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Hyaluronic Acid Induces Survival and Proliferation of Human Myeloma Cells through an Interleukin-6-mediated Pathway Involving the Phosphorylation of Retinoblastoma Protein*

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Hydrophobic acid induces survival and proliferation of human myeloma cells through an interleukin-6-mediated pathway involving the phosphorylation of retinoblastoma protein.

Originating from a post-switch memory B cell or plasma cell compartment in peripheral lymphoid tissues, malignant myeloma cells accumulate in the bone marrow of patients with multiple myeloma. In this favorable microenvironment their growth and survival are dependent upon both soluble factors and physical cell-to-cell and cell-to-extracellular matrix contacts. In this report we show that hyaluronan (HA), a major non-protein glycosaminoglycan component of the extracellular matrix in mammalian bone marrow, is a survival and proliferation factor for human myeloma cells. The effect of HA is mainly mediated through a gp 80-interleukin 6 (IL-6) receptor pathway by a CD44-independent mechanism, suggesting that HA retains and concentrates IL-6 close to its site of secretion, thus favoring its autocrine activity. In addition, we show that HA-mediated survival and proliferation of myeloma cells is associated with a down-regulation in the expression of p27kip1 cyclin-dependent kinase inhibitor and a hyper-phosphorylation of the retinoblastoma protein (pRb). These data suggest that HA could be an important component in the myeloma cell physiopathology in vivo by potentiating autocrine and/or paracrine IL-6 activities.

It is well established that cell growth and signal transduction are regulated coordinately by growth factors and adhesive interactions between cells and the extracellular matrix (1, 2). In this regard, most of normal cells require a physical contact with a substrate to grow and survive (3). However this cellular interaction may be reduced or lost at terminal stages of tumor development (3, 4). Different components of the extracellular matrix such as members of the integrin family (5) or hyaluronan (HA) (6, 7) have been shown to play a critical role in this process. HA is the major non-protein glycosaminoglycan component of the extracellular matrix in mammalian bone marrow (8–10). It is the principal ligand for the widely distributed cell surface glycoprotein molecule CD44 (7, 11). Association between HA and CD44 has been implicated in many physiological processes involving cell to cell or cell to extracellular matrix interactions. In particular, binding of HA to CD44 is a costimulatory signal in the activation of human T cell (12). In the same way, interaction between HA and CD44 has been shown to play a role during normal or autoimmune responsiveness by regulating murine B cell effector functions (13). It was also demonstrated that HA stimulates the growth and differentiation of CD34+ umbilical cord blood cells into mature eosinophils (14). In addition to the standard form of CD44 molecule (CD44s), the alternative splicing of at least 10 small exons, numbered v1 to v10, generates different variant isoforms (15). The overexpression of several CD44 splice variants in a variety of malignant tumors correlates with tumor aggressiveness. This supports the notion that interaction between CD44 and HA may play an important role in tumor growth and dissemination (16–20). For example, a strong expression of CD44v6 correlates with a shorter survival of patients with acute myeloid leukemia or with non-Hodgkin’s lymphoma (21, 22). It has been shown that CD44 function promotes metastatic mammary carcinoma cell survival in invaded tissue in correlation with an ability to bind and internalize HA (23). Overexpression of human CD44 promotes lung colonization during micrometastasis of murine fibrosarcoma cells (24). In vivo tumor formation by human lymphoma Namalwa cells can be suppressed by a soluble human CD44-immunoglobulin fusion protein (25). More recently, binding of HA to CD44 has been shown to reverse blockage of differentiation of human acute myeloid leukemia (AML) cells providing a new therapy way in AML (26). On the other hand, HA could act through a CD44-independent pathway. Indeed, it was suggested that HA stimulates growth of murine megakaryocyte progenitors by modifying the activity of several growth-regulating factors (27). In addition, interleukin-1 (IL-1), IL-2, and IL-6 could bind glycosaminoglycans (28), suggesting that this binding is likely to retain and concentrate the cytokines close to their site of secretion, thus favoring autocrine and paracrine activities. In the same way, it has been shown that IL-3 and granulocyte macrophage colony-stimulating factor bind to glycosaminoglycans, suggesting that small amounts of growth factors synthesized by stromal cells can act in a paracrine pathway (8, 29). More recently, hyaluronan has been shown to be a potent activator of dendritic cells from CD44-deficient mice, demonstrating that HA receptors are not required to mediate all the biological effects of HA (30).

