Insulin-Like Growth Factor-I Receptor Expression Regulates Neuroblastoma Metastasis to Bone

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Abstract
Neuroblastoma is a pediatric tumor that preferentially metastasizes to bone. Patients with bone metastases have a mortality rate >93%, indicating a need for novel treatment targets. Our laboratory has shown that type I insulin-like growth factor receptor (IGF-IR) expression and activation regulate neuroblastoma cell proliferation, motility, invasion, and survival, and that expression of the IGF-IR correlates with neuroblastoma tumorigenicity. Bone expresses large amounts of IGF ligands, and the IGF system is required for normal bone physiology. The current study addresses the role of the IGF system in neuroblastoma metastasis to bone. Upon reaching the bone marrow through the circulation, neuroblastoma cells must dock at the bone marrow endothelium, extravasate into the bone microenvironment, and destroy bone tissue to allow for tumor growth. This report examines the effects of high IGF-IR expression on neuroblastoma cell interaction with bone. The current data show that neuroblastoma cells with high IGF-IR expression, either endogenously or through transfection, adhere to human bone marrow endothelial cells and subsequently migrate toward both IGF-I and human bone stromal cells. High IGF-IR-expressing neuroblastoma cells adhere tightly to bone stromal cells, flatten, and extend processes. When neuroblastoma cells are injected directly into the tibiae of mice, those cells with increased IGF-IR form both osteolytic lesions within the tibiae and secondary tumors within other sites. These results support the hypothesis that IGF-IR expression in neuroblastoma cells increases tumor cell interaction with the bone microenvironment, resulting in greater formation of metastases.

Introduction
Neuroblastoma accounts for 10% of all pediatric cancers (1), and advanced-stage neuroblastoma in children over 1-year old is largely incurable using current treatment protocols (2). Disseminated disease leads to a fatal outcome, and children with bone metastasis have a <7% survival rate (2, 3). Treatment protocols aimed at preventing or decreasing bone metastasis are needed to decrease childhood mortality due to neuroblastoma.

One family of factors potentially involved in neuroblastoma metastatic progression is the insulin-like growth factor (IGF) family. The IGF family consists of two ligands, IGF-I and IGF-II, whose known actions occur through type I IGF receptor (IGF-IR) activation and signaling (4). IGF-IR overexpression promotes tumor growth, progression, invasion, and metastasis (4), whereas disruption of IGF-IR expression reverses the transformed phenotype (5). Increased IGF-I, IGF-II, and IGF-IR expression is present in neuroblastoma (6), and IGF-I or IGF-II coupled to IGF-IR promotes neuroblastoma cell survival and growth (7), whereas IGF-IR inhibition induces tumor regression in mice (8).

Our work focuses on the role of the IGF-IR in neuroblastoma tumor progression and metastasis. IGF-IR promotes neuroblastoma motility (9), migration (10), and invasion (11), steps necessary for dissemination of tumor cells. The "seed and soil" hypothesis of metastasis, developed by Steven Paget, states that the molecular profile of a tumor cell (seed) must match the molecular profile of the organ (soil) for secondary tumor formation (12). Increased IGF-IR expression occurs in highly tumorigenic cells (13), and IGF ligands are produced within bone (14). Therefore, the hypothesis underlying the current study is that neuroblastoma cell "seed" expression of IGF-IR and the bone "soil" secretion of IGF-I support neuroblastoma metastasis formation in bone.

In the current study, neuroblastoma cells with high IGF-IR expression migrate through endothelium toward IGF-I and human bone stromal (HBS) cells, prevented by IGF-IR inhibitors. High IGF-IR–expressing neuroblastoma cells adhere tightly to bone stromal cells and target bone in vivo. Neuroblastoma cells with increased IGF-IR form osteolytic lesions within the tibiae and secondary tumors within other sites. These results support the hypothesis that IGF-IR expression increases neuroblastoma tumor cell interaction with the bone microenvironment, resulting in metastasis formation.

Materials and Methods
Materials. Tissue culture supplies were purchased from BD Biosciences (Bedford, MA). Chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). The IGF-IR inhibitor NVP-AEW541 was provided by Novartis Institute of Biomedical Research, Inc. (Cambridge, MA). The IGF-IR neutralizing antibody (clone 33255) and control IgG were purchased from R&D Systems, Inc. (Minneapolis, MN).

Cell culture and transfection. SHEP and IMR-32 cells were kindly provided by Dr. Valerie Castle at the University of Michigan and maintained in DMEM (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% calf serum (Hyclone, Logan, UT). SHEP/vector and SHEP/IGF-IR cells (characterized previously in ref. 15) were grown in DMEM + 10% calf serum containing 250 μg/ml G418 (Life Technologies, Carlsbad, CA). Human bone marrow endothelial (HBME) cells, human aortic endothelial cells (HAEC), and human dermal microvascular endothelial cells (HDMVEC) were kindly provided by Dr. Kenneth Pienta at the University of Michigan and maintained in DMEM containing 10% fetal bovine serum (FBS). HS-5 HBS cells were purchased (American Type Culture Collection, Manassas, VA) and grown in DMEM + 10% FBS. All cell lines were incubated at 37°C in a humidified atmosphere with 10% CO2. Cells were routinely subcultured using trypsin-EDTA (Invitrogen).

