Intra-abdominal hyaluronan concentration in peritoneal fluid of horses with sudden signs of severe abdominal pain

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Objective—To determine hyaluronan concentrations in peritoneal fluid from healthy horses and horses with sudden signs of severe abdominal pain and to identify the cellular sources of hyaluronan within the peritoneal cavity.

Animals—7 client-owned horses that were evaluated for sudden signs of severe abdominal pain, 6 healthy teaching horses, and 13 euthanized horses (11 with no abdominal disease and 2 that had undergone abdominal surgery 2 weeks previously for a different study).

Procedures—Abdominal fluid was collected from the client-owned and teaching horses. Hyaluronan concentrations were determined with an ELISA. Equine mesothelial cells were aseptically harvested from euthanized horses immediately after euthanasia, cultured, and processed for western blot immunoassays to detect expression of the following mesothelial cell markers: cytokeratins 8 and 18, vimentin, calretinin, mesothelin, and CD44. A reverse transcriptase–PCR assay was used to detect genetic expression of hyaluronan synthase-2 (HAS-2) from cultured and native equine tissue.

Results—The mean ± SD abdominal hyaluronan concentration in peritoneal fluid from horses with signs of abdominal pain (1,203.3 ± 46.3 ng/mL) was significantly greater than that in healthy horses (228.4 ± 167.3 ng/mL). Harvested cells were maintained, and immunoblotting analyses confirmed expression of the mesothelial markers. Gene expression of HAS-2 from cultured mesothelial cells and fibroblasts was confirmed.

Conclusions and Clinical Relevance—Peritoneal hyaluronan concentration was much higher in horses with severe abdominal pain than in healthy horses. Cultured equine mesothelial cells and fibroblasts can produce hyaluronan through HAS-2. Future investigation should focus on establishing the effect of exogenous hyaluronan administration on mesothelial cell function in horses with abdominal disease. (*Am J Vet Res* 2011;72:1666–1673)

Intra-abdominal adhesions are important postoperative complications that develop after celiotomy in horses, resulting in future episodes of abdominal pain.[1] Horses that develop such adhesions generally have a poor long-term prognosis.[2] For all horses undergoing surgery for small intestinal disease, the proportion that requires additional surgery or are subsequently euthanized is as high as 22%.[3] However, the risk of adhesion formation can be reduced by handling tissues atraumatically, controlling hemorrhage, minimizing bacterial contamination, limiting the introduction of foreign material, preventing tissue dessication, ensuring timely surgical intervention, and aggressively managing any postoperative endotoxemia or ileus. Because these preventive measures can be challenging to fully achieve in clinical situations, additional prophylactic measures have been developed to combat adhesion formation, including the application of lubricants to the surface of the bowel at the time of surgery.[4,5] These treatments may have direct effects on mesothelial cells.

Abbreviation

HAS Hyaluronan synthase

The mesothelium is an extensive monolayer, the function of which is essential to the health of the abdominal cavity. It maintains a virtually frictionless environment within the abdomen that facilitates bowel motility. Additionally, mesothelial cells are capable of rapid migration after serosal injuries[6] and modulate serosal inflammation via the production and secretion of various pro- and anti-inflammatory mediators.[6–10] Proposed means of mesothelial surface reconstitution include the migration of mesothelial cells, which is in part facilitated by endogenous hyaluronan, the predominant glycosaminoglycan secreted by mesothelial cells.

Preservation of the peritoneal mesothelium is important for preventing adhesion formation, and pharmacological manipulation is a method for maintaining mesothelial integrity. Two therapeutic agents used to decrease abdominal adhesion formation are exogenous hyaluronan[11] and bioabsorbable hyaluronate-carboxymethylcellulose.[12] Presumably, use of these agents provides a lubricated barrier to prevent the forma-

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tion of fibrin on disrupted serosal surfaces; however, other mechanisms of action may be responsible for the therapeutic benefit provided, particularly in the case of hyaluronan.

Synthesis of hyaluronan has been identified in human mesothelia, and 3 membrane-bound HAS isoforms (HAS-1, HAS-2, and HAS-3) have been detected at the inner face of the plasma membrane of mesothesial cells.[13] To date, only mRNA for HAS-2 has been identified in tissue of equine origin.[14] Additionally, only HAS-1 and -3 (and not HAS-2) have been found in equine cumulus cells.[15] After hyaluronan is produced, hyaluronan can bind 2 main classes of cell surface receptors: CD44 and the receptor for hyaluronan-mediated motility. Subsequent to receptor binding, various physiologic events are initiated, including cell migration, cell adhesion, and cell proliferation,[16–18] all of which are vital to the wound healing process. The CD44 receptor is present in equine lymphocytes, serosa, peritoneum, omentum, and mesentery[a]; however, the precise biological importance of CD44 expression in horses has yet to be determined.

