ABSTRACT. In multiple myeloma, a large number of growth factors (IL-6, IGF-1, FGF, HGF and HB-EGF) are involved in promoting myeloma cell growth. In the present study, a serum-free, cytokine-free, collagen-based assay, which does not allow the generation of spontaneous myeloma colonies, was used to identify the clonogenic growth factors for fourteen myeloma cell lines. IL-6 is the only clonogenic factor able to stimulate both CD45+ and CD45- myeloma cell lines, generating myeloma colonies from 10 out of 14 myeloma cell lines. Using a pharmacological Erk inhibitor, we show that the Erk/MAPK pathway is involved in IL-6-induced clonogenicity of CD45+, but not CD45- myeloma cell lines. In contrast to IL-6, the other growth factors (IGF-1, FGF, HGF and HB-EGF) stimulate only some myeloma cell lines, but always CD45-, and less effectively than IL-6. Among them, IGF-1 is the most potent, generating myeloma colonies from five out of eight CD45- myeloma cell lines. Finally, the capacity of IGF-1 and FGF to stimulate the clonogenicity of CD45- myeloma cells correlates with their ability to stimulate the Erk/MAPK pathway. We conclude that CD45 expression plays a crucial role in determining signaling and proliferation of human myeloma cell responses to IL-6, IGF-1 and other growth factors. The poor outcome of CD45- myeloma patients could be related to the capacity of CD45-myeloma cells to take advantage of multiple growth factors.

Keywords: multiple myeloma, clonogenicity, CD45, IL-6, IGF-1
USA and Pr. B. Van Camp, Belgium respectively. The XG-1, XG-2, XG-6, NAN-1, NAN-4 and MDN HMCLs had been previously established in our laboratory from peripheral blood samples or pleural effusion of patients with MM (see table 1) [12], and were cultured in the presence of 3 ng/ml of r-IL-6 (Novartis, Basel, Switzerland). All HMCL expressed CD138 (table 1) [12]. Cell lines were maintained in RPMI-1640 medium supplemented with 10% FCS, 2 mM glutamine, antibiotics and 5x10⁻³M 2-βME.

**Monoclonal antibodies (mAbs) and reagents**

Human recombinant IGF-1 was purchased from Sigma (St Louis, MI, USA). Human recombinant IL-6 was kindly provided by Novartis. Human recombinant FGF, HB-EGF and HGF were purchased from Preprotech (Rockhill, NJ, USA). Anti-phospho-p44/42 MAP kinase, anti-p44/42 MAP kinase and anti-phospho-Akt (Ser 473) are from Cell Signaling (Ozyme, Saint Quentin Yvelines, France). U0126 and wortmannin are from Alexis Biochemicals (Carlsbad, CA, USA).

**Immunofluorescence analysis**

Cells (0.5 x10⁶) were incubated with different PE-conjugated mAb or anti-CD45-FITC (Beckman Coulter, Marseilles) for 20 min at 4°C. The different PE-conjugated mAb were anti-CD138, anti-CD126 from Beckman Coulter, Marseilles, France and anti-CD38, anti-IGF-1R, anti-CD45RA and anti-CD45RB from BD, Biosciences, Le Pont de Claix, France. After two washes, cells were fixed in 1% formaldehyde. Flow cytometry analysis was performed on a FACSCalibur using the CELLQuest program (Becton Dickinson, San Jose, CA, USA). The fluorescence ratio was determined by dividing the mean fluorescence intensity by the mean fluorescence intensity of the respective control.

**Myeloma cell colony-forming assay**

Myeloma cells (10³ cells) were plated in 1ml IMDM serum-free, cytokine-free, human purified collagen-based, semi-solid medium (stem III, StemAlpha SA, France) in triplicate (330 μL/well), in 4-well plates and grown for 15 days. For cytokine-stimulated assays in the presence or not of inhibitors, cytokines and/or inhibitors were mixed with the cell suspension in IMDM before addition of the collagen. The gels were harvested on glass slides, dried and stained with May-Grünwald-Giemsa. Colonies were counted on triplicate gels by microscopy. The number of colonies was expressed as an average per 10³ cells.