Western immunoblotting. Western blot analyses were done as previously described (16). Cell lysates were collected using modified...
radioimmunoprecipitation assay buffer containing 20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% sodium deoxycholate, and 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 100 µg/ml phenylmethylsulfonyl fluoride. Fifty micrograms protein were loaded and subjected to SDS-PAGE. After transfer to nitrocellulose membranes (Hybond, Amersham Biosciences Corp., Piscataway, NJ), the membranes were incubated in blocking solution containing 5% milk (Carnation, nonfat dry) dissolved in TBS containing 0.1% Tween 20 for 2 hours at room temperature. Blots were incubated with anti-IGF-IR primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-phospho-Akt (Cell Signaling Technology, Inc., Beverly, MA) or anti-phospho-extracellular signal-regulated kinase (Erk) 1/2 (Cell Signaling Technology) diluted 1:1000 in blocking solution overnight at 4 °C. After rinsing, blots were incubated with horseradish peroxidase (HRP)—conjugated goat-anti-mouse IgG or goat-anti-rabbit IgG secondary antibody (1:1000; Santa Cruz Biotechnology) for 1 hour at room temperature. Blots were developed with the Phototope-HRP Western Blot Detection kit (Cell Signaling Technology) and exposed to Hyperfilm-ECL film (Amersham Biosciences). Blots shown are one of at least three independent experiments done.

For dot blotting, HBME and HBS cells were grown to near confluence in a six-well plate. Growth medium was replaced with serum-free DMEM for 24 hours, and conditioned medium was analyzed for IGF-I per our published protocol (17). Recombinant human IGF-I (kindly provided by Cephalon, Inc., Westchester, PA) was used as a positive control.

Reverse transcriptase PCR. Quantitative PCR (qPCR) was done using the Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA). RNA was isolated from six-well plates using TRIzol Reagent (Invitrogen) according to the instructions of the manufacturer and cDNA was produced using the avian myeloblastosis virus-reverse transcriptase kit (Promega, Madison, WI) with oligo-dT primer per the recommendations of the manufacturer. The cDNA integrity was determined by conventional PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For qPCR, aliquots of 2 ng cDNA were combined with IGF-IR primers (150 mmol/L each) and SYBR green master mix in final volume of 25 µL in a SmartCycler (Cepheid, Sunnyvale, CA). The IGF-IR primers set used was designed to produce a 180 bp product. Primer sequences are as follows: forward 5'-CATGTTGGAGAAACACCACTT-3', reverse 5'-ACATTTTCTGGCACGGCGTTTG-3' (18). PCR was carried out in a SmartCycler (Cepheid) for 35 cycles. The presence of PCR final products was confirmed by separation on a 1.2% TAE agarose gel. The number of cycles necessary to reach threshold were determined for each cell line and compared with GAPDH. Experiments were done in triplicate.

Attachment assays. Quantitative attachment assays were done as previously described (19). Bone cells (5 x 10⁴/mL) were placed in 96-well plates and grown to confluence. SHEP, SHEP/IGF-IR, or IMR32 human neuroblastoma cell lines were released with nonenzymatic Cell Dissociation Solution (Sigma, St. Louis, MO), collected, and resuspended in DMEM and layered onto the HBS cell monolayer at a density of 3 x 10⁵ cells per well. At desired time points, cells were fixed using 4% paraformaldehyde for 20 minutes at room temperature, followed by rinsing with 0.1 mol/L PBS (pH 7.4). A POLARON sputter coater was used to coat the membranes with gold using an ion current of 20 mA and 1.5 kV for 90 seconds. Membranes were imaged using an AMRAY 1000B scanning electron microscope and SEMICAPS 2000A imaging system.

Migration assays. For transendothelial migration, HBME or HBS cells were plated in the bottom chamber of a 12-well plate and grown to near confluence. HBME cells were grown to a monolayer on the upper surface of collagen-coated 3-µm transwell filters. Before placing the transwell into the 12-well plate, the preplated HBME and HBS cells were incubated in serum-free medium for 24 hours. HBME cell-coated transwells were then placed in each well of the 12-well plate. SHEP SHEP/IGF-IR, and IMR32 cells were dissociated in trypsin-EDTA for 5 minutes, then centrifuged for 5 minutes at 3,000 rpm (1,877 x g) in a Sorval RT6000B centrifuge in the presence of 2% calf serum to inactivate the trypsin. Cells were rinsed, resuspended in DMEM, and 6 x 10⁵ cells in 100 µL DMEM were plated on top of the HBME cells. After 48 hours, nonmigrating cells were removed from the upper surface of the membrane, whereas migrating cells on the bottom surface were stained, eluted, and quantitated as previously described (11). Ten percent calf serum in DMEM was used as a positive control chemotactrant; DMEM only was used as a negative control. Optical densities were measured at 540 nm on a Multiskan Ascent plate reader (Labsystems, Franklin, MA).

