Interleukin-6 in the Bone Marrow Microenvironment Promotes the Growth and Survival of Neuroblastoma Cells

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Abstract
Neuroblastoma, the second most common solid tumor in children, frequently metastasizes to the bone marrow and the bone. Neuroblastoma cells present in the bone marrow stimulate the expression of interleukin-6 (IL-6) by bone marrow stromal cells (BMSC) to activate osteoclasts. Here we have examined whether stromal-derived IL-6 also has a paracrine effect on neuroblastoma cells. An analysis of the expression of IL-6 and its receptor, IL-6R, in 11 neuroblastoma cell lines indicated the expression of IL-6 in 4 cell lines and of IL-6R in 9 cell lines. Treatment of IL-6R–positive cells with recombinant human IL-6 resulted in signal transducer and activator of transcription-3 and extracellular signal–regulated kinase-1/2 activation. Culturing IL-6R–positive neuroblastoma cells in the presence of BMSC or recombinant human IL-6 increased proliferation and protected tumor cells from etoposide-induced apoptosis, whereas it had no effect on IL-6R–negative tumor cells. In vivo, neuroblastoma tumors grew faster in the presence of a paracrine source of IL-6. IL-6 induced the expression of cyclooxygenase-2 in neuroblastoma cells with concomitant release of prostaglandin-E2, which increased the expression of IL-6 by BMSC. Supporting a role for stromal-derived IL-6 in patients with neuroblastoma bone metastasis, we observed elevated levels of IL-6 in the serum and bone marrow of 16 patients with neuroblastoma bone metastasis and in BMSC derived from these patients. Altogether, the data indicate that stromal-derived IL-6 contributes to the formation of a bone marrow microenvironment favorable to the progression of metastatic neuroblastoma. [Cancer Res 2009;69(1):329–37]

Introduction
It has become increasingly apparent that factors that influence the progression of cancer cells originate not only from genetic and epigenetic alterations in malignant cells but also from interactions between tumor cells and nonmalignant cells in the tumor microenvironment (1–3). The tumor microenvironment not only influences the growth of primary tumors but also plays a critical role in the development of distant metastasis, a role initially recognized more than 100 years ago by Paget (4). The bone marrow and the bone, which are among the most common sites of metastasis in cancer, provide a soil that is particularly favorable to the progression of cancer cells. They are a reservoir of numerous stimulatory cytokines and growth factors and provide a sanctuary against the cytotoxic effects of chemotherapy (5, 6). The bone marrow contains two distinct populations of stem cells that contribute to cancer progression. The first consists of hematopoietic stem cells located in the endosteal niche. When mobilized toward the vascular niche, these cells mature into vascular endothelial growth factor receptor (VEGFR)-1– and VEGFR-2–expressing cells, which are recruited by the primary tumor where they contribute to inflammation and vascularization. VEGFR-1–positive cells also colonize distant organs where they form premetastatic niches (7). The second population is made of mesenchymal stem cells. These cells give rise to a broad spectrum of stromal cells, including osteoblasts, osteocytes, chondrocytes, smooth muscle cells, adipocytes, fibroblasts, myofibroblasts, and cardiac muscle cells, and have the capacity to repair injured tissues (8, 9). These cells express a variety of cell surface-associated markers like STRO-1, CD105, CD44, and CD166 (10, 11). Their role in cancer progression is still poorly understood.

Neuroblastomas are biologically heterogeneous tumors of neural crest origin and represent the second most common solid tumor in children (12). Despite major progress in our understanding of the biology of this type of cancer and in the treatment of these patients with intensive myeloablative chemotherapy, bone marrow transplantation, and retinoic acid therapy (13), metastasis remains the leading cause of morbidity and mortality. It is present in ~60% of children with neuroblastoma at the time of diagnosis, with the most common sites of metastasis being the bone marrow, bone, and liver (14). Bone metastasis in neuroblastoma is characterized by the presence of osteolytic lesions caused by an increase in osteoclast activation (15). We have previously reported that most neuroblastoma cells do not produce osteoclast-activating factors like parathyroid hormone–related peptide or receptor activator of nuclear factor-κB ligand, which are typically produced by metastatic breast cancer cells, but stimulate the production of interleukin-6 (IL-6) by bone marrow stromal cells (BMSC), which are a potent activator of osteoclasts (16).

IL-6 is a pleiotropic cytokine that exerts its effect through interaction with the IL-6 receptor complex composed of a α-chain subunit (IL-6Rα/gp80) and a β-chain subunit (gp130; refs. 17, 18). Whereas many cells express gp130, the expression of the IL-6Rα/gp80 provides the specificity of the response to IL-6 (17). Binding of IL-6 to its heterodimeric receptor leads to conformational changes in the gp130 subunit, which, through activation of Janus kinases (Jak), activates members of the signal transducer and activator of transcription (STAT) family of proteins and the extracellular signal–regulated kinase (Erk)-1/2 pathway (19–21). IL-6Rα/gp80 can be present in a soluble form (sIL-6R) generated by
either alternate splicing or proteolytic shedding of the membrane-associated receptor. It acts as an agonist and potentiates the activity of IL-6 (22). Cells lacking IL-6Ra/gp80 can therefore become sensitive to IL-6 in the presence of sIL-6R (23).