Multiple myeloma is a neoplasm characterized by the accumulation of malignant plasma cells in the bone marrow compartment, where the microenvironment seems to be favorable for their growth and survival (31). The survival and prolifera-
tion of myeloma cells may be dependent upon both soluble factors and physical cell-to-cell contact between myeloma cells and stromal cells as well as interactions with the bone marrow extracellular matrix. In particular, IL-6, which is mainly produced by the stromal environment, is a major survival and proliferation factor for malignant plasma cells both in vitro and in vivo (32–35). IL-6 production by stromal cells from patients with multiple myeloma has been shown partly mediated by cell surface molecules such CD56, fibronectin, and especially CD44 (36, 37), suggesting that CD44 could be important in the physiopathology of multiple myeloma. Indeed, the expression of v9 containing CD44 isoforms is related to a short overall survival in multiple myeloma (38, 39). In addition, the expression of the standard form of CD44 is strongly decreased on myeloma plasma cells and nonmalignant B cells in affected bone marrow of myeloma patients (39). On the same way, an abnormally low or high concentration of HA in the serum of patients with multiple myeloma is associated with a significantly shorter median survival than those with an intermediate HA concentration (40).

Based on these observations, we have analyzed the ability of HA to promote growth and survival of myeloma cells. Human myeloma cell lines obtained from patients with the terminal phase of the disease (41) represent a good model to study the biology of tumor stem cells that are present in patients with chronic disease because they are still dependent on the addition of exogenous IL-6 to grow in vitro, similar to primary myeloma cells. In this report, we demonstrate that HA acts as a survival and proliferation factor of myeloma cells through an IL-6 autocrine pathway. These effects are partly mediated by a CD44-independent mechanism, suggesting that HA could retain and concentrate IL-6 near the plasma cells, favoring an autocrine loop. We also show that HA-mediated proliferation of myeloma cells is associated with a down-regulation in the expression of p27kip1 cyclin-dependent kinase inhibitor and a hyperphosphorylation of the retinoblastoma protein (pRb). Because HA is a major component of the bone marrow extracellular matrix, these data support the idea that HA could play a major role in the survival and proliferation of myeloma cells in vivo.

MATERIALS AND METHODS

Cell Cultures—XG-1, XG-2, and XG-6 human myeloma cell lines (HMCL) were obtained from patients with terminal disease, as described (41). The survival and growth of these cell lines are completely dependent upon the addition of exogenous IL-6. The cell lines were cultured in the presence of 5% normal human recombinant human IL-6 (Sandoz, Vienna, Austria) in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 5 × 10^−5 M 2-mercaptoethanol. HA from umbilical cord and rooster comb and chondroitin sulfate A and B were purchased from Sigma Aldrich and ICN Biomedicals.

Antibodies—Monoclonal anti-p27kip1 antibody (clone 57) was obtained from Transduction Laboratories, and monoclonal anti-CD44 antibody (clone J-173) was obtained from Immunotech, France. Monoclonal anti-pRb antibody was obtained from Pharmingen International. A3 blocking anti-CD44 monoclonal antibody was obtained from Dr. M-S. Sy (42). Monoclonal antibody against α-tubulin (clone B-5-1-2) was purchased from Sigma Aldrich. The BR3 anti-gp130 antibody was a generous gift from Dr. J. Brochier. Detection of IL-6 was performed with the IL-6 enzyme-linked immunosorbent assay purchased from Beckman Coulter-Immunotech.

Detection of Apoptotic Cells—Apoptotic cells were detected using fluorescein isothiocyanate-labeled annexin V method (FITC-annixin-V, Roche Molecular Biochemicals). Annexin V has a high affinity for phosphatidyserine present on the outer cytoplasmic membrane of apoptotic cells (43). Cells were washed, labeled with Annexin-V-Fluos according to the manufacturer's recommendations, and analyzed by flow cytometry.

Cell Cycle Distribution Analysis—The cell cycle distribution of XG cell lines was assessed by flow cytometry analysis by propidium iodide (PI) and bromodeoxyuridine (BrdUrd) double-staining. The cells were incubated for 30 min at 37°C in a medium containing 10 μg BrdUrd and then collected by centrifugation, washed twice with phosphate buffered saline (PBS), and fixed in 70% ethanol for 20 min at room temperature. After two washes with PBS, cells were resuspended in 50 μl of 3 N HCl, 0.5% Tween 20 and incubated for 20 min at 20°C to denature the DNA. The cells were then recovered by centrifugation, resuspended in 250 μl of 10 mM sodium tetraborate to neutralize the reaction, washed twice with PBS, 0.05% Tween 20, and incubated with 20 μl of anti-BrdUrd-FITC according the manufacturer's recommendations.

Flow Cytometry Analysis—The binding of HA on myeloma cells and the expression of CD44 molecules were quantitated by direct immunofluorescence staining using HA conjugated to fluorescein (HA-FTTC) or by a monoclonal antibody to the human CD44 (Immunotech, Marseille, France). 5 × 10^5 cells were washed twice with PBS supplemented with 1% (v/v) FBS. The cells were resuspended in 30 μl of PBS, 1% FBS containing HA-FTTC or CD44-FTTC monoclonal antibodies and were incubated for 45 min at 4°C. The cells were then washed twice and resuspended in 400 μl of PBS. Fluorescence analysis was performed with a FACScan fluorescence-activated cell sorter (Becton Dickinson). The nonspecific binding of the FITC conjugates was determined in control samples using a mouse IgG1-FITC negative control (Immunotech, France). The cell preparations were analyzed by size, and 10^4 cells were evaluated for the percentage of positive cells and their fluorescence intensity.