Intratibial injections. Six-week-old severe combined immunodeficient (SCID) female mice (Jackson Labs, Bar Harbor, ME) were anesthetized with a 100 µL i.p. injection of 3:1 ketamine/xylazine solution diluted in normal saline. IMR32, SHEP, SHEP/IGFIR, or SHEP/vector cells (5 x 10⁵) were injected into the right proximal tibia (100 µL total volume injected), whereas 100 µL DMEM was injected into the contralateral proximal tibia (sham injection). After 7 weeks, animals were anesthetized and radiographs taken with a Faxitron X-ray machine (Faxitron X-ray Corp., Wheeling, IL). Each animal was scored as positive or negative for the presence of notable radiolucent lesions (extending from the site of injection to the outer edge of the tibia) upon examination of radiographs by two independent investigators. Animals were then sacrificed by cervical dislocation. Presence of gross tumors within the flank was scored for each animal preceding the harvest of tibias (both tumor cell–injected and contralateral, medium-only injected), liver, lungs, and eyes for histologic analysis.

Histology. To analyze tibias histologically, both the injected tibias and the control (contralateral, PBS injected) tibias were stripped of overlying soft tissues and fixed in 10% formalin for 48 hours. Tibias were decalcified using 14% EDTA for 72 hours; rinsed in 5%, 10%, and 20% sucrose in phosphate buffer; embedded longitudinally in OCT; and sectioned (10 µm). For histologic evaluation, every fifth section was stained with H&E (Sigma) per the protocol of the manufacturer. Sections were examined by at least three independent, unbiased scorers, including one veterinarian and two pathologists.

Staining with tartrate-resistant acid phosphatase (TRAP) was done to identify differentiated osteoclasts. Sections adjacent to those used for H&E staining were stained with acid phosphatase and tartrate solution for 1 hour at 37°C (using Sigma procedure number 387) and counterstained with methyl green. Osteoclasts were identified as large TRAP-positive cells.

Intracardiac injections. Six-week-old SCID female mice (Jackson Labs) were anesthetized with isoflurane. Intraventricular injection of either 1 x 10⁵ or 2 x 10⁵ tumor cells in SCID mice was followed 6 weeks later by radiography, gross examination, and tissue analysis as described for intratibial injections.

Statistical analyses. All quantitative assays were subject to one-way ANOVA analysis with a Tukey’s posttest done using GraphPad Prism 4 software (GraphPad Software, Inc., San Diego, CA). Mean values of at least three independent experiments were included in analyses. Error bars indicate SE for all graphs.

Results
IGF-IR expression in neuroblastoma cell lines. Recently, we showed increased IGF-IR protein expression in neuroblastoma cells with tumorigenic potential (13). Based on those data, four neuroblastoma cell lines were chosen for our current experiments: IMR32, a highly aggressive cell line expressing high endogenous IGF-IR; SHEP, a nonaggressive cell line expressing little IGF-IR; and...
SHEP cells transfected with either human IGF-IR (SHEP/IGF-IR) or pSFFVneo vector control (SHEP/vector). To directly compare expression between these lines, IGF-IR expression was examined via either QPCR (Fig. 1A) or Western immunoblotting (Fig. 1B). SHEP cells express little IGF-IR, both at the RNA (Fig. 1A) and protein (Fig. 1B) levels. QPCR analysis for IMR32 RNA shows a decrease in the number of cycles necessary to reach threshold compared with SHEP (mean = 8.667; \( P < 0.05 \)), indicating a higher amount of IGF-IR RNA (Fig. 1A). SHEP/IGF-IR cells, as expected, show an additional increase in IGF-IR RNA expression (mean decrease in number of cycles necessary to reach threshold = 20.33; \( P < 0.01 \); Fig. 1A). The IGF-IR protein expression level is considerably higher in both IMR32 and SHEP/IGF-IR cells than in SHEP cells alone (Fig. 1B). SHEP/vector cells have equal IGF-IR expression to parental SHEP cells (Fig. 1) and consistently display an in vitro phenotype identical to untransfected SHEP cells (13, 20), therefore, the remaining in vitro experiments within this study are done using SHEP, IMR32, and SHEP/IGF-IR cells.

**Neuroblastoma cell docking to bone endothelial cells.** Once tumor cells reach an organ site through the circulation, tumor cells attach to the endothelium in a process known as docking, which allows subsequent migration through the endothelium (12). To assess the role of IGF-IR expression on neuroblastoma cell docking, SHEP, IMR32, and SHEP/IGF-IR cell adhesion to HBME cells was measured quantitatively and qualitatively (Fig. 2A). Quantitative analysis was done using a calcein in vitro attachment assay, as described in Materials and Methods (19). Significantly more IMR32 cells than SHEP cells adhere to HBME cells (15.5% versus 3.2%, \( P = 0.0084 \); Fig. 2A). SHEP/IGF-IR cells also show increased adhesion to HBME cells compared with SHEP (10.1% versus 3.2%), although this difference just fails to reach statistical significance (\( P = 0.0517 \)).

**Figure 1.** IGF-IR expression is increased in tumorigenic neuroblastoma cells. A, total RNA was isolated from SHEP, IMR32, SHEP/vector, and SHEP/IGF-IR cells with Trizol (Invitrogen) using the protocol of the manufacturer. Real-time QPCR for IGF-IR was done using the published primers 5'-CGATGTGTGAGAAGACCACCA-3' (forward) and 5'-ACATTTCCTGGCCACCGGTTT-3' (reverse; ref. 18), followed by CYBR green fluorescence analysis using an ABI Prism 7700. The number of cycles needed to reach threshold was determined, normalized to GAPDH, and the ratio was calculated compared with SHEP. B, whole cell lysates were collected from SHEP, IMR32, SHEP/vector, and SHEP/IGF-IR cells. Lysates were run on a SDS-PAGE gel and Western immunoblotted for the IGF-IR.

**Figure 2.** IGF-IR-expressing neuroblastoma cells dock at HBME cells. A, HBME cells (5 x 10^5/mL) were placed in 96-well plates and grown to confluency. SHEP, SHEP/IGF-IR, and IMR32 human neuroblastoma lines were released with nonenzymatic Cell Dissociation Solution (Sigma); cells were then collected and resuspended in DMEM. Calcein AM (5 \( \mu L \), Life Technologies) was added to 1 x 10^5 cells and incubated at 37°C for 30 minutes with occasional agitation. Cells were then processed per the protocol of the manufacturer. Final fluorescence was taken as the ratio of adherent cell fluorescence to total cell fluorescence. Columns, average of six wells per experiment for three separate experiments; bars, SE. *, \( P < 0.05 \); **, \( P < 0.01 \), for each condition compared with control. For scanning electron microscopy, HBME cells were grown to confluence on 3 \( \mu m \) filters in DMEM + 10% calf serum. SHEP, SHEP/IGF-IR, and IMR32 cells were added to the medium, and after 6 hours, samples were rinsed and detached tumor cells were prepared for scanning electron microscopy per standard protocol (19). B, HBME, HAEC, or HDMVEC cells (5 x 10^6/mL) were placed in 96-well plates and grown to confluence. IMR32 human neuroblastoma lines were released with nonenzymatic Cell Dissociation Solution; cells were collected and resuspended in DMEM. Five microliters calcein AM were added to 1 x 10^5 cells and incubated at 37°C for 30 minutes with occasional agitation. Cells were then processed per the protocol of the manufacturer. Final fluorescence was taken as the ratio of adherent cell fluorescence to total cell fluorescence. Columns, average of four wells per experiment for three separate experiments; bars, SE. *, \( P < 0.05 \); **, \( P < 0.01 \), for each condition compared with HBME.

Qualitative assessment of neuroblastoma cell attachment to HBME cells is done using scanning electron microscopy. Very few tumor cells were visualized on the HBME monolayer at 2 and 4 hours (data not shown). Therefore, our qualitative comparisons of tumor cell adhesion to endothelial cells were done at 6 hours. SHEP cells remain rounded on the endothelial cell monolayer with few, thin cellular processes apparent (Fig. 2A, top right). In contrast, IMR32 and SHEP/IGF-IR cells (Fig. 2A, bottom) have multiple processes extended from the cell periphery and attached to the endothelial cell layer. SHEP cells also show extensive membrane blebbing (Fig. 2A), which suggests that lack of an adherencessupporting substrate may promote cell death within these cells. Together, these data show that IGF-IR expression promotes neuroblastoma cell docking to bone marrow endothelial cells.
Prostate carcinoma cells capable of forming bone metastases preferentially bind to HBME cells over other types of endothelial cells (21). Adhesion assays comparing neuroblastoma attachment to endothelial cells of different origin were done to determine if neuroblastoma cells show the same specificity. Tumorigenic neuroblastoma cells expressing high levels of IGF-IR (IMR32 cells) were plated onto HBME cells, HAEC, or HDMVEC as described above. IMR32 neuroblastoma cells preferentially bind to HBME cells over HAEC (38.4% versus 15.5%, \( P = 0.0136; \) Fig. 2B) or HDMVEC (38.4% versus 11.8%, \( P = 0.0018; \) Fig. 2B).

**Neuroblastoma cell migration through bone endothelium.** Following docking, tumor cells migrate through the endothelium toward stromal elements of bone, which provide a rich source of IGF-I (22). To recapitulate this situation in culture, a trans-endothelial migration assay was done as described in Materials and Methods. IMR32 cells show an 8-fold increase in migration through HBME cells toward HBS cells (\( P = 0.0002; \) Fig. 3A, left), and SHEP/IGF-IR cells migrate in 6-fold greater number than SHEP toward HBBS cells (\( P = 0.0025; \) Fig. 3A, left). The proliferation rate of SHEP/IGF-IR and IMR32 cells in HBS-conditioned medium is <2-fold increased over SHEP and SHEP/vector cells at 2 days (data not shown), indicating that the increased number of cells detected on the bottom surface of the filter is due primarily to migration, not proliferation.

Our hypothesis is that migration through endothelial cells toward stromal cells is due, in part, to local IGF-I secretion by bone...

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**Figure 3.** Increased IGF-IR expression stimulates transendothelial migration. A, HBME or HBS cells were plated in the bottom chamber of a 12-well plate and allowed to grow to near confluence. HBME cells were plated on the upper surface of collagen-coated 3 \( \mu \)m transwell filters in DMEM and also grown to near confluence. SHEP, SHEP/IGF-IR, and IMR32 cells were plated on top of the HBME cells. Cells were allowed to migrate for 48 hours, then nonmigrating cells were removed from the upper surface of the membrane, whereas migrating cells on the bottom surface were stained, eluted, and quantitated as previously described (11). Columns (left), average of three experiments; bars, SE. *, \( P < 0.0025, \) for each condition compared with SHEP. For dot plotting (middle), HBME and HBS cells were grown to near confluence in a six-well plate. Growth medium was replaced with serum-free DMEM for 24 hours; conditioned medium was then collected, concentrated, and analyzed for IGF-I via dot blotting per our published protocol (17). PC, positive 10 nmol/L IGF-I control. For migration toward IGF-I, SHEP and IMR32 cells were plated on collagen-coated 3 \( \mu \)m transwell filters in DMEM + 0.1% serum until cells attached. Medium was then changed to DMEM in the upper chamber and DMEM + 0.3, 1, 3, or 10 nmol/L IGF-I or 10% calf serum in the lower chamber. Columns (right), average of three experiments; bars, SE. *, \( P < 0.01, \) for each condition compared with unstimulated controls. B, HBME cells were plated on the upper surface of collagen-coated 3 \( \mu \)m transwell filters in DMEM and grown to near confluence. IMR32 cells were plated on top of the HBME cells or directly onto the transwell surface in either DMEM or DMEM + 0.3 \( \mu \)m NVP. Cells were allowed to migrate for 48 hours, then nonmigrating cells were removed from the upper surface of the membrane, whereas migrating cells on the bottom surface were stained, eluted, and quantitated as previously described (11). Columns, average of three experiments; bars, SE. *, \( P < 0.05 \) for each condition, compared with non-NVP condition. For immunoblotting (IB), IMR32 cells were treated with 10 nmol/L IGF-I + increasing doses of NVP-AEW541 as indicated. Western immunoblotting was done for phospho-Akt, phospho-Erk1/2, or IGF-IR. Immunoprecipitation (IP) for the IGF-IR was also done, followed by anti-phospho-tyrosine immunoblotting. C, IMR32 cells treated with or without 5 \( \mu \)g/mL MAB391 anti-human IGF-IR monoclonal antibody or 5 \( \mu \)g/mL control IgG antibody were plated in a transendothelial migration assay and migration toward calf serum, 10 nmol/L IGF-I, or HBS cells analyzed as in (A). Columns, average of three experiments; bars, SE. *, \( P < 0.01; \) **, \( P < 0.001 \) for each condition compared with the corresponding untreated condition. For Western immunoblotting, IMR32 cells were treated with 10 nmol/L IGF-I + increasing doses of the IGF-IR monoclonal antibody as indicated. Immunoblotting was done for phospho-Akt, phospho-Erk1/2, or GAPDH.
cells. HMBE secrete detectable IGF-I, but at levels 10-fold less than HBS cells (Fig. 3A, center). Neuroblastoma with increased IGF-IR expression (IMR32) migrate toward IGF-I in a dose-dependent fashion, up to 23-fold higher than control medium alone (Fig. 3A, right). Low IGF-IR-expressing neuroblastoma cells (SHEP) do not migrate in response to IGF-I (Fig. 3A, right). We previously reported that SHEP/IGF-IR cells have increased migration toward IGF-I (10). These data support our hypothesis that IGF-I facilitates IGF-IR–expressing neuroblastoma migration through endothelium toward underlying bone components.

To verify IGF-IR involvement in neuroblastoma transendothelial migration, neuroblastoma cells were incubated with a specific IGF-IR inhibitor, NVP-AEW541 (Fig. 3B). NVP-AEW541 is a highly selective small-molecule inhibitor of IGF-IR, developed and kindly provided by the Novartis Institutes for Biomedical Research, which prevents in vivo tumor progression (23–25). Our results show that at 0.3 μmol/L, a concentration that effectively inhibits both IGF-IR autophosphorylation and downstream signaling through Akt and Erk1/2 in neuroblastoma cells (Fig. 3B, right; ref. 26), transendothelial cell migration is also blocked (Fig. 3B, left). A monoclonal neutralizing antibody to the IGF-IR prevents Akt and Erk1/2 downstream signaling in IMR32 cells, with significant inhibition at the 5.0 μg/mL dose (Fig. 3C, right). This dose prevents transendothelial migration toward calf serum, IGF-I, and HBS cells with similar efficacy (56%, 65%, and 68%, respectively), whereas a control nonspecific IgG has no significant effect (Fig. 3C, left).

**Neuroblastoma cell adhesion to bone stromal cells.** Our working hypothesis predicts that neuroblastoma cells with increased IGF-IR expression interact with bone stromal elements, subsequently promoting osteolytic lesion formation. To test this hypothesis, adhesion to HBS cells was assessed. IMR32 cells adhere in 24-fold greater numbers than SHEP (P = 0.0026; Fig. 4A). Once again, SHEP/IGF-IR cells show a trend toward increased attachment, with 10-fold more SHEP/IGF-IR cells adhering than SHEP; however, this fails to reach statistical significance (P = 0.0982; Fig. 4A). Qualitatively, the results parallel the quantitative data. Increased numbers of cells bind to the stromal cell monolayer at earlier time points; therefore, our qualitative comparisons for neuroblastoma cell attachment to HBS cells are done at 4 hours (Fig. 4B-D). SHEP cells (Fig. 4B) remain unattached or loosely attached to the stromal cell monolayer. By 4 hours, IMR32 (Fig. 4C) and SHEP/IGF-IR (Fig. 4D) cells adhere to and extend multiple processes into the stromal cell monolayer. SHEP/IGF-IR and IMR32 cells become markedly flattened, demonstrating additional membrane contact between the two cell types.