The observation that neuroblastoma cells increase the expression of IL-6 by BMSC raised the question whether IL-6, in addition to activating osteoclasts, could also have a paracrine effect on tumor cells within the bone marrow microenvironment. Supporting this concept, we show in this paper that neuroblastoma cells respond to IL-6 and that IL-6 stimulation provides them with a proliferative and a survival advantage.

Materials and Methods

Cell cultures. Eleven cell lines with and without MYCN amplification were obtained from Dr. C. Patrick Reynolds (Childrens Hospital Los Angeles, Los Angeles, CA) with the exception of NB-19 cells, which were obtained from RIKEN (BioResource Center). SAOS-2 and MG-63 human osteosarcoma cells were purchased from American Type Culture Collection. CHLA-255/Luc expressing the firefly luciferase reporter gene were used as previously reported (24). CHLA-255 cells overexpressing human IL-6 (hIL-6) were obtained by infecting cells with the viral supernatant of 293 FT cells expressing a lentivirus (pLenti4/TO/V5-DEST vector; Invitrogen) in which hIL-6 cDNA was inserted using LR clonase. IL-6-expressing cells were selected in the presence of zeocin. These cells produced an average of 2.6 ng hIL-6/mL over 24 h. Human BMSC were either purchased from ALLiCells LLC or obtained from bone marrow samples of patients with neuroblastoma enrolled by the Children's Oncology Group. These samples were infiltrated with neuroblastoma cells (20% in sample NB-208 and 80% in sample NB-209). Mononuclear cells were separated by density gradient centrifugation over Ficoll-Hypaque using a human mesenchymal stem cell enrichment cocktail according to a previously reported procedure (25).

Reagents. Recombinant human IL-6 (rhIL-6), rhIL-6, and mouse monoclonal antibodies (mAb) against IL-6Ra/gp80 and gp130 were purchased from R&D Systems. Rabbit polyclonal antibodies against phospho-STAT-3, STAT-3, phospho-Erk1/2, and Erk1/2 were purchased from Cell Signaling Technology, Inc. Mouse mAb against β-tubulin and nonspecific mouse IgG were obtained from Sigma Aldrich. An unconjugated antihuman STRO-1 mouse mAb was purchased from R&D Systems. Phycocyanin-conjugated antihuman CD166, FITC-conjugated antihuman CD44, unconjugated antihuman CD34, and unconjugated anti-CD105 mouse mAb were purchased from R&D Systems. Phycoerythrin-conjugated antihuman CD166, FITC-conjugated antihuman STRO-1 mouse mAb was purchased from R&D Systems and nonspecific mouse IgG were obtained from Sigma Aldrich. An anti-human IL-6 antibody (AF-206-NA, 1:100 dilution) and gp130 and phycoerythrin-conjugated mouse IgG were purchased from BD Biosciences and used for fluorescence-activated cell sorting (FACS) analysis. The mitogen-activated protein kinase/Erk kinase-1 inhibitor PD98059 and the Jak/STAT-3 inhibitor AG490 were purchased (BD Biosciences) and used for fluorescence-activated cell sorting (FACS) analysis. The mitogen-activated protein kinase/Erk kinase-1 inhibitor PD98059 and the Jak/STAT-3 inhibitor AG490 were purchased (BD Biosciences) and used for fluorescence-activated cell sorting (FACS) analysis. The mitogen-activated protein kinase/Erk kinase-1 inhibitor PD98059 and the Jak/STAT-3 inhibitor AG490 were purchased (BD Biosciences) and used for fluorescence-activated cell sorting (FACS) analysis. The mitogen-activated protein kinase/Erk kinase-1 inhibitor PD98059 and the Jak/STAT-3 inhibitor AG490 were purchased (BD Biosciences) and used for fluorescence-activated cell sorting (FACS) analysis. The mitogen-activated protein kinase/Erk kinase-1 inhibitor PD98059 and the Jak/STAT-3 inhibitor AG490 were purchased (BD Biosciences) and used for fluorescence-activated cell sorting (FACS) analysis. The mitogen-activated protein kinase/Erk kinase-1 inhibitor PD98059 and the Jak/STAT-3 inhibitor AG490 were purchased (BD Biosciences) and used for fluorescence-activated cell sorting (FACS) analysis. The mitogen-activated protein kinase/Erk kinase-1 inhibitor PD98059 and the Jak/STAT-3 inhibitor AG490 were purchased (BD Biosciences) and used for fluorescence-activated cell sorting (FACS) analysis. The mitogen-activated protein kinase/Erk kinase-1 inhibitor PD98059 and the Jak/STAT-3 inhibitor AG490 were purchased (BD Biosciences) and used for fluorescence-activated cell sorting (FACS) analysis. The mitogen-activated protein kinase/Erk kinase-1 inhibitor PD98059 and the Jak/STAT-3 inhibitor AG490 were purchased (BD Biosciences) and used for fluorescence-activated cell sorting (FACS) analysis. The mitogen-activated protein kinase/Erk kinase-1 inhibitor PD98059 and the Jak/STAT-3 inhibitor AG490 were purchased (BD Biosciences) and used for fluorescence-activated cell sorting (FACS) analysis. The mitogen-activated protein kinase/Erk kinase-1 inhibitor PD98059 and the Jak/STAT-3 inhibitor AG490 were purchased (BD Biosciences) and used for fluorescence-activated cell sorting (FACS) analysis. The mitogen-activated protein kinase/Erk kinase-1 inhibitor PD98059 and the Jak/STAT-3 inhibitor AG490 were purchased (BD Biosciences) and used for fluorescence-activated cell sorting (FACS) analysis. The mitogen-activated protein kinase/Erk kinase-1 inhibitor PD98059 and the Jak/STAT-3 inhibitor AG490 were purchased (BD Biosciences) and used for fluorescence-activated cell sorting (FACS) analysis. The mitogen-activated protein kinase/Erk kinase-1 inhibitor PD98059 and the Jak/STAT-3 inhibitor AG490 were purchased (BD Biosciences) and used for fluorescence-activated cell sorting (FACS) analysis. The mitogen-activated protein kinase/Erk kinase-1 inhibitor PD98059 and the Jak/STAT-3 inhibitor AG490 were purchased (BD Biosciences) and used for fluorescence-activated cell sorting (FACS) analysis. The mitogen-activated protein kinase/Erk kinase-1 inhibitor PD98059 and the Jak/STAT-3 inhibitor AG490 were purchased (BD Biosciences) and used for fluorescence-activated cell sorting (FACS) analysis. The mitogen-activated protein kinase/Erk kinase-1 inhibitor PD98059 and the Jak/STAT-3 inhibitor AG490 were purchased (BD Biosciences) and used for fluorescence-activated cell sorting (FACS) analysis. The mitogen-activated protein kinase/Erk kinase-1 inhibitor PD98059 and the Jak/STAT-3 inhibitor AG490 were purchased (BD Biosciences) and used for fluorescence-activated cell sorting (FACS) analysis. The mitogen-activated protein kinase/Erk kinase-1 inhibitor PD98059 and the Jak/STAT-3 inhibitor AG490 were purchased (BD Biosciences) and used for fluorescence-activated cell sorting (FACS) analysis.

Flow cytometry. Analyses were done using a FACScan flow cytometer and the data were analyzed using the Cell Quest software (BD Biosciences). SAOS-2 human osteosarcoma cells (positive for IL-6Ra/gp80 and gp130) and NIH 3T3 (negative) cells were used as control.

Immunofluorescence. Cells were cultured in Lab-Tek II 8 chamber slides for 48 h at two different densities (2 × 10⁴ and 1 × 10⁴ per well). Cells were washed and fixed with 4% formaldehyde in PBS for 10 min and permeabilized with 0.1% Triton X-100, 15% FCS in PBS for 5 min, before being incubated with a goat anti-human IL-6 antibody (AF-206-NA, 1:100 dilution, R&D Systems) overnight at 4°C. These sections were sequentially incubated in the presence of a secondary Cy3-conjugated donkey anti-goat antibody and a FITC-conjugated horse anti-mouse IgG antibody at 1:300 dilution for 45 min in the dark at room temperature. After washing with 0.1% Triton X-100 in PBS thrice, slides were mounted with 6-diamidino-2-phenylindole (DAPI) containing Vectashield medium (Vector Laboratories) and photographed under a Zeiss fluorescent microscope. Analysis was also done on paraffin-embedded sections (4 μm) of bone marrow biopsies from five patients with neuroblastoma bone marrow metastasis. Sections were deparaffinized in xylene, rehydrated, and treated with an antigen unmasking solution (citrate buffer, pH 6.0; Vector Laboratories) for 10 min at 95°C. These sections were sequentially incubated in the presence of a goat anti-human IL-6 antibody and a mouse anti-human tyrosine hydroxylase mAb (dilution 1:1000; Pel-Freez, Arkansas, LLC). After washing, the slides were incubated in the presence of a FITC conjugated horse anti-mouse IgG antibody (dilution 1:2000) and a Cy3-conjugated donkey anti-goat IgG (dilution 1:800). The slides were mounted in DAPI containing Vectashield medium.

Animal experiments. Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were injected s.c. with 5 × 10⁵ of CHLA-255/Luc mixed with 5 × 10⁵ CHLA-225/LI-6 or 5 × 10⁵ CHLA-255/control neuroblastoma cells in the left and right posterior thoracic side. After 4 wk, mice were examined for bioluminescence as previously described (24). Animal experiments were done according to a protocol approved by Institutional Animal Care and Use Committee of Childrens Hospital Los Angeles.

Statistical analysis. All assays were done in triplicate. Comparisons between two groups were done by the unpaired Student t test and one-way ANOVA using the Tukey method of multiple comparisons. All reported P values are two sided.
Results

Neuroblastoma cells express IL-6R in the absence of IL-6 and sIL-6R. To explore whether IL-6 could have a paracrine or autocrine effect on neuroblastoma cells, we first examined the expression of IL-6Rα/gp80, gp130, IL-6, and sIL-6R in 11 human neuroblastoma cell lines by flow cytometry (IL-6Rα/gp80 and gp130) and ELISA (IL-6 and sIL-6R). The data (Table 1; Supplementary Fig. S1A) indicated the presence of the ubiquitous gp130 receptor protein in all cell lines and the presence of the IL-6Rα/gp80 protein in nine cell lines. In contrast, IL-6 was detected in unconcentrated serum-free conditioned medium of only four cell lines, and in two of these cell lines [CHLA-171 and SK-N-BE(2)] the levels were <10 pg/mL. sIL-6R was not detected. Concentration of the conditioned medium to 10^2/C2 did not result in the detection of IL-6 or sIL-6R in cell lines for which these proteins were undetected in unconcentrated serum-free medium (data not shown). These data were validated in five cell lines [CHLA-171, CHLA-255, SMS-SAN, SK-N-BE(2), and NB-19] by RT-PCR and Western blot analysis (Supplementary Fig. S1B and C).

Neuroblastoma cells respond to exogenous IL-6. We then tested the response of these neuroblastoma cell lines to exogenous IL-6 by examining the effect of rhIL-6 on STAT-3 and Erk1/2 activation, the two major signaling pathways downstream of IL-6. In CHLA-255 cells, we observed activation of STAT-3 and Erk1/2 at concentrations of rhIL-6 ranging from 10 to 100 ng/mL with a maximum between 30 and 50 ng/mL of rhIL-6 (Fig. 1A). Activation of STAT-3 and Erk1/2 occurred as early as 5 minutes after exposure to rhIL-6 and was maximal at 30 minutes (Fig. 1B). A similar activation of STAT-3 and Erk1/2 was observed with CHLA-171, SMS-SAN, and SK-N-BE(2), but not with NB-19 cells that did not

### Table 1. Expression of IL-6Rα/gp80, gp130, IL-6, and sIL-6R in human neuroblastoma cell lines

<table>
<thead>
<tr>
<th>NB cell line</th>
<th>MYCN status</th>
<th>gp130, % of cells</th>
<th>IL-6Rα/gp80, % of cells</th>
<th>IL-6, pg/mL</th>
<th>sIL-6R, pg/mL</th>
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<tbody>
<tr>
<td>CHLA-42</td>
<td>N</td>
<td>49.83</td>
<td>73.07</td>
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<tr>
<td>CHLA-90</td>
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<td>99.95</td>
<td>99.81</td>
<td>274</td>
<td>0</td>
</tr>
<tr>
<td>CHLA-119</td>
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<td>68.10</td>
<td>97.81</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CHLA-171</td>
<td>N</td>
<td>68.89</td>
<td>96.48</td>
<td>3.1</td>
<td>0</td>
</tr>
<tr>
<td>CHLA-255</td>
<td>N</td>
<td>99.41</td>
<td>97.80</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LAN-6</td>
<td>N</td>
<td>96.48</td>
<td>91.20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NB-19</td>
<td>N</td>
<td>69.48</td>
<td>0.40</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SH-SY-SY</td>
<td>N</td>
<td>98.76</td>
<td>0.40</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SK-N-BE(2)</td>
<td>A</td>
<td>82.64</td>
<td>92.62</td>
<td>6.6</td>
<td>0</td>
</tr>
<tr>
<td>SK-N-RA</td>
<td>N</td>
<td>85.00</td>
<td>98.67</td>
<td>236</td>
<td>0</td>
</tr>
<tr>
<td>SMS-SAN</td>
<td>A</td>
<td>97.46</td>
<td>95.38</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE: The expression of gp130 and IL-6Rα/gp80 was determined by flow cytometry as shown in Supplementary Fig. S1A. Expression of IL-6 and sIL-6R was determined by ELISA, and the data represent the mean concentrations in serum-free medium after 24 h. N, MYCN nonamplified; A, MYCN amplified.

Figure 1. IL-6 activates STAT-3 and Erk1/2 in IL-6R–positive CHLA-255 neuroblastoma cells.

A, expression of phospho-STAT-3 (pSTAT-3), STAT-3, phospho-Erk1/2 (pErk1/2), and Erk1/2 examined by Western blot analysis in total cell lysates (20 μg) of CHLA-255 cells collected 15 min after treatment with rhIL-6 at indicated concentrations. As positive control (PC), we used lysates from IFN-γ–treated HeLa cells (Cell Signaling Technology). B, cell lysates (20 μg) of CHLA-255 cells treated with rhIL-6 (10 ng/mL) for the indicated times were collected and examined for pSTAT-3, STAT-3, phospho-Erk1/2, and Erk1/2 by Western blot. C, CHLA-255 cells were treated with sIL-6R (250 ng/mL), AG490 (50 μmol/L), or an anti–IL-6R antibody (2 μg/mL) before being exposed to rhIL-6 (20 ng/mL) for 15 min. Cell lysates were then examined for pSTAT-3 and STAT-3 expression by Western blot as in A and B. IL-6–treated SAOS-2 cells were used as positive control. D, same experiment as in C, but cells were treated with PD98059 (100 μmol/L) in lieu of AG490 and examined for the expression of phospho-Erk1/2 and Erk1/2. The data in A to D are representative of one experiment from three separate experiments showing similar results.
express IL-6α/gp80 (Supplementary Fig. S2A and B). Activation of STAT-3, but not Erk1/2, was enhanced by the addition of sIL-6R (250 ng/mL), whereas activation of both signaling pathways was inhibited by a blocking antibody against IL-6α/gp80. STAT-3 activation was abrogated in the presence of AG490 (50 μmol/L), and Erk1/2 activation was blocked by PD98059 (100 μmol/L; Fig. 1C and D). Altogether, the data indicate that treatment of neuroblastoma cells that express IL-6α/gp80 and gp130 with rhIL-6 stimulates signaling pathways known to be downstream of its receptor, which suggests the presence of a functional receptor.

IL-6 produced by BMSC stimulates the proliferation of IL-6R-positive, but not IL-6R-negative, neuroblastoma cells. Because we had previously shown that human neuroblastoma cells stimulate the expression of IL-6 by BMSC in cocultures (16), we initially explored whether BMSC would affect the proliferation of neuroblastoma cells in an IL-6-dependent manner. For these experiments, we selected CHLA-255 (IL-6R positive) and NB-19 (IL-6R negative) cells. We observed that CHLA-255 cells cocultured in a transwell chamber in the presence of BMSC proliferated at a faster rate than when cultured alone (Fig. 2A). Consistent with our previous report (16), we detected a progressive increase in IL-6 production in the supernatant of the cocultures (Supplementary Fig. S3A). Confirming that the growth stimulatory effect of BMSC was mediated by IL-6, we found an absence of growth stimulation in the presence of a blocking antibody against IL-6α/gp80 (Fig. 2A). In contrast, coculturing IL-6α/gp80-deficient NB-19 cells with BMSC had no effect on their rate of proliferation. We then documented that rhIL-6 (10 ng/mL) stimulated the growth of CHLA-255 cells both in the presence (Fig. 2B) and in the absence of serum (Supplementary Fig. S3B). The growth stimulatory effect of rhIL-6 was dose dependent at concentrations ranging between 1 and 10^4 pg/mL and was neutralized in the presence of a blocking mAb against IL-6α/gp80 (Fig. 2C). In contrast, and as anticipated, rhIL-6 had no growth stimulatory effect on NB-19 cells (Fig. 2C). To confirm that IL-6 stimulated cell proliferation, we

![Figure 2](https://example.com/figure2.png)

**Figure 2.** BMSC stimulate neuroblastoma cell proliferation in an IL-6-dependent manner. A, BMSC (2.5 × 10^4 per well) and CHLA-255 cells (left) or NB-19 cells (right; 1 × 10^5 per well) were cocultured in transwell tissue culture plates. The culture medium was changed on day 4 and, where indicated, an anti–IL-6R antibody (2 μg/mL) was added on days 0 and 4. Cells were counted after trypsinization. Points, average cell numbers per well of triplicate samples; bars, SD. B, CHLA-255 cells were cultured in the presence of serum with or without rhIL-6 (10 ng/mL) added on days 0, 2, and 4. Cells were counted at the indicated times. Points, average cell numbers per dish of triplicate dishes; bars, SD. C, CHLA-255 and NB-19 cells were cultured for 4 d with rhIL-6 at indicated concentrations. An anti–IL-6R antibody (2 μg/mL) or control mouse IgG (0.2 μg/mL) was added to the culture medium of CHLA-255 cells on days 0 and 2. The number of viable cells was determined with an MTT assay as described in Materials and Methods. Points, mean percent of the control (no IL-6) at day 4 from triplicate samples; bars, SD. D, NOD/SCID mice were s.c. injected in the right side with CHLA-255/Luc cells and CHLA-255/IL-6 cells and in the left side with CHLA-255/Luc and CHLA-255/vector (control) cells. Left, representative bioluminescence data on three mice obtained 4 wk after tumor cell injection. Right, columns, mean luminescence intensity values obtained from seven mice; bars, SD. Representative of two separate experiments showing similar results.
examined its effect on cell cycle and BrdUrd incorporation. These experiments revealed an increased percent of BrdUrd-positive cells in the presence of 10 ng/mL of IL-6 without an increase in apoptotic (BrdUrd-negative, 7-AAD–positive) cells (Supplementary Fig. S3C). This growth stimulatory effect appeared to be dependent on Erk1/2 activation because it was inhibited in the presence of PD98059 (Supplementary Fig. S3D) that blocked Erk1/2 phosphorylation. However, we could not rule out the possibility that STAT-3 activation provided an alternate signaling pathway because this inhibitor, which blocked the proliferative effect on CHLA-255, inhibited both Erk1/2 and STAT-3 phosphorylation. We finally tested whether IL-6 could also have an autocrine growth stimulatory activity on neuroblastoma cells that express both the receptor and the cytokine. For this experiment, we tested the effect of a blocking anti–IL-6Rα/gp80 antibody on the proliferation of SK-N-RA cells that express IL-6R and IL-6 (Table 1). The data indicated a significant inhibition of proliferation in the presence of the blocking antibody when compared with cells incubated in the presence of a nonspecific mouse IgG (Supplementary Fig. S3E). Altogether, these data point to IL-6 having a growth stimulatory effect on neuroblastoma cells in vitro.

Paracrine IL-6 stimulates the growth of CHLA-255 neuroblastoma cells in vivo. We then tested whether IL-6 could also stimulate the proliferation of IL-6R–positive neuroblastoma cells in vivo. For these experiments, we coinjected s.c. in NOD/SCID mice CHLA-255/Luc cells with CHLA-255 cells expressing IL-6 as a paracrine source of hIL-6 and used luciferin-induced bioluminescence to determine the effect of paracrine hIL-6 (made by CHLA-255/IL-6 cells) on the proliferation of CHLA-255/Luc cells. The data indicated a 5-fold increase in the average luminescence intensity when CHLA-255/Luc cells were coinjected with CHLA-255/IL-6 when compared with coinjection with CHLA-255/vector control cells (Fig. 2D). The data point to IL-6 having a growth-stimulatory activity on human neuroblastoma cells in vivo as in vitro.

IL-6 protects neuroblastoma cells from drug-induced apoptosis. It has been previously reported that the bone marrow microenvironment is a known sanctuary for tumor cells and protects them from the cytotoxic effect of chemotherapeutic agents (5). We therefore asked the question whether IL-6 could contribute to this effect by protecting neuroblastoma cells from drug-induced cytotoxicity. For these experiments, we selected etoposide, a podophyllotoxin derivative and topoisomerase inhibitor used in the conventional treatment of patients with advanced neuroblastoma (26). We first showed that etoposide induced apoptosis and increased caspase-3 activity in CHLA-255 cells in a dose-dependent manner (Fig. 3A). Selecting a concentration of etoposide of 0.25 μg/mL, we then examined whether the apoptotic effect of etoposide on neuroblastoma cells would be altered by the presence of BMSC. For this experiment, we compared CHLA-255 (IL-6R–positive) and NB-19 (IL-6R–negative) cells. The data (Fig. 3B) revealed a trend to a decrease in etoposide-induced apoptosis when CHLA-255 cells were cocultured with BMSC that, however, was not statistically significant but was eliminated on addition of an anti–IL-6Rα/gp80 blocking antibody. There was no similar trend with NB-19 cells. Coculturing CHLA-255 cells with BMSC also inhibited the levels of caspase-3 activity in CHLA-255 cells exposed to etoposide (Supplementary Fig. S3A). Considering that etoposide also induced apoptosis in BMSC and therefore may have decreased the production of IL-6 by these cells, the data suggested that IL-6 could have a protective effect on etoposide-induced apoptosis.
To confirm this possibility, we examined the effect of rhIL-6 on etoposide-induced apoptosis in CHLA-255 and NB-19 cells. The data showed that exposure of CHLA-255 to rhIL-6 before treatment with etoposide significantly inhibited apoptosis, whereas it had no significant effect on NB-19 cells (Fig. 3C; Supplementary Fig. S4B). The data thus indicate that through the production of IL-6, BMSC protect IL-6R-positive neuroblastoma cells from the cytotoxic effect of etoposide.

Cyclooxygenase-2 induced by IL-6 in neuroblastoma cells provides an amplification loop for IL-6 expression. Because we had previously reported that the stimulation of IL-6 expression in BMSC by neuroblastoma cells is adhesion independent, we looked for the presence of a soluble factor in the medium of neuroblastoma cells that stimulates IL-6 expression. IL-6 has been shown to stimulate cyclooxygenase-2 (Cox-2) expression and, concomitantly, prostaglandin E2 (PGE2), a secreted product of Cox-2 activity. PGE2 is also known to stimulate IL-6 expression (27). Therefore, we tested whether IL-6 would affect Cox-2 expression in neuroblastoma cells. This experiment indicated an absence of expression of Cox-2 in neuroblastoma cells under basal conditions, but an induction of expression in the presence of rhIL-6 (10 ng/mL) that was abrogated in the presence of an anti–IL-6Rα/gp80 blocking antibody (Fig. 4A). Induction of Cox-2 expression by IL-6 was concomitantly associated with an increase in the amount of PGE2 secreted in the culture medium (Fig. 4B). We then showed that treatment of BMSC with PGE2 increased the expression of IL-6 in a dose-dependent manner (Fig. 4C). The data are consistent with the hypothesis that the induction of Cox-2 by IL-6 in neuroblastoma cells contributes to an amplification loop where IL-6-stimulated neuroblastoma cells secrete PGE2, further increasing IL-6 production by BMSC. To confirm this possibility, we tested whether blocking PGE2 production in IL-6–treated neuroblastoma cells by celecoxib would suppress their capacity to induce expression in BMSC. The data (Fig. 4D) indicated a dose-dependent inhibition of PGE2 in the supernatant of celecoxib-treated CHLA-255 cells and a loss of IL-6 mRNA expression in BMSC incubated in the presence of the supernatant of celecoxib-treated CHLA-255 cells. Celecoxib also inhibited the amount of IL-6 present in the supernatant of the cocultures.

IL-6 is produced by BMSC derived from patients with metastatic bone marrow disease and is increased in the serum and bone marrow of patients with neuroblastoma bone metastasis. The data reported thus far were generated with established neuroblastoma cell lines cultured in vitro. We considered that it was important to obtain evidence that IL-6 also contributed to neuroblastoma progression in patients. To accomplish this, we first examined the expression of IL-6 in bone marrow biopsies obtained from five patients with neuroblastoma bone metastasis by dual immunofluorescence to confirm the stromal origin of IL-6. The data (Fig. 5A) indicated that IL-6 was expressed in tyrosine hydroxylase–negative stromal cells located around tyrosine hydroxylase–positive tumor cells.
not possible to show that these cells represent mesenchymal stem cells. We therefore decided to isolate BMSC from the bone marrow of two patients with metastatic disease. After isolation and passage in culture, we obtained adherent cells that expressed several mesenchymal markers like STRO-1 (76.2%), CD105 (89.4%), CD166 (99.9%), and CD44 (99.4%) as determined by FACS analysis. These cells also expressed IL-6 as documented by dual-immunofluorescence (Supplementary Fig. S4). IL-6 was detected in the culture medium of these cells after several passages in culture, and its concentration was increased by 2- to 3-fold in the presence of 50× concentrated culture medium from CHLA-255 cells (Fig. 5B). These data provide evidence that BMSC are a source of IL-6 in the bone marrow of patients with neuroblastoma. Additionally, evidence that induction of IL-6 in the bone marrow microenvironment is an important mediator of neuroblastoma progression and bone metastasis was obtained by measuring the levels of IL-6 in 16 patients with neuroblastoma bone metastasis (Fig. 5C). Whereas we did not detect IL-6 in the serum and bone marrow supernatant of normal individuals, the mean levels of IL-6 were 97.9 pg/mL in the bone marrow supernatant ($n = 8$) and 14.6 pg/mL in the serum ($n = 16$) of these patients. Altogether, these data, providing evidence that IL-6 is increased in patients with metastatic neuroblastoma, support our in vitro observations and are consistent with the concept that stromal-derived IL-6 in the bone marrow contribute to a microenvironment that promotes the proliferation and survival of neuroblastoma cells.

**Discussion**

In this article, we show that IL-6 expressed by BMSC has a paracrine effect on neuroblastoma cells, stimulating their proliferation and protecting them from drug-induced apoptosis. We also provide evidence for the presence of a Cox-2–dependent amplification loop that enhances the expression of IL-6 by BMSC in the presence of neuroblastoma cells. Finally, supporting a role for IL-6 in patients with neuroblastoma bone metastasis, we document that BMSC isolated from patients with neuroblastoma bone marrow metastasis express IL-6 and that patients with neuroblastoma bone metastasis have elevated levels of IL-6 in their serum and bone marrow.

It has been well recognized, in particular in multiple myeloma, that the interaction between stromal cells and tumor cells in the bone marrow contributes to tumor progression (6). For example, adhesion of myeloma cells to BMSC through members of the cell adhesion molecules family of proteins or integrins induces the expression of IL-6 by BMSC (28). Our data show a similar contribution of BMSC and IL-6 to the progression of neuroblastoma in the bone marrow, but point to an important difference. Whereas in myeloma the induction of IL-6 by BMSC requires cell-cell contact, in neuroblastoma contact between tumor cells and BMSC is not required. Our data indicate that the release of PGE2 by IL-6–stimulated neuroblastoma cells is one of the soluble factors that contribute to the production of IL-6 by BMSC by contributing to an amplification loop. However, other soluble factors can contribute, and we have recently shown that galectin-3–binding
protein, a glycosylated protein produced by neuroblastoma cells that binds to galectin-3, a receptor present on BMSC, also stimulates IL-6 expression by BMSC (29).

The effect of IL-6 on neuroblastoma cell proliferation has been the subject of previously conflicting reports. A growth-promoting activity of IL-6 and sIL-6R in human and murine neuroblastoma cells was initially reported (30, 31), whereas other investigators reported that MYCN overexpression down-regulates IL-6 in the SH-EPO007 neuroblastoma cell line. They also showed that IL-6 does not inhibit neuroblastoma cell proliferation but inhibits endothelial cell proliferation via the STAT-3 pathway and VEGF-induced neovascularization in the rabbit cornea assay (32). Our data, which show an increase in cell proliferation and an increase in BrdUrd incorporation in association with Erk1/2 activation, are consistent with IL-6 having a growth stimulatory effect on neuroblastoma cells. The growth-promoting activity of IL-6 was also shown in vivo in mice cojected with CHLA-255/Luc and CHLA-255/IL-6 cells. Whether IL-6 could inhibit endothelial cell proliferation and angiogenesis was, however, not explored. We also show that PD98059, which inhibits Erk1/2, but not STAT-3, activation, prevents the growth stimulatory activity of IL-6. This suggests that the growth stimulatory effect of IL-6 on neuroblastoma cells is, at least in part, mediated through the Erk1/2 pathway, as was previously reported in multiple myeloma (20). However, because we also observed inhibition of growth stimulation by AG490, which inhibited both Erk1/2 and STAT-3, we cannot rule out the possibility that STAT-3 provides an alternate pathway. In B cells and renal cell carcinoma, IL-6 stimulates proliferation in a STAT-3-dependent manner (33, 34). Our data indicate that in most cases, IL-6 has a paracrine effect on neuroblastoma cells (i.e., its source is not the tumor cells), but that in some cases, it can have an autocrine effect (i.e., its source is also in the tumor cells) as shown in SK-N-RA cells.

The protective effect of IL-6 on drug-induced apoptosis is reported here for the first time in neuroblastoma. Several IL-6-mediated signaling pathways have been implicated in chemoresistance (28, 35, 36). IL-6 induces survival by transcriptionally up-regulating the X-linked inhibitor of apoptosis in cholangiocarcinoma cells (37). In multiple myeloma and colorectal cancer, STAT-3 activation up-regulates the expression of survival proteins like survivin, cyclin D1, Bcl-XL, and Mcl-1 (38). The protective activity of IL-6 on drug-induced apoptosis may also involve regulation in the expression of multidrug resistance transporters (39). The mechanism by which IL-6 protects neuroblastoma cells from drug-induced apoptosis is not known at this point, but is currently being actively investigated in our laboratory.

Supporting a role for stromal-derived IL-6 in patients with neuroblastoma bone metastasis, we showed in bone marrow biopsies that IL-6 is expressed by cells in the bone marrow stroma and not by tumor cells. We also show that BMSC isolated and cultured from the bone marrow of two patients with metastatic disease express IL-6 and that the expression is enhanced in the presence of supernatant from neuroblastoma cells. The data thus support the concept that BMSC are an important source of IL-6 in the bone marrow, but do not exclude the possibility that other nonmalignant cells also contribute. Further evidence supporting a role of IL-6 in bone metastasis in neuroblastoma was obtained by finding elevated levels of IL-6 in the serum and bone marrow of patients with neuroblastoma bone metastasis. Elevated levels of IL-6 in the serum of patients with other cancers, such as myeloma, melanoma, Hodgkin’s lymphoma, and prostate and colon carcinomas, have been reported to correlate with a more severe outcome (40–43). Whether IL-6 levels in patients with neuroblastoma are indicators of poor clinical outcome will require a larger study. Altogether, the data indicate that stromal-derived IL-6 in patients with neuroblastoma marrow metastasis is an important contributor to tumor progression.

The data raise the possibility that IL-6 could be a valuable therapeutic target in patients with high-risk neuroblastoma. Several agents targeting IL-6 have been developed and some are currently being tested in clinical trials in inflammatory diseases and malignancies. Tocilizumab, a humanized antibody against IL-6R (44), has been successfully used in patients with rheumatoid arthritis (45) and in children with systemic onset of juvenile rheumatoid arthritis (46). It is approved for the treatment of Castleman’s disease, a lymphoproliferative disease associated with elevated levels of IL-6. More recently, a genetically engineered form of this antibody suitable for delivery by gene therapy has been shown to be effective in an IL-6–dependent multiple myeloma cell line in vivo (47). mAb against IL-6 is also currently being tested in clinical trials in myeloma in combination with melphalan and dexamethasone (48). Targeting IL-6–mediated Jak2/STAT-3 with small molecules, such as capsacin and SD1008, is another approach currently being tested in preclinical models of multiple myeloma (49) and ovarian cancer (50). Our data support the testing of IL-6–targeted therapies in clinical trials in children with advanced neuroblastoma.

Disclosure of Potential Conflicts of Interest

Y.A. DeClerck: consultant/advisory board, Serono. The other authors disclosed no potential conflicts of interest.

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