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, but the pathophysiologic effects of GDF15 in this setting remain undefined. GDF15 has been described in numerous solid tumors but never in hematologic malignancies. In this study, we report that GDF15 significantly increases survival of stroma-dependent multiple myeloma cells including primary multiple myeloma cells. In particular, GDF15 conferred resistance to melphalan, bortezomib, and to a lesser extent, lenalidomide in both stroma-dependent and stroma-independent multiple myeloma cells. Akt-dependent signaling was critical to mediate the effects of GDF15, whereas Src and extracellular signal-regulated kinase 1/2 signaling pathways were not involved. Given these results, we tested the clinical significance of plasma concentrations of GDF15 (pGDF15) in 131 patients with multiple myeloma 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 than patients with low pGDF15 levels. Our findings suggest that tumor microenvironment-derived GDF15 is a key survival and chemoprotective factor for multiple myeloma cells, which is pathophysiologically linked to both initial parameters of the disease as well as patient survival.

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Introduction

Multiple myeloma is a clonal plasma cell malignant disease that accounts for 13% of hematologic 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) but 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 multiple myeloma microenvironment (5–12).

Nevertheless, multiple myeloma 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 multiple myeloma cells but also their microenvironment (14). A better understanding of the mechanism by which the multiple myeloma microenvironment affects the disease is still required to define new therapeutic targets.

Multiple myeloma cells proliferate in close contact with cells from the bone marrow microenvironment. Bone marrow-mesenchymal stem cells (BM-MSC) are the only long lived cells of the bone marrow microenvironment. They secrete survival factors for multiple myeloma 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 multiple myeloma, we reported that BM-MSCs grown without multiple myeloma cells had an abnormal gene expression profile: 145 genes were differentially expressed between BM-MSCs from patients with multiple myeloma and normal subjects. In particular, we found that growth differentiation factor 15 (GDF15) was increased in BM-MSCs from patients with multiple myeloma (21).

GDF15 is a 40-kDa propeptide that is cleaved to release a 25-kDa circulating protein (22). It was first described as a divergent member of the human TGF-\(\beta\) superfamily (23). GDF15 has a broad activity, as indicated by the diversity of...
the nomenclature (MIC-1, PTGF-β, PLAB, PDF, NAG-1, and 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 biologic role of GDF15 in cancer growth is challenging. Some studies have documented its protumorigenic role (31, 32) and others have shown antitumorigenic activity (22, 23, 33). The role of GDF15 in hematologic malignancies has not yet been documented.

To determine the potential impact of GDF15 overexpression by BM-MSCs from patients with multiple myeloma, we assessed the bioactivity of GDF15 on multiple myeloma cells under serum-free culture conditions. We sought correlates of plasma concentration of GDF15 (pGDF15) in patients with multiple myeloma with biologic and clinical parameters of the disease. We found that GDF15 is a survival and chemoprotective factor for multiple myeloma 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 multiple myeloma cell line was a generous gift from Dr. Harashima (ref. 35; Fujisaki Cell Center, Hayashibara Biochemical Laboratories) in 2007. Cryopreserved MOLP-6 cells were defrosted and grown in BM-MSCs in RPMI-1640 medium with 10% fetal calf serum and 10 μg/mL ciprofloxacin. BM-MSCs were obtained from healthy bone marrow 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 multiple myeloma cell line was purchased from American Type Culture Collection 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 2 multiple myeloma cell lines were tested and authenticated before starting the study (morphologic analysis, immunophenotype, carotype, stroma dependence, mycoplasma, and EBV detection). Bone marrow from 4 patients with newly diagnosed multiple myeloma was obtained by sternal puncture after patients gave their written informed consent. Primary bone marrow myeloma cells were purified with use of magnetic anti-human CD138 microbeads (Miltenyi Biotec), and multiple myeloma BM-MSCs were obtained from the CD138-negative fraction and prepared as previously described (21).

Cell cultures

All culture experiments were carried out in SynH serum-free culture medium, an Iscove-based fully defined culture medium containing human albumin without insulin (ABCell-Bio; ref. 12).

Cell survival. MOLP-6, MM1.S, and primary multiple myeloma cells were plated at 50 × 10⁶ cells per 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 pretreated overnight with an Akt-1/2 inhibitor (124018) at 1 μmol/L (Merck) before adding 200 ng/mL GDF15 for 24 hours.