Determination of Amount of Endogenous HA Associated with the Cell Layer—The amount of endogenous HA associated with the cell layer was quantified by indirect immunofluorescence staining with a biotinylated hyaluronic acid-binding protein (HABP-biot) (Calbiochem). 5 × 10^5 cells were washed twice with PBS supplemented with 3% (v/v) FBS. The cells were resuspended in 100 μl of PBS, 3% FBS containing 10 μg/ml HABP-biot for 4 h at 4°C. The cells were then washed twice with PBS. The HABP-biot-labeled cells were revealed with streptavidin conjugated to phycoerythrin (Immunotech, France). The nonspecific binding of the phycoerythrin conjugate was determined in control samples using streptavidin-phycoerythrin alone. The cell preparations were analyzed by size, and 10^4 cells were evaluated for the percentage of positive cells and their fluorescence intensity.

Proliferation Assay—The cells were cultured in 96-well microtiter plates with various concentrations of IL-6 or HA. Cultures were made in triplicate. 8 h before stopping the cultures, 0.5 μCi/well of [3H]thymidine (specific activity: 25 Ci/mM, ICN France, Orsay, France) was added, and the [3H]thymidine incorporation was determined as previously described (45). Western Blotting Analysis—Cells (1 × 10^6) were resuspended in 50 μl of SDS-polyacrylamide-loading buffer (10 mM Tris-HCl, pH 6.8, 1% SDS, 5 mM EDTA, and 50% glycerol) and incubated 5 min at 90°C. The proteins were fractionated on a 10% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. After a blocking step, the membrane was incubated with the appropriate antibody and then developed using a chemiluminescent detection system (ECL, Amersham Pharmacia Biotech).

Immunofluorescence Analysis—Immunofluorescence was performed with anti-CD44 monoclonal antibodies (diluted 1/200). To this aim, cells were collected by centrifugation, resuspended in PBS, and plated on polysine-coated slides. The cells were fixed for 5 min in PBS containing 5% formaldehyde. CD44 was detected with an anti-CD44 monoclonal antibody conjugated to fluorescein. Slides were viewed using a Leica microscopic, and image files were processed with the Adobe Photoshop program.

Statistical Analysis—The means percentages of apoptotic cells were determined for the different culture conditions, and the statistical significance was evaluated by using the Student t test for pairs.

RESULTS

HA Antagonizes the Apoptosis Induced by the Removal of IL-6 on XG-1, XG-2, and XG-6 Myeloma Cell Lines—to investigate the effect of HA on myeloma cell survival, the XG-1, XG-2, and XG-6 cell lines, whose survival and proliferation are dependent on addition of exogenous IL-6 (41), were starved of
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IL-6 and then cultured with various concentrations of HA or with 5 ng/ml IL-6. Because cells underwent necrosis when cultured after 4 days without IL-6 (46), the percentage of apoptotic cells was evaluated on day 3 by flow cytometry analysis with the annexin V-staining method. For XG-2, apoptosis was evaluated on day 4 because this cell line is less sensitive to IL-6 removal. As shown in Fig. 1A, 42% of XG-6 and 29% of XG-1 myeloma cells died by apoptosis within 3 days upon removal of IL-6, and 33% of XG-2 died within 4 days. The apoptosis was blocked by the addition of IL-6. These data are consistent with the differences in the IL-6 dependence previously described for each cell line (41). The addition of HA significantly reduced the percentage of apoptotic cells on the three cell lines tested (Fig. 1A and Table I). Interestingly, the reduction in the number of apoptotic cells is more efficient for the XG-6 cell line, which exhibited the higher sensitivity to IL-6 depletion. This experiment was reproduced several times with three HA preparations of two different origins. The mean values and the statistical significance of these experiments are presented in Table I.

The survival activity of HA began to be detected with 5 μg/ml HA (Fig. 1B). An optimal survival effect was obtained for each cell line with HA concentrations ranging between 50 and 80 μg/ml (Fig. 1B). Because HA preparations may be usually contaminated with chondroitin sulfate A and B, we have tested the effect of these two sulfated glycosaminoglycans on the survival of myeloma cells. In the same experimental conditions, the survival of myeloma cells was unaffected by the addition of each of these components (data not shown). In addition, no significant loss in HA-mediated survival activity was observed with HA previously incubated for 10 min at 95 °C, and no presence of IL-6 was detected in HA preparations by using an IL-6 immunoassay (data not shown). These data indicate that the survival of myeloma cells in the presence of HA preparations was due to HA and not to a contaminating protein or glycan. We have then analyzed the kinetics of survival induction using the XG-6 cell line and a HA concentration of 80 μg/ml. As shown in Fig. 1C, HA-induced survival was clearly observed after 48 h of culture, 29% of apoptosis without HA versus 20% with HA, whereas no significant effect was detected at 24 h of culture. The maximal effect was observed at 96 h (32% versus 32%).