**Neuroblastoma cells target bone in vivo.** Collectively, our data indicate that neuroblastoma cells with increased IGF-IR interact with bone components in vitro. To test the ability of neuroblastoma cells with high IGF-IR expression to target bone in vivo, 1 × 10⁶ or 2 × 10⁶ IMR32 cells were injected into the left ventricle of SCID mice, placing tumor cells within the circulation. Six weeks postinjection, radiological and histologic examinations were done. Tumor cells are detected in the epiphysial areas of long bones in all six injected animals (Fig. 5B) as visualized by plain radiographs with increased tibial area (1.1 and 1.32 cm²) compared with un.injected animals (tibial area: 0.72 cm²; Fig. 5A). Histologic assessment reveals tumor cells within the bone, liver, and adrenal in injected animals (Fig. 5B), but not in control animals (Fig. 5A). These data confirm our hypothesis that IGF-IR-expressing neuroblastoma cells target to bone.

**Figure 4.** IGF-IR–expressing neuroblastoma cells attach to HBS cells. A, HBS cells (5 × 10⁵/mL) were placed in 96-well plates and grown to confluence. SHEP, SHEP/IGF-IR, and IMR32 human neuroblastoma lines were released with nonenzymatic Cell Dissociation Solution (Sigma), cells were collected and resuspended in DMEM. Five microliters calcein AM (Life Technologies) were added to 1 × 10⁵ cells and incubated at 37°C for 30 minutes with occasional agitation. Cells were then processed per the protocol of the manufacturer. Final fluorescence was calculated as described previously. Columns, average of six wells per experiment for three separate experiments; bars, SE. *, P < 0.01 for each condition, compared with control. B to D, HBS cells were grown to confluence on 3 μm filters in DMEM + 10% calf serum. SHEP (B), SHEP/IGF-IR (C), and IMR32 (D) cells were added to the medium, and after 4 hours samples were rinsed and attached tumor cells were prepared for scanning electron microscopy per standard protocol (19). Arrows, processes beginning to form.

**Neuroblastoma cells form osteolytic lesions in vivo.** Neuroblastoma forms osteolytic lesions in patients and experimental animal models (27, 28). To examine the role of IGF-IR expression on neuroblastoma cell interaction with bone in vivo, tumor cells were directly injected into the tibiae of mice, and gross examination, radiography, and histochemical analysis were done. Table 1 summarizes the results. All of the IMR32-injected animals had tumors within bone and evidence of gross metastases in the liver. Animals injected with SHEP or SHEP/vector cells had no signs of tumor formation. Three of five SHEP/IGF-IR–injected animals had tumors, although of smaller volume than IMR32-injected animals. Interestingly, SHEP/IGF-IR–injected animals showed signs of intraorbital, not liver, metastasis. These data indicate that intratibial injection of IGF-IR–expressing cells (i.e., IMR32 and SHEP/IGF-IR) produces tumors within bone and two major sites of neuroblastoma metastases (29), the liver and eye, all of which produce IGF-I (30).

Radiographs indicate osteolysis in the tibia of IMR32-injected animals (Fig. 6B), with less bone density visible in the head of the tumor cell injected tibia, and an adjacent soft tissue mass apparent, likely due to complete penetration of tumor cells through the external surface of the bone. H&E staining clearly shows the presence of tumor within the IMR32-injected tibia (Fig. 6B), in contrast to the contralateral, DMEM-injected tibia, which has normal architecture (Fig. 6A). The control side shows normal bone tissue architecture, with the cartilage intact at the epiphyseal plate and spongy bone visible on both sides (Fig. 6A). In contrast, the IMR32-injected side shows tumor growth in both ossification centers, although the cartilage remains intact (Fig. 6B). Two of the three tumor-bearing animals injected with SHEP/IGF-IR cells showed signs of osteolytic lesions. The remaining animal seemed to
only have a soft tissue mass in the flank, indicating that the cells were either not actually injected into the tibia or the injected volume flowed back out of the punctured tibia after injection.

TRAP staining identifies differentiated osteoclasts (27, 31). Control tibias have TRAP-positive cells in the normal bone tissue (Fig. 6C, arrows), as expected during normal bone turnover. IMR32-injected tibias show increased TRAP staining within the bone tissue, particularly near the bone-tumor interface (Fig. 6D). TRAP-positive cells are also detected within the tumor tissue (Fig. 6D, arrows), suggesting that the tumor has enveloped normal bone cells. These results indicate that increased IGF-IR expression leads to neuroblastoma tumor and osteolytic lesion formation in vivo, likely through increased osteoclast differentiation.

Discussion

Bone is a metastatic site for several tumors, including breast carcinoma (32), prostate carcinoma (33), and neuroblastoma (34). Neuroblastoma patients with secondary tumors in bone have a survival rate of <7% (3), and bone metastasis results in factors affecting the quality of life, including severe pain, fractures, and hypercalcemia (35). Improvement in neuroblastoma patient survival requires the identification of molecular targets for treatment based on a thorough understanding of the metastatic process. Bone is rich in IGF ligands (14), and highly tumorigenic neuroblastoma cells have increased IGF-IR expression (13), suggesting that the IGF system may be involved in neuroblastoma bone metastasis and prove useful as a therapeutic target in bone metastatic disease.

