Bioactivity and prognostic significance of growth differentiation factor GDF15 secreted by bone marrow mesenchymal stem cells in multiple myeloma

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Abstract

Overexpression of growth differentiation factor 15 (GDF15) by bone marrow mesenchymal stem cells occurs widely in patients with multiple myeloma (MM), but the pathophysiological effects of GDF15 in this setting remain undefined. GDF15 has been described in numerous solid tumors but never in haematological malignancies. In this study, we report that GDF15 significantly increases survival of stroma-dependent MM cells including primary MM cells. In particular, GDF15 conferred resistance to melphalan, bortezomib, and to a lesser extent, lenalidomide in both stroma-dependent and stroma-independent MM cells. Akt-dependent signaling was critical to mediate the effects of GDF15, whereas Src and ERK1/2 signaling pathways were not involved. Given these results, we tested the clinical significance of plasma concentrations of GDF15 (pGDF15) in 131 MM patients and found that it correlated with disease prognosis. Specifically, patients with high levels of pGDF15 had lower probabilities of event-free and overall survival 30 months after diagnosis, compared to patients with low pGDF15 levels. Our findings suggest that tumor microenvironment-derived GDF15 is a key survival and chemoprotective factor for MM cells which is pathophysiologically linked to both initial parameters of the disease as well as patient survival.
Introduction

Multiple myeloma (MM) is a clonal plasma-cell malignant disease that accounts for 13% of hematological cancers (1). The disease is fatal and the median survival is 5 years (2), but this survival has significantly increased with the introduction of novel therapies, (3) with some patients surviving more than 10 years (4). Prognostic factors reflecting tumor burden, tumor damage in organs and tissues, and intrinsic characteristics including genetic abnormalities have been described, but so far, none of these specifically reflect the MM microenvironment (5-12).

Nevertheless, MM has been a prototypic disease model for the study of interactions between the microenvironment and malignant cells (13) and has led to the development of novel drugs such as immunomodulatory drugs and proteasome inhibitors, which target not only MM cells but also their microenvironment (14). A better understanding of the mechanism by which the MM microenvironment affects the disease is still required to define new therapeutic targets.

MM cells proliferate in close contact with cells from the bone marrow (BM) microenvironment. BM mesenchymal stem cells (BM-MSCs) are the only long-lived cells of the BM microenvironment. They secrete survival factors for MM cells and cytokines that promote osteoclastogenesis and angiogenesis (15-18). Abnormalities of BM-MSCs have recently been reported (19-21). In patients with newly diagnosed MM, we reported that BM-MSCs grown without MM cells had an abnormal gene expression profile: 145 genes were differentially expressed between BM-MSCs from MM patients and normal subjects. In particular, we found that growth differentiation factor 15 (GDF15) was increased in BM-MSCs from MM patients (21).

GDF15 is a 40-kDa pro-peptide that is cleaved to release a 25-kDa circulating protein (22). It was first described as a divergent member of the human TGF-β superfamily (23). GDF15 has a broad activity, as indicated by the diversity of the nomenclature (MIC-1, PTGF-β, PLAB, PDF, NAG-1, PL74). In healthy subjects, GDF15 is highly expressed in the placenta, with serum concentration increasing during pregnancy (24, 25). GDF15 concentration is notably increased in the serum of patients with glioma, prostate, colorectal or pancreatic cancer (26-29), and the factor was recently described as belonging to a series of 20 biomarkers that best define the malignant phenotype of numerous tumors (30). Understanding the biological role of GDF15 in cancer growth is challenging. Some studies have documented its pro-tumorigenic role (26, 28-32), and
others have shown anti-tumorigenic activity (22, 33, 34). The role of GDF15 in haematological malignancies has not yet been documented.

To determine the potential impact of GDF15 overexpression by BM-MSCs from MM patients, we assessed the bioactivity of GDF15 on MM cells under serum-free culture conditions. We sought correlates of plasma concentration of GDF15 (pGDF15) in MM patients with biological and clinical parameters of the disease. We found that GDF15 is a survival and chemoprotective factor for MM cells and show correlates of pGDF15 to initial parameters of the disease and to patient survival.

**Materials and Methods**

**Cell samples**
The MOLP-6 stroma-dependent MM cell line was a generous gift from Dr. Harashima (35) (Fujisaki Cell Center, Hayashibara Biochemical Laboratories, Okayama, Japan) in 2007. Cryopreserved MOLP-6 cells were defrosted and grown on BM-MSCs in RPMI-1640 medium with 10% fetal calf serum and 10 µg/mL ciprofloxacin. BM-MSCs were obtained from healthy BM by culturing cells harvested from filters used to remove ossicles and aggregates from the allograft, and prepared as previously described (21). The MM1.S stroma-independent MM cell line was purchased from ATCC in 2009. Cryopreserved MM1.S cells were defrosted and grown at 37°C and 5% CO2 in RPMI-1640 medium with 10% fetal calf serum and 10 µg/mL ciprofloxacin. These two MM cell lines were tested and authenticated before starting and during the study (morphologic analysis, immunophenotype, caryotype, stroma-dependence, mycoplasma and EBV detection). BM from 4 patients with newly diagnosed MM was obtained by sternal puncture after patients gave their written informed consent. Primary BM myeloma cells were purified with use of magnetic anti-human CD138 microbeads (Miltenyi Biotec), and MM BM-MSCs were obtained from the CD138-negative fraction and prepared as previously described (21).

**Cell cultures**
All culture experiments were performed in Syn H serum-free culture medium, an Iscove-based fully defined culture medium containing human albumin without insulin (ABCell-Bio) (12).

**Cell survival.** MOLP-6, MM1.S and primary MM cells were plated at 50 x 10³ cells/mL with 0, 10, 100 or 200 ng/mL recombinant human GDF15 (R&D systems). Cells were grown at 37°C and 5% CO₂. At day 1 of culture, viable cells were counted by Trypan blue exclusion assay. In a separate set of experiments, MOLP-6 cells were pre-treated overnight with an Akt-1/2 inhibitor (124018) at 1 µM (Merck) before adding 200 ng/mL GDF15 for 24 hr.

**Chemoprotection assay.** MOLP-6 and MM1.S cells were plated at 50 x 10³ cells/mL with or without 200 ng/mL GDF15 for 24 hr. Cells were grown at 37°C and 5% CO₂. The following drugs were added to the cell cultures for one day: 8 µM melphalan (Sigma), 8 nM bortezomib (Millennium Pharmaceuticals) or 100 µM lenalidomide (Celgene). Appropriate controls (cells treated with dimethyl sulfoxide or buffer alone) were included. In a separate set of experiments, both MM cell lines were pre-treated overnight with an Akt-1/2 inhibitor (124018) at 1 µM before applying the above mentioned procedure. Viable cells were counted by Trypan blue exclusion assay. Percentage cell survival in cells pre-treated with GDF15 +/- Akti versus non-pre-treated cells was obtained, and percentage of control cell survival (without Akti or GDF15 pre-treatment and without any drugs) was then calculated.

**Akt phosphorylation**

MOLP-6, MM1.S and primary MM cells were treated with 20 ng/mL GDF15 (R&D systems) or 50 ng/mL recombinant human interleukin 6 (IL-6) (R&D systems) in serum-free medium for 15 min (a set of experiments was performed in 10% calf serum conditions). Cells were fixed for 15 min, washed, permeabilized for 30 min with use of an intracellular stain kit (BD Pharmingen) and stained with a PE-conjugated anti-pT308 or S473 Akt MoAb (BD Biosciences) for 30 min. Akt phosphorylation was detected by flow cytometry (EPICS XL-MCL, Beckman Coulter). Live cells, which could be clearly distinguished in forward versus sideways scatter plots, were gated, and the median fluorescence intensity (MFI) was determined. Fold changes of MFI for each condition were defined as the fraction (MFI of measured sample) / (MFI of isotype control). A set of experiments with an Akt-1/2 inhibitor (124018, 1 µM) was performed to verify inhibition of Akt phosphorylation.
Src and ERK1/2 phosphorylation
MOLP-6 and MM1.S cells were treated with 20 ng/mL GDF15 (R&D systems) in serum-free medium for 15 min. Cells were fixed for 15 min, washed, permeabilized for 30 min with use of an intracellular stain kit (BD Pharmingen) and stained with an Alexa-Fluor 488-conjugated anti-pY416 Src (Millipore) for 60 min or PE-conjugated anti-pT202/pY204 ERK1/2 MoAb (BD Biosciences) for 30 min. Src or ERK1/2 phosphorylation was detected by flow cytometry (EPICS XL-MCL).

Patient and healthy individual plasma samples
Bone marrow plasma. BM of 24 patients with newly diagnosed MM was obtained by sternal puncture after patients gave their written informed consent. BM from 5 healthy individuals was obtained from the remaining sample used for quality control during the preparation of allogeneic hematopoietic grafts. BM plasma was obtained after centrifugation of BM. The median age of patients with MM and healthy subjects was 57±10 years and 45±13 years, and the sex ratio (M:F) 1.5 and 1.3, respectively.

Blood plasma. Peripheral blood plasma from 131 patients with newly diagnosed MM was obtained after patients gave their written informed consent. Patients' characteristics are in Table 1. The median age of patients was 59±10 years, and the sex ratio (M:F) 1.4. These 131 patients were enrolled in different Intergroupe Françophone du Myélome treatment trials: 53 received conventional chemotherapy, including melphalan and prednisone, and 78 high-dose chemotherapy. Patients with Durie Salmon disease stage I were symptomatic and thus treated. Blood plasma from 13 healthy volunteers was also collected after written agreement; the median age was 52±3 years and the sex ratio (M:F) 1.2.

GDF15 measurement
Quantification of GDF15 in supernatants and plasma involved use of DuoSet ELISA for human GDF15 (R&D systems) following the manufacturer’s protocol. All samples were assayed in duplicate. Intra-assay reproducibility was evaluated in 3 independent experiments. Each assay involved 6 replicates of 3 plasma samples containing low, median and high values of GDF15. The calculated overall intra-assay coefficient of variation was 11%, 7% and 7%, respectively. Inter-assay reproducibility was evaluated in 5 independent experiments. Each assay involved a
duplicate of the same 3 plasma samples containing low, median and high values of GDF15. The calculated overall inter-assay coefficient of variation was 15%, 4% and 4%, respectively.

**Statistical analysis**
Statistical analysis of categorical variables was tested with the chi-square test or Fisher’s exact test and that of continuous variables with the Student’s t test, then Mann-Whitney U test. All tests were two-tailed. Correlation between pGDF15 level and initial biological parameters was tested by Spearman correlation. A P <0.05 was considered statistically significant.

Survival was evaluated for the 131 MM patients by pGDF15 level. The threshold value of pGDF15 (0.50 ng/mL), defining low and high levels, was defined as mean + 3SD of pGDF15 of healthy subjects. The duration of event-free survival (EFS) was calculated for all patients from the date of diagnosis to the time of disease progression, relapse, or death. Kaplan-Meier curves for EFS and overall survival (OS) were plotted and compared by the log-rank test. Prognostic factors for survival were determined by the Cox proportional-hazards model for univariate and multivariate analysis. As possible prognostic factors, age, International Staging System (ISS) (7), β2-microglobulin level, haemoglobin level, lactate deshydrogenase (LDH) activity, C-reactive protein (CRP) level, and presence or absence of deletion of chromosome 13 (del13) were included in the regression model. For continuous variables, classical cut-off values were selected.

**Results**

**GDF15 increases survival of a stroma-dependent MM cell line and primary MM cells**
We first measured the survival of a stroma-dependent (MOLP-6) and a stroma-independent (MM1.S) MM cell lines, and primary MM cells under serum-free conditions supplemented with titrated concentrations of GDF15. Without stromal cells, only 43.9% ± 3.1% of MOLP-6 cells and 51.2% ± 5.8% of primary MM cells survived at day 1 of culture, whereas 77.4% ± 10.2% of MM1.S cells survived. GDF15 increased the MOLP-6 cell and the primary MM cells survival: 200 ng/mL of GDF15 yielded up to 70.3 ± 5.6% viable MOLP-6 cells (P< 0.005) and 74.0 ± 5.9% viable primary MM cells (P< 0.05). By contrast, GDF15 did not significantly increase
MM1.S cell survival: 89.7 ± 10.2% of MM1.S cells survived with 200 ng/mL of GDF15 ($P= 0.2$) (Fig. 1).

**GDF15 triggers Akt phosphorylation in a stroma-dependent MM cell line and primary MM cells**

MM cell survival involves intracellular signaling cascades such as the Akt pathway (36). We thus wondered whether GDF15 triggered the Akt phosphorylation on T308 and S473 residues from MOLP-6 and MM1.S cells and from purified primary MM cells from 4 patients in serum-free culture conditions. Intracellular immunostaining followed by flow cytometry showed that GDF15 could trigger T308 and S473 Akt phosphorylation in MOLP-6 cells ($P < 0.0005$ and $P < 0.05$, respectively), whereas treatment with an IL-6 control did not (Fig. 2A). GDF15 was still effective on Akt phosphorylation in serum conditions (Supplementary Fig.1). By contrast, neither GDF15 nor IL-6 was able to induce phospho-Akt T308 and S473 in MM1.S cells (Fig. 2C), reflecting their constitutive activation of Akt (Supplementary Fig.2). In primary MM cells, GDF15 induced T308 and, although to a lower extent, S473 Akt phosphorylation ($P < 0.05$ and non-significant, respectively), whereas IL-6 induced only T308 phosphorylation (Fig. 2B). Therefore, GDF15 enhances Akt phosphorylation and activity in MOLP-6 and primary MM cells but not MM1.S cells. Overnight pre-treatment of MOLP-6 cells with an Akt-1/2 inhibitor (124018, 1 µM) inhibited GDF15-induced phospho-Akt (Fig. 3A) and abrogated the GDF15-induced survival increase ($P < 0.05$) (Fig. 3B).

Of note, GDF15 didn’t induce phosphorylation of Src and ERK1/2 in both MM cell lines (Supplementary Fig.3 and 4).

**GDF15 confers drug resistance to melphalan, bortezomib and lenalidomide in a stroma-dependent and stroma-independent MM cell line**

Using the same culture conditions as above, we asked whether GDF15 was chemoprotective against drugs classically used in MM treatment. DMSO alone did not affect MM cell survival (data not shown). In drug-treated cultures, the proportion of control MOLP-6 cell survival was increased when the cells were pre-treated with GDF15 (melphalan: 70.0 ± 10.1% vs. 48.8 ± 6.6%, $P < 0.01$; bortezomib: 91.0 ± 10.6% vs. 67.6 ± 7.5%, $P < 0.01$; lenalidomide: 43.0 ± 7.6% vs. 31.1 ± 3.8%, non-significant) (Fig. 4A). Similar results were obtained with MM1S cells (melphalan: 80.0 ± 24.3% vs. 30.0 ± 6.7%, $P < 0.01$; bortezomib: 81.5 ± 17.5% vs. 54.0 ± 8.0%,
P< 0.05; lenalidomide: 20.7 ± 7.8% vs. 5.6 ± 1.4%, non-significant) (Fig. 4B). Thus, GDF15 decreases chemotherapy-induced cytotoxicity of the 3 drugs in both MM cell lines. Overnight pre-treatment of MOLP-6 cells with an Akt-1/2 inhibitor (124018, 1 µM) tended to abrogate the GDF15-induced drug resistance (melphalan: 52.4 ± 5.7% vs. 70.0 ± 10.1%, P<0.05; bortezomib: 69.4 ± 1.4% vs. 91.0 ± 10.6%, P< 0.05; lenalidomide: 32.7 ± 6.3% vs. 43.0 ± 7.6%, non-significant) (Fig. 4A). On the contrary, Akt-1/2 inhibitor had no significant effect on the GDF15-induced drug resistance for MM1.S cells (Fig. 4B).

**GDF15 is not produced by MM cells themselves**

Because GDF15 has been described to be produced by tumors cells themselves in solid cancer, we measured simultaneously the concentration of GDF15 in supernatants of primary BM-MSCs and MM cells from 3 patients with newly diagnosed myeloma. Whereas the concentration of GDF15 ranged from 4.10^{-3} to 8.10^{-3} pg/cell for their BM-MSCs, we didn’t detect any GDF15 in the corresponding MM cells supernatants. We found similar results with both MM cell lines, MOLP-6 and MM1.S cells (Supplementary Fig. 5). Hence GDF15 is a specific factor of microenvironment in myeloma.

**Plasma concentration of GDF15 (pGDF15) increases with MM disease stage**

Because GDF15 is oversecreted by BM-MSCs from MM patients relative to healthy subjects (21) and confers in vitro survival and chemoresistance to MM cells, we next wondered whether the concentration of GDF15 was also higher in BM plasma from MM patients than from healthy subjects. We measured the concentration of GDF15 in BM plasma from MM patients (n= 16) and healthy subjects (n=5), and found it significantly higher in MM patients (0.95 ± 0.92 ng/mL vs. 0.41 ± 0.19 ng/mL; P< 0.001). Among patients with MM, the mean concentration of GDF15 was significantly higher for the 9 patients with ISS stage II and III disease (1.26 ± 0.16 ng/mL) than for the 7 patients with stage I disease (0.57 ± 0.20 ng/mL) (P< 0.001) (Fig. 5A).

In order to study the concentration of GDF15 in a larger cohort of subjects, and because blood samples are easier to collect than BM samples, we next measured the concentrations of GDF15 in BM and blood plasma in 24 patients with MM. We found a correlation coefficient of 0.98 (Supplementary Fig. 6), so for the rest of the study, we tested only blood plasma.
The plasma concentration of GDF15 (pGDF15) was measured in blood from 131 additional patients with newly diagnosed MM and 13 healthy subjects. Demographic and clinical characteristics for the MM patients are in Table 1. Mean pGDF15 was significantly higher for MM patients (0.90 ± 1.10 ng/mL) than for healthy subjects (0.25 ± 0.08 ng/mL) (P < 0.001). In addition, it was significantly higher for patients with ISS stage III disease (2.10 ± 2.04 ng/mL) than stage II disease (0.81 ± 0.47 ng/mL) (P < 0.003) and for patients with stage II disease than stage I disease (0.49 ± 0.24 ng/mL) (P < 0.001) (Fig. 5B).

**pGDF15 level is correlated with initial MM parameters**

For the 131 patients with MM, we searched for a correlation between pGDF15 and initial biological factors of the disease. We found a significant positive correlation between pGDF15 level and β2 microglobulin level, creatinemia, calcemia and serum monoclonal protein level and a significant inverse correlation with levels of haemoglobin and albumin. pGDF15 level was not correlated with BM plasma cell frequency, LDH activity or CRP level (Supplementary Fig. 7).

Mean pGDF15 was calculated according to Durie Salmon (DS) disease stage, presence or absence of del13 and bone status. Mean pGDF15 was significantly higher in patients with DS stage III than DS stage II or I (1.31 ± 1.55 vs. 0.68 ± 0.38 or 0.53 ± 0.37 ng/mL, P < 0.02). Mean pGDF15 was also significantly higher in patients with than without del13 (1.13 ± 1.41 vs. 0.64 ± 0.38 ng/mL, P < 0.004). Finally, pGDF15 was significantly higher in patients with than without osteolytic lesions (1.05 ± 1.26 vs. 0.66 ± 0.46 ng/mL, P < 0.02).

**pGDF15 level is correlated with survival**

Survival was evaluated for the 131 patients with MM by pGDF15. Among the 131 patients, 50 had pGDF15 < 0.50 ng/mL (mean 0.33 ± 0.10; “pGDF15<sub>low</sub> patients”) and 81 had pGDF15 ≥ 0.50 ng/mL (mean 1.25 ± 1.26; “pGDF15<sub>high</sub> patients”). For the 131 patients with MM, the median follow-up from time of diagnosis was 27 months (range 1 to 60 months). The median duration of EFS was 33 months (range 1 to 60 months) and the median duration of OS was not reached. For the 50 pGDF15<sub>low</sub> patients, the median duration of EFS was 39 months (range 5 to 60 months), and the probability of EFS and OS 30 months after diagnosis was 80% and 97%, respectively. Among the 81 pGDF15<sub>high</sub> patients, the median duration of EFS was 29 months (range 1 to 60 months) and the probability of EFS and OS 30 months after diagnosis was 50%
and 75%, respectively. EFS and OS were significantly longer for pGDF15\textsuperscript{low} patients ($P<0.0045$ and $P<0.013$, respectively) (Fig. 6). None of the 26 patients with pGDF15 $<0.34$ ng/mL died, and median duration of EFS for these patients was $>60$ months; among these patients were 18 with ISS disease stage I and 8 stage II, as well as 11 with DS stage I, 5 stage II and 10 stage III (data not shown).

**Prognostic factors of event-free survival**

We analysed the impact of prognostic factors (age, ISS stage, $\beta$2-microglobulin level, haemoglobin level, LDH activity, CRP level, and presence or absence of del13) on EFS for the 131 patients with MM. On univariate analysis, EFS was significantly related to pGDF15 ($P=0.003$), age ($P=0.003$) and $\beta$2-microglobulin level ($P=0.02$). On multivariate analysis, EFS was related to only pGDF15 ($P=0.04$) and age ($P=0.001$).

**Discussion**

Our study demonstrates that functionally GDF15 is a survival and chemoprotective factor for MM cells, and that clinically pGDF15 is related to initial parameters of the disease and survival. This study provides new insights into the mechanism by which the abnormal MM microenvironment affects the pathophysiology and the prognosis of MM. Here, we investigated the significance of one of MM microenvironment dysfunctions, the overexpression of GDF15 by BM-MSCs (21). Although the overexpression of GDF15 has been described in numerous solid malignancies (26, 29, 32, 37), its precise implication in tumors remains unknown. GDF15 may have different functions in cancer, such as being a tumor suppressor during early oncogenesis but a promoter at later stages (22, 38). GDF15 has never been studied in haematological malignancies.

Here, we first determined the role of GDF15 on MM cell survival *in vitro*. This involved experiments under serum-free conditions to avoid components present in serum, in particular insulin-like growth factor type 1 (IGF-1), which might confound interpretation of the results (12). GDF15 increased cell survival in the MOLP-6 stroma-dependent MM cell line, confirming our previous work (21) but in more stringent culture conditions. Moreover, GDF15 was able to
significantly increase cell survival in primary MM cells. Although these results contrast with GDF15-induced apoptosis of prostate cancer cells (33), they are notably consistent with those of GDF15 supporting malignant glioma cell proliferation (26). We previously reported that BM-MSCs from patients with MM induced an overgrowth of MOLP-6 cells when compared to BM-MSCs from normal subjects (21). To determine if the overproduction of GDF15 by MM BM-MSCs is involved in this overgrowth, we performed MOLP-6 cells and MM BM-MSCs coculture experiments involving MSCs from 4 patients with MM that were transfected either with a control plasmide, or with a shRNA targeting GDF15. The number of MOLP-6 cells after 7 days of co-culture was significantly decreased ($P<0.05$) when MSCs were transfected with shRNA GDF15 (Supplementary Fig. 8). Although this decrease is moderate, it suggests that the overproduction of GDF15 participates to the MOLP-6 overgrowth we previously observed with MM BM-MSCs. We did not find any significant survival advantage with GDF15 in the MM1.S stroma-independent MM cell line. Of note, no overgrowth of stroma-independent cell line had been observed with MM BM-MSCs in our previous work (21). Interestingly, GDF15 induced Akt phosphorylation in MOLP-6 and primary MM cells but not in MM1.S cells. In addition, pre-treating MOLP-6 cells with an Akti-1/2 inhibitor abrogated GDF15-induced survival increase. These results support the presence of an Akt-dependent survival mechanism, which is consistent with the observation from Kim et al. (31), who found that GDF15 activates Akt signaling in human breast and gastric cancer cells. The contribution of the Akt pathway to the growth of MM cells has been extensively studied (36, 39-41). Functionally, the Akt pathway is implicated in cell-cycle and apoptosis regulation in MM cells. Akt is phosphorylated in BM biopsies from MM patients, notably because of BM microenvironment-derived cytokines such as IL-6 or IGF-1 (42). Our data indicate that GDF15 also contribute to triggering the Akt pathway in MM cells. The absence of IL-6 effect on Akt signaling in MOLP-6 cells was expected since IL-6 has no bioactivity on their survival (35). Its marginal effect in primary MM cells in our serum-free conditions is due to the fact that the bioactivity of this cytokine is highly dependent on IGF-1 presence (12). The lack of significant effect of IL-6 and GDF15 on MM1.S stroma-independent cells survival reflects constitutive activation of Akt (41). Apart from Akt, GDF15 has been described to activate ERK1/2 in a SNU-216 gastric cancer cells (31) and Src in SK-BR-3 breast cancer cells (43). In our culture conditions, GDF15 couldn’t induce phosphorylation of ERK1/2
in both MM cell lines, suggesting that the Ras/Raf/MEK/MAPK pathway wouldn’t be involved. In addition, GDF15 had no bioactivity on Src in MOLP-6 and MM1.S cells.

GDF15 conferred drug resistance in MOLP-6 and MM1.S cells to three drugs classically used in MM treatment (melphalan, bortezomib and, to a lower extent, lenalidomide). These data are consistent with previous observations (44) showing that GDF15 protects prostate cancer cells against the cytotoxic effect of docetaxel and mitoxanthrone. Because the GDF15-mediated chemoprotection of MOLP-6 cells was abrogated when the cells were pre-treated with an Akti-1/2 inhibitor, we suggest the presence of an Akt-dependent chemoprotection mechanism. For MM1.S cell line, this effect might be Akt-independent. Other signaling cascades activated by cytokines or growth factors in MM cells may be involved, including JAK/Stat3, Wnt, NFkB pathways (1, 14) and underlying other cellular processes which might influence chemoresistance (drug efflux, cell death, genotoxic sensing, DNA repair, proteasome activity…). The differences of biological profile between MOLP-6 and MM1.S cells and the similarities between MOLP-6 and primary MM cells pinpoint the importance of the stroma dependence of MM cell lines chosen as in vitro models to study the impact of microenvironment on tumour.

We next showed that GDF15 is increased in BM plasma from MM patients. Although our previous studies indicated that this increase reflected GDF15 overproduction by MM BM-MSCs, macrophages might also contribute to total GDF15 level. Macrophages are able to secrete GDF15 (23) and constitute abundant components of MM microenvironment, able to protect MM cells against drug-induced apoptosis (45). However, as compared with its production in solid tumors, GDF15 is not produced by the malignant MM cells themselves but specifically by their microenvironment.

Although GDF15 has been described in many solid tumors, a lot remains to be uncovered on its biology; in particular GDF15 receptor is still unknown today. There is some evidence for SMAD pathway activation, suggesting GDF15 may act through a TGF-β superfamily (46). A recent study identified GDF15 as an acute phase modifier of CCR2/TGF-βRII-dependent inflammatory responses to vascular injury (47). On the other side, Kim and al. elegantly demonstrated that GDF15 induces the transactivation of ErbB2 tyrosine kinase in SK-BR-3 breast and SNU-216 gastric cancer cells (31). We didn’t find any expression of TGF-βRII or ErbB2 on both MM cell lines and primary MM cells (data not shown), suggesting that GDF15 receptor also remains to be discovered in MM.
In order to determine whether the GDF15 concentration level increase was indicative of the severity of the disease in MM patients, and because we found that the concentrations of GDF15 in BM and blood plasma in 24 MM patients were correlated, we next measured the plasma concentration of GDF15 (pGDF15) in 131 patients with newly diagnosed MM. The pGDF15 level increase was correlated with prognosis, as was reported for patients with prostate, colorectal and endometrial cancers (27, 28, 48). Finally, we found a strong relation between pGDF15 level and survival to 30 months in MM patients.

This study allows to gain a better understanding into the mechanism by which the abnormal microenvironment affects the pathophysiology and the prognosis of MM. Microenvironment has become a therapeutic target that cannot be ignored in MM. However, the identification of specific targets into this tumoral microenvironment is urgently needed for the development of next-generation therapies. Although further work need to be done to characterize GDF15 biology, we suggest that GDF15 participates in the control of minimal residual disease, possibly by maintaining in a chemoprotective niche an undetectable pool of MM cells causing the relapse. Because of the moderately minor phenotype displayed by GDF15-knockout mice (49, 50), therapeutic strategy specifically targeting GDF15 might be conceivable. In this regard, future studies from our laboratory will assess GDF15 as one of them for therapeutic strategies in MM.

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Table 1. Characteristics of the 131 patients with newly diagnosed multiple myeloma

<table>
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<tr>
<th>Characteristics</th>
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<tbody>
<tr>
<td>Age (yr)</td>
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<td>Serum light chains (no. of patients (%))</td>
<td></td>
</tr>
<tr>
<td>kappa</td>
<td>81 (62)</td>
</tr>
<tr>
<td>lambda</td>
<td>47 (36)</td>
</tr>
<tr>
<td>non secretory</td>
<td>3 (2)</td>
</tr>
<tr>
<td>International Staging System (no. of patients (%))</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>58 (44)</td>
</tr>
<tr>
<td>II</td>
<td>47 (36)</td>
</tr>
<tr>
<td>III</td>
<td>26 (20)</td>
</tr>
<tr>
<td>Albumin level (g/L)</td>
<td>40±6</td>
</tr>
<tr>
<td>Beta2-microglobulin level (mg/L)</td>
<td>4.2±5.9</td>
</tr>
<tr>
<td>Haemoglobin level (g/dL)</td>
<td>11±2</td>
</tr>
<tr>
<td>Bone marrow plasma cells (% of cells)</td>
<td>32±22</td>
</tr>
<tr>
<td>Calcemia (mmol/L)</td>
<td>2.4±0.3</td>
</tr>
<tr>
<td>Creatininemia (µmol/L)</td>
<td>97±36</td>
</tr>
<tr>
<td>Serum lactate deshydrogenase activity (IU/L)</td>
<td>353±89</td>
</tr>
<tr>
<td>Deletion of chromosome 13 (no. of patients (%))</td>
<td></td>
</tr>
<tr>
<td>no</td>
<td>58 (44)</td>
</tr>
<tr>
<td>yes</td>
<td>73 (56)</td>
</tr>
<tr>
<td>Lytic bone lesions (no. of patients (%))</td>
<td></td>
</tr>
<tr>
<td>no</td>
<td>46 (35)</td>
</tr>
<tr>
<td>yes</td>
<td>85 (65)</td>
</tr>
<tr>
<td>Treatment (no. patients (%))</td>
<td></td>
</tr>
<tr>
<td>Melphalan-Prednisone</td>
<td>53 (40)</td>
</tr>
<tr>
<td>High Dose Chemotherapy</td>
<td>78 (60)</td>
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</table>
Figure Legends

Figure 1. Effect of GDF15 on survival of MOLP-6 stroma-dependent cells (A), primary MM cells (B) and MM1.S stroma-independent cells (C). Cells were cultured for 1 day with GDF15 (1, 10, 100 and 200 ng/mL). Experiments were carried out under serum-free conditions. Percentage cell survival in control versus GDF15-treated cells was measured by Trypan blue exclusion assay. Data represent mean +/- SD of 4 independent experiments performed in duplicate. NT: no treatment. *, $P<0.05$; **, $P<0.005$

Figure 2. Effect of GDF15 on T308 and S473 Akt phosphorylation in MOLP-6 stroma-dependent cells (A), primary MM cells (B) and MM1.S stroma-independent cells (C). Cells were pre-treated or not with GDF15 (20 ng/mL) or interleukin 6 (IL-6) (50 ng/mL) for 15 min, and stained for pT308 or pS473 Akt and isotype control for flow cytometry. Experiments were carried out under serum-free conditions. Live cells, which could be clearly distinguished in forward versus sideways scatter plots, were gated, and the median fluorescence intensity (MFI) was determined. Fold changes of MFI for each condition were defined as the fraction (MFI of measured sample) / (MFI of isotype control). Data represent mean +/- SD of 4 independent experiments. *, $P<0.05$; ***, $P<0.0005$.

Figure 3. Effect of Akt-1/2 inhibitor (Akti-1/2) pre-treatment on GDF15-induced MOLP-6 Akt phosphorylation (A) and survival (B). A, representative experiment where cells were pre-treated or not with Akti 1/2 (1 µM), incubated with GDF15 and stained for pT308 Akt for flow cytometry. Isotype control staining (black line) and pT308 Akt (grey line) are shown on histograms. B, cells were pre-treated or not overnight with Akti-1/2 (1 µM) and then treated or not with GDF15 (200 ng/mL) for 1 day. Experiments were carried out under serum-free conditions. Percentage cell survival in control versus Akti-1/2 pre-treated cells was obtained by Trypan blue exclusion assay. Results are expressed according to percentage of control cell survival (without Akti-1/2 pre-treatment and without GDF15). Data represent mean +/- SD of three independent experiments performed in duplicate. *, $P<0.05$. 


Figure 4. Effect of GDF15 on chemoprotection in MOLP-6 (A) and MM1.S (B) cell lines. Cells were pre-treated or not with GDF15 (200 ng/mL) and then treated with melphalan (8 µM), bortezomib (8 nM) or lenalidomide (100 µM) for 1 day. In a separate set of experiments, both MM cell lines were pre-treated overnight with an Akti-1/2 at 1 µM before applying the above mentioned procedure. Experiments were carried out under serum-free conditions. Percentage cell survival in cells pre-treated with GDF15 +/- Akti versus non-pre-treated cells was measured by Trypan blue exclusion assay. Results are expressed according to percentage of control cell survival (without Akti or GDF15 pre-treatment and without any drugs). Data represent mean +/- SD of five (MOLP-6) (A) and three (MM.1S) (B) independent experiments performed at least in duplicate. NS: non-significant. *, P<0.05; **, P<0.01.

Figure 5. GDF15 concentration measured by ELISA in bone marrow (BM) plasma (A) and blood plasma (B) in healthy subjects and in MM patients. Data represent mean +/- SD. *, P<0.003; **, P<0.001.

Figure 6. Kaplan-Meier plot of survival to 30 months in 131 MM patients. 50 patients with pGDF15 level < 0.5 ng/mL and 81 patients with pGDF15 level ≥ 0.5 ng/mL. A, event-free survival. B, overall survival.
Figure 1.

A) **

% MOLP-6 cell survival

<table>
<thead>
<tr>
<th>GDF15 concentration</th>
<th>NT</th>
<th>1 ng/mL</th>
<th>10 ng/mL</th>
<th>100 ng/mL</th>
<th>200 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>% MOLP-6 cell survival</td>
<td>40</td>
<td>45</td>
<td>50</td>
<td>60</td>
<td>70</td>
</tr>
</tbody>
</table>

B) 

% primary MM cell survival

<table>
<thead>
<tr>
<th>GDF15 concentration</th>
<th>NT</th>
<th>1 ng/mL</th>
<th>10 ng/mL</th>
<th>100 ng/mL</th>
<th>200 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>% primary MM cell survival</td>
<td>40</td>
<td>50</td>
<td>60</td>
<td>70</td>
<td>80</td>
</tr>
</tbody>
</table>

C) 

% MM1.S cell survival

<table>
<thead>
<tr>
<th>GDF15 concentration</th>
<th>NT</th>
<th>1 ng/mL</th>
<th>10 ng/mL</th>
<th>100 ng/mL</th>
<th>200 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>% MM1.S cell survival</td>
<td>40</td>
<td>50</td>
<td>60</td>
<td>70</td>
<td>80</td>
</tr>
</tbody>
</table>
Figure 2.

A) **Fold MFI pT308 Akt**

- **Control**
- **IL-6**
- **GDF15**

B) **Fold MFI pS473 Akt**

- **Primary MM cells**

C) **Fold MFI pT308 Akt**

- **MM1.S cell line**
Figure 3.

A) MOLP-6 + GDF-15

No pre-treatment

Akti-1/2 pre-treatment (1 µM)

Fold increase 2.78

Fold increase 1.06

B) no pre-treatment

Akti-1/2 pre-treatment (1 µM)

% of control MOLP-6 survival

NT

GDF15 (200 ng/mL)
Figure 4.

A) Akti (1µM) + GDF15 pre treatment (200ng/mL)

B) GDF15 pre-treatment (200 ng/mL)

% of control MOLP-6 cell survival

0 20 40 60 80 100 120

Melphalan (8 µM) Bortezomib (8 nM) Lenalidomide (100 µM)

no pre-treatment

GDF15 pre-treatment (200 ng/mL)

Akti (1µM) + GDF15 pre treatment (200ng/mL)
Figure 5.

A)

**GDF15 concentration in BM plasma (ng/mL)**

- **Healthy subjects** n=5
- **Patients with MM** n=16
- **Patients with MM ISS I** n=7
- **Patients with MM ISS II/III** n=9

B)

**GDF15 concentration in blood plasma (ng/mL)**

- **Healthy subjects** n=13
- **Patients with MM** n=131
- **Patients with MM ISS I** n=58
- **Patients with MM ISS II** n=47
- **Patients with MM ISS III** n=26

Legend:
- 
- **Healthy subjects**
- **Patients with MM**
- **Patients with MM ISS I**
- **Patients with MM ISS II/III**

Statistical significance:
- **p < 0.01**
- **p < 0.05**
Figure 6.

A) Event-free survival

- pGDF15 < 0.5 ng/mL
- pGDF15 ≥ 0.5 ng/mL

P = 0.0045

B) Overall survival

- pGDF15 < 0.5 ng/mL
- pGDF15 ≥ 0.5 ng/mL

P = 0.013
Bioactivity and prognostic significance of growth differentiation factor GDF15 secreted by bone marrow mesenchymal stem cells in multiple myeloma

Jill Corre, Elodie Labat, Nicolas Espagnolle, et al.

Cancer Res  Published OnlineFirst February 2, 2012.

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