Comparative Medicine
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Vol 53, No 2
April 2003
Pages 207-212

Vascular Leak Syndrome of Sprague-Dawley Rats in a Mandibular Distraction Osteogenesis Study

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Vascular leak syndrome (VLS) is a common and often fatal sequela of multiple bone traumas, and of infectious, toxic, and allergic insults in human patients. Although an animal model for VLS has not been fully established, rats have shown sensitivity to the syndrome that approximates that of the human population. We describe cases of VLS in three-month-old adult and one-month-old Sprague-Dawley rats in an osteogenesis study aimed at optimizing correction of bone hypoplasias and other craniofacial deformities in children, using a mandibular distraction device. In the study reported here, VLS was diagnosed in 46% of the rats that were necropsied after dying or being euthanized early, subsequent to mandibular osteotomy, a procedure that involves minimal bone trauma. The gross and histologic findings, as well as the clinical course of VLS in the rats of the osteogenesis study, were similar to those of documented human cases. Hence, the rat may be a useful animal model to help characterize the physiologic and molecular events that accompany this syndrome.

Vascular leak syndrome (VLS) is a serious and common complication of either trauma or surgery involving the long bones in humans. The lungs being affected first, the disease is also called "lung capillary leak," and is reported as such in 30% of multiple trauma patients (1-4). In 34 to 66% of cases with respiratory complications (5), the patients die, as VLS triggers the pathologic changes of acute respiratory distress syndrome (ARDS), which is characterized principally by acute lung failure. VLS also has an important systemic component responsible for the high mortality of the disease in which edema spreads beyond the lungs and quickly leads to hypotension, blood hypercoagularity, and eventually, disseminated intravascular coagulation (DIC). At best, ARDS progresses to a proliferative phase, with remodeling of lung tissue, most often resulting in fibrosis and emphysema instead of complete recovery (6-8). Vascular leak syndrome is also seen in any circumstances involving a strong inflammatory or immune response, including those that are iatrogenic, such as cytokine therapy for cancer (9). Despite the great importance of VLS, the precise pathogenesis of the syndrome has not been established, but several, non-mutually exclusive mechanisms have been proposed (9-12). Therefore, it has been difficult to prevent the syndrome in susceptible patients, including laboratory animals undergoing procedures involving surgical bone manipulation. Although reports of VLS in animals, whether accidental or induced (9, 13, 17), are few, such reports are valuable, given the need for an adequate model for the disease. The following cases of VLS developed in Sprague-Dawley rats used in a mandibular distraction osteogenesis study, the goal of which was optimization of a protocol to correct hypoplasias or other craniofacial deformities in children (14, 15). The precise incidence of confirmed VLS cases in this animal population is unknown since some of the rats died shortly after the bone surgery and were not necropsied. However, gross and histologic findings, as well as the timing of death after surgery were consistent with VLS in 46 to 54% of cases that were necropsied. Therefore, the incidence of VLS in this population was similar to that in humans with bone trauma, if we assume that the necropsied animals were representative of the population that died unexpectedly. However, human VLS cases tend to be associated with bone trauma resulting from multiple bone injury or from the surgery to repair such injuries, rather than from elective procedures, which would be expected to induce significantly less tissue damage (1, 2, 5, 7). Therefore, the high incidence of VLS in the rats of this study that underwent a minor procedure would suggest that rats are particularly sensitive to this syndrome.

Received: 8/21/02. Revision requested: 10/16/02. Accepted: 12/16/02.
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Materials and Methods

Animals. Sixty-six one-month-old and 255 three-month-old male Sprague-Dawley rats (Animal Technologies Ltd, Fremont, Calif.) were studied. All rats were housed in isolator cages in a modified specific-pathogen-free (SPF) facility, and were fed a standard irradiated rodent diet (Picolab Rodent diet 20 #5053, pellets or Meal, LabDiet, St. Louis, Mo.) and water ad libitum. Disease surveillance in this SPF facility includes a sentinel program, consisting of two sentinels per rack, that receive dirty bedding from all other cages on that rack. Serologic testing is done on the sentinels every six weeks, and full necropsy is done every three months. The pathogens excluded from this facility are murine hepatitis virus, murine parvovirus, epizootic diarrhea of infant mice, *Mycoplasma* spp., rat parvovirus, sialodacryoadenitis virus, rat coronavirus, and ecto- and endoparasites. The housing, care, and experimental protocol strictly followed humane guidelines and were approved by the Institutional Animal Care and Use Committee at the University of Washington.