Chemoprotection assay. MOLP-6 and MM1.S cells were plated at 50 × 10⁵ cells per mL with or without 200 ng/mL GDF15 for 24 hours. Cells were grown at 37°C and 5% CO₂. The following drugs were added to the cell cultures for 1 day: 8 μmol/L melphanal (Sigma), 8 nmol/L bortezomib (Millennium Pharmaceuticals), or 100 μmol/L lenalidomide (Celgene). Appropriate controls (cells treated with dimethyl sulfoxide or buffer alone) were included. In a separate set of experiments, both multiple myeloma cell lines were pretreated overnight with an Akt-1/2 inhibitor (124018) at 1 μmol/L before applying the above mentioned procedure. Viable cells were counted by Trypan blue exclusion assay. Percentage cell survival in cells pretreated with GDF15 ± Akti versus nonpretreated cells was obtained, and percentage of control cell survival (without Akti or GDF15 pretreatment and without any drugs) was then calculated.

Akt phosphorylation

MOLP-6, MM1.S, and primary multiple myeloma 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 minutes (a set of experiments was carried out in 10% calf serum conditions). Cells were fixed for 15 minutes, washed, permeabilized for 30 minutes with use of an intracellular stain kit (BD Pharmingen) and stained with a phycoerythrin (PE)-conjugated anti-pT308 or S473 Akt mAb (BD Biosciences) for 30 minutes. 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 μmol/L) was carried out 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 minutes. Cells were fixed for 15 minutes, washed, permeabilized for 30 minutes with use of an intracellular stain kit (BD Pharmingen) and stained with an Alexa Fluor 488–conjugated anti-pY416 Src (Millipore) for 60 minutes or PE-conjugated anti-pT202/pY204 ERK1/2 mAb (BD Biosciences) for 30 minutes. Src or extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation was detected by flow cytometry (EPICS Xl-MCL).

Patient and healthy individual plasma samples

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

**Blood plasma.** Peripheral blood plasma from 131 patients with newly diagnosed multiple myeloma 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) was 1.4. These 131 patients were enrolled in different Intergroupe Francophone du Myelome treatment trials: 53 received conventional chemotherapy, including melphalan and prednisone, and 78 high-dose chemotherapy. Patients with Durie Salmon stage I and 1.3, respectively.

**Bone marrow.** Bone marrow from 5 healthy individuals was obtained from the puncture after patients gave their written informed consent; the median age was 52 ± 3 years and the sex ratio (M:F) was 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. Interassay 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 interassay coefficient of variation was 15%, 4%, and 4%, respectively.

**Statistical analysis**
Statistical analysis of categorical variables was tested with the $\chi^2$ test or the Fisher exact test and that of continuous variables with the Student $t$ test, then Mann–Whitney U test. All tests were 2 tailed. Correlation between pGDF15 level and initial biologic parameters was tested by Spearman correlation. A $P$ value less than 0.05 was considered statistically significant.

Survival was evaluated for the 131 patients with multiple myeloma by pGDF15 level. The threshold value of pGDF15 (0.50 ng/mL), defining low and high levels, was defined as mean ± 3 SD 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; ref. 7), B2-microglobulin level, hemoglobin level, lactate dehydrogenase (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 multiple myeloma cell line and primary multiple myeloma cells**
We first measured the survival of a stroma-dependent (MOLP-6) and a stroma-independent (MM1.S) multiple myeloma cell lines and primary multiple myeloma 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 multiple myeloma 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 multiple myeloma 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 multiple myeloma cells.

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

<table>
<thead>
<tr>
<th>Category</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>Age, y</td>
<td>59 ± 10</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>76/55</td>
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<tr>
<td>Durie Salmon stage [no. of patients (%)]</td>
<td>31 (24)</td>
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<tr>
<td>Serum heavy chains [no. of patients (%)]</td>
<td>79 (60)</td>
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<tr>
<td>Serum light chains [no. of patients (%)]</td>
<td>81 (62)</td>
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<tr>
<td>ISS [no. of patients (%)]</td>
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<td>Aliment level, g/L</td>
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<tr>
<td>B2-microglobulin level, mg/L</td>
<td>4.2 ± 5.9</td>
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<tr>
<td>Hemoglobin level, g/dL</td>
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<tr>
<td>Bone marrow plasma cells (% of cells)</td>
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<td>Calcemia, mmol/L</td>
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<td>Creatininemia, pmol/L</td>
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<td>Serum LDH activity, IU/L</td>
<td>353 ± 89</td>
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<td>del13 [no. of patients (%)]</td>
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<tr>
<td>Lytic bone lesions [no. of patients (%)]</td>
<td>46 (35)</td>
</tr>
<tr>
<td>Treatment [no. of patients (%)]</td>
<td>Melphalan/prednisone 53 (40)</td>
</tr>
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<td></td>
<td>High-dose chemotherapy 78 (60)</td>
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myeloma cells ($P < 0.05$). In contrast, GDF15 did not significantly increase MM1S cell survival: 89.7% ± 10.2% of MM1S cells survived with 200 ng/mL of GDF15 ($P = 0.2$; Fig. 1).

