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A novel rat model for the study of intraosseous metastatic spine cancer

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Object. Although metastatic spinal disease constitutes a significant percentage of all spinal column tumors, an accessible and reproducible animal model has not been reported. In this study the authors describe the technique for creating an intraosseous spinal tumor model in rats and present a functional and histological analysis.

Methods. Eighteen female Fischer 344 rats were randomized into two groups. Group 1 animals underwent a transabdominal exposure and implantation of CRL-1666 breast adenocarcinoma into the L-6 vertebral body (VB). Animals in Group 2 underwent a sham operation. Hindlimb function was tested daily by using the Basso-Beattie-Bresnahan scale. Sixteen days after tumor implantation, animals were killed and their spines were removed for histological assessment. Statistical analysis was performed using the Wilcoxon signed-rank test.

By Day 15 functional analysis showed a significant decrease in motor function in Group 1 animals (median functional score 2 of 21) compared with Group 2 rats (median functional score 21 of 21) ($p = 0.0217$). The onset of paraparesis in Group 1 occurred within 14 to 16 days of surgery. Histopathological analysis showed tumor proliferation through the VB and into the spinal canal, with marked osteolytic activity and spinal cord compression.

Conclusions. Analysis of these findings demonstrates the consistency of tumor growth in this model and validates the utility of functional testing for onset of paresis. This new rat model allows for the preclinical evaluation of novel therapeutic treatments for patients harboring metastatic spine disease.

Key Words • **animal model** • **spinal metastasis** • **breast metastasis** • **rat**

Between 5 and 10% of all patients with cancer will suffer from metastatic spinal cancer during the course of their disease. Twenty-two percent of patients with breast cancer, 15% with lung cancer, and 10% with prostate carcinomas are known to suffer symptomatic spinal cord compression.[7] Despite the clinical impact of metastatic spinal cancer, there are currently no practical, reproducible, and reliable animal models in which this disease can be studied. An effective animal model would allow for the exploration, characterization, and comparison of various treatment methodologies in a preclinical context.

In the few cases in which researchers have attempted to mimic the effects of intraosseous spinal tumors, the tumor induction methodology has been paraspinal injection. This model was reported by Ushio, et al.,[8,9] in 1977, and its primary limitation is that it does not allow for standardization of the magnitude/pathology of tumor infiltration. For this reason, the onset of paraplegia ranges from 3 to 4 weeks, limiting any temporal resolution when treatments are applied. Additionally, the very nature of an epidural injection is fundamentally different from metastatic disease, which arises from within the VB.

Based on the frequency of spinal metastases from primary breast tumors, an experimental breast adenocarcinoma tumor cell line, CRL-1666, was selected for this study to establish its growth potential after intraosseous implantation and to correlate it with functional and histological progression parameters. The CRL-1666 line has been previously used in animal models of breast cancer.[2–6,10,11] This tumor line exhibits histopathological and biological characteristics that are similar to those exhibited by human breast adenocarcinomas.

In this study we present a reproducible rat model for spinal metastatic tumors, describe the methodology, determine its pathological progression, and present a functional correlation of spinal cord compression and loss of motor function after intraosseous tumor implantation.

Materials and Methods

Animals and Experimental Design

Eighteen 10-week-old female Fischer 344 rats (Charles River Laboratories, Wilmington, MA) weighing 180 to 220 g were used

Abbreviations used in this paper: Ba-Be-Br = Basso-Beattie-Bresnahan; PMMA = polymethylmethacrylate; VB = vertebral body.

for this experiment. Animals were randomized into two experimental groups: nine rats in Group 1 underwent surgery and implantation of a portion of the CRL-1666 tumor, and nine in Group 2 underwent a sham surgery without tumor implantation. Animals were maintained in standard facilities, four rats per cage, and given free access to Baltimore city water and rodent chow. All experimental protocols were approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine.

Anesthesia Composition

Animals were anesthetized with an intraperitoneal injection of 3 ml/kg of a stock solution composed of ketamine hydrochloride 25 mg/ml, xylazine 2.5 mg/ml, and 14.25% ethyl alcohol in 0.9% NaCl.

