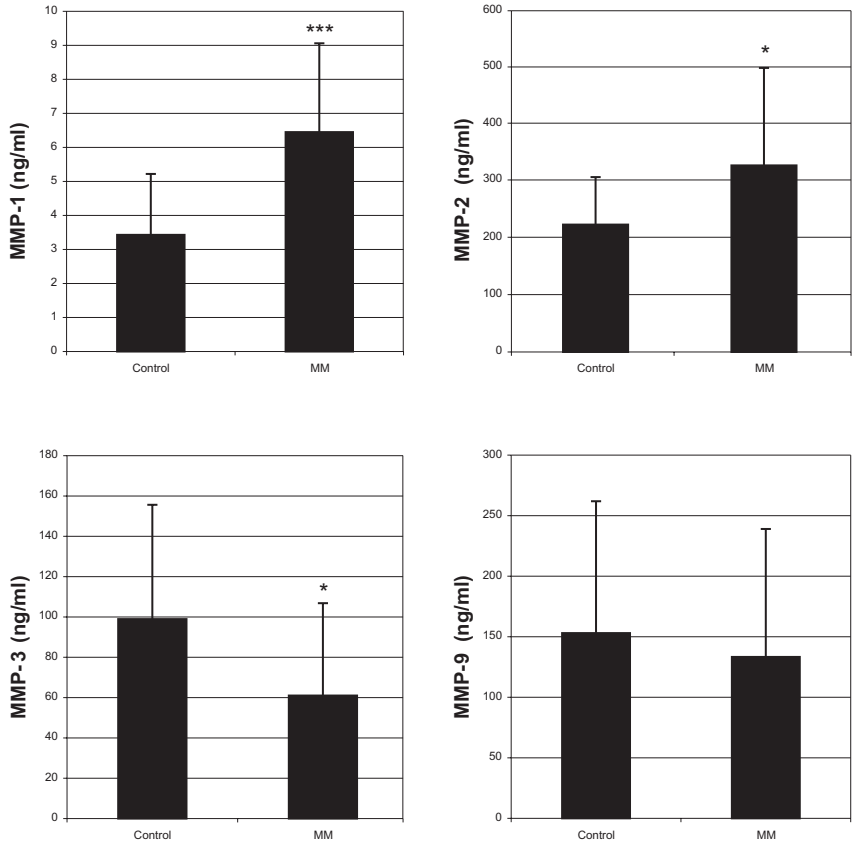

Fig. 3. Detection of MMP-1, MMP-2, MMP-3, and MMP-9 by ELISA in supernatants of serum-free cultures of bone marrow adherent cells of MM patients (20 subjects) and controls (23 subjects). Statistically significant compared with controls at *0.05, **0.01, and ***0.001, in the Mann-Whitney U-test.

Cytokine production by MM and normal bone marrow adherent cells

Spontaneous and LPS- or NDV-induced cytokine production was measured in MM and control bone marrow adherent cells by the ELISA method. Both cultures spontaneously released low but detectable levels of IL-10 and IL-11. No IFN-α and TNF-α was detected in non-induced cultures. The IL-10 level was significantly higher in MM-derived bone marrow adherent cells than in controls, in contrast to IL-11, the level of which was higher in control cultures (Fig. 4). The production of IL-10 and IL-11 was stimulated by LPS, but their levels were significantly higher in MM bone marrow adherent cells than in controls. NDV was a good inducer of cytokine production, and the levels of TNF-α, IFN-α, IL-10, and IL-11 were significantly higher in MM-derived bone marrow adherent cells than in controls.

DISCUSSION

Evidence that MMPs play a functional role in pathological processes is well documented. Both MMP-2 and MMP-9, apart from degradation of denatured collagen, fibronectin, and elastin, play a special role in tumor invasion and metastasis [11]. It has been reported that a significant increase in MMP activity occurs in the bone marrow environment of patients with MM [15]. Myeloma cells constitutively secrete MMP-9 and are able to induce the activation of latent MMP-2 (pro-MMP-2) secreted by the bone marrow environment via MMP-7 (matrilysin), which is also constitutively produced by myeloma cells [3, 30].