GDF15 triggers Akt phosphorylation in a stroma-dependent multiple myeloma cell line and primary multiple myeloma cells

Multiple myeloma 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 MM1S cells and from purified primary multiple myeloma 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. S1). In contrast, neither GDF15 nor IL-6 was able to induce p-Akt T308 and S473 in MM1S cells (Fig. 2C), reflecting their constitutive...
activation of Akt (Supplementary Fig. S2). In primary multiple myeloma cells, GDF15 induced T308 and, although to a lower extent, S473 Akt phosphorylation ($P < 0.05$ and nonsignificant, respectively), whereas IL-6 induced only T308 phosphorylation (Fig. 2B). Therefore, GDF15 enhances Akt phosphorylation and activity in MOLP-6 and primary multiple myeloma cells but not MM1.S cells. Overnight pretreatment of MOLP-6 cells with an Akt-1/2 inhibitor (124018, 1 μmol/L) inhibited GDF15-induced p-Akt (Fig. 3A) and abrogated the GDF15-induced survival increase ($P < 0.05$; Fig. 3B).

Of note, GDF15 did not induce phosphorylation of Src and ERK1/2 in both multiple myeloma cell lines (Supplementary Figs. S3 and S4).

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

Using the same culture conditions as above, we asked whether GDF15 was chemoprotective against drugs classically used in multiple myeloma treatment. Dimethyl sulfoxide alone did not affect multiple myeloma cell survival (data not shown). In drug-treated cultures, the proportion of control MOLP-6 cell survival was increased when the cells were pretreated 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%, nonsignificant; Fig. 4A). Similar results were obtained with MM.1S cells (melphalan: 80.0% ± 24.3% vs. 30.0% ± 6.7%, $P < 0.05$; bortezomib: 81.5% ± 17.5% vs. 54.0% ± 8.0%, $P < 0.05$; lenalidomide: 20.7% ± 7.8% vs. 5.6% ± 1.4%, nonsignificant; Fig. 4B). Thus, GDF15 decreases chemotherapy-induced cytotoxicity of the 3 drugs in both multiple myeloma cell lines. Overnight pretreatment of MOLP-6 cells with an Akt-1/2 inhibitor (124018, 1 μmol/L) 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%, nonsignificant; 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 multiple myeloma 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 multiple myeloma cells from 3 patients with newly diagnosed myeloma. Whereas the concentration of GDF15 ranged from $4 \times 10^{-7}$ to $8 \times 10^{-5}$ pg per cell for their BM-MSCs, we did not detect any GDF15 in the corresponding multiple myeloma cells supernatants. We found similar results with both multiple myeloma cell lines, MOLP-6 and MM1.S cells (Supplementary Fig. S5). Hence, GDF15 is a specific factor of microenvironment in myeloma.

**pGDF15 increases with multiple myeloma disease stage**

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

To study the concentration of GDF15 in a larger cohort of subjects, and because blood samples are easier to collect than bone marrow samples, we next measured the concentrations of GDF15 in bone marrow and blood plasma in 24 patients with multiple myeloma. We found a correlation coefficient of 0.98 (Supplementary Fig. S6), so for the rest of the study, we tested only blood plasma.

The pGDF15 was measured in blood from 131 additional patients with newly diagnosed multiple myeloma and 13 healthy subjects. Demographic and clinical characteristics for the patients with multiple myeloma are in Table 1.
pGDF15 was significantly higher for patients with multiple myeloma (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 multiple myeloma parameters

For the 131 patients with multiple myeloma, we searched for a correlation between pGDF15 and initial biologic factors of the disease. We found a significant positive correlation between pGDF15 level and β2-microglobulin level, creatinema, calcemia, and serum monoclonal protein level and a
significant inverse correlation with levels of hemoglobin and albumin. pGDF15 level was not correlated with bone marrow plasma cell frequency, LDH activity, or CRP level (Supplementary Fig. S7).