Surgical placement of mandibular distraction device and distraction procedure. Two days before surgery, powder

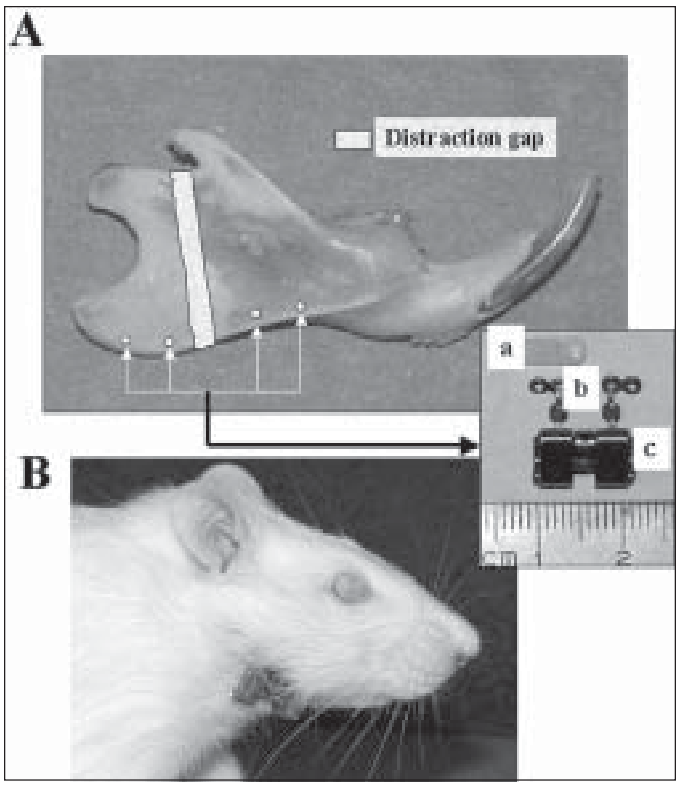

Figure 1. (A) Landmarks for mandibular distraction device. Inset (a) methylmethacrylate block; (b) two Luhr L-shaped microplates; (c)Leone jackscrew. (B) Rat fitted with mandibular distraction device.

and pelleted diet were made available to the rats. On the day of surgery, rats were anesthetized with ketamine sulfate (60 to 70 mg/kg of body weight) and xylazine HCl (8 to 13 mg/kg) administered intraperitoneally. Prophylactic antibiotics (cefazolin sodium, 10 mg/kg, i.p.) were given before surgery (all of the above supplied by Cardinal Health, Dublin, Ohio). Animals were positioned in lateral recumbency, the hair over the mandibular area was clipped, and skin was prepared in an aseptic manner.

A 10-mm transverse skin incision was made along the ventral border of the right hemi-mandible. A 5-mm incision was made into the masseter muscle, which was then elevated to expose the mandibular angle, sigmoid notch, and mandibular body caudal to the third molar. The lingual border of the mandibular angle also was freed of its muscular attachment. Since the bone is extremely thin in that area, a pre-fabricated 7 × 3 × 1-mm methylmethacrylate block (Fig. 1A, inset a) was placed against the lingual surface of the mandibular angle for additional support. The distraction device (Fig. 1, inset) was positioned on the lateral surface of the mandible near the ventral border, and consisted of two Luhr L-shaped microplates (0.8 mm, 5 holes) and a Leone jackscrew (0.2 mm per quarter turn, Fig. 1A, insets, b and c). Using four self-tapping microscrews (0.8 × 3 mm; Fig. 1A, white dots), the cranial microplate was secured to the body of the mandible. Likewise, the caudal microplate and the methylmethacrylate block were secured with microscrews on either side of the angle of the mandible.