**Immunoblot analysis**

Cells (4x10⁶) were resuspended in lysis buffer (10 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 2 mM Na₃VO₄, 1 mM NaF, 2 μg/mL aprotinin, leupeptin 1 μg/mL and 0.5% NP40). After 40 min on ice, lysates were cleared by centrifugation at 12 000 x g for 30 min at 4°C. Protein concentration was measured using bicinchoninic acid (BCA protein assay, Pierce Rockford, IL, USA). One hundred μg of proteins were loaded for each lane. The proteins were separated by 10% SDS-PAGE and then electrotransferred to PVDF membranes. Western blot analysis was performed using standard techniques with ECL detection (Roche, France).

**Statistical analysis**

The Fisher’s test was used for statistical analysis.

**RESULTS**

*IL-6 is a clonogenic factor for both CD45+ and CD45- human myeloma cell lines (HMCL), whereas IGF-1 and other growth factors (FGF, HGF and HB-EGF) are clonogenic only for CD45- HMCL*

This study was designed to compare the capacity of the different myeloma cell growth factors to act as clonogenic factors for HMCL in a collagen-based assay. The serum-free and cytokine-free, collagen-based assay was designed not to allow the generation of spontaneous myeloma colonies in the absence of exogenous growth factors, and, with

<table>
<thead>
<tr>
<th>HMCL</th>
<th>Isotype</th>
<th>Sample</th>
<th>CD138</th>
<th>CD38</th>
<th>CD45</th>
<th>CD45RA</th>
<th>CD45RO</th>
<th>CD45RB</th>
<th>CD126</th>
<th>CD221</th>
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<tr>
<td>LP1</td>
<td>IgG λ</td>
<td>PB</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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</tr>
<tr>
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<td>IgA k</td>
<td>PE</td>
<td>+</td>
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<tr>
<td>JIM-3</td>
<td>IgA</td>
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<td>IgA k</td>
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<td>IgG λ</td>
<td>PB</td>
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<td>IgE λ</td>
<td>PB</td>
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<td>-</td>
<td>86%</td>
<td>86%</td>
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</table>

Expression of each molecule was evaluated by a single immunofluorescence assay. Positive expression of a molecule is defined by the level of intensity with a ratio > 2 (the ratio is obtained by the ratio of fluorescence divided by the fluorescence of an isotopic control).

- : not expressed, +: expressed as 100%, otherwise the% of positive cells is given. NS: non-secreting, PB: peripheral blood, PE: pleural effusion.
the exception of RPMI-8226 (10% of clonogenic cells) and L363 (2% of clonogenic cells), no other HMCL was able to generate significant numbers of colonies (> 1% of clonogenic cells) spontaneously. Fourteen HMCL were studied, six expressed CD45 on a majority of cells (> 80%), and eight were lacking CD45 expression on a majority of cells (> 50%). The observed CD45 expression corresponded to the CD45RB isoform expression associated with either CD45RA or RO (n = 4) or both CD45RA and CD45RO (n = 2). IL-6 generated myeloma colonies in 10 out of the 14 HMCL. The clonogenicity ranged from 7% to 50% (figure 1). Notably, IL-6 did not enhance the spontaneous colony formation of RPMI-8226. In contrast to IL-6, IGF-1 generated colonies in five out of the 14 HMCL, inducing a weak effect (< 3% of clonogenic cells) for L363 cells. This was not due to a lack of IGF-1R expression since all 14 HMCL expressed the IGF-1R (table 1) [13]. The ability of FGF to stimulate colony formation was restricted to LP1 and RPMI-8226 HMCL (figure 1). HGF and HB-EGF had a very weak stimulating effect on colony formation of LP1, RPMI-8226 and L363 (< 2% of clonogenic cells) and of LP1 (3% of clonogenic cells), respectively. Interestingly, the capacity of IGF-1, FGF, HGF and HB-EGF to stimulate clonogenicity was restricted to CD45- HMCL, whereas IL-6 was a clonogenic factor for both CD45- and CD45+ HMCL. Finally, IGF-1 had a broader range of activity than FGF, HGF and HB-EGF as it was able to stimulate the clonogenicity in more HMCL. Moreover, the number of colonies generated by IGF-1 was greater compared to FGF, HGF or HB-EGF, except for LP1, where FGF generated more (36% of clonogenic cells with FGF versus 22% of clonogenic cells with IGF-1).

