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Role for interleukin-6 and insulin-like growth factor-I via PI3-K/Akt pathway in the proliferation of CD56$^-$ and CD56$^+$ multiple myeloma cells

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Objectives. **Several studies including ours have suggested that lack of CD56 in multiple myeloma (MM) defines a unique patient subset with poorer prognosis. However, the mechanism underlying this aggressive behavior of CD56$^-$ MM has not been well elucidated. Interleukin-6 (IL-6) or insulin-like growth factor I (IGF-I) induce proliferation of MM cells. In this study, we report about the relationship between CD56 expression and responsiveness to these cytokines.**

Methods. **We sorted out both CD56$^-$ and CD56$^+$ fractions from MM cell lines such as KMS-21-BM and U-266, and investigated their different responsiveness to IL-6 or IGF-I. Furthermore, we compared the effects of these cytokines on the regulation of cell-cycle distribution between CD56$^-$ and CD56$^+$ cells.**

Results. **Although CD56$^-$ cells in both KMS-21-BM and U-266 cells responded significantly to IL-6, CD56$^+$ cells did not. Ki-67$^+$ cells in the CD56$^-$ cells were significantly increased by IL-6. Western blotting showed that IL-6 phosphorylated Akt, and upregulated and downregulated the level of cyclin D1 and p27 protein in the CD56$^-$ KMS-21-BM cells, respectively. LY-294002 completely blocked these effects of IL-6. On the other hand, Ki-67$^+$ cells in the CD56$^+$ cells did not respond to IL-6. Anti-IGF-I mAb significantly reduced Ki-67$^+$ cells only in the CD56$^+$ cells. IGF-I phosphorylated Akt and upregulated cyclin D1 in the CD56$^+$ KMS-21-BM cells, which was completely blocked by LY294002.**

Conclusions. **These results suggest that CD56$^-$ and CD56$^+$ MM cells could be stimulated by IL-6 and IGF-I, respectively, via PI3-K/Akt pathway, and provide useful information for anti-cytokine therapies.**

The interaction of multiple myeloma (MM) cells with bone marrow stroma cells (BMSCs) is considered to play an important role in the regulation of proliferation of MM cells [1–3]. It is mainly mediated by the cell-surface adhesion molecules [4,5]. MM cells have been reported to express several kinds of surface adhesion molecules, such as CD11a (lymphocyte function–associated antigen-I, LFA-I) [4–6], CD49d (very late antigen-4, VLA-4), CD49e (VLA-5), CD44, CD45, CD54 (intercellular adhesion molecule-I, ICAM-I) [4,5,7–9], CD56 (neural cell adhesion molecule, NCAM) [8–10], and CD138 (syndecan-I) [11–13]. Some of them have been discussed with their prognostic significance. A lack of LFA-1 was reportedly correlated with higher proliferative activity in MM [14]. The MM cells lacking VLA-5 were reported to be characterized by an immature morphology, a higher proliferative activity, interleukin-6 (IL-6) responsiveness, the extramedullary

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involvement, and a greater resistance to chemotherapy [7]. CD45 expression has also been identified in a proliferative fraction with higher IL-6 responsiveness [15,16]. However, the majority of MM cells were reported to lack LFA-1 [14] or CD45 [12]. Another report also demonstrated that it is difficult to determine VLA-5 expression in living cells in MM [17]. Therefore, these molecules are actually difficult to use as a prognostic factor, and a more practical molecule to predict prognosis is needed.

Several studies reported that CD56 was frequently expressed on MM cells; an absence of CD56 expression on MM cells correlated with the acute progression of the disease and extramedullary involvement [10,18,19]. We previously reported that $CD56^-$ MM was associated with shorter survival, higher β_2-microglobulin level, lower platelet counts, and a higher prevalence of Bence Jones protein type MM, renal insufficiency, and plasmablastic morphology than $CD56^+$ MM [20]. However, the biological characters underlying these aggressive clinical behaviors of $CD56^-$ MM have not been well elucidated.

IL-6 and insulin-like growth factor I (IGF-I) often induce proliferation of MM cell lines and freshly isolated MM cells [21–30]. The BMSCs produce high concentrations of these cytokines and promote tumor expansion in paracrine fashion [1,31]. Although CD56 mediates cell-to-cell interaction between MM cells and BMSCs [32], there have not been any reports about the relationship between CD56 expression and responsiveness to IL-6 and IGF-I. In the present study, we sorted out $CD56^-$ and $CD56^+$ fractions from MM cell lines and investigated the importance of CD56 expression in the susceptibility to these cytokines. Furthermore, we compared the effects of these cytokines on the regulation of cell-cycle distribution and its related molecules, such as cyclin D1, p27, and phosphorylated Akt between $CD56^-$ and $CD56^+$ MM cells.