Finally, an osteotomy was performed from the sigmoid notch down to the ventral border of the mandible and between the two plates. Hole drilling and osteotomy were performed by using a diamond burr on an electric drill set at a lower speed (10,000 rpm),

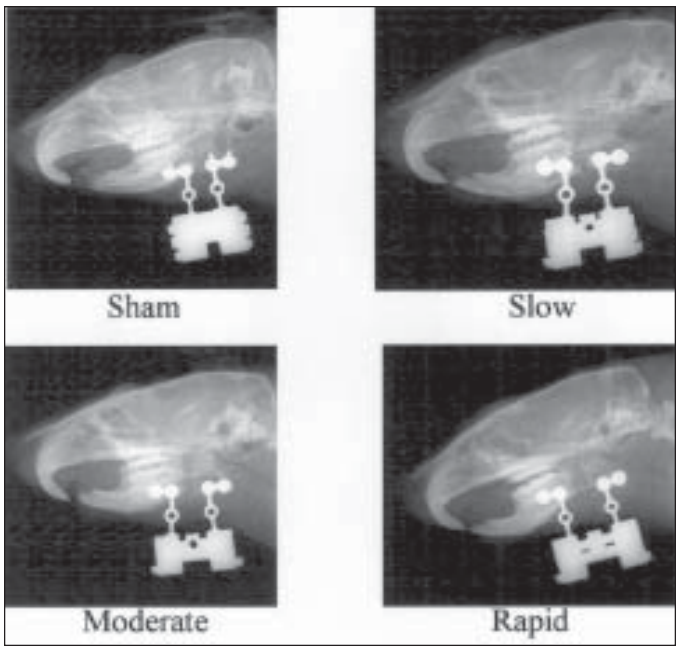

Figure 2. Radiographs of mandibles of rats at the late consolidation stage after osteotomy with distraction devices in place. Clockwise from upper left, after slow, moderate, and rapid distraction.

and the site was copiously irrigated with 0.9% sodium chloride, to minimize any possible thermal effect on the rate of healing of the bone. The incision was closed in two layers, using size 4.0 chromic gut and a simple continuous pattern for the muscular layer, and a simple interrupted pattern for the skin.

The animals recovered from anesthesia on a heating pad. Fluids (10 to 20 ml of lactated Ringers' solution, s.c.) were given immediately following surgery, and buprenorphine (0.1 mg/kg i.m.; Cardinal Health, Dublin, Ohio) was also given after surgery. Rats were fed a powdered diet for 10 days, then were gradually weaned to the pelleted diet. Rats were weighed daily after implantation of the device, until they were euthanized.

Following a three-day postsurgical latency period, rats were randomized to one of four groups: a sham group, whose implanted devices were not distracted, and three other groups, whose distraction devices were activated at rates of 0.2 mm (slow distraction), 0.4 mm (moderate distraction) or 0.6 mm (rapid distraction) per day from postoperative day 3 to day 7. The schedule for sacrifice was baseline (day 3), mid-distraction (day 6), early consolidation (day 10), mid-consolidation (day 24), and late consolidation (day 38). Figure 2 is composed of radiographs of the device at the late consolidation stage after slow, moderate, and rapid distraction.

Clinical pathologic examination and necropsy. A complete blood count (CBC) and serum biochemical analysis (Phoenix Laboratories, Seattle, Wash.) were done on blood collected by cardiac puncture and submitted for one rat that had antemortem signs of DIC. Of 321 rats, 29 died unexpectedly on the day of surgery or on the first postoperative day. Complete necropsy and histologic examination were done on 13 (45%) of these rats (seven rats were presented before death because of illness, and six rats were presented after death). The remaining 16 rats either died on the day of surgery without recovering from anesthesia or were found dead in the cage and were not necropsied. Rats that became ill and were submitted live for necropsy were

Table 1. Summary of necropsy findings in rats after placement of mandibular distraction device