Little data exist regarding the function of mesothelial cells in the abdomens of horses, despite the wealth of information available for other animal species.[19–23] A more thorough understanding of mesothelial cell migration in equine peritonea and the pathogenesis of adhesion formation at a cellular level will aid in the prevention or treatment of postoperative abdominal adhesions. However, to our knowledge, the presence and production of hyaluronan within healthy and abnormal equine abdominal fluid have not been reported. Furthermore, an equine peritoneal mesothelial cell line has not been established nor have techniques for harvesting and maintaining primary equine mesothelial cells. If the abdominal source of hyaluronan were identified in horses, then its effect on mesothelial cell migration or its role in fibrinolysis could then be investigated in vitro. The purpose of the study reported here was to determine whether differences existed in the concentration and site of hyaluronan production in peritoneal fluid from healthy horses and horses with sudden signs of severe abdominal pain.

Materials and Methods

Animals—Thirteen horses were used for measurement of hyaluron concentration in peritoneal fluid: 6 healthy horses from the Kansas State University teaching herd and 7 client-owned horses that had been admitted to the veterinary teaching hospital for sudden signs of severe abdominal pain and underwent exploratory celiotomy. An additional 13 horses that had been donated to the veterinary teaching hospital and had been euthanized for reasons unrelated to the study were used for the harvesting of mesothelial cells. Eleven of the donated horses had no history of abdominal disease, and the other 2 had undergone abdominal surgery for an unrelated study 2 weeks previously. The Institutional Animal Care and Use Committee of Kansas State University approved all study procedures involving horses.

Peritoneal fluid collection and analysis—Samples of peritoneal fluid were aseptically collected from healthy, standing teaching horses and diseased horses via an 18-gauge needle into serum-separation and EDTA-treated tubes and from horses undergoing celiotomy via a 12-mL syringe during surgery. Samples from standing horses were obtained from a point approximately 10 cm caudal to the xyphoid process on the midline of the ventral aspect of the abdomen horses. Peritoneal fluid was analyzed for total nucleated cell count and total protein concentration as well as gross appearance. A blood sample was also collected from all horses via jugular venipuncture at the of abdominocentesis. Duration of signs of abdominal discomfort, location of the intestinal lesion, and outcome of the surgery (euthanized or discharged) were recorded for the client-owned horses that underwent an exploratory laparotomy.

Plasma was harvested from blood samples, and peritoneal fluid and plasma samples were stored at –80°C for later analysis. A 96-well commercially available ELISA[b] was used for the determination of hyaluronan concentration in thawed samples of plasma and peritoneal fluid as described elsewhere.[21] All samples were analyzed in triplicate. The assay was performed according to manufacturer's specifications with the standards provided. The reported intra-assay and interassay coefficients of variation for testing of equine plasma samples are 4.9% and 11%, respectively.[24]

Mesothelial cell harvesting and culture—Equine mesothelial cells were harvested aseptically immediately after horses were euthanized. In preparation, cadavers were positioned in dorsal recumbency, and a ventral midline approach was used to open the abdomen. Three techniques were used to harvest cells: mesenteric sack (n = 10 horses), omental explant (2), and mesenteric explant (3). The mesenteric sack technique consisted of a purse-string suture preplaced in a circular portion of mesentary between 2 arterial arcades of the midjejunum. The mesentery was sharply transected outside the suture line, and the purse-string suture was tightened, leaving a small opening to the lumen of the sack. This sack technique was developed specifically to expose just the mesothelium to digestive enzymes and limit their contact with submesothelia tissue, which may contain fibroblasts. Eight mesenteric sack samples were harvested from each horse in which this technique was used, yielding 80 samples. Omental and mesenteric explants were obtained by isolating and sharply transecting 1 sample of each tissue type (approx 10 × 10 cm) from each horse. All samples were washed and immediately transported to the laboratory in PBS solution.