HA Is a Survival Factor for Human Myeloma Cells

Because IL-6 is the major survival and proliferation factor for malignant plasma cells both in vitro and in vivo (32–35), we have investigated the ability of HA to support proliferation of myeloma cells in the absence of IL-6. To this aim, the cell cycle distribution of XG-6 cell line incubated for 72 h with or without IL-6 (5 ng/ml) or in the presence of 80

FIG. 1. HA is a survival factor for human myeloma cells. A, the XG-1 and XG-6 cell lines were cultured for 72 h and XG-2 for 96 h in culture medium supplemented with 10% FBS in the presence or absence of IL-6 (5 ng/ml) or in the presence of HA (80 μg/ml). Apoptotic cells were detected in flow cytometry by the annexin V staining method. Flow histograms are shown for each cell line cultured in the three different conditions. In the histograms the abscissa represents the fluorescence intensity, and Counts represents the relative cell number. For each experimental condition the percentage of apoptotic cells (M1 gate in each histogram) is indicated under the histogram. B, the XG-1, XG-2, and XG-6 cells were cultured for 72 h in culture medium supplemented with 10% FBS in the presence of different concentrations of HA. The histogram represents the percentage of apoptotic cells for each concentration of HA for the three cell lines, XG-6 (△), XG-1 (○), and XG-2 (□). C, time course of HA-induced survival. The XG-6 cells were incubated with HA (80 μg/ml) for the indicated times (0, 24, 48, 72, 96 h). The percentage of apoptotic cells determined as previously described are presented for the cells cultured with IL-6 (○), with HA (△), and without IL-6 and HA (□).

| Cell lines | +IL-6 | -IL-6 | -IL-6+HA | n | p     |
|------------|------|------|---------|---|------|
| XG-1       | 15.6 ± 1.9 | 31.4 ± 1.9 | 20.6 ± 3.5 | 6 | 0.0002 |
| XG-2       | 14.5 ± 1.0 | 30.0 ± 2.9 | 24.5 ± 2.7 | 4 | 0.0156 |
| XG-6       | 8.5 ± 1.7 | 47.5 ± 3.7 | 28.9 ± 4.2 | 9 | 0.0001 |
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![Fig. 2. HA is a proliferation factor for human myeloma cells. XG-6 cell line incubated for 72 h in culture medium supplemented with 10% FBS in the presence (A) or absence (B) of IL-6 (5 ng/ml) or in the presence (C) of HA (80 μg/ml). The cell cycle distribution of cells was assessed in flow cytometry by PI and BrdUrd double-staining method. Flow dot plots and histograms are shown for each experimental condition. In the dot plot, FL1-height (FL1-H) represents the fluorescence intensity of BrdUrd staining, and FL2-height (FL2-H) represents the fluorescence intensity of PI staining. In the histogram, the cellular DNA content in each of the experimental condition is represented by FL2-height (PI staining), and Counts represents the relative cell number. The percentage of cells in S phase (number of cells in the upper and lower right quadrants in the dot plot) and G0/G1 (number of cells in the upper and lower left quadrants in the dot plot) phases of the cell cycle are indicated underneath each histogram.

![Fig. 3. Time course of HA-induced cell cycle redistribution. XG-6 cells were incubated with (○) or without (■) IL-6 (5 ng/ml) or with HA (▲) (80 μg/ml) for the indicated times (0, 24, 48, 72 h). The number of cells in the S phase of the cell cycle was determined as described in Fig. 2.]

μg/ml HA were assessed by flow cytometry analysis with PI and BrdUrd double-staining. Cells were analyzed by size, and 2 × 10⁶ cells were evaluated for their fluorescence intensity. To better show the cell cycle distribution, the non-apoptotic cells were gated and analyzed. In the presence of IL-6, 39% of cells (measured by the number of cells in the upper and lower right quadrants in each dot plot) were in the S phase of the cell cycle (Fig. 2A). Removal of IL-6 promoted an accumulation of cells in the G1 phase (number of cells in the lower left quadrants in each dot plot; Fig. 2B), with a strong diminution of the number of cells in the S phase (11%), as previously described (46). In culture medium where HA was substituted for IL-6, a large increase in the percentage of cells in the S phase (22%) was observed concomitantly with a diminution of cells in the G1 phase (Fig. 2C). These data demonstrated that HA is a survival and proliferation factor for myeloma cells. Interestingly, the kinetics of HA-restored cell cycle distribution (Fig. 3) was very similar to the kinetics of HA-induced survival, suggesting that HA-mediated survival of myeloma cells is coupled to the regulation of cell cycle progression. Similar data were obtained with the XG-1 cell lines (data not shown).