Mean pGDF15 was calculated according to Durie Salmon disease stage, presence or absence of del13, and bone status. Mean pGDF15 was significantly higher in patients with Durie Salmon stage III than Durie Salmon 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 multiple myeloma by pGDF15. Among the 131 patients, 50 had pGDF15 less than 0.50 ng/mL (mean 0.33 ± 0.10; "pGDF15<sub>low</sub> patients") and 81 had pGDF15 0.50 ng/mL or more (mean 1.25 ± 1.26; "pGDF15<sub>high</sub> patients"). For the 131 patients with multiple myeloma, the median follow-up from time of diagnosis was 27 months (range, 1–60 months). The median duration of EFS was 33 months (range, 1–60 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, 5–60 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, 1–60 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<sub>low</sub> patients (P < 0.0045 and P < 0.013, respectively; Fig. 6). None of the 26 patients with pGDF15 less than 0.34 ng/mL died, and median duration of EFS for these patients was more than 60 months; among these...
On multivariate analysis, EFS was related to only pGDF15 stage, as well as 11 with Durie Salmon stage I, 5 stage II, and 10 stage patients were 18 with ISS disease stage I and 8 stage II, as previously observed with multiple myeloma BM-MSCs in our previous work (21) but in more stringent culture conditions. Moreover, GDF15 was able to significantly increase cell survival in primary multiple myeloma 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 multiple myeloma induced an overgrowth of MOLP-6 cells when compared with BM-MSCs from normal subjects (21). To determine whether the overproduction of GDF15 by multiple myeloma BM-MSCs is involved in this overgrowth, we carried out MOLP-6 cells and multiple myeloma BM-MSCs coculture experiments involving MSCs from 4 patients with multiple myeloma that were transfected either with a control plasmid or with a short hairpin RNA (shRNA)-targeting GDF15. The number of MOLP-6 cells after 7 days of coculture was significantly decreased ($P < 0.05$) when MSCs were transfected with shRNA GDF15 (Supplementary Fig. S8). Although this decrease is moderate, it suggests that the overproduction of GDF15 participates to the MOLP-6 overgrowth we previously observed with multiple myeloma BM-MSCs. We did not find any significant survival advantage with GDF15 in the MM1.S stroma-independent multiple myeloma cell line. Of note, no overgrowth of stroma-independent cell line had been observed with multiple myeloma BM-MSCs in our previous work (21). Interestingly, GDF15 induced Akt phosphorylation in MOLP-6 and primary multiple myeloma cells but not in MM1.S cells. In addition, pretreating 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 and colleagues (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 multiple myeloma cells has been extensively studied (36, 39–41). Functionally, the Akt pathway is implicated in cell-cycle and apoptosis regulation in multiple myeloma cells. Akt is phosphorylated in bone marrow biopsies from patients with multiple myeloma, notably because of bone marrow microenvironment-derived cytokines such as IL-6 or IGF-1 (42). Our data indicate that GDF15 also contribute to triggering the Akt pathway in multiple myeloma cells. The absence of IL-6 effect on Akt signaling in MOLP-6 cells was expected as IL-6 has no bioactivity on their survival (35). Its marginal effect in primary multiple myeloma 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

Prognostic factors of EFS

We analyzed the impact of prognostic factors (age, ISS stage, β2-microglobulin level, hemoglobin level, LDH activity, CRP level, and presence or absence of del13) on EFS for the 131 patients with multiple myeloma. On univariate analysis, EFS was significantly related to pGDF15 ($P = 0.003$), age ($P = 0.003$), and β2-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 shows that functionally GDF15 is a survival and chemoprotective factor for multiple myeloma 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 multiple myeloma microenvironment affects the pathophysiology and the prognosis of multiple myeloma. Here, we investigated the significance of one of multiple myeloma 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 hematologic malignancies.