Mesenteric sacks were filled with warmed (37°C) saline (0.9% NaCl) solution containing 0.25% trypsin-EDTA,[c] the purse-string suture was tightened, and the samples were incubated at 37°C with gentle agitation. The duration of incubation was determined empirically and ranged from 15 to 45 minutes, with more dissociation of mesothelial cells noticed after 30 to 45 minutes of incubation than after a shorter period. Dissociation was stopped with a 50% dilution of culture medium (to be described later). Dissociated cells were transferred to a cell culture flask[d] (25 cm^2). Cells were maintained with a culture medium containing medium M199[e] supplemented with 10% fetal bovine serum,[f] penicillin (100 U/mL) and streptomycin (100 mg/mL),[e] L-gluta-

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mine (2 µg/mL),[c] insulin (5 µg/mL),[e] and hydrocortisone (0.4 µg/mL).[c]

Omental and mesenteric specimens were placed into cell culture flasks (25 cm^2) and incubated with prewarmed saline solution containing 0.25% trypsin-EDTA (5 mL) with agitation for 15 minutes at 37°C. Tissues were removed, and trypsin-EDTA was neutralized with equal parts of culture medium. The solution containing cells, trypsin-EDTA, and medium was transferred to a 15-mL conical tube and centrifuged at 300 × *g* for 5 minutes. Pelleted cells were suspended in culture medium and seeded onto cell culture flasks.

All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Cells were grown to a confluent monolayer, with the medium replaced 24 hours after the initial seeding. Medium was replaced every 48 to 72 hours afterward. Light photomicroscopy was performed on live cells by use of an inverted, phase-contrast microscope[g] with attached camera[h] and digital software[i] on a daily basis. The interval to achieve confluence (monolayer of cells covering the bottom of the entire tissue flask) was documented.

Subcultures—Once cell monolayers were confluent, additional passages of cells were obtained by discarding the medium and adding 1 mL of 0.25% trypsin-EDTA to each cell culture flask. After 2 minutes, the trypsin-EDTA and lifted cells were removed and placed immediately into a new cell culture flask (25 cm^2) along with 5 mL of culture medium. The original tissue flask was washed 3 times with 5 mL of PBS solution, and culture medium was replaced (5 mL). Samples were maintained subsequently at 37°C in a humidified atmosphere containing 5% CO_2.

To minimize fibroblast contamination of mesothelial cell cultures, plates were examined for areas of fibroblastic overgrowth on a daily basis. Fibroblasts were identified on the basis of cell morphology by means of light microscopy. The areas of fibroblast growth were scraped with a cell scraper. Afterward, 1 mL of 0.25% trypsin-EDTA solution was added to each cell culture flask and allowed to remain for approximately 1 minute, during which time fibroblast lifting was monitored continuously. Trypsin-EDTA and fibroblasts were aspirated and placed into new cell culture flasks with 5 mL of culture medium. These subcultured fibroblasts were used for further analyses. The original culture flask was washed with 3 cycles of 5 mL of culture medium to aid in the removal of any loose fibroblasts that remained, filled again with 5 mL of culture medium, and returned to the incubator.

Reverse transcriptase–PCR assay of HAS-2—To determine relative expression of mRNA coding for HAS-2, RNA was extracted from cultured equine peritoneal mesothelial cells, cultured equine peritoneal fibroblasts, and freshly isolated native equine mesenteric, ovarian, and synovial tissue by use of a commercially available kit[j] and stored at –80°C. Tissues were disrupted, lysed, and homogenized in the provided lysis buffer with the addition of β-mercaptoethanol (10 µL/mL of lysis buffer). Quality and quantity of RNA were measured through determination of the A_{260}:A_{280} ratio with a spectrophotometer.[k] Sample aliquots were frozen at –80°C.

The expression of HAS-2 was analyzed with the PCR assay with real-time reporting. Equine-specific primers for HAS-2 and 18S rRNA were used as described elsewhere.[14] Analyses were conducted by use of cultured mesothelia and fibroblasts from 3 horses. Reverse transcriptase–PCR assay was performed on a DNA amplification system[l] by use of an asymmetric cyanide dye[m] and a commercial kit.[n] Each reaction was performed with 100 ng of RNA. The thermocycling protocol was as follows: 1 cycle at 48°C for 45 minutes and 94°C for 2 minutes, followed by 40 cycles at 94°C for 30 seconds, 55°C for 1 minute, and 68°C for 2 minutes. The PCR products were processed with a commercially available kit,[o] and results of gel electrophoresis of these products confirmed that there was a single product for each primer pair. The purified PCR products were sequenced with dye terminator cycle sequencing method by use of a kit[p] and a genetic analysis system.[q] Product identity was verified by comparison with the sequence reported for equine HAS-2 (National Center for Biotechnology Information Entrez accession No. AY56582).