HA-induced Survival and Proliferation of Myeloma Cell Lines Is Partly CD44-independent—HA is the main ligand for the cell surface glycoprotein CD44 (7, 11), and most of the biological properties of HA are mediated by its binding to CD44 molecules (47). However, a lower expression of the standard form of CD44 associated with expression of various variant CD44 isoforms is observed on myeloma plasma cells, suggesting that an abnormal CD44-signaling pathway and/or CD44-mediated cellular adhesion is involved in multiple myeloma (38, 39). These data prompted us to analyze the ability of HA to bind myeloma cells via CD44 molecules. The percentage of HA binding cells was quantitated by labeling cells with HA conjugated to fluorescein (HA-FITC) (44). To this aim, XG cells were incubated 45 min on ice with HA-FITC, and the frequency of HA-binding cells was quantitated by labeling cells with HA conjugated to fluorescein (44). To this aim, XG cells were incubated 45 min on ice with HA-FITC, and the frequency of HA-positive cells was determined with a flow cytometer. As shown in Fig. 4A, XG-1 and XG-2 cells bound HA-FITC, and this binding was strongly decreased when cells were incubated with the A3 anti-CD44 antibody that blocks the binding of HA to CD44 before HA-FITC addition (Fig. 4B). These data demonstrated that HA-FITC binding was essentially mediated by cell surface CD44 molecules. Surprisingly, even though they are very sensitive to the survival and proliferation activity of HA, the XG-6 cells do not bind HA-FITC efficiently. These data are consistent with the fact that XG-6 has lost the capacity to stimulate the production of IL-6 by osteoblastic cell lines through a CD44-mediated pathway, suggesting the absence or the weak presence of functional CD44 molecules (36). To test whether the CD44 molecules expressed by the XG-6 cell line need to be activated to bind HA-FITC efficiently, we have tested the ability of TNFα and IL-6 to potentiate HA-FITC binding. In fact, TNFα is known to be the most efficient factor able to activate CD44 to bind HA (48), and TNFα has been shown to be a survival factor for myeloma cells (49). In our experimental conditions, neither TNFα nor IL-6 could enhance HA-FITC binding on all our cell lines. There was no direct correlation between cellular binding of HA and its biological activity. According to that, HA-induced survival was not affected when the cells were incubated in the presence of blocking A3 anti-CD44 antibody (data not shown).

High levels of HA binding to CD44 require reorganization of the cytoskeleton proteins and clustering of CD44 on the cell surface (44). We therefore investigated whether the weak ability of XG-6 to bind HA-FITC is due to a lack of CD44 expression or in a deficiency in CD44-clustering formation. XG-6 cells were incubated with an anti-CD44 antibody conjugated to fluorescein and stained with FITC-conjugated anti-CD44 antibody. The percentage of cells that bind HA-FITC was determined by flow cytometry. The results showed that HA-FITC binding was significantly decreased in the presence of blocking anti-CD44 antibody (data not shown).

HA-mediated survival of myeloma cells is coupled to the regulation of cell cycle progression. Similar data were obtained with the XG-1 cell lines (data not shown).

HA-induced Survival and Proliferation of Myeloma Cell Lines Is Partly CD44-independent—HA is the main ligand for the cell surface glycoprotein CD44 (7, 11), and most of the biological properties of HA are mediated by its binding to CD44 molecules (47). However, a lower expression of the standard form of CD44 associated with expression of various variant CD44 isoforms is observed on myeloma plasma cells, suggesting that an abnormal CD44-signaling pathway and/or CD44-mediated cellular adhesion is involved in multiple myeloma (38, 39). These data prompted us to analyze the ability of HA to bind myeloma cells via CD44 molecules. The percentage of HA binding cells was quantitated by labeling cells with HA conjugated to fluorescein (HA-FITC) (44). To this aim, XG cells were incubated 45 min on ice with HA-FITC, and the frequency of HA-positive cells was determined with a flow cytometer. As shown in Fig. 4A, XG-1 and XG-2 cells bound HA-FITC, and this binding was strongly decreased when cells were incubated with the A3 anti-CD44 antibody that blocks the binding of HA to CD44 before HA-FITC addition (Fig. 4B). These data demonstrated that HA-FITC binding was essentially mediated by cell surface CD44 molecules. Surprisingly, even though they
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Because IL-6 is a major survival and proliferation factor for myeloma cells, we then investigated whether the survival effect of HA could be mediated by IL-6. To this aim, XG-1 and XG-6 cells were incubated in the presence of neutralizing IL-6 antibody or antibody directed against the gp80 IL-6 binding chain of IL-6 receptor. In these experimental conditions, the survival effect of HA was completely abolished (Fig. 7). These data suggested that HA survival effect on myeloma cells was partly mediated through an IL-6 autocrine process. However, in the absence of HA, the autocrine secretion of IL-6 is not efficient to promote long time cell survival. In addition, HA alone has no effect because its activity is completely abolished with antibodies neutralizing IL-6 activity (Fig. 7), suggesting that both HA and IL-6 are required for an optimal effect. We have therefore compared the activity of HA on XG-6 cell line in the presence of different concentrations of exogenous IL-6. For a better sensitivity, the proliferation activity of HA was quantified by [3H]thymidine incorporation. As shown in Fig. 8A, the effects of HA and exogenous IL-6 are synergistic for the very low concentrations of IL-6 (<20 ng/ml). For the higher concentrations, the effects of HA are masked by the more efficient activity of exogenous IL-6. By considering the rate of proliferation with HA alone, we determined that the proliferative activity of HA was equivalent to a concentration of ~8 pg/ml IL-6. For a low concentration of exogenous IL-6 (5 pg/ml), an increase in the rate of proliferation was observed by adding HA concentrations ranging between 0.5 and 15 μg/ml (Fig. 8B). An optimal effect was obtained with HA concentrations greater than 15 μg/ml.

HA Induces Survival of Myeloma Cells through an IL-6 Autocrine Pathway—Because IL-6 and other cytokines that activate signaling cascades through gp130 are the major survival factors for myeloma cells (51), we investigated whether the anti-apoptotic effect of HA on myeloma cells was mediated through a gp130 activation. To this aim, XG-1 and XG-6 cells were incubated for 72 h in the presence of 80 μg/ml HA with or without 10 μg/ml of a neutralizing (BR3) anti-gp130 monoclonal antibody previously reported to block the signaling activities of IL-6, IL-11, ciliary neurotrophic factor, and oncostatin M/leukemia inhibitory factor (52). As shown in Fig. 7, the survival effect of HA was completely inhibited by the BR3 antibody. Interestingly, the anti-gp130 antibody enhanced apoptosis induced by the removal of IL-6, suggesting the existence of an autocrine loop acting through a gp130-signaling pathway. These data clearly indicate that the myeloma cell survival activity of HA was dependent on gp130 transducer activation.

Finally, we investigated the possibility that XG-6 already has endogenous HA occupying cell surface receptors, preventing the binding of HA-FITC. The amount of endogenous HA associated with the cell layer was quantified by staining cells with a HABP-biot known to bind hyaluronan specifically and strongly (50). To this aim, XG-1 and XG-6 cells were incubated for 4 h at 4 °C with HABP-biot, and the frequency of HABP-biot-positive cells was determined by flow cytometry after staining with streptavidin-phycoerythrin conjugate. Interestingly, XG-6 cells exhibited a higher concentration of membrane-associated hyaluronan accessible to the probe compared with the XG-1 cells (Fig. 6). The specificity of the staining was controlled by preincubating HABP-biot with soluble HA (5 μg/1 μg HABP-biot) for 2 h at 4 °C (Fig. 6). These data suggest the possibility that the binding of HA-FITC on XG-6 cells might be masked by the abundant concentration of endogenous membrane-associated HA and can explain why we found no correlation between the ability of myeloma cells to bind HA-FITC and the myeloma cell survival activity of HA.

HA Modulates the Expression of p27kip1 Cyclin-dependent Kinase Inhibitor and the Phosphorylation Status of pRb—Cell cycle progression induced by growth factors through G1 phase requires inactivation of the pRb by phosphorylation involving both cyclin D-cdk4/6 and cyclin E-cdk2 complexes (53–55). In particular, the activation of cyclin E-cdk2 seems to be due to the inhibition of the expression of the p27kip1 cyclin inhibitor rather than in variations of cyclin expression itself (56). In addition, growth factor stimulation of cyclin D and E requires cell anchorage or interaction to the extracellular matrix (57), suggesting that HA-induced cell cycle progression could be associated with regulation of cyclin D and E. The fact that cell cycle progression induced by IL-6 in IL-6-deprived ANBL-6 and KAS-6/I myeloma cells has been shown associated with an hyperphosphorylation of pRb pleads for this hypothesis (58). We therefore examined the modulation of p27kip1 in myeloma cell lines. As shown in Fig. 8A, the effects of HA and exogenous IL-6 are synergistic for the very low concentrations of IL-6 (<20 ng/ml). For the higher concentrations, the effects of HA are masked by the more efficient activity of exogenous IL-6. By considering the rate of proliferation with HA alone, we determined that the proliferative activity of HA was equivalent to a concentration of ~8 pg/ml IL-6. For a low concentration of exogenous IL-6 (5 pg/ml), an increase in the rate of proliferation was observed by adding HA concentrations ranging between 0.5 and 15 μg/ml (Fig. 8B). An optimal effect was obtained with HA concentrations greater than 15 μg/ml.

FIG. 4. Binding of HA on human myeloma cell. A, the XG-1, XG-2, and XG-6 cells were incubated for 45 min on ice with HA-FITC. Fluorescence analysis was performed with a FACScan fluorescence-activated cell sorter (Becton Dickinson). The cell preparations were analyzed by size, and 10^4 cells were evaluated for the percentage of positive cells and their fluorescence intensity. B, XG-2 binding of HA-FITC (continuous line) is strongly abolished when the cells were incubated in the presence of blocking anti-CD44 antibody before the addition of HA-FITC (hatch line).
cells during HA-mediated proliferation. To this aim, total protein extracts from XG-1, XG-2, and XG-6 cells deprived in IL-6 and then cultured with or without HA were analyzed by immunoblotting with a specific p27kip1 antibody. As expected, the accumulation of cells in G1 phase of the cell cycle by the removal of IL-6 resulted from a high expression of p27kip1 (Fig. 9A). This expression was repressed by the addition of HA (80 μg/ml) in the cell lines very sensitive to IL-6 depletion, XG-1

Fig. 6. Total amount of endogenous HA associated with the cell layer. A, The XG-1 and XG-6 cells were incubated for 4 h at 4°C with HABP-biot. HABP-biot-labeled cells were revealed with streptavidin conjugated to phycoerythrin. Fluorescence analysis was performed with a FACSScan fluorescence-activated cell sorter (Becton Dickinson). The cell preparations were analyzed by size, and 10^5 cells were evaluated for the percentage of positive cells and their fluorescence intensity. For each cell line, the binding of HABP-biot (continuous line) was strongly abolished when HABP-biot was preincubated with soluble HA (5 μg/1 μg HABP-biot) (hatched line).

Fig. 7. HA induces survival of myeloma cells through an IL-6 autocrine pathway. The XG-1 and XG-6 cell lines were cultured for 72 h in medium containing 5 ng/ml IL-6 or 80 μg/ml HA in the presence or absence of either blocking gp130 antibody (BR3), blocking anti-gp80 antibody (M195), or neutralizing IL-6 antibody (BE8). Apoptotic cells were detected in flow cytometry by the annexin V staining method. The histograms represent the percentage of apoptotic cells for each experimental condition.
and XG-6, whereas no significant variation was observed for the XG-2 cell line. These data were consistent with the fact that XG-2 is less sensitive to the removal of IL-6.

The up-regulation of $p27^{kip1}$ expression by HA prompted us to examine the possibility that HA-induced proliferation of myeloma cells resulted from differential phosphorylation of pRb. As shown in Fig. 9B, the depletion of IL-6 induced the hypophosphorylation of pRb in the XG-6 cell line, as revealed by the accelerated rate of pRb electrophoretic migration, as previously described (59). 72 h stimulation of the cells with HA resulted in the appearance of the hyperphosphorylated form of pRb. A minor part of pRb remained hypophosphorylated, which is consistent with the fact HA is less efficient than the large amount of exogenous IL-6 used to induce growth and survival of myeloma cells.

**DISCUSSION**

The ability of transformed cells to avoid apoptotic pathways confers to them a selective growth and survival advantage and an enhanced metastatic capacity (5). Originating from post-switch or plasmocytoma cells, malignant myeloma cells develop in bone marrow that supports their survival and growth. Multiple myeloma is characterized by a very slow proliferation rate, suggesting that the accumulation of plasma cells in bone marrow could be due to a resistance to apoptotic process. This observation is very important because it shows the necessity for plasma cells to interact physically or by means of soluble factors with the stromal matrix to survive. Although IL-6 is the major survival and proliferation factor for myeloma cells (34, 60), additional factors have been shown to promote myeloma cell survival or proliferation in the absence of IL-6, such as interferon $\alpha$ (46, 61), tumor necrosis factor $\alpha$, and insulin like growth factor 1 (62).

In this report, we demonstrated that HA, the major nonprotein glycosaminoglycan component of the extracellular matrix in mammalian bone marrow (8–10), stimulates the survival and growth of myeloma cell lines cultured in the absence of exogenous IL-6. An optimal survival effect was obtained for each cell line with HA concentrations ranging between 50 and 80 mg/ml. The effect of HA is more pronounced on cells exhibiting a higher sensitivity to IL-6 removal. We demonstrated that the myeloma cell survival activity of HA preparation was due to HA. Indeed, no survival effect was observed with the sulfated glycosaminoglycans chondroitin sulfate A and B, which can usually contaminate HA preparation. In addition, no significant loss in the HA-mediated survival activity was observed when HA was heated for 10 min at 95 °C, excluding that the survival activity of HA preparations could be due to a contamination by cytokines or growth factors. Using antibodies neutralizing the gp130 transducer, we found that HA promoted myeloma cell survival and proliferation through an activation of gp130. This is not surprising because IL-6 and other cytokines that activate signaling cascades through gp130 are major survival factors for myeloma cells (51). In addition, we have previously shown that some of these cell lines may produce...
oncostatin M or IL-6, suggesting that HA could act by inducing or potentiating an autocrine loop of activation. Indeed, HA was previously shown to induce bone marrow macrophages to secrete IL-6 and to stimulate the expression of IL-1β, TNFα, and insulin like growth factor 1 mRNA transcription in macrophages (63). Using neutralizing IL-6 antibody or blocking antibody directed against the gp80 IL-6 receptor, we showed that the HA survival effect on myeloma cells was mainly mediated through an IL-6 autocrine process. For macrophage and other cell lineages, HA activity was mediated by binding to CD44 cell surface molecules. However, HA could induced cytokine secretion through a CD44-independent pathway (64). On the other hand, it was suggested that HA stimulates growth of murine megakaryocyte progenitors by modifying the activity of several growth-regulating factors (27). This hypothesis could explain why we found no correlation between the ability of myeloma cells to bind HA and the myeloma cell survival activity of HA. In particular, we failed to detect HA-FITC binding to XG-6 cells, even though these cells were the most sensitive to HA-induced survival and proliferation and expressed a large density of CD44 molecules. In addition, HA-induced survival was not affected when these cells were incubated in the presence of blocking anti-CD44 antibody, and no detectable production of IL-6 was detected by using an IL-6 immunosassay, suggesting that HA could protect and concentrate IL-6 near the plasma cells and potentiate the autocrine activity of IL-6 in a CD44-independent way. Several previously published data plead for this hypothesis. Indeed, now an increasing number of cytokines and interleukins are known to bind selectively on glycosaminoglycans (28, 65), allowing a restricted diffusion of these small soluble glycoproteins away from tissue microenvironments of secretion and favoring autocrine and paracrine rather than endocrine activity. In particular, IL-6 has been shown to bind selectively at a physiological ionic strength on various glycosaminoglycans such as hyaluronan, heparin, dermatan, and dextran sulfate (28, 66). Interestingly, chondroitin sulfates, which have no effect on the survival of myeloma cells, poorly bind IL-6 (28, 66), suggesting that the binding of IL-6 to hyaluronan could be critical in its survival activity. More recently, hyaluronan has been shown to be a potent activator of dendritic cells. HA-induced dendritic cell maturation does not involve the HA receptor CD44 or the receptor for hyaluronan-mediated motility. Indeed, dendritic cells from CD44-deficient mice and wild type mice both responded similarly to HA stimulation in the absence of detectable receptors for hyaluronan-mediated motility (30). These data demonstrated that HA receptors are not required to mediate all the biological effects of HA. However, we cannot exclude that the important concentration of endogenous membrane-associated hyaluronan observed on XG-6 cell line may interfere with the binding of HA-FITC to another HA-related receptor.

Growth arrest of cells that accumulated in the G2 phase of the cell cycle by contact inhibition or mitogen withdrawal is associated with a high level of p27kip1 cyclin-dependent kinase inhibitor expression (67). The inhibition of cyclin-dependent kinase activity by p27kip1 results in a hypophosphorylation of pRb (53, 55, 56, 68). In addition, phenomena such as apoptosis, which cooperatively depend on the cell cycle machinery for their proper execution, may be influenced by modulation in the expression of the p27kip1. In our study, we demonstrated that HA-mediated survival and proliferation of myeloma cells is correlated with a down-regulation in the expression of p27kip1 cyclin-dependent kinase inhibitor. According to the fact that cyclin-dependent kinase inhibitor functions by inhibiting cyclin-dependent kinase-mediated phosphorylation of pRb, we showed that HA-induced proliferation of myeloma cells resulted from a hyperphosphorylation of pRb. Interestingly, transgenic mice in which pRb was inactivated developed slowly growing tumors with high rates of apoptosis (69), suggesting a control of apoptosis by the cell cycle machinery. The fact that the loss of pRb function has been shown to trigger the p53 apoptotic pathway supports this idea (70). In contrast, HA inactivation of pRb by hyperphosphorylation is associated with a decrease in the rate of apoptosis of myeloma cells. This apparent paradigm could be explained by the frequent alteration of p53 pathway observed on myeloma cells. In particular, a strong increase in the percentage of cells with mutation in the p53 gene was observed in the leukemic terminal stage of the disease (71). In addition, a strong and constitutive expression of double minute 2 (MDM2) protein, which facilitates G1 to S phase transition by activation of E2F-1 and enhances cell survival by suppressing p53 function, was observed in multiple myeloma (72, 73). In the same way, MDM2 gene expression is associated with poor prognostic features, poor response to chemotherapy, and short survival (72). These data and our report strengthen the idea that HA could play a crucial role in the myeloma cell physiopathology in vivo.

Further investigations are needed to clarify the mechanisms of HA-induced survival and growth of myeloma cells. The present findings are likely very important in the physiopathology of multiple myeloma in vivo. Indeed, HA is a main component of the bone marrow extracellular matrix in human, and high and low levels of serum HA were recently documented in patients with multiple myeloma in association with a poor prognosis. In vivo, myeloma cells from patients with chronic myeloma survive close to bone marrow stromal cells and are embedded in extracellular matrix molecules such as HA. Tumor cells from patients with chronic myeloma poorly proliferate in vivo. When these cells are purified and cultured in vitro, they rapidly die. Thus, HA and the extracellular matrix could be a critical survival factor working in synergy with signals given by stromal cells. This explains, in part, why myeloma cells accumulate in the bone marrow of patients with multiple myeloma in the earlier stages of the disease.

Immunotherapy approach in the multiple myeloma comes up against the fact that, in the earlier stage of the disease, malignant plasma cells present in the bone marrow environment of patients are characterized by a very slow proliferation rate and the incapability to survive and grow in vitro. Our data suggest that HA could be useful in immortalizing these cells to allow the development of such therapy.

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