Here, we first determined the role of GDF15 on multiple myeloma cell survival in vitro. This involved experiments under serum-free conditions to avoid components present in serum, in particular insulin-like growth factor 1 (IGF-1), which might confound interpretation of the results (12). GDF15 increased cell survival in the MOLP-6 stroma-dependent multiple myeloma cell line, confirming our previous work (21) but in more stringent culture conditions. Moreover, GDF15 was able to significantly increase cell survival in primary multiple myeloma 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 multiple myeloma induced an overgrowth of MOLP-6 cells when compared with BM-MSCs from normal subjects (21). To determine whether the overproduction of GDF15 by multiple myeloma BM-MSCs is involved in this overgrowth, we carried out MOLP-6 cells and multiple myeloma BM-MSCs coculture experiments involving MSCs from 4 patients with multiple myeloma that were transfected either with a control plasmid or with a short hairpin RNA (shRNA)-targeting GDF15. The number of MOLP-6 cells after 7 days of coculture was significantly decreased ($P < 0.05$) when MSCs were transfected with shRNA GDF15 (Supplementary Fig. S8). Although this decrease is moderate, it suggests that the overproduction of GDF15 participates to the MOLP-6 overgrowth we previously observed with multiple myeloma BM-MSCs. We did not find any significant survival advantage with GDF15 in the MM1.S stroma-independent multiple myeloma cell line. Of note, no overgrowth of stroma-independent cell line had been observed with multiple myeloma BM-MSCs in our previous work (21). Interestingly, GDF15 induced Akt phosphorylation in MOLP-6 and primary multiple myeloma cells but not in MM1.S cells. In addition, pretreating 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 and colleagues (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 multiple myeloma cells has been extensively studied (36, 39–41). Functionally, the Akt pathway is implicated in cell-cycle and apoptosis regulation in multiple myeloma cells. Akt is phosphorylated in bone marrow biopsies from patients with multiple myeloma, notably because of bone marrow microenvironment-derived cytokines such as IL-6 or IGF-1 (42). Our data indicate that GDF15 also contribute to triggering the Akt pathway in multiple myeloma cells. The absence of IL-6 effect on Akt signaling in MOLP-6 cells was expected as IL-6 has no bioactivity on their survival (35). Its marginal effect in primary multiple myeloma 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 may act through a TGF-β/some evidence for SMAD pathway activation, suggesting that the particular GDF15 receptor is still unknown today. There is evidence that GDF15 could not induce phosphorylation of ERK1/2 in both multiple myeloma cell lines, suggesting that the Ras/Raf/MEK/MAPK pathway would not be involved. In addition, GDF15 had no bioactivity on Src in MOLP-6 and MMLS cells.

GDF15 conferred drug resistance in MOLP-6 and MMLS cells to 3 drugs classically used in multiple myeloma treatment (melphanal, 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 mitoxantrone. Because the GDF15-mediated chemoprotection of MOLP-6 cells was abrogated when the cells were pretreated with an Akt-1/2 inhibitor, we suggest the presence of an Akt-dependent chemoprotection mechanism. For MMLS cell line, this effect might be Akt independent. Other signaling cascades activated by cytokines or growth factors in multiple myeloma cells may be involved, including Janus-activated kinase (JAK)/Stat3, Wnt, NF-κB pathways (1, 14), and underlying other cellular processes that might influence chemoresistance (drug efflux, cell death, genotoxic sensing, DNA repair, proteasome activity). The differences of biologic profile between MOLP-6 and MMLS cells and the similarities between MOLP-6 and primary multiple myeloma cells pinpoint the importance of the stroma dependence of multiple myeloma cell lines chosen as in vitro models to study the impact of microenvironment on tumor.

We next showed that GDF15 is increased in bone marrow plasma from patients with multiple myeloma. Although our previous studies indicated that this increase reflected GDF15 overproduction by multiple myeloma BM-MSCs, macrophages might also contribute to total GDF15 level. Macrophages are able to secrete GDF15 (23) and constitute abundant components of multiple myeloma microenvironment and able to protect multiple myeloma cells against drug-induced apoptosis (45). However, as compared with its production in solid tumors, GDF15 is not produced by the malignant multiple myeloma 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 colleagues elegantly showed that GDF15 induces the transactivation of ErbB2 tyrosine kinase in SK-BR-3 breast and SNU-216 gastric cancer cells (31). We did not find any expression of TGF-βRII or ErbB2 on both multiple myeloma cell lines and primary multiple myeloma cells (data not shown), suggesting that GDF15 receptor also remains to be discovered in multiple myeloma.

To determine whether the GDF15 concentration level increase was indicative of the severity of the disease in patients with multiple myeloma, and because we found that the concentrations of GDF15 in bone marrow and blood plasma in 24 patients with multiple myeloma were correlated, we next measured the pGDF15 in 131 patients with newly diagnosed multiple myeloma. 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 patients with multiple myeloma.

This study allows to gain a better understanding into the mechanism by which the abnormal microenvironment affects the pathophysiology and the prognosis of multiple myeloma. Microenvironment has become a therapeutic target that cannot be ignored in multiple myeloma. 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 multiple myeloma 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 a novel target for therapeutic strategies in multiple myeloma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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