Western blot assay—Immunoblot assays were performed to identify protein expression of mesothelial cell markers from the cultured cells that had a morphology consistent with mesothelia cells (cytokeratins, calretinin, and mesothelin) and fibroblasts (vimentin). Proteins of interest included pancytokeratin (mouse anti-human pancytokeratin clone C-11 monoclonal antibody[d]), cytokeratin 8 and 18 (mouse anti-human cytokeratin 8 and 18 monoclonal antibody[d]), vimentin (mouse anti-vimentin clone V9 monoclonal antibody[d]), calretinin (rabbit anti-human calretinin polyclonal antibody[r]), CD44 (rat anti-human CD44S[s]), and mesothelin (mouse anti-human mesothelin monoclonal antibody[d]). Although these antibodies are not equine specific, cross-reactivity was confirmed and controlled through use of protein harvested from rat epithelial cell lines (IEC-6), equine heart tissue, rat brain and heart tissues, and lysis buffer[s] to ensure antibodies identified a protein of interest at the reported electrophoretic mobility of the expected target.

Confluent monolayers of equine mesothelial cells and fibroblasts were harvested for protein analysis. Cells were washed several times with chilled PBS solution and placed in lysis buffer for total cell protein isolation. Cell lysate protein concentrations were determined with a bicinchoninic acid assay.[t] Aliquots were stored at –80°C until used for immunoblotting.

Protein (20 µg) was suspended in SDS gel loading buffer with and without 2-mercaptoethanol for reducing and nonreducing conditions, respectively. Protein was then loaded and resolved electrophoretically on a 4% to 12% SDS–polyacrylamide gel for subsequent wet transfer to a nitrocellulose membrane.[u] Membranes underwent blocking of nonspecific binding sites with 5% nonfat dry milk in tris-buffered saline solution containing 1.0% Tween 20, for 1 hour at room temperature (approx 20°C). After blots were washed 3 times for 5 minutes with PBS solution containing 0.1% Tween 20, they were incubated with primary antibodies overnight at 4°C. Membranes were washed 3 times for 15 minutes in PBS solution containing 0.1% Tween 20 and in-

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cubated with horseradish peroxidase–conjugated goat anti-mouse[v] or goat anti-rabbit IgG[w] for 1 hour at room temperature.

Antibodies were detected via enhanced chemiluminescence by use of a chemiluminescent substrate.[x] Prestained color mobility standards were resolved in each gel and used to estimate the molecular mass associated with immunoreactive bands. Membranes were stripped[y] and again probed with a rabbit anti-actin antibody[z] for 1 hour at room temperature, followed by incubation horseradish peroxidase–conjugated goat anti-rabbit IgG for 1 hour at room temperature. Again, antibodies were detected via enhanced chemiluminescence. Densitometric analysis was performed by use of computer software[aa] to determine the immunoreactivity of each target protein. Labeling intensity of the target proteins was normalized to the density of actin in the respective lanes.

Statistical analysis—Statistical comparisons were made between healthy and ill horses for hyaluronan concentration within plasma and peritoneal fluid, total protein, total nucleated cell count, and age by means of *t* tests of unequal variances. Results of immunoblot assays were also evaluated with paired *t* tests of unequal variances to assess differences in protein expression between cell types on the basis of morphology. Data are reported as mean ± SD. Values of $P \leq 0.05$ were considered significant.

Results

Animals—Signalment and outcome were summarized for client-owned horses with sudden signs of severe abdominal pain (**Table 1**).

Peritoneal hyaluronan concentrations—Mean ± SD abdominal hyaluronan concentrations in peritoneal fluid differed significantly between the 7 horses with abdominal pain (1,203.3 ± 46.3 ng/mL) and the 6 healthy horses (228.4 ± 167.3 ng/mL). Plasma hyaluronan concentrations, however, did not differ. Compared with the mean value in horses without abdominal disease (1.0 ± 0.4 g/dL), mean peritoneal total protein concentrations in diseased horses (2.9 ± 0.4 g/dL) were significantly higher. No difference was detected between the mean peritoneal total nucleated cell counts of the clinically normal horses (2,650 ± 1,024 cells/μL) and diseased horses (4,040 ± 1,789 cells/μL).

Peritoneal mesothelial cells—Viable mesothelial cells were obtained from 3 of the 10 horses in which the mesenteric sack technique was used. Confluent monolayers were obtained from 2 samples, with 1 at day 22 (from 1 horse with peritonitis of 6 days' duration from a previous surgical procedure and from a healthy horse; **Figure 1**). Fibroblastic contamination of mesothelial cells was observed after 3 separate harvests mesenteric sacks (**Figure 2**). Viable cells were obtained in omental and mesenteric explant harvests with trypsin-EDTA exposure times of 15 minutes (n = 3 horses). Omental explant samples yielded confluent monolayers 5 days after harvest for culturing (n = 2 horses), whereas the mesenteric explant harvest did not result in cells that reached a confluent monolayer during the same time frame (3).

Cell morphology—From omental explant samples, the yield of equine peritoneal mesothelial cells was extremely high and the primary flasks were populated densely at seeding. The cells appeared microscopically as large clusters and adopted a fibroblastic, spindle-shaped appearance after initial seeding, becoming polygonal upon confluence. Confluent monolayers were obtained after 4 to 5 days of culturing, with the cells becoming polygonal by day 7. Two additional passages of cells were obtained by day 10 with similar intervals to confluence. The omental explant culture method yielded a fairly pure population of mesothelial cells represented by no endothelial cell contamination (which would be characterized by the formation of capillary-like tubules) and minimal fibroblast contamination.

The morphological characteristics of the mesenteric sack samples differed depending on the disease status of the horses. In samples obtained from a horse with peritonitis of 6 days' duration at the time of tissue harvest, the initial fibroblastic morphology of the

Table 1—Signalment, ailment, outcome, and peritoneal fluid characteristics of 7 horses evaluated for sudden signs of severe abdominal pain.

Signalment	Lesion	Outcome	Gross appearance of peritoneal fluid	TNCC (X 10^3 cells/L)	TP (g/dL)	Colic duration (h)	Hyaluronan (ng/mL)
2-year-old female QH	360° large colon volvulus	Euthanized	Clear	0.9	2.0	12	1,255
11-year-old female QH	Ileal impaction	Discharged from hospital	Turbid	14.0	2.2	12	1,135
9-year-old castrated male Arabian	Large colon displacement, severe gas distention, proximal enteritis	Discharged from hospital	Turbid	3.0	4.3	12	1,204
3-year-old castrated male QH	Large colon displacement	Discharged from hospital	Clear	1.1	2.5	10	1,196
6-year-old castrated male QH	Epiploic foramen entrapment; no resection	Discharged from hospital	Serosanguineous	0.7	1.8	6	1,176
17-year-old female Appaloosa	Strangulating lipoma	Discharged from hospital	Serosanguineous	4.8	3.0	6	1,146
16-year-old female Andalusian	Strangulating lipoma	Euthanized	Serosanguineous	3.8	4.1	24	1,309

QH = Quarter Horse. TNCC = Total nucleated cell count in peritoneal fluid. TP = Total protein concentration in peritoneal fluid.
Data for individual horses are provided to show the variation among horses with respect to the reported characteristics.

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mesothelial cells was similar to that of the peritoneal mesothelial cells harvested from omental tissue; however, the interval to confluence and a polygonal appearance was longer, at 22 days. Mesenteric cells were harvested successfully from 2 additional horses; however, the cells were larger. Mesothelial cells harvested from a horse with no abdominal disease grew to confluence at 39 days. Cells harvested from a horse euthanized because of an incarcerated small intestine in the gastrosplenic space became senescent (characterized by a loss of proliferation and an increase in cell size as identified over time via microscopy). Fibroblast contamination was morphologically evident in samples from 2 of the 3 horses when samples were collected with the mesenteric sack technique; however, there was no evidence of endothelial contamination.

One mesenteric explant sample (clinically normal horse with no history of abdominal disease) yielded a higher seeding density than did the mesenteric sack samples. The initial cell appearance was polygonal with a gradual increase in cell size, consistent with senescence. No fibroblast or endothelial cell contamination was evident as determined on the basis of microscopic inspection of cell morphology.

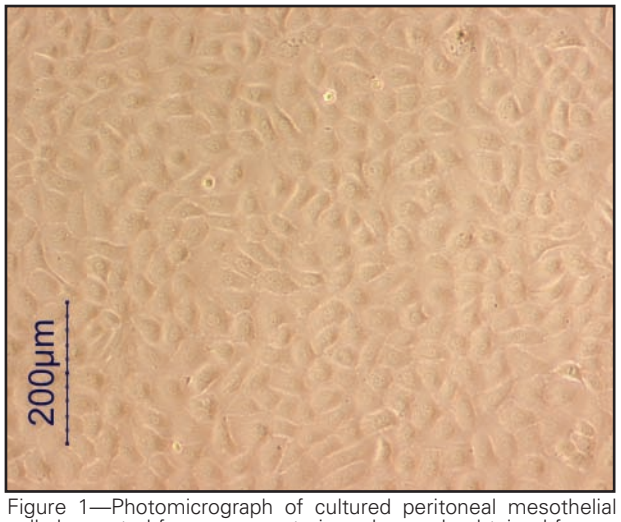

Figure 1—Photomicrograph of cultured peritoneal mesothelial cells harvested from a mesenteric sack sample obtained from a horse with peritonitis. Monolayer confluence was obtained at 22 days. Bar = 200 µm.

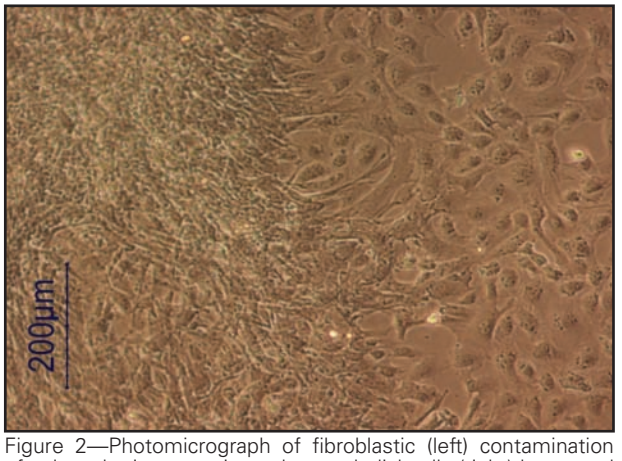

Figure 2—Photomicrograph of fibroblastic (left) contamination of cultured primary peritoneal mesothelial cells (right) harvested from an omental explant sample obtained from a horse with peritonitis. Cell types were identified on the basis of morphology and results of protein expression markers. Bar = 200 µm.

Reverse transcriptase–PCR assay analysis—Primary cell lines typically yielded high-quality RNA (range for mesothelial cells, 426.1 to 447.8 ng/µL; range for fibroblasts, 278.8 to 293.0 ng/µL; range for A_{260}:A_{280} ratio, 1.95 to 1.97, with 2.0 considered pure). Gene expression of HAS-2 by cultured equine mesothelial cells and fibroblasts was confirmed by reverse transcriptase–PCR assay with the expected product size of 486 kbp. Native mesenteric, synovial, and ovarian tissue cells (positive control samples) also had expression of HAS-2. Sequencing for all RNA sources confirmed the presence of HAS-2 with a 96% to 100% homology for this segment, compared with the published equine sequences.

Western blot assay of mesothelial cell markers—Western blot immunoassay of mesothelial cells harvested from mesenteric sacks and omental explants confirmed expression of cell markers pancytokeratin, cytokeratin 8 and 18, calretinin, mesothelin, and vimentin. Pancytokeratin immunoreactivity was observed as a single, dense band with a mobility of 58 kDa. There was significantly more relative protein expression of pancytokeratin within the cultured mesothelial cells than in cultured fibroblasts. Antibodies applied to detect cytokeratin 8 and 18 revealed 2 dominant bands, as expected. One band indicated a molecular mass between 50 and 75 kDa, and the other was between 30 and 50 kDa. There was more relative protein expression of these cytokeratins in mesothelial cells, although a significant (P = 0.09) difference was not found. A dominant 69-kDa band was detected with anti-mesothelin antibody. There was significantly more relative protein expression of mesothelin within cultured mesothelial cells than within cultured fibroblasts. Anti-vimentin antibody labeled a single band with an apparent mass of 50 kDa. Conversely, there was no difference in relative protein expression of vimentin within cultured fibroblasts versus mesothelial cells. Mesothelial cells were probed with an antibody to detect calretinin. Consistently, a faint band was detected at 69 kDa in the mesothelial cell lane, which is the reported mobility for the prema-

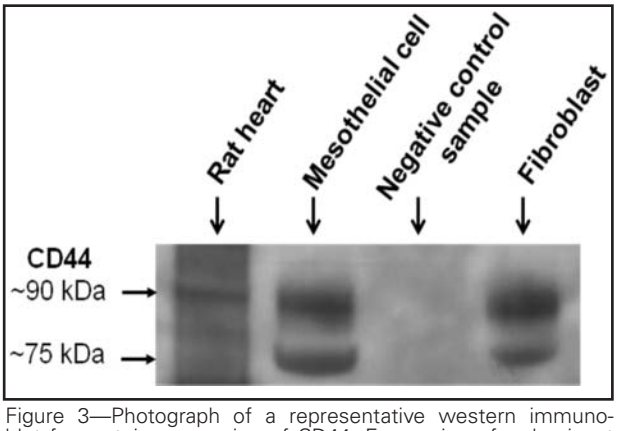

Figure 3—Photograph of a representative western immunoblot for protein expression of CD44. Expression of a dominant 90-kDa band for CD44 appears in the lanes for mesothelial cells and fibroblasts. Lysis isolation buffer served as a negative control sample. ~ = Approximately.

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ture protein. However, a dense band was detected at 29 kDa in the lane for rat brain cells, which presumably represented the molecular mass of the mature mesothelin protein. Antibodies applied to detect CD44 yielded a dense band at 90 and 70 kDa (**Figure 3**) within the lanes for mesothelial cells and fibroblasts.

Discussion

In the study reported here, peritoneal hyaluronan concentrations were compared between healthy horses and horses with sudden, severe abdominal pain. Findings indicated that hyaluronan and total protein concentrations are higher in diseased horses, which is consistent with previous clinical findings that total protein concentration increases prior to an increase in total nucleated cell counts in the peritoneal fluid from horses with moderate to severe signs of abdominal pain.[24,25] Additionally, our findings are similar to those reported for other species in experimental and clinical studies[19,20] that demonstrated an increase in hyaluronan with severe inflammation of the abdominal cavity. Moreover, human peritoneal mesothelial cells increase hyaluronan production when exposed to inflammatory cytokines[21,22] or after in vitro wounding (trauma) of human peritoneal mesothelial cell monolayers.[18] Increases in peritoneal hyaluronan concentrations might therefore be expected in situations of severe inflammation, regardless of the dominant pathological process.

Increases in peritoneal hyaluronan concentrations were not accompanied by increases in plasma hyaluronan concentrations in our study, indicating that the increase in hyaluronan concentration in the diseased peritoneal cavity was attributable to local hyaluronan production. This finding is also similar to findings in humans with severe inflammation in whom it was determined that most, if not all, of the peritoneal hyaluronan was derived from mesothelial cells.[22] An upregulation of HAS expression in mesothelial cells reportedly occurs in response to peritoneal cytokines and proinflammatory mediators released in humans with peritonitis.[22] It was not possible to determine whether the increase in hyaluronan concentration found in diseased horses in the present study was a response to inflammatory cytokines, although the data suggested that this may be true. The presence of proinflammatory mediators within the peritoneal fluid from horses with ischemic intestines has been reported.[24]

The ELISA used to measure hyaluronan concentration in the present study has been used with serum and plasma to assess the degree of liver fibrosis and cirrhosis in humans with chronic liver disease.[26] It was also used to determine plasma hyaluronan concentrations in 120 horses.[27] In that study, the mean hyaluronan concentration in plasma was 89 ng/mL, which is less than the concentrations detected in the present study. Plasma hyaluronan concentrations of 25 resting horses were reported to range from 190 to 760 ng/mL when a specific radioimmunoassay was used,[28] and those values are greater than those of the healthy horses and less than those of the horses with abdominal disease in the present study. There appears to be a large but reproducible interindividual variability in plasma hyaluronan concentrations among healthy horses. We found no differences in plasma hyaluronan concentrations between diseased and healthy horses; however, significant differences were detected within peritoneal fluid, which suggests that hyaluronan plays a local role in severe equine abdominal disease.

Hyaluronan, an important mediator of inflammation, enhances in vitro wound healing[18] and is an important modulator of peritoneal mesothelial cell migration in humans.[18,29–31] This glycosaminoglycan is synthesized at the inner face of the cellular plasma membrane and is extruded into the extracellular space as it is produced.[13] Human mesothelial cells are prolific hyaluronan producers and express all 3 HAS isoforms.[18] To determine whether HAS-2 is present in equine mesothelial cells, we attempted to develop an equine mesothelial cell harvesting technique as well as to harvest RNA from native tissue. Cultured cells yielded a PCR product of expected size and sequence when probed with HAS-2 primers. Native omental and mesenteric tissues are primarily comprised of mesothelial cells; however, connective tissue and other cell types are present. It is difficult to determine the exact cell types that are responsible for hyaluronan production from full-thickness harvests. Hyaluronan synthase-2 was expressed by cultured fibroblasts, mesenteric native tissue, and cultured mesothelial cells from horses. Because mesothelial cells are the primary lining of the peritoneal cavity, the increase in hyaluronan concentration detected in the peritoneal fluid from the diseased horses likely originated from those mesothelial cells. Additional studies are necessary to determine whether peritoneal mesothelial cells are the primary source of hyaluronan and to determine whether HAS-1 and -3 are also expressed by mesothelial cells in horses.

Three techniques for isolation and maintenance of a primary cell line of equine peritoneal mesothelial cells were used in the present study. Methods included creation of mesenteric sacks and explants of omental and mesenteric tissues. Isolation of human mesothelial cells from omental specimens is an established, reproducible technique.[32] Cultured human and rat mesothelial cells have a heterogeneous phenotype that has been characterized morphologically as polygonal to elongated.[33–35] Consistent with other findings,[35] mesothelial cells from the omental explant and mesenteric sack samples from a horse with peritonitis appeared multipolar or even elongated during the initial stages of growth. When grown to confluence, these cells became polygonal in appearance and morphologically distinct from the spindle-like connective tissue fibroblasts.

Of the techniques used in the present study, the omental explant method provided the highest yield and shortest time to confluence of mesothelial cells. Samples collected from the horse with peritonitis (used in another study[22] 2 weeks prior) grew to confluence rapidly, which may have been attributable to stimulation by the inflammatory process. In general, mesenteric mesothelial cells were larger in size (senescent) with a prolonged interval to confluence, compared with omental samples. Additional research is necessary to determine the usefulness of the primary equine mesothelial cell line to establish the role of these cells during inflammation, wound healing, fibrinolysis, transport, and hyaluronan biosysnthesis.

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Characterization of primary cell lines relies on cell morphology and the cells' ability to produce proteins that are expressed in native cells of the same type. Protein expression of calretinin, pancytokeratin, cytokeratins 8 and 18, vimentin, mesothelin, and CD44 have been used in human and equine studies.[19,36–39] We chose various antibodies to specifically detect vimentin, pancytokeratin, cytokeratins 8 and 18, and CD44 in mesothelial cells. The outcome suggested strongly that the primary cultured cells were characterized accurately as mesothelial cells. Equine-specific antibodies against these proteins are not available commercially; however, the results support the hypothesis that these epitopes are highly conserved and can be used to characterize mesothelial cells across species.

Findings in the present study suggested hyaluronan of peritoneal origin may be an important element in wound healing within the abdomen following abdominal surgery in horses. Gaining knowledge of the interrelationship between mesothelial cells and their production of hyaluronan will be useful in determining how to prevent and treat adhesions following surgery.

a. Frees K, Gaughan E, Lillich J, et al. The presence of hyaluronan receptors on equine serosa and peritoneum (abstr), in *Proceedings*. 11th Annu Meet Am Coll Vet Surg 2001;7.
b. Corgenix Inc, Westminster, Colo.
c. Invitrogen Corp, Grand Island NY.
d. PRIMARIA Tissue Culture Flask, Becton Dickinson Labware, Franklin Lakes, NJ.
e. Sigma-Aldrich Inc, St Louis, Mo.
f. GIBCO, Invitrogen Corp, Grand Island, NY.
g. Nikon Eclipse TS100, Nikon USA, El Segundo, Calif.
h. Nikon DXM-1200, Nikon USA, El Segundo, Calif.
i. Nikon ACT-1, Nikon USA, El Segundo, Calif.
j. RNeasy Plus Mini Kit, QIAGEN Sciences, Germantown, Md.
k. Nanodrop ND-1000 Spectrophotometer, NanoDrop Technologies Inc, Rockland, Del.
l. SmartCycler, Cepheid, Sunnyvale, Calif.
m. SYBR green I, Applied Biosystems, Foster City, Calif.
n. SuperScript III Platinum One-Step qRT-PCR System, Invitrogen, Grand Island, NY.
o. QIAquