LETTER TO THE EDITOR

Neighboring adipocytes participate in the bone marrow microenvironment of multiple myeloma cells

Multiple myeloma (MM) is a hematological malignancy, characterized by the accumulation of monoclonal plasma cells in the bone marrow (BM). MM disease progression has been recently recognized as the result of an evolving crosstalk between different cell types within the BM. Although genetically abnormal plasma cells define the tumor compartment itself, the surrounding and interwoven stroma provides the supporting framework of the tumor. This framework includes extracellular matrix proteins, secreted growth factors and cellular interactions with fibroblasts, macrophages, endothelial cells, osteoblasts and osteoclasts.[1] Little attention has been given to another cell type present in the BM cavity: namely the adipocyte. These are absent in the BM of a new-born individual; however, their number increases with advancing age, resulting in adipocytic deposits occupying up to 70% of the BM cavity in elderly persons. MM is typically a disease of the elderly with a median age of diagnosis of 65 years and the incidence increases with age. Knowing that with advancing age, the BM cavity is filled with adipocytes and that MM cells closely interact with their neighboring cells, we assumed functional interactions between BM adipocytes and MM cells. We studied these interactions using the 5T33MM model and the human MM5.1 cell line. In this study, we further tried to characterize the secreted cytokines and explored the potential role of leptin in mediating the effects of adipocytes. We finally evaluated the expression of leptin receptor on both murine and human MM cells and tried to correlate this with different clinical parameters.

From the observation that MM cells, at interstitial disease stages, can be found in close contact with adipocytes, functional interactions between these cells are reasonable and prompted us to start *in vitro* tests. The murine BM adipocyte cell line 14F1.1 (obtained from Professor Zipori D, Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot, Israel) and primary isolated human adipocytes were used. The 14F1.1 cells initially have a fibroblastoid morphology, but after reaching confluence they start to differentiate towards adipocytes.[2] Addition of an adipogenic cocktail (2 μM insulin, 0.5 mM 3-isobutyl-1-methyl-xanthine and 1 μM dexamethasone) finally results in a homogenous fat cell population (95% of the cells containing large Oil Red O-positive lipid droplets). For confirmation with human MM cells, primary cultured BM adipocytic cells were used. These were isolated from femoral BM samples, obtained from patients undergoing hip surgery at the CHU Hospital (Liege, Belgium) after giving informed consent. Cells from femoral BM biopsies were isolated by digestion with bacterial collagenase for 1 h 30 min at 37°C. Floating adipocytes were separated from hematopoietic and stromal cells (sedimented cells) by centrifugation for 10 min at 700 r.p.m. and then filtered on a 200 μm membrane and washed twice with phosphate-buffered saline.[3]

To investigate the effect of secreted soluble factors on MM cell proliferation, we added normal and concentrated conditioned medium (CM) from BM fibroblasts, BM adipocytes and peripheral adipocytes to 5T33MMvv cells and measured their DNA synthesis in a ^{3}H-thymidine incorporation assay.[4] Under each condition a significant increase (up to two-, threefold) could be detected when compared to control, but there was no difference observed between the BM fibroblasts, peripheral and BM adipocytes (Figure 1a) as stimulants. Direct cell–cell contact between MM and adipocytic cells increased the DNA-synthesis of murine 5T33MM cells about threefold (Figure 1b). These results could be confirmed using human MM5.1 cells and cultured human BM adipocytes (results not shown). Adhesion to the 14F1.1 adipocytic cell line finally protected the 5T33MMvv cells against apoptosis, assessed by FACS analysis of caspase-3 activity (Figure 1c), whereas soluble factors had no effect (results not shown). We subsequently investigated an involvement of adipocytes in cell migration by measuring the cells migrating to concentrated CM from adipocytic and stromal cells using a Transwell (Coster Elscolab, Kruibeke, Belgium) system. A significant increase of migration towards this CM could be seen (Figure 1d), but a similar increase was observed towards peripheral adipocytes or BM fibroblasts. Using the human adipocytic and myeloma cells, we confirmed these effects on MM cell migration (results not shown).

From the previous experiments, it is clear that BM adipocytes secrete both MM growth factors and chemokines. To identify the cytokines secreted by BM adipocytes, we analyzed the supernatant of 14F1.1 cells by multi-analyte profile (MAP) testing (Rules-Based Medicine, Austin, TX, USA). Three independent samples were analyzed in duplicate. The obtained results can be found in Table 1. The s.d. obtained from duplicate measurements of the same sample were on average 7.5%. Interexperimental deviations were however larger, mainly owing to variables like cell passage, level of confluence, concentration efficacy, et cetera. This prompted us to use the obtained results for a qualitative inventory rather than a quantitative one. Looking for cytokines, differentially expressed by adipocytes, we were interested in the adipokine leptin. By showing that the expression of leptin receptor resulted in an inferior response to an initiated therapy with thalidomide, Kumar *et al.*[5] provided a first evidence of the possible importance of the leptin–leptin receptor axis in MM disease. To our knowledge, this growth factor was however not further studied in MM disease. Reverse transcription polymerase chain reaction (RT-PCR) (Figure 2a) was performed for qualitative measurement of leptin expression and demonstrated a specific band of mRNA of the BM adipocytic cells and peripheral fat tissue, whereas this signal could not be detected in the mRNA of 5T33MMvv cells or BM fibroblasts. A highly sensitive enzyme-linked immunosorbent assay (ELISA) (R&D systems, Abingdon, UK) confirmed the secretion of leptin by 14F1.1 cells and 3T3L.1 cells (530 and 603 pg/ml respectively), whereas no leptin could be found in the supernatants of MM cells or fibroblasts. To exert its functions, leptin has to bind to its receptors. In mice, six different leptin receptor isoforms have been described. The long isoform
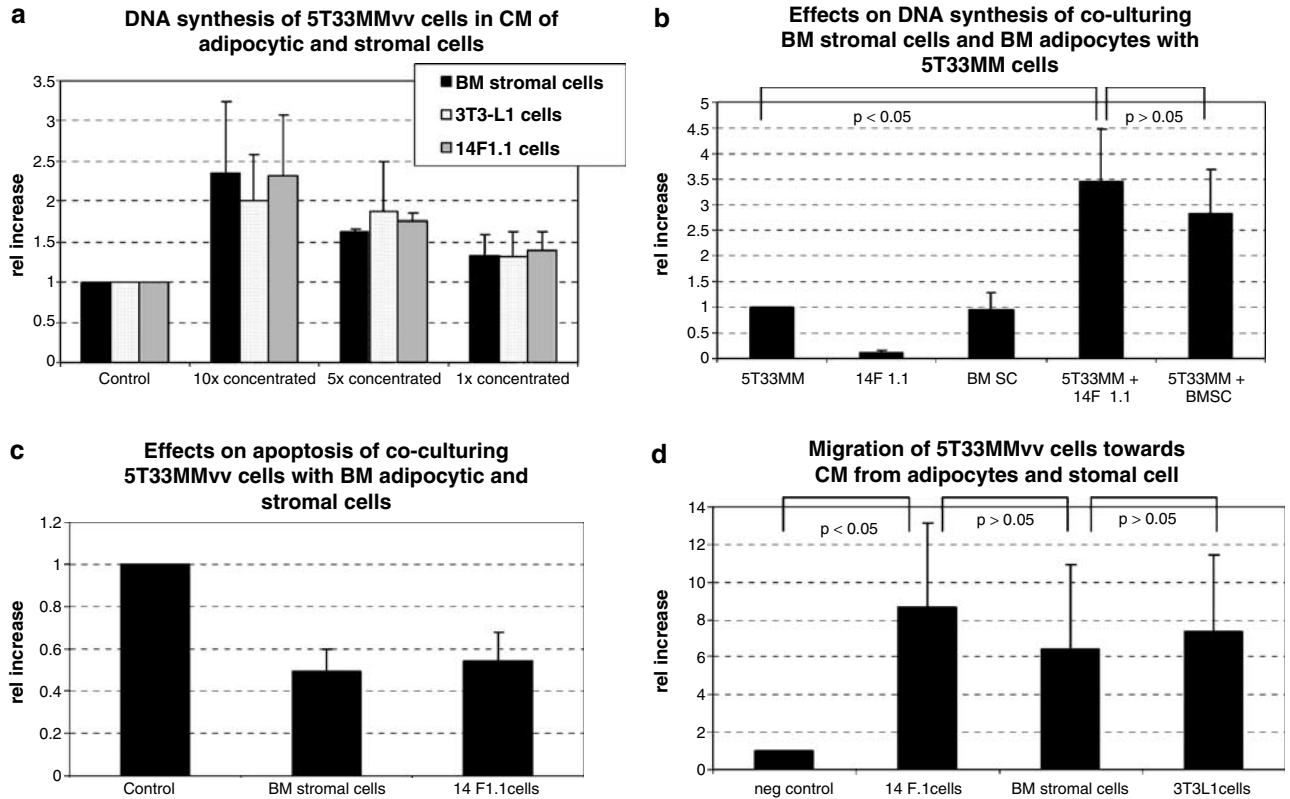
a

DNA synthesis of 5T33MMvv cells in CM of adipocytic and stromal cells

Legend: ■ BM stromal cells, □ 3T3-L1 cells, ▨ 14F1.1 cells

y-axis: rel increase (0 to 3.5)
x-axis: Control, 10x concentrated, 5x concentrated, 1x concentrated

b

Effects on DNA synthesis of co-ulturing BM stromal cells and BM adipocytes with 5T33MM cells

$p < 0.05$, $p > 0.05$
y-axis: rel increase (0 to 5)
x-axis: 5T33MM, 14F 1.1, BM SC, 5T33MM + 14F 1.1, 5T33MM + BMSC

c

Effects on apoptosis of co-culturing 5T33MMvv cells with BM adipocytic and stromal cells

y-axis: rel increase (0 to 1.2)
x-axis: Control, BM stromal cells, 14 F1.1cells

d

Migration of 5T33MMvv cells towards CM from adipocytes and stomal cell

$p < 0.05$, $p > 0.05$, $p > 0.05$
y-axis: rel increase (0 to 14)
x-axis: neg control, 14 F.1cells, BM stromal cells, 3T3L1cells

Figure 1 BM adipocytic cells support proliferation and migration of MM cells whereas they inhibit MM cell apoptosis. The supernatants of the BMSC, 3T3L-1 and 14F1.1 stimulated the proliferation in a same degree and in a dose-dependent manner (**a**). Cell–cell contact between MM and adipocytic cells significantly increased the proliferation of 5T33MM cells (**b**) and protected against apoptosis (**c**), but there was no significant difference between BM adipocytes and BMSC. An enhanced migration of murine MM cells towards 10 times concentrated CM of BM adipocytic cells could be seen (**d**). These assays were performed in triplicate and results are shown as mean±s.d. of three independent tests.

Table 1 Cytokines, growth factors and chemokines secreted by BM stromal cells and BM adipocytes, analyzed by multiplex analysis

	14F1.1 (s.d.)	BMSC (s.d.)
IL-1β	25.1 (3.53)	27.8 (2.83)
IL-6	1.17 (0.44)	9.67 (8.82)
IL-10	21.9 (9.79)	18.9 (7.14)
IL-12p70	12.1 (2.74)	8.5 (0.57)
IL-18	16.7 (4.28)	21.8 (10.1)
Leptin	935.4 (206)	5.2 (1.85)
Oncostatin M	16.1 (1.9)	15.2 (1.45)
Stem cell factor	231.3 (1.61)	22.3 (13.4)
VCAM-1	2911 (633)	4860 (608)
TNF-α	13.72 (0.52)	11.7 (2.3)
VEGF	7346 (6415)	1650 (2007)
FGF-9	146.3 (27)	85.48 (43.2)
FGF-basic	31.1 (5.24)	19.48 (3.9)
LIF	5.44 (1.45)	6.60 (1.2)
MCP-1	1833 (1237)	1547.50 (102.5)
MCP-3	3558 (2012)	2550.00 (989.9)
MIP-1α	5.33 (0.59)	12.50 (3.9)
MIP-1β	12.68 (0.64)	27.25 (23.4)
MIP-1γ	5963 (1701)	888.40 (114)

Abbreviations: BMSC, bone marrow stromal cells; FGF, fibroblast growth factor; IL, interleukin; s.d., standard deviation; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.
Concentrations are given in pg/ml and are the result of three independent analyses.

(OB-Rb) is the one that has complete signaling capabilities and is expressed mainly in the hypothalamus but also on many other cell types. Figure 2a illustrates that both Ob-Ra and OB-Rb sequences were present in the mRNA of 5T33MMvv cells. Since isolated 5T33MMvv cells may contain stromal contamination, a RT-PCR was also performed on 5T33MMvt cells, a clonally identical *in vitro* growing variant and thus 100% pure MM, generating the same results (data not shown). Subsequently, functional studies were performed using recombinant murine leptin. At concentrations from 30–250 ng/ml leptin, there was only a moderate increase (maximum factor: 1.35) in DNA synthesis (Figure 2b), whereas at higher concentrations a decrease in the proliferative response of 5T33MM cells to leptin was noted. This might be explained by a potential down-regulation of the leptin receptor at higher concentrations of leptin. In the same concentration range, interleukin-6 (IL)-6 increased 5T33MMvv cell proliferation by a factor 1.6. Adding leptin to IL-6 or IGF-1, only resulted in additive effects and no synergistic effects (results not shown). To prove the contribution of leptin in the stimulating effects of BM adipocytes on MM cells, 5T33MM cells were incubated with a blocking antibody against leptin receptor, leading to a decrease in the induced DNA synthesis with $19 \pm 7.5\%$ (Figure 2c). To confirm the expression of the leptin receptor on human MM cells, we performed immunohistochemical stainings on sections of 50 different BM biopsies from newly diagnosed MM patients. We
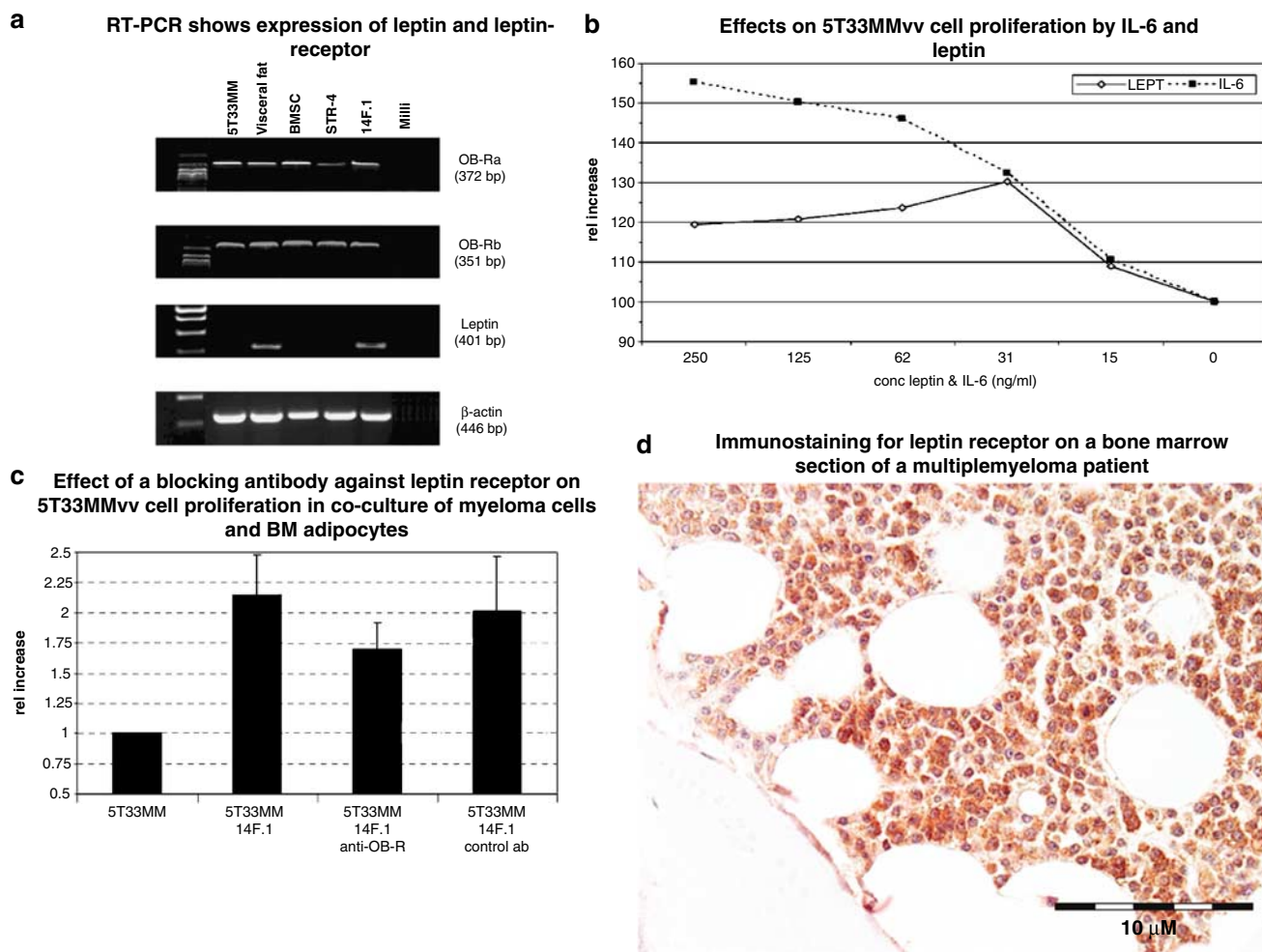
a RT-PCR shows expression of leptin and leptin-receptor

b Effects on 5T33MMvv cell proliferation by IL-6 and leptin

c Effect of a blocking antibody against leptin receptor on 5T33MMvv cell proliferation in co-culture of myeloma cells and BM adipocytes

d Immunostaining for leptin receptor on a bone marrow section of a multiplemyeloma patient

Figure 2 Presence of leptin receptor on 5T33MM*vv* cells and effects of leptin on MM cell proliferation. (**a**) RT-PCR confirmed the expression of leptin by BM and peripheral fat cells (third row). Both short (first row) and long isoform (second row) of leptin receptor were expressed by 5T33MM*vv* cells as well as BM endothelial and BM adipocytes. (**b**) The addition of recombinant leptin resulted in a small but significant increase in 5T33MM cell proliferation (factor 1.3) is illustrated. To proof the contribution of leptin in the stimulating effects of BM adipocytes on MM cells, 5T33MM cells were incubated with a blocking antibody against leptin receptor (**c**). This incubation decreased the induced DNA synthesis with $19 \pm 7.5\%$) (**d**) illustrates the positive staining for OB-R upon MM cells. Here MM cells are surrounded by adipocytes.

observed strong Ob-R immunoactivity in MM cells in 50% of the biopsies. Figure 2d illustrates a positive staining in a BM section. Expression of the leptin receptor could not be correlated to different prognostic parameters such as Salmon & Durie staging, β2-microglobulin, CRP, sedimentation rate, renal failure or global survival. Kaplan–Meier analysis of the progression free survival after the first therapy showed that the different times to progression tended to differ between the groups ($P = 0.090$). Patients with a weak expression of leptin receptor had a longer time to progression (25.33 months, s.d. = 5.44) compared to patients with a strong leptin receptor (16.55 months, s.d. 2.66). An analysis of the patient's response to a given treatment showed a higher response (complete or partial) rate in patients with a weak OB-R expression (66%) compared to patients with strong OB-R expression (44%) ($P = 0.170$). There was no significant difference in first-line treatment between the two subgroups.

From the results obtained in the current study, we conclude that BM adipocytes are able to contribute in the BM microenvironment of MM disease by affecting proliferation, apoptosis and migration. These effects were comparable to those of BM fibroblasts, suggesting that BM adipocytes have equivalent functions as fibroblasts. As MM cells further invade the BM and have a diffuse growth pattern, BM adipocytes however disappear during disease development, whereas other stromal cells (endothelial cells, fibroblasts) remain present and are activated. These results suggest that the role of BM adipocytes is mainly restricted to the initial stages of the disease before a remodeling of the BM microenvironment has occurred. The expression of different growth factors, chemokines and angiogenic factors, known to be involved in MM disease development further strengthens this hypothesis of a possible involvement of adipocytes in MM disease.

In our experiments, no significant difference could be found between the stimulatory effects of CM from peripheral or BM adipocytic cells. A hallmark of MM cells is their predominant localization in the BM of the patients during the main course of their disease. Our group earlier showed that this selective presence of MM cells in the marrow is owing to a combination of a selective homing to and a selective survival and growth in the BM.[6] Because of this restricted distribution of MM cells to the BM microenvironment, the interactions of MM cells in close

contact to BM adipocytes are likely to be more important than interactions with adipocytic cells in peripheral organs and than the systemic circulation of lower concentrations of adipokines. To our knowledge, no MM growth has been reported in the peripheral fat tissue.

Adipocytes secrete specific cytokines, playing a central role in different biological and physiological processes. Leptin, a product of the obese gene, is such a multifunctional cytokine predominantly produced by adipocytes. In recent years, different studies reported the effects of leptin on cancer cell growth, migration and invasion, suggesting that this hormone is capable of promoting an aggressive cancer phenotype. By RT-PCR and ELISA, we showed that adipocytes were the only cells within the MM microenvironment that secreted leptin. Leptin was earlier demonstrated as a cytokine that supported leukemic development in different models. In the biology of MM disease, a possible functional involvement of leptin was not published hitherto. In the interactions between BM adipocytes and MM cells, leptin does not seem to be a predominant growth factor, as the addition of leptin resulted only in a minor increase of MM cell proliferation and addition of a neutralizing antibody against leptin receptor in cocultures of 5T33MM cells and BM adipocytes, only partially inhibited the proliferation induced by adipocytes. These results suggest that other growth factors, secreted by adipocytes are equally or even more important. MAP profile showed that BM adipocytes secrete growth factors produced by other stromal cells and known to be involved in MM disease, such as vascular endothelial growth factor, basic fibroblast growth factor , stem cell factor, VCAM-1, IL-1β, IL-6, IL-10 and IL-12, oncostatin-M and tumor necrosis factor alpha (TNF)-α. To identify the pivotal cytokines in the interactions between BM adipocytes and MM cells, further investigations are warranted.

Two recent studies already described the involvement of leptin and leptin receptor in MM disease. A first study indicated that leptin serum levels were increased in MM patients at diagnosis compared to control patients. Although leptin levels did not increase with advancing MM stages, leptin levels decreased following treatment.[7] Recent gene expression studies revealed that expression of leptin receptor on MM cells was predictive for a patients' response to a treatment with thalidomide.[5] In our patient population, we found a heterogeneous expression of leptin receptor, but we were not able to find a significant correlation between leptin receptor expression and disease state, prognostic parameters or overall survival. However, patients with weak leptin receptor expression tended to have a longer progression-free survival compared to patients with strong leptin expression.

In summary, we believe that BM adipocytes should no longer be considered as passive bystander cells, as they are able to contribute in the BM microenvironment of MM disease by affecting proliferation, apoptosis and migration. Leptin takes part in these processes by affecting proliferation. These results strengthen the hypothesis that BM adipocytes are metabolically active cells that participate in the different biological processes within the BM.[8]

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