Based on our previous work (13), two human neuroblastoma cell lines, one with very low (SHEP) and one with high (IMR32) endogenous expression of IGF-IR, were chosen for the current investigation. Differences in IGF-IR expression were confirmed at both the mRNA and protein level and compared with an IGF-IR–transfected cell line (SHEP/IGF-IR) and the corresponding vector-only control (SHEP/vector; Fig. 1). Untransfected SHEP cells do not display a tumorigenic phenotype in vitro and cannot form tumors in nude mice (36), whereas IMR32 cells are tumorigenic, both in vitro and in vivo (37). This suggests that IGF-IR expression may correlate with tumorigenesis and metastatic potential, which is true for a number of human cancers (38). For this study, nontumorigenic SHEP cells transfected with IGF-IR are compared with tumorigenic IMR32 cells with high endogenous IGF-IR to determine the relative contribution of the IGF-IR in neuroblastoma cell interaction with bone.

Bone tissue is a reservoir for IGF-I ligands, and these ligands are critical for both formation and maintenance of the skeleton (22).

Table 1. Nude mice injected intratibially with 1 × 10^7 SHEP, IMR32, SHEP/vector, and SHEP/IGF-IR cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor formation</th>
<th>Osteolytic lesions</th>
<th>Gross metastases?</th>
</tr>
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<tbody>
<tr>
<td>SHEP</td>
<td>0/5</td>
<td>0/5</td>
<td>No</td>
</tr>
<tr>
<td>IMR32</td>
<td>5/5</td>
<td>5/5</td>
<td>Yes, liver metastases</td>
</tr>
<tr>
<td>SHEP/vector</td>
<td>0/5</td>
<td>0/5</td>
<td>No</td>
</tr>
<tr>
<td>SHEP/IGF-IR</td>
<td>3/5</td>
<td>2/5</td>
<td>Yes, intraorbital</td>
</tr>
</tbody>
</table>

NOTE: Mice were sacrificed after 7 weeks. Radiographs were taken and examined for osteolytic lesions. Upon necropsy, animals were scored as positive for tumor formation if a tumor mass was visible on the hindflank of the animal. Animals were scored as positive for osteolytic lesion formation if notable radiolucent images were seen extending from the injection site in the proximal tibia to the outside border of the tibia. Several organs were harvested and examined for tumor nodules indicating metastases, including the liver, lung, and eyes. Tumor lesions were confirmed by examining two to three H&E-stained sections per animal.
IGFs within the bone microenvironment are available locally through secretion by stromal cells and osteoblasts and through release from bone matrix due to osteoclast activity (22). One of the first steps in secondary tumor formation in bone is tumor cell docking to the bone marrow endothelium. Tumor cells loosely adhere to endothelial cells during docking before extravasation into the local bone microenvironment, where they interact with cellular and matrix components of bone (19, 39). Neuroblastoma cells with high IGF-IR expression preferentially adhere to an HBME monolayer in greater numbers than those cells with low IGF-IR expression (Fig. 2). Docking to bone endothelium is extensively investigated in other tumors metastatic to bone, including prostate carcinoma and multiple myeloma. In prostate carcinoma, cells that express IGF-IR preferentially bind to HBME cells (19, 33), a process controlled in part by local growth factors produced by bone stromal cells (19). In multiple myeloma, the IGF-IR is also implicated in tumor cell adhesion to bone, and blocking the interaction between the two cell types prevents multiple myeloma homing to bone (40). These studies support our contention that IGF-IR expression enhances the ability of tumor cells to adhere to endothelial cells before transendothelial migration.

Following docking to endothelium, tumor cells migrate and/or invade into the bone microenvironment in response to secreted molecules, including IGF-1 (41). Neuroblastoma cells with high IGF-IR expression that form osteolytic lesions in vivo migrate toward HS-5 bone stromal cells, which secrete high levels of IGF-I (Fig. 3). Preventing IGF-IR–mediated signaling in high IGF-IR–expressing neuroblastoma cells (IMR32) with a neutralizing antibody or the small-molecule inhibitor NVP-AEW541 significantly decreases transendothelial migration (Fig. 3), suggesting that IGF-IR receptor activity is necessary for this process. This inhibitor and the closely related compound NVP-ADW742 are effective in several tumors, including multiple myeloma (42), fibrosarcoma (23), musculoskeletal tumors (24), small-cell lung carcinoma (43), and epithelial ovarian cancer (25). In breast cancer cell lines, both endothelial cell adhesion and transendothelial migration determine the ability of the cells to form osteolytic lesions, irrespective of differences in proliferation (44). Multiple myeloma cells that form tumors within bone also migrate through the endothelium before attachment to the bone stroma (45). Although numerous factors are produced by bone stroma that affect migration, IGF-I may play a prominent role for some tumor cell types (19, 35, 46). IGF-I is a bone marrow–derived chemoattractant for multiple myeloma cells (47), resulting in increased transendothelial migration (48). In vitro, IGF-I increases neuroblastoma (49), prostate carcinoma (50), and breast carcinoma cell migration (51, 52). Therefore, stromal cell–derived IGF-I may allow increased neuroblastoma cell transendothelial migration.

Subsequent to migration through endothelium, tumor cells must adhere to other bone components, including bone stromal cells, to produce osteolytic lesions (46). Those neuroblastoma cells that form bone metastases in vivo (SHEP/IGF-IR and IMR32) adhere much more strongly to HBS cells than nontumorigenic SHEP cells (Fig. 4). These data agree with other reports in the literature. Multiple myeloma cells, upon interaction with bone marrow endothelial cells, up-regulate the IGF-IR to increase attachment to bone marrow stromal cells (47). Adhesion of multiple myeloma cells to bone stromal cells confers a proliferative and survival advantage to those cells, promoting osseous tumor formation (53). These studies suggest that IGF-IR on the neuroblastoma tumor cell surface may promote tumor cell adhesion to bone cellular and matrix components, allowing for bone tumor formation. Our in vitro results indicate that the most substantial effect of IGF-IR overexpression is an increase in migration (Figs. 2-4). This further supports the role of IGF-IR in bone metastasis, as prostate cancer cell migration and invasion through HBME monolayers most accurately reflects bone metastatic potential (54). Finally, our data indicate that high IGF-IR–expressing neuroblastoma cells preferentially target to bone, as these cells attach to HBME cells over other endothelial cell types, and intraventricular injection of these cells results in tumor formation within bone (Fig. 5).

Neuroblastoma tumor cells interact with bone in vivo, producing osteolytic lesions and tumor growth (28). In the current study, the ability of IGF-IR expression to regulate osteolytic lesion formation was tested. Tumorigenic IMR32 neuroblastoma cells, which express high levels of IGF-IR (Fig. 1), form tumors within bone when injected into the tibia (Fig. 6). In contrast, SHEP cells, expressing little IGF-IR (Fig. 6), do not form tumors within bone (Table 1). However, simply overexpressing the IGF-IR in SHEP cells through transfection results in tumor formation within the bone, indicating that IGF-IR expression in part determines the ability of tumor cells to interact with bone to produce osteolytic lesions. Intratibial injection of IGF-IR–expressing cells (i.e., IMR32 and SHEP/IGF-IR) produces additional tumors in two major sites of neuroblastoma metastases (29), the liver and the eye, both of which produce IGF-I (30). Although IGF-IR overexpression promotes bone tumor formation, the tumor take rate and tumor size do not reach that of IMR32 cells. Recent studies have indicated that expression of the chemokine receptor CXCR4, implicated in tumor cell targeting, is up-regulated in primary neuroblastoma tumors and plays a role in...
Our preliminary results show that CXCR4 is up-regulated in IMR32 cells, but not in SHEP/IGF-IR cells, suggesting that this may be a pathway acting in parallel with the IGF-IR in neuroblastoma metastasis. We are currently examining the relative contribution of these two systems to neuroblastoma bone metastasis through small interfering RNA silencing and pharmacologic inhibition.

When high IGF-IR-expressing neuroblastoma cells form tumors within bone, increased numbers of TRAP-positive cells, indicative of mature osteoclasts, are detected (Fig. 6). Several tumor types, including breast carcinoma, prostate carcinoma, and other neuroblastoma models, produce osteolytic lesions in both human patients and mouse models (27, 28, 32, 33). Neuroblastoma-induced osteolytic lesions occur through increased osteoclast differentiation rather than through effects on osteoblasts, and bisphosphonate treatment, which prevents osteoclast maturation, decreases neuroblastoma-induced osteolytic lesions (27, 28).

Several recent studies indicate IGF system involvement in osteolytic lesions. IGF-IR signaling is involved in multiple myeloma bone tumor formation, and bone lesions are ameliorated through targeted therapy against the IGF-IR (42). IGF signaling also promotes bone lesion formation in prostate carcinoma (57). Potential mechanisms involved in neuroblastoma osteolytic lesion formation include direct modulation of cytokines (58) or stimulation of osteoclast-activating factor release from bone components (59). IGF-I increases cytokine production within bone cells both in vitro and in vivo (60), and a selective small-molecule IGF-IR kinase inhibitor significantly decreases osteoclast-activating factors within bone (42). We are currently investigating the effects of IGF-IR expression and activation on these stimulators of osteoclastogenesis.

In summary, neuroblastoma cells expressing high levels of the IGF-IR interact with the bone microenvironment and form osteolytic lesions, likely through increased osteoclast differentiation. These data suggest that targeting the IGF-IR, which has recently gained new attention as a treatment option (61), may prove beneficial as an adjuvant treatment for aggressive neuroblastoma and provide rationale for further investigation into mechanisms of bone metastasis regulated by the IGF system.

Acknowledgments


Grant support: Program for Understanding Neurological Diseases, the Juvenile Diabetes Research Foundation Center for the Study of Complications in Diabetes, and NIH grants RO1 NS36778 and RO1 NS8849.

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We thank Judy Boldt for excellent secretarial assistance, Dr. Kelli Sullivan for aid in overseeing animal tissue harvest and histochemical analysis, Dr. Francesco Hoffmann from Novartis Institutes for Biomedical Research for supplying NVP-AEW541, Dr. Laurie Mc Caul for performing intracardiac injections, Dr. Jinlu Dai for teaching us intratrab injection protocols, and Chris Strayhorn from the University of Michigan Dental School for bone sectioning.

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