Recommended Instruments

The surgeries were performed under direct vision by using a Zeiss operating microscope. Dumont No. 7 forceps, Dumont No. 5 forceps, scissors, spring scissors (Fine Science Tools, San Francisco, CA), suction (via a Pasteur pipette connected to a laboratory vacuum system), and a hand-held cautery device were used in all procedures. The instruments were sterilized in the autoclave prior to the procedure and were placed over sterile drapes during surgery.

Tumor Line/Flank Tumor

The CRL-1666 mammary adenocarcinoma cell line (also known as the 13762 MAT B III) was selected for this model (American Type Culture Collection, Manassas, VA).[10] This rat cell line was initially established at the EG&G Mason Research Institute (Bethesda, MD) from a transplantable rat ascites tumor derived from the 13762 solid mammary adenocarcinoma. The cells, which are only loosely adherent, are maintained in cell culture in Dulbecco modified Eagle medium with 10% fetal bovine serum, streptomycin (80.5 pg/ml), penicillin (base 80.5 U/ml), and 1% L-glutamine (all products from Gibco BRL, Grand Island, NY). Cells are maintained in a humidified atmosphere of 5% CO_2 at 37°C, grown to a concentration of 1 million cells/ml, and then diluted in medium (~ every 3 days).

To obtain a solid tumor piece for implantation, carrier animals (Fischer 344 rats) were anesthetized as described in *Anesthesia Composition* and subcutaneously injected with 10^6 CRL-1666 cells into the flank. At 10 days postinjection the animals were killed by intraperitoneal injection of pentobarbital (120 mg/kg), and the tumor was resected and placed in 0.9% NaCl sterile solution. Tumor segments measuring approximately 0.7 × 0.7 × 0.7 mm are appropriate for implantation.

Surgical Technique

After induction of anesthesia, the abdomen was shaved and prepared with a betadine swab. A 3-cm midline skin incision was made, centered between the iliac crests, the underlying abdominal muscles were exposed, and a small 0.25-cm superficial incision was made in the midline between the two rectus abdominis muscles and extended deeper until an opening of the abdominal cavity was created. The abdominal muscles were elevated to separate the bowels from the abdominal wall, and scissors were used to extend the opening superiorly and inferiorly. The margins of the abdominal muscle opening ended slightly before the margins of the skin incision (to allow for separate closure of both tissue layers; Fig. 1).

Prior to the application of retraction, the vascular bundle containing the aorta and vena cava were identified along the dorsal wall of the abdominal cavity. Sustained retraction of the rat's bowels to provide adequate exposure to the dorsal portion of the abdominal cavity while avoiding lethal bowel perforations proved to be a challenge. To achieve adequate exposure, blunt dissection of the adipose tissue and bowels was conducted using two sterile cotton-tipped applicators. Once the aorta was observed, a retractor was placed in the abdominal cavity (Fig. 1). Traditional self-retaining retractors were not appropriate for this procedure because intestinal trauma was frequently caused by the "teeth," and the retraction achieved was not static, which allowed the intestinal matter and adipose tissue to regularly block the exposure. To overcome these challenges, we fashioned a retractor composed of a cylindrical plastic wall, measuring 2 cm in diameter and 1 cm in height (Fig. 2).

To expose the VB, the point at which the aorta bifurcates into the external iliac arteries is located, and the lateral region to the right of the vessels was bluntly dissected to expose the lateral walls of the aorta/vena cava. A landmark for this blunt dissection is the left medial iliac lymph node that serves as the lateral border of the dissection. The dissection continued dorsally (deep) until the underlying bone was observed. The landmark for the L-6 VB is the deep circumflex iliac artery, which arises from the lateral wall of the aorta, approximately 0.3 cm superior to the aortic bifurcation (Fig. 3). The Dumont No. 7 forceps were then used to mobilize the tissue beneath the aorta and vena cava and gently retract the vascular bundle to the left. Simultaneously, the area just inferior to the deep circumflex iliac vessel is cauterized, exposing the underlying bone inferiorly until the intervertebral disc is seen.

A high-speed surgical drill (Dental Drill; Aseptico, Woodinville, WA) was used in concert with a 1-mm burr to drill the cavity into the VB for tumor implantation. The cavity is made on the inferior

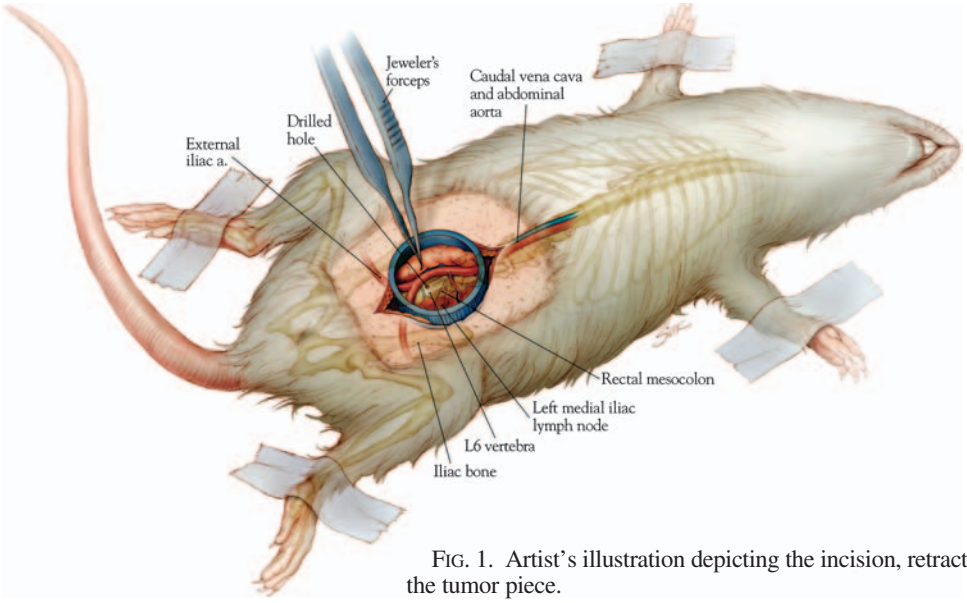

FIG. 1. Artist's illustration depicting the incision, retraction, and exposure for implantation of the tumor piece.

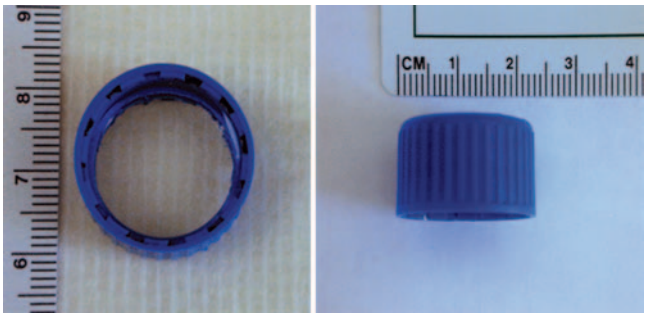

FIG. 2. Photographs showing the novel abdominal retractor.

border of the VB, approximately 0.5 mm superior to the intervertebral disc (Fig. 4), at a depth of approximately 1 mm. A tumor section is chosen such that it can fit entirely inside the cavity, without any protuberances (Fig. 3A–C).

The tumor is subsequently sealed into the bone with the use of Surgical Simplex (Stryker, Mahwah, NJ), also known as PMMA. The PMMA is packaged in two parts, as a powder and as a liquid; combining the two produces the bone cement. The sealing procedure is as follows. First a plug of PMMA is created (with 3 mg of PMMA mixed with 30 μl of PMMA) and allowed to harden slightly before being placed as a plug into the mouth of the tumor-containing hole. Approximately 15 mg of PMMA powder is then placed on top of the VB's anterior surface and approximately 15 μl of liquid PMMA is titrated with a pipette until the second layer of PMMA hardens to form a reinforcing barrier (Fig. 3A–C). Once the intraosseous VB cavity is sealed, the abdominal muscles are approximated using a 3.0 Vicryl running suture (Ethicon, Somerville, NJ), and the skin is closed with surgical autoclips. The rats are observed and allowed to awaken fully in their cages before returning to their holding facilities. Buprenorphine (0.01 mg/kg) was subcutaneously administered for pain relief.