It has been reported that the adherent layer established in LTBMCs from normal donors secretes latent forms of MMP-9 and MMP-2 under serum-free conditions [25]. Our experiments have confirmed the observation that bone marrow adherent cells from normal donors release both pro-MMP-9 and pro-MMP-2, but we also detected that the level of MMP-2 (total) was significantly higher in MM bone marrow adherent cells than in the control cells. It has already been reported that MMP-2 and MMP-9 are produced by osteoblasts and osteoclasts [22, 24, 26]. As the number of osteoclasts and plasma cells was significantly higher in the cultures of bone marrow adherent cells derived from MM patients than in the controls, we can speculate that at least a part of the increased level of MMP-2 was due to the presence of these cells in the culture. In our study, the level of MMP-9 was comparable in both groups. Our results differed from those obtained by Barille et al. [2], who did not detect any enzymatic activity corresponding to MMP-9 in gel zymography of the supernatants from BMSCs derived from MM patients. It should be stressed, however, that they used BMSCs at a higher passage level (3–4) than the bone marrow adherent cells in our experiments (1st passage level). It is possible that

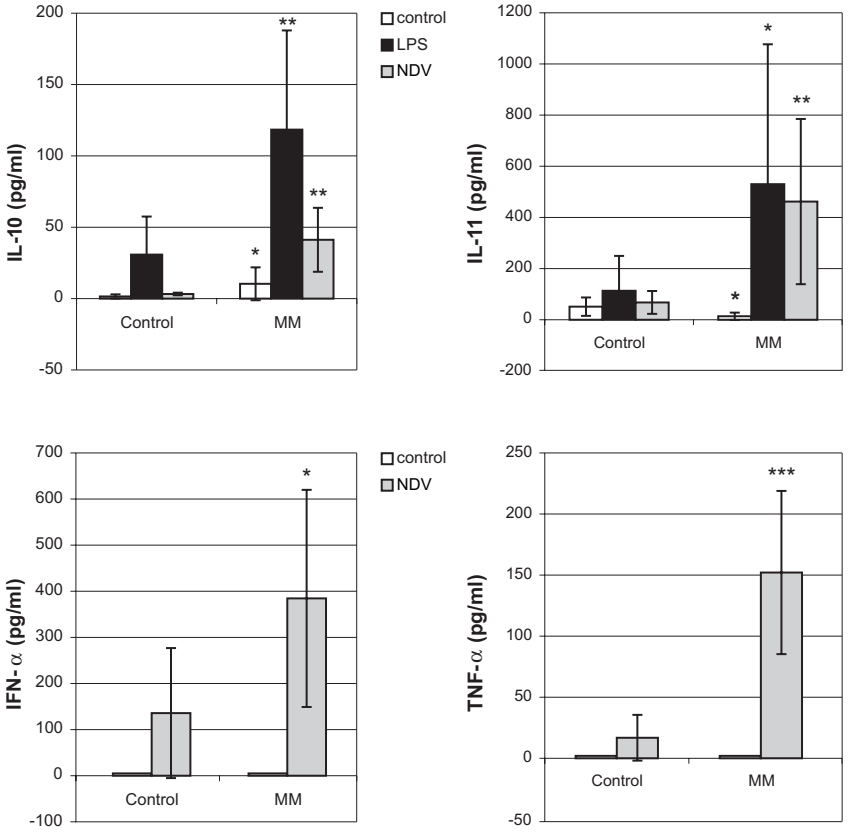

Fig. 4. Cytokine (IL-10, IL-11, IFN-α, TNF-α) production in bone marrow adherent cells spontaneously and after induction with LPS or NDV. Statistically significant in comparison to control at *0.05, **0.01, and ***0.001, in the Mann-Whitney U-test.

other cell types, but not stromal fibroblasts, are involved in the MMP-9 production in our experiments. In supernatants from MM and control cultures of bone marrow adherent cells, gel zymography revealed additional enzyme activity corresponding to the 80-kDa activated form of MMP-9. These results are in agreement with the results of Vacca et al. [30]. Moreover, three supernatants from MM cultures exhibited 28-kDa enzyme activity, probably a latent form of MMP-7. As no correlation with the number of plasma MM cells present in these bone marrow adherent cells was detected, we can suspect that this enzyme activity was not released by MM cells, but by other cells present in the cultures, such as fibroblasts, macrophages, or endothelial cells.

In ELISA, significantly higher levels of MMP-1 and MMP-2 released by MM bone marrow adherent cells were detected compared with control cultures. It has already been reported that BMSCs can secrete MMP-1 and that malignant plasma cell adhesion to stromal cells can upregulate its production [8]. MMP-1 is known to initiate the bone resorption process by degradation of type I collagen, which in a denatured form becomes a substrate for MMP-2, an enzyme involved not only in bone resorption, but also in tumor metastasis [20, 28].

The two forms of stromelysin, MMP-3 and MMP-10, have been reported to take part in bone remodeling. They share 82% sequence homology, but exhibit differences in cellular synthesis and inducibility by cytokines and growth factors. In the developing human bone marrow, active MMP-3 expression has been detected in osteoblasts and in the matrix surrounding osteolytic lacunae, while MMP-10 has been detected mainly in sites of bone resorption. Moreover, in situ zymography has demonstrated that MMP-3 is produced in a matrix-bound proenzyme form and may act as a reservoir for later activation [5]. In our study we did not detect an activated form of MMP-3 by gelatin zymography. However, in ELISA we discovered that cultures of adherent cells derived from MM patients released significantly less MMP-3 into the culture medium. We can only speculate that this phenomenon may be associated with a decreased *in vitro* activity of osteoblasts derived from bone marrow of MM patients and a decreased potential to form bone.

Evidence is growing that a network of cytokines operates in the growth progression and dissemination of multiple myeloma. This network includes IL-1β, IL-6 and its soluble receptor, TNF-α, IL-10, and IL-11 [21]. Each of them seems to be involved in a different manner in the biology of normal and malignant plasma cells. It has been our goal to examine whether bone marrow adherent cells may, due their secretory properties, be involved in the biology of MM cells. In our study we found that bone marrow adherent cells from MM patients released, spontaneously and also after LPS and NDV stimulation, a significantly higher level of IL-10. It is known that IL-10 functions as a growth factor for MM plasma cells due to the modulation of the expression of other cytokines and cytokine receptors. IL-10 has been shown to upregulate the expression of IL-11 receptors on MM cells. Moreover, IL-10 is a potent MM cell growth factor, as well as an inhibitor of IL-6 production. Overproduction of IL-10 by bone marrow adherent cells of MM patients has not yet been described and seems to be an intrinsic property of bone fibroblasts or monocytes/macrophages, as IL-10 levels correlate negatively with plasma cell number in bone marrow [21]. IL-10 has been described as an inhibitory factor for osteogenic differentiation in mouse bone marrow and an inhibitor of collagene type I synthesis as well as of osteocalcin and mineralized ECM. Therefore, overproduction of IL-10 by bone marrow adherent cells of MM patients may result in downregulation of osteoblast formation [31].

In contrast to IL-10, the spontaneous production of IL-11 by bone marrow adherent cells of MM patients in our study was significantly lower in comparison with healthy controls. It has already been described that IL-11 is synthesized in BMSCs and that it is an anti-inflammatory cytokine against the action of IL-1 and TNF-α, both of which are bone resorbance-inducing cytokines [29]. Therefore, decreased spontaneous release of IL-11 may be involved in the pathogenesis of bone loss in multiple myeloma [29]. However, after LPS and NDV induction, bone marrow adherent cells from MM patients released significantly more IL-11 than controls. IL-11 has been also shown to stimulate osteoclast formation, so its overproduction under inflammatory conditions (mimicked by LPS or NDV presence) may be involved in osteoclastogenesis and bone resorption in MM patients.

In multiple myeloma, the role of TNF-α is controversial, as it is able to stimulate both the growth and apoptosis of plasma malignant cells, and it stimulates the expression of receptor-activator NF-κB ligand (RANKL) in stromal cells, which is a fundamental component of inflammatory osteolysis [32, 33]. It has already been shown that bone marrow cultures of patients with bone marrow disease secrete more TNF-α than cultures of patients without bone disease and that TNF-α induces IL-6 release as well as NF-κB activation in BMSCs [1, 16]. Increased release of TNF-α in bone marrow adherent cells of MM patients after LPS and NDV stimulation can be considered as the mechanism by which, under inflammatory conditions, TNF-α may be involved in bone osteolysis. Moreover, it has been shown that TNF-α promotes bone resorption by activating mature osteoclasts and inducing their differentiation. TNF-α has also been described as a potent MM plasma cell growth factor *in vitro* and as a potent inducer of IL-6 production in MM cells [17]. Therefore it can also participate in the development of MM lesions in bones.

IFN-α has been reported to either suppress or promote MM plasma cell growth. Some *in vitro* studies using freshly collected cells from patients or established MM cell lines have shown that IFN-α may stimulate their growth. This effect is probably caused by IFN-α--stimulated autocrine production of IL-6 [18, 19, 21]. Thus, IFN-α used in MM therapy might allow a rapid

emergence of IL-6-producing myeloma subclones and worsen the disease. Such a phenomenon might also occur under inflammatory conditions when IFN-α is overproduced in bone marrow environment and may support the development of new malignant plasma cell subclones. Moreover, it has been found that IFN-α may inhibit hematopoiesis *in vitro*, supported by bone marrow stroma [19]. Thus, IFN-α overproduction under inflammatory conditions may be responsible for hematological abnormalities observed in MM patients.

In summary, bone marrow adherent cell cultures of MM patients, which are composed mainly of fibroblasts, monocytes/macrophages, and endothelial cells, over--secreted MMPs such as MMP-1 and MMP-2 and cytokine IL-10 compared with cultures derived from healthy persons. In contrast, the spontaneous production of IL-11 and secretion of MMP-3 were significantly lower than in the controls. After LPS and NDV induction, bone marrow adherent cells of MM patients over--produced IFN-α, TNF-α, IL-10, and IL-11. The abnormalities observed in metalloproteinase and cytokine production seem to occur not only in malignant plasma cell growth, but also in the process of bone osteolysis in multiple myeloma.

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