IL-6-induced colony formation involves the MAPK pathway in CD45+ but not in CD45- HMCL

IL-6 triggers both the Ras/MAP kinase and the JAK/STAT pathways, the latter promoting MM cell survival. Since, IL-6 is a clonogenic factor for both CD45+ and CD45-HMCL, we next examined whether the Erk pathway was involved in clonogenicity, using the pharmacological MEK1/2 inhibitor, U0126. U0126 inhibited the IL-6-induced clonogenicity of all CD45+ HMCL tested with an
inhibition ranging from 36% to 67% (mean value \( m = 53\% \)) (figure 2). In contrast, U0126 only reduced the clonogenicity (16%) of one out of five CD45-HMCL tested. Of note, U0126 strongly increased the IL-6-induced clonogenicity of NCI-H929 (243% increase) (figure 2). Altogether, these results demonstrated that the Erk/MAPK cascade was significantly involved in IL-6-induced clonogenicity in CD45+HMCL but not CD45-HMCL \((p < 0.05)\) (Fisher’s test).

Significant Erk phosphorylation induction in response to IGF-1 or FGF correlates with the capacity to generate colony formation

We searched for a correlation between clonogenicity and the signaling pathway activated by IGF-1 and FGF in MM cells. We and others, have recently demonstrated that PI-3 kinase pathway activation was induced in all HMCL in response to IGF-1. However, the magnitude of Akt phosphorylation in response to IGF-1 was greater in CD45- than in CD45+HMCL \([8]\). Finally, the strong activation of the PI3-kinase pathway in all CD45- HMCL does not seem to be sufficient to explain the IGF-1-restricted clonogenicity of five out of the eight CD45- HMCL. Thus, we focused on Erk phosphorylation induced by IGF-1 and FGF. Under IGF-1 stimulation, Erk phosphorylation was induced in L363, JIM-3, LP1 and RPMI-8226 CD45-HMCL (figure 3A), whereas induction of Erk phosphorylation was undetectable in NAN-4, XG-1, XG-6 and MDN CD45+ HMCL. A kinetic study of Erk phosphorylation in a CD45-HMCL (L363) indicated that the ERK response was similar between 15 min to 120 min under IGF-1 stimulation (figure 3B). Similar kinetics in a CD45+HMCL (NAN-4) confirmed that the absence of ERK induction of CD45+ HMCL was not due to a difference in kinetic response, but to a total absence of response. Altogether, these results demonstrated that the ERK response to IGF-1 is significantly different between CD45- HMCL and CD45+ HMCL \((p < 0.05)\). Of interest, the ERK phosphorylation induced by IGF-1 was completely restricted to the CD45- HMCL able to generate myeloma colonies in the presence of IGF-1. The same analysis was performed with FGF, demonstrating that induction of ERK phosphorylation by FGF is restricted to LP1 and RPMI-8226 (figure 3C). Thus, the activation of the MAPK pathway by IGF-1 or FGF correlated with the capacity of these factors to generate myeloma colonies. Taken together, these results highlight the importance of the ERK/MAPK pathway in IGF-1- or FGF-induced clonogenicity of CD45- HMCL.

IL-6 and growth factors do not co-operate to induce clonogenicity, whereas IGF-1 and HGF can synergize to induce colony formation

Since cross-talk between IL-6R and IGF-1R has been demonstrated \([14]\), we next evaluated the potential of IGF-1 and IL-6 in combination, to generate myeloma cell colonies. In all HMCL tested \((n = 5)\), the number of myeloma cell colonies induced by IGF-1 and IL-6 combination was never higher than that induced by IL-6 alone, indicating that IL-6 and IGF-1 do not co-operate in colony formation induction (figure 4). Similarly, we found that the combination of IL-6 with the other growth factors (FGF, HB-EGF and HGF), did not co-operate to induce clonogenicity (result not shown). Consistent with these results, in CD45- HMCL, where both cytokines induced the Erk/MAPK pathway, the combination of IL-6 and IGF-1 did not result in an additive effect of Erk phosphorylation (figure 5B). Finally, we analyzed the effect of IGF-1 in combination with the other growth factors (FGF, HB-EGF and HGF); we found that the combination of IGF-1 and HGF synergized in inducing clonogenicity. As illustrated
in figure 5A, a marked increase in clonogenicity was observed with the combination of IGF-1 and HGF compared to IGF-1 alone (324 ± 1 colonies versus 134 ± 4 colonies) for NCI-H929. Consistent with this, IGF-1 in combination with HGF, and compared to IGF-1 alone, was associated with a marked increase in the levels of both Erk phosphorylation (324% increase) and Akt phosphorylation (133% increase) (figure 5B).

**DISCUSSION**

In the present study, we have described a serum-free, cytokine-free, collagen-based assay that identified the capacity of an isolated cell to self-renew only when the right growth factor or combination of growth factors were present. This assay also allowed the prioritization of specific clonogenic factors for HMCL. Furthermore, we utilized a panel of heterogeneous HMCL reflecting the genetic diversity of clinical MM. With the exception of RPMI-8226, none of the HMCL retains the capacity to self-renew and proliferate in the absence of cytokines or growth factors. We identified IL-6 as a ubiquitous clonogenic factor for human MM cells that acted independently of their CD45 phenotype. Of note, all HMCL expressed the IL-6R except JJJ-3, which is one of the two HMCL
serum-free, collagen-based, semi-solid stem colony formation. Synergistic effect of the combination of IGF-1+HGF on NCI-H929 dependent proliferation [17, 18]. Moreover, translocation of CD45 to lipid rafts is also required to confer the ability to respond to IL-6. This is consistent with our observation that all the CD45+ HMCL expressed the CD45RB isoform of CD45, which, unlike CD45RA, is able to translocate to lipid rafts [19]. Thus, both the Ras/MAP kinase pathway and src activation are necessary for the IL-6-induced proliferation and clonogenicity of CD45+ HMCL, whereas IL-6 is a clonogenic factor for CD45- HMCL, independent of these pathways. Further investigations are necessary to elucidate the signaling pathway responsible for the latter observation. In this respect, a working hypothesis will be that IL-6 induces the generation of an autocrine clonogenic factor in CD45- HMCL. Although this factor has not yet been identified, we can already exclude the growth factors tested in this study. Furthermore, the inability of IGF-1 inhibition to prevent IL-6-induced clonogenicity, and the ability of Baff to induce colony formation in only a limited number of HMCL (LP1, L363 and RPMI-8226) (data not shown) would argue against a role for an autocrine production of these factors in IL-6-induced clonogenicity. Interestingly, our study demonstrated that whereas CD45+ HMCL could benefit from IL-6 only, CD45- myeloma cells responded to both IL-6 and a range of other growth factors (IGF-1, FGF, HB-EGF, HGF) to generate myeloma colonies. The capacity of IGF-1 to generate myeloma colonies correlated with the induction of Erk/MAPK signaling and activation of the PI 3-kinase pathway. Indeed, IGF-1 was unable to activate the Erk/MAPK pathway or induce colony formation in CD45+ HMCL. Moreover, in the CD45-HMCL, IGF-1 synergized with HGF to induce colony formation, as well as Erk/MAPK and Akt activation. Finally, it has been previously reported that IL-6 and growth factors co-operate to induce MM cell growth [13, 20, 21]. However, in our semi-solid assay, we observed that IL-6 did not synergize with IGF-1, FGF, HB-EGF or HGF, and indeed the combination of IL-6 and IGF-1 was inhibitory. In accordance with these data, in cell lines where IGF-1 or IL-6 induced Erk activation, this activation was never enhanced with IL-6 and IGF-1 combination. In accordance with these data, in cell lines where IGF-1 or IL-6 induced Erk activation, this activation was never enhanced with IL-6 and IGF-1 combination. A more detailed analysis of cross-talk between these factors will help us to understand these results.

In conclusion, CD45 is essential for the control of signaling and proliferation of human myeloma cells in response to IL-6, IGF-1 and other growth factors. The poor outcome of CD45- myeloma patients could be related to the capacity of CD45- myeloma cells to take advantage of multiple growth factors. Thus, treatment strategies for CD45- patients should combined the disruption of signaling induced by both IL-6 and IGF-1, and other growth factors.

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REFERENCES


Figure 5
Synergistic effect of the combination of IGF-1+HGF on NCI-H929 colony formation. A) Myeloma cells (10^3) were seeded per ml of serum-free, collagen-based, semi-solid stem III medium containing the indicated growth factor and grown for 15 days. Gels were then dried and stained with MGG, and MCF was scored. Values represent the mean ± SD of three experiments of duplicate cultures. B) The synergistic effect of the combination of IGF-1+HGF on colony formation is mediated by a stronger activation of both PI 3K/Akt and Erk/MAPK pathways. Eighteen-hour, serum-starved cells for were treated or not with IGF-1, IL-6, IGF-1+IL-6, HGF or IGF-1+HGF for 30 minutes. Equivalent amounts of cell lysates were separated by SDS-PAGE, then immunoblotted with anti-phospho-ERK or anti-phospho Akt antibodies. Protein loading was controlled with an anti-ERK total.

Figure 5