Sham Surgery

In the nine rats undergoing sham surgery, all of the aforementioned steps were undertaken, expect for insertion of the tumor. In these animals, after the cavity was established, it was sealed with PMMA as described.

Functional Assessment and Statistical Analysis

The Ba-Be-Br Scale[1] was used to evaluate the hindlimb motor function. Briefly, rats are placed in an open field, allowed to become accustomed to their surroundings, and observed for 4 minutes. Based on the characteristics of the rat's gait, a score between 0 and 21 is assigned according to preestablished criteria. Scores ranging from 14 to 21 indicate paw rotation and dragging of the toes during the initial stages of tumor progression (with 21 representing totally normal performance); those ranging from 8 to 13 indicate the progression of neurological deterioration as they reflect the measurement of frequency of stepping and quality of coordination; and scores ranging from 0 to 7 indicate the movement of isolated joints, reflecting the advanced stages of spinal cord compression (a score of 0 reflects the absence of spontaneous hindlimb movement). The animals were evaluated by two observers blinded to their treatment group, the scores obtained were averaged, and the mean intergroup scores were compared using the Wilcoxon signed-rank test to establish statistical significance.

Tissue Processing

Once the animals became paraparetic (Ba-Be-Br Scale score range 0–3), they were killed by an overdose of pentobarbital (120 mg/kg intraperitoneally injected). The spines were harvested, fixed in 10% formalin for 24 hours, embedded in paraffin, and sectioned at a thickness of 10 μm for H & E staining.

Results

Functional Progression

Eighteen rats were used in this study: nine received CRL-1666 tumor implants and nine underwent a sham surgery. Two animals, one from each group, were excluded from the study because of surgery-related complica-

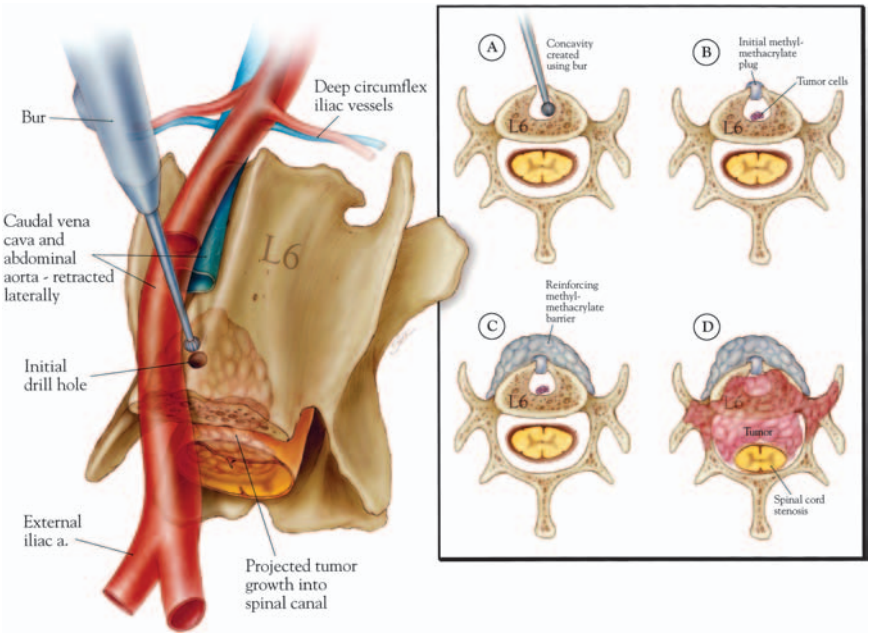

FIG. 3. Artist's rendering of the landmarks for the L-6 VB. *Inset:* The drilling and sealing procedure: the concavity is created using the burr (A); the initial PMMA plug is inserted (B); the reinforcing PMMA barrier is applied (C); and as the tumor grows, it causes spinal cord compression (D). A = artery.

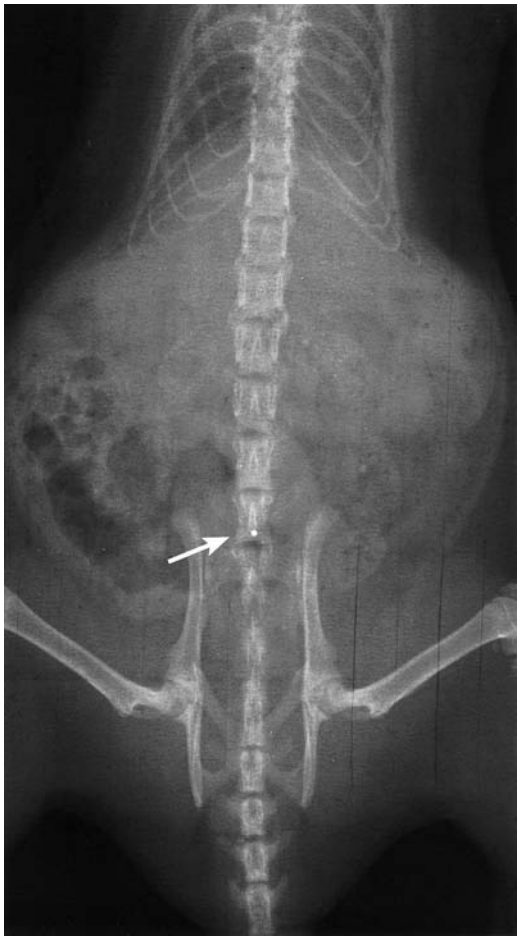

FIG. 4. Radiograph demonstrating the location of the hole *(arrow)*. The landmarking was made by inserting a small radiopaque fragment into a drilled hole.

tions: one animal died of infection 1 day postoperatively and one died of hemorrhage 10 days postoperatively. The graph in Fig. 5 depicts the median Ba-Be-Br Scale score for each treatment group over time. Whereas 15 days postoperatively the median Ba-Be-Br Scale score was 2 for the tumor group, that for the sham group was 21 ($p = 0.0217$, 95% confidence level, Wilcoxon signed-rank test).

Histopathological Examination

Examination of cross-sections from the spines showed an aggressive infiltrative pattern, with marked osteolytic activity. The tumors consistently infiltrated the spinal canal through the VB and compressed the spinal cord as shown in Fig. 6. The tumors showed extensive areas of hemorrhage and necrosis and exhibited cellular atypia.

Discussion

In this study we describe a new model of metastatic spine tumors in rats, describing the technique for tumor implantation and the pathological and functional findings. We found that animals implanted with the breast adenocarcinoma CRL-1666 experience hindlimb paraparesis in 14 to 16 days, with a consistent decrease in Ba-Be-Br Scale scores and that animals undergoing sham surgery recover uneventfully and maintain maximal Ba-Be-Br Scale scores throughout the study.

Based on the median Ba-Be-Br Scale score we found that almost 50%, 75%, and 100% of the animals in which tumors were implanted can be expected to suffer from paraparesis by Days 14, 15, and 16, respectively. This tightly grouped decrease in Ba-Be-Br Scale scores indicates a high degree of standardization and provides encouragement for future in vivo efficacy studies of novel treatments for metastatic tumors.

The progressive neurological dysfunction observed in the tumor-treated animals indicates that there are three gross stages: an initial stage wherein the functional score parallels that of the control animals, a brief onset phase wherein some deficit is observed, and thereafter a rapid decompensation into paraparesis. These phases resemble the natural clinical progression that occurs in humans.

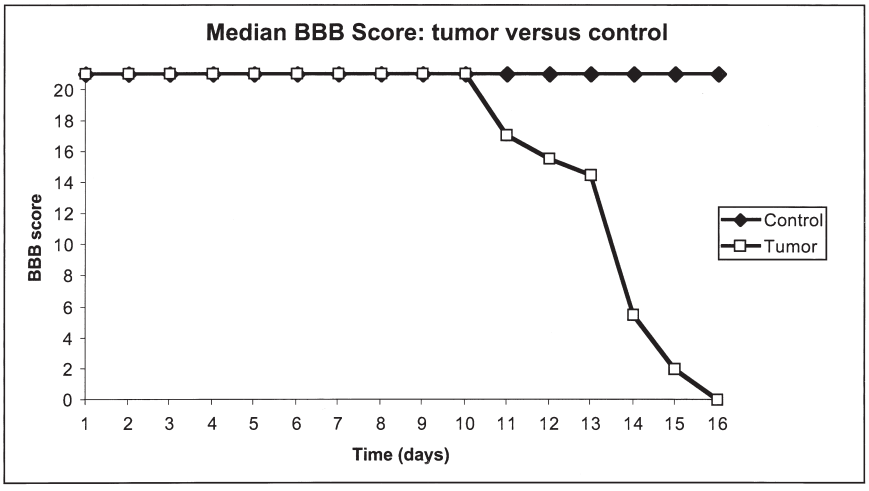

FIG. 5. Graph depicting the median Ba-Be-Br (BBB) Scale score of each treatment group over time.

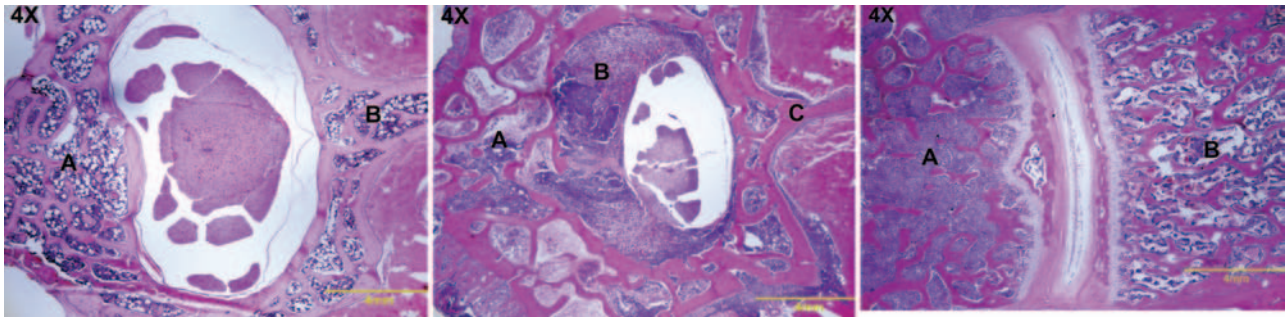

FIG. 6. Photomicrographs. *Left:* Control group spine cross-section: VB (A); spinous process (B). *Center:* Tumor group spine cross-section. VB (A); spinal canal filled with tumor cells that are infiltrating through the VB (B); spinous process (C). *Right:* A coronal section of tumor group VBs. An L-6 VB saturated with tumor (A); L-5 VB, free of tumor (B). H & E, bar = 4 mm.

Analysis of the H & E–stained cross-sections showed that the CRL-1666 cell line has marked osteolytic activity and that the tumor infiltrates through the VB and occupies more than 50% of the spinal column. Evaluation of the coronal sections demonstrates that the tumor cells are exclusively limited to the implanted level if animals are killed immediately after onset of paraparesis (Fig. 6 *right*).

Surgery-related complications specific to the technique were observed only in two animals. One animal died of a hemorrhagic complication possibly due to weakening of the aortic wall created during manipulation of the vessel. Shortly after surgery, the other animal presented with peritoneal infection, which resulted from perforation of the bowel and subsequent autopsy examination–confirmed sepsis. These complications can be minimized by careful surgical techniques.

Conclusions

This new model for intraosseous metastatic spinal disease provides reliable and reproducible findings that mimic the human condition. Our methodology for CRL-1666 cell line application can be applied to implantation of other experimental tumors, such as prostate and lung tumors, that frequently invade the spinal column. An in vivo model for this disease opens the way for further characterization of the pathobiological nature of this tumors in the spine and allows for preclinical testing of novel therapeutics.

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