Materials and methods

Reagents

IL-6, IGF-I, LY294002, Quercetin, and anti-IGF-1 monoclonal antibodies (mAbs) were purchased from Sigma Chemical Co. (St Louis, MO, USA). LY294002 was dissolved in ethanol at a stock solution of 10^{-1} mol/L and diluted to working concentration before use. The final concentration of ethanol in the culture medium never exceeded 0.1% (vol/vol). The treatment with 0.1% of ethanol affected neither the amount of Ki-67 nor cell-cycle distribution, compared with those without ethanol. Fluorescein isothiocyanate (FITC)-labeled anti-CD11a, CD28, CD44, Ki-67 mAbs and phycoerythrin (PE)-labeled anti-CD49d, CD49e, CD54, CD56, CD126, CD130, CD221, Cy5-CD138 mAbs were purchased from Pharmingen (San Diego, CA, USA). FITC-anti-CD38 and CD45 mAbs were purchased from Coulter Immunotech (Miami, FL, USA). These mAbs were used according to the manufacturer's instructions.

Cell culture

The U-266 human MM-derived cell line was obtained from American Type Culture Collection (Rockville, MD, USA). KMS-21-BM was previously established from IgD -λ type MM patient [33]. Cells were cultured in control medium containing RPMI 1640, L-glutamine, antibiotics, and 10% fetal calf serum (Gibco, Grand Island, NY, USA) in the presence or absence of IL-6 (2 ng/mL for KMS-21-BM and 10 ng/mL for U-266, respectively). Cell proliferation was assessed by trypan blue dye exclusion assay. After staining with PE-CD56 and Cy5-CD138, $CD56^-CD138^+$ and $CD56^+CD138^+$ populations in the U-266 and KMS-21-BM cells were isolated by the cell sorter (EPICS Elite; Beckman Coulter, Fullerton, CA, USA). Purity of the sorted cells from both cell lines was more than 95%. IGF-I levels in culture supernatants were measured using enzyme-linked immunosorbent assay (ELISA) development reagents (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's recommendations.

Ki-67 antigen and cell-cycle analysis

The expression of Ki-67 antigen, which relates to the proliferation of MM, was examined in the $CD56^-$ and $CD56^+$ MM cells as described by Kawano et al. [16]. The $CD56^-$ and $CD56^+$ cells sorted from KMS-21-BM or U-266 were cultured at an initial concentration of 2×10^5 cells/mL with the control medium containing 0.1% of ethanol, IL-6 (2 ng/mL in KMS-21-BM or 10 ng/mL in U-266), and/or LY294002 (10 μM) for 24 hours. Furthermore, to avoid the toxic effect of ethanol, we also examined Quercetin (20 μM), which is water-soluble phosphatidylinositol 3-kinase (PI3-K) inhibitor, instead of LY294002. The $CD56^-$ and $CD56^+$ cells from KMS-21-BM or U-266 were also cultured in the control medium containing goat IgG (isotype-matched control to anti-IGF-I mAb), IGF-I (100 ng/mL), or anti-IGF-I mAb (25 μg/mL) for 24 hours. After cultivation, they were fixed with 70% ethanol at 4°C for 10 minutes. After washing, FITC-anti-Ki-67 was added to the cell pellet, which incubated at 4°C for 60 minutes. After the cells were washed, 250 μL of phosphate-buffered saline and 25 μL of RNase (Sigma) were added to the cell pellet. The expression of Ki-67 and cell-cycle profile were analyzed by flow cytometry with propidium iodide (PI) staining (100 μg/mL) (Sigma).

Western blot analysis

Protein extracts from KMS-21-BM cells that were prepared with lysis buffer (50 mM Tris-HCl [pH 8.0], 170 mM NaCl, 0.5% NP-40, 50 mM NaF, 10 μg/mL leupeptin, and PMSF) were loaded on a 12.5% SDS-polyacrylamide gel, electrophoresed, and then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Richmond, CA, USA). After blocking with 2% nonfat dry milk, the membranes were incubated with the rabbit polyclonal antibodies to human cyclin D1 and p27 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). For the analysis of Akt phosphorylation, the membranes were blocked in 4% bovine serum albumin (Gibco), and incubated with anti-phospho-specific Akt (serine 473) antibody (Cell Signaling, Beverly, MA, USA). Then, they were incubated with peroxidase-conjugated goat anti-rabbit IgG (Amersham Life Science, Buckinghamshire, UK). After a wash, immunocomplexes were visualized by chemiluminescence (ECL kit, Amersham Life Science). To ensure equal protein loading, a similar experiment was performed using anti-actin or total Akt antibody as an internal control.