				Gross necropsy findings						Timing of death of necropsied rats (days post-surgery)			
Year	No. of rats	Unexpected deaths after placement of device* (% of total rats in study)	Rats necropsied†	Pulmonary edema/WBC infiltration	Hydrothorax	Edema in other organs	Enterocolitis	DIC	Other‡	< 3	7-10	>10	Unknown
2000	104	18 (17.3 %)	9	4	3	2	0	0	4	4	0	4	1
2001	217	4 (1.8 %)	4	1	0	0	3	1	0	0	3	1	0
Total	321	22 (6.85 %)	13	5	3	2	3	1	4	4	3	5	1

*Animals that died on the day of surgery (before or after recovery from anesthesia), were found dead in the cage, or were euthanized because of illness after placement of the mandibular distractor.
†All animals necropsied had been fitted with the mandibular distractor. Some of the unexpected deaths (6 in 2000 and 1 in 2001) occurred before rats underwent surgery and were not included here.
‡Surgery-related deaths or euthanasia because of infection.

euthanized by CO_2 inhalation. Collected tissues were fixed in neutral-buffered 10% formalin for 24 to 48 h, paraffin embedded, sectioned at 4-µm thickness, and dried on slide plates overnight at 38 to 40°C. Slides were stained, using hematoxylin and eosin (H&E), on a Shandon liner stainer.

Case Report

Twenty four of 104 (23%) and 5 of 217 (2.3%), died unexpectedly in 2000 and 2001, respectively. As indicated (Table 1), 18 (17.3%) of these rats died after mandibular osteotomy in 2000, and nine of these were necropsied. In 2001, 4 (1.8%) of these rats died after mandibular osteotomy, and all were necropsied. Pathologic changes were observed in other organs besides the lungs, and histopathologic findings are summarized in Table 1. The presence of one or more of the following signs resulted in a diagnosis of VLS: pulmonary edema, hydrothorax, and presence of edema in other organs, such as the pancreas and kidneys. Therefore, the diagnosis was made in six of the 13 cases (46%) necropsied in 2000 and 2001. In addition, four of the six cases were known to have died within three days of surgery, which is a typical clinical course for VLS; one rat died 21 days after surgery; and the exact date of death in relation to the surgery was unknown for one rat. These last two rats were from the slow distraction and sham group, respectively, and the first four did not undergo distraction since they died within three days of placement of the device. We, therefore, have no reason to suspect that presence or speed of distraction was a factor contributing to development of VLS. Results of histologic examination confirmed VLS diagnoses, as typical lung sections had substantial congestion in capillaries, infiltration of the parenchyma with white blood cells (WBC), presence of neutrophils and albumin in dilated lymphatic vessels (lymphatic sumps), and a few fibrin deposits in alveoli (Fig. 3). In addition, other organs, such as kidneys (Fig. 4), and pancreas (Fig. 5), were affected with congestion, edema, and WBC infiltration. Bacteriologic culture of lung tissue from these rats did not yield any growth.

In those rats not diagnosed with VLS, the clinical and necropsy findings were as follows. Three rats with no signs of respiratory tract dysfunction were sacrificed between seven and 10 days because of intractable diarrhea. Necropsy revealed WBC infiltration in the lungs, consistent with either beginning or resolving VLS or pneumonia. One rat died 19 days after placement of the distraction device and, therefore, 12 days after cessation of distraction (slow), with clinical signs and histopathologic findings consistent with DIC (Fig. 6). This animal presented with dehydration, lethargy, hematuria, ataxia, and slight hind limb paresis. Blood was collected and results of a CBC and serum biochemical analysis were consistent with dehydration (high hematocrit and hemoglobin and BUN concentrations), inflammation (absolute neutrophilia and monocytosis, with high globulin concentration), and DIC (high fibrin degradation products).

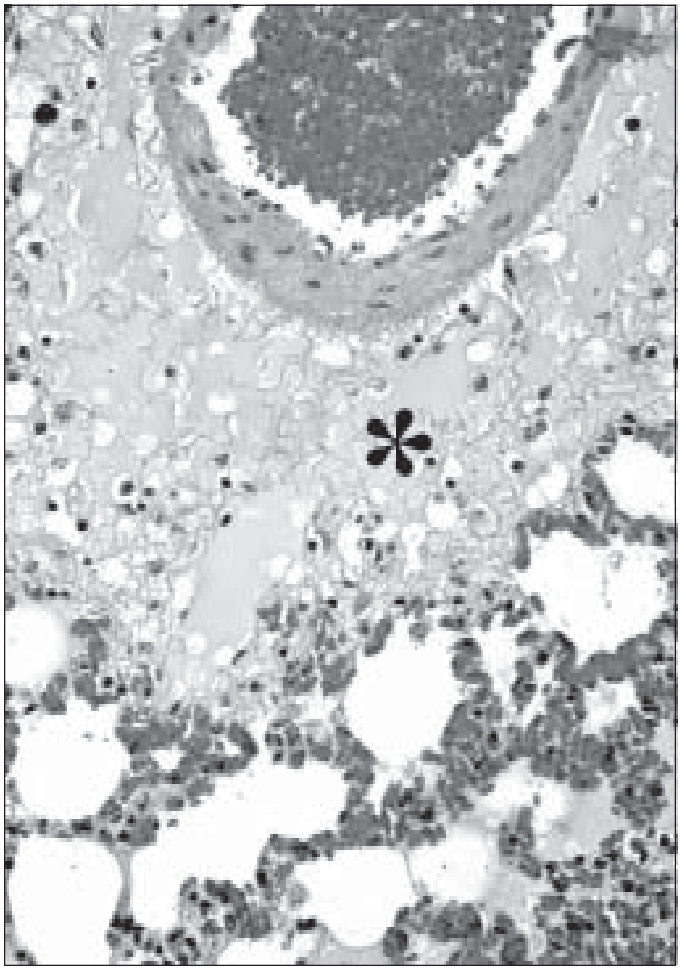

Figure 3. Photomicrograph of a section of lung tissue from a rat with vascular leak syndrome (VLS). Notice lymphatic sumps surrounding pulmonary vessel in lower right (asterisk) and diffuse mild interstitial white blood cell (WBC) infiltration, alveolar capillary congestion, and alveolar flooding with WBC and fibrin in lymphatic sumps. H&E stain; magnification 400×.

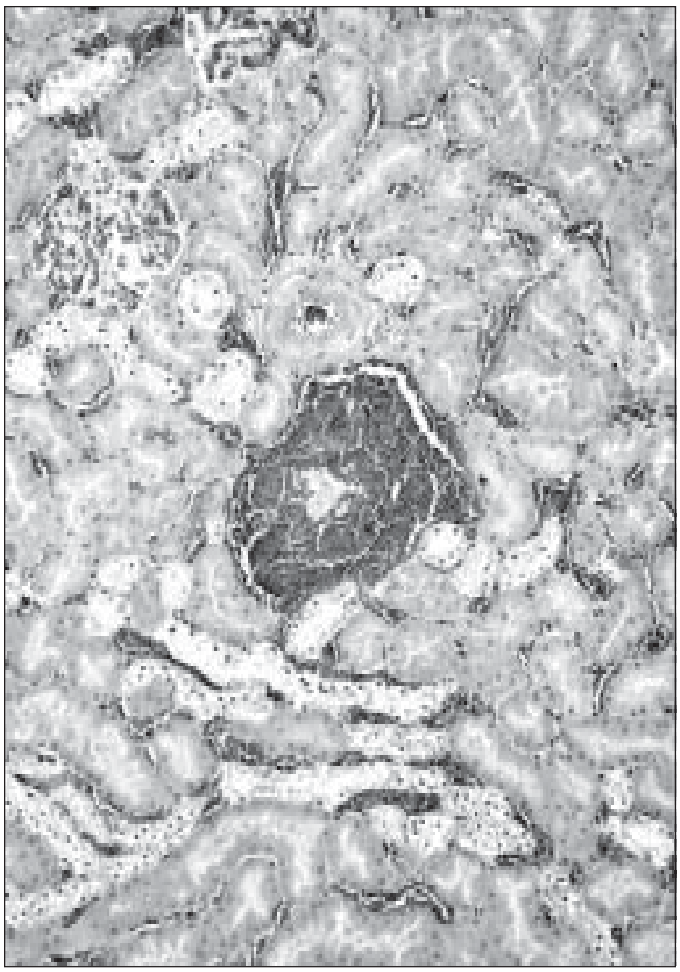

Figure 4. Photomicrograph of a section of kidney tissue from a rat affected with VLS. Notice diffuse interstitial congestion, with multifocal tubular degeneration and necrosis. H&E stain; magnification 200×.

Necropsy also revealed hemorrhage in the bladder, small intestine, cecum, brain, and spinal cord; histologic examination revealed WBC infiltration and thrombi in the lungs. Finally, one rat was euthanized following infection of the osteotomy site, and two others died without recovering from anesthesia, without pathologic changes consistent with VLS or DIC.

Discussion

Vascular leak syndrome is triggered by any local or systemic insult to the immune system that stimulates the inflammatory cascade; therefore, this insult can be of infectious, toxic, autoimmune, allergic, or even chemotherapeutic origin. In the study reported here, a number of factors could have potentially contributed to development of VLS in the rats, especially in the year 2000, when most of the unexpected deaths occurred. As time went on, surgeons became more proficient with the procedure. Therefore, the duration of the surgery and the dosage of anesthetic drugs progressively decreased (ketamine sulfate was decreased from 70 to 60 mg/kg, i.p., and xylazine was decreased from 13 to 8 mg/kg). Likewise, the amount of trauma done to the attachments of the masseter and medial pterigoid muscles reportedly decreased as well. Every effort was made to minimize such variables, but they were difficult to avoid entirely, as they would be in any study spanning several years. Their role in the

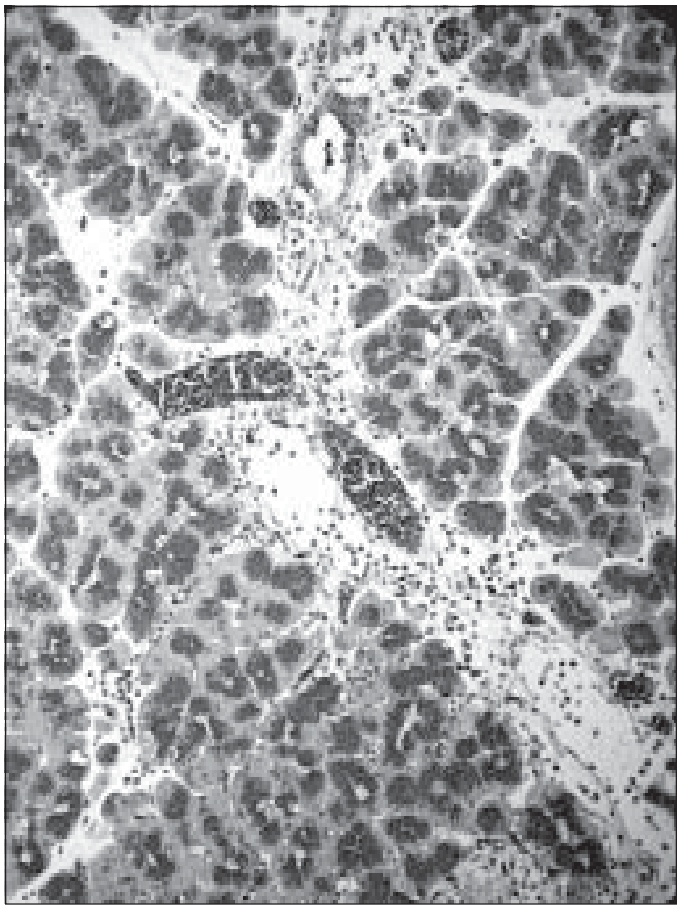

Figure 5. Photomicrograph of a section of pancreatic tissue from a rat affected with VLS. Notice diffuse interstitial edema, congestion, and WBC infiltration. H&E stain; magnification 100×.

onset of VLS is difficult to quantify because the precise mechanism of the syndrome is yet unknown. The key pathologic event in VLS is an increase in permeability of the microvasculature, first in the lungs, and later, in other organs. We saw evidence of pulmonary edema in at least six (46%) of the rats in the osteogenesis study that were necropsied, and presence of edema in other organs was noticed in two (15.3%) of these necropsied cases.

The marked change in vascular permeability can be the result of direct or indirect destruction of endothelial cells, this latter involving creation of temporary full-length migration pores. Alternatively, endothelial cells can contract and thereby enlarge vascular pores, which would also markedly alter fluid movement (16). Seepage of fluid and albumin into lung parenchyma and eventually into other tissues causes a number of abnormalities, such as impairment of surfactant synthesis, pulmonary hypertension, pulmonary congestion, and alveolar flooding, resulting in decreased lung compliance and gas exchange efficiency. In its end stage, generalized edema can trigger systemic hypotension, blood hypercoagularity, and eventually shock or DIC. Histologic findings in the rats of the osteogenesis study that suffered an early demise were consistent with this pathogenesis. The findings described in this study were similar to lesions reported in Wistar rats, in which VLS was induced by administration of immunotoxins, and included accumulation of fluid in peribronchovascular spaces, thickened alveolar walls, and infiltration of mononuclear inflammatory cells. In contrast to these lesions commonly found in

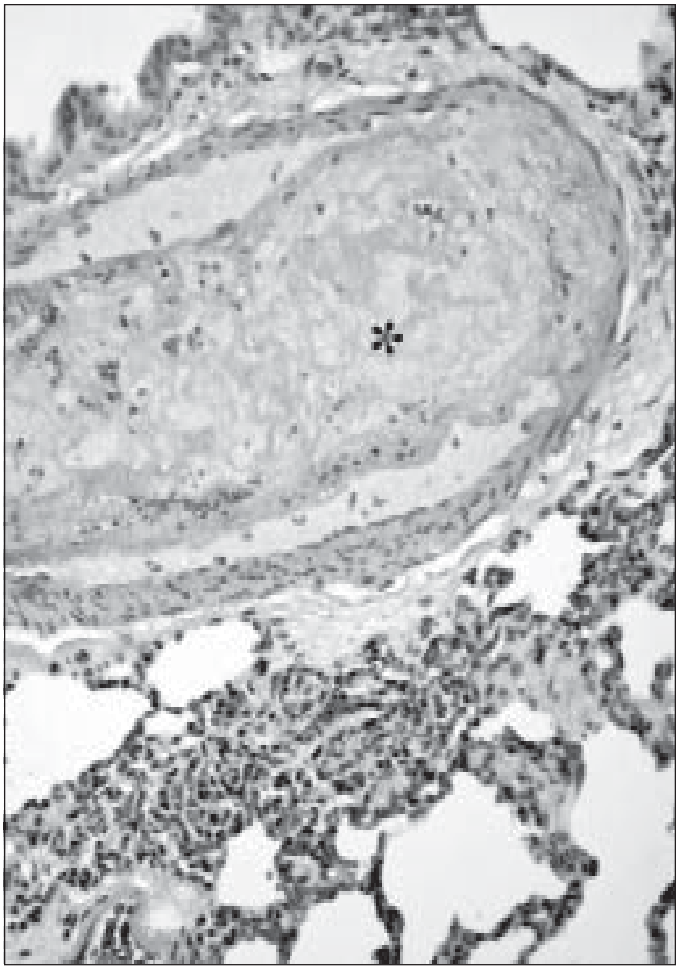

Figure 6. Photomicrograph of a section of lung tissue from a rat affected with disseminated intravascular coagulation with or without VLS. Notice large thrombus (asterisk) within pulmonary vessel. H&E stain; magnification 200×.

cases of VLS, fibroblast infiltration in the lungs, remodeling, fibrosis, and eventually, emphysema are other, less frequently seen end points of VLS; likewise, such lesions were not seen in the rats of the osteogenesis study we describe, and to our knowledge, have not been reported in literature about animal models of VLS.

The paucity of studies using rats as models of VLS induced by bone trauma is surprising because the sensitivity of rats to the syndrome is similar to that of human beings and higher than that of mice. The authors of one clinical study using rats attempted to predict the incidence of VLS, depending on the mode of fixation of bone fractures. In that study (13), Willis and coworkers determined that any type of internal fixation, as opposed to external fixation, would increase capillary permeability in the lungs. In addition, they reported that pins placed inside the medullary cavity of unreamed bones were subsequently associated with higher neutrophil activity in lung tissues, a counter-intuitive observation, since reaming induces additional trauma to the bone. However, neutrophil activity was measured in lung tissue from rats that were euthanized before actually developing VLS, so neither neutrophil activity as a predictor of VLS nor internal fixation as a cause of VLS could be fully established from that study.

The physiologic changes associated with VLS are better understood due to retrospective studies of human patients experiencing the phenomenon during intensive care (7, 8). Investigators are now focusing on the molecular mechanisms responsible for the increase in pulmonary vascular permeability. Cytokine-mediated events are likely since VLS is a common sequela of interleukin 2 (IL-2) immunotherapy for cancer and IL-2-induced VLS has been documented in rats (18). The $CD4^+$ T lymphocytes usually produce IL-2 when activated by IL-1, an early inflammatory mediator. Interleukin 2 then activates cytotoxic T cells ($CD8^+$), double-negative T cells (DN,) natural killer (NK) cells, and lymphokine-activated killer (LAK) cells into expressing CD44, an adhesion molecule (9, 10, 19). Through CD44, these cells adhere to endothelial cells directly or via hyaluronic acid, an important ligand for CD44, and destroy endothelial cells and basal lamina.

A role for CD44 in VLS is also suggested by CD44 knockout mice, which are less sensitive to VLS, and provide an animal model with resistance to this syndrome (9). Fujita and co-workers (16) suggested a different hypothesis regarding the molecular pathogenesis of VLS that involves LAK cells. After activation by IL-2, LAK cells adhere to endothelial cells through microvilli rather than through CD44. The LAK cells then penetrate the cytoplasm of endothelial cells through temporary membrane-lined pores, and finally through defects in the basement membrane into the perivascular space. Creation of the pores results in damage to endothelial cells characterized by cytoplasmic edema, vacuoles, and myelin figures. The damage, rather than the pores, is responsible for the increased permeability of the microvasculature. Another study by Ito and co-workers (12) indicated that tachykinin, bradykinin, and histamine, through specific airway receptors, could also mediate vascular leakage. Finally, a third possible mechanism involves thrombin-mediated contraction of endothelial cells through rearrangement of F-actin fibers (through increase in intra-cellular calcium), and phosphorylation of myosin light chains by rho-kinase (11).

Although those studies suggest potential disease mechanisms, a more complete understanding of the pathophysiologic mechanism(s) of VLS at the molecular level will be necessary to be able to make clinical decisions toward minimizing its incidence and severity. In the osteogenesis study reported here, a variety of factors, including experience of the surgeon, age of the animals, and refinement of the anesthetic protocol, may have influenced the incidence of VLS. Such factors may explain, in ways that need to be further characterized, the difference in incidence of postsurgical illness or death from 2000 to 2001 (from 17.3% of rats dying after placement of distraction device to 1.8%). Nevertheless, the high incidence of VLS cases in a study involving bone trauma in rats suggests high sensitivity of rats to this syndrome, and their suitability to serve as an animal model to study VLS, an important syndrome in human patients.

Acknowledgments

We thank S. Dowling, D. Liggitt, P. Treuting, and C. Karr-May for their expertise and contributions. The project of mandibular distraction osteogenesis in the rat was supported by NIDCR grant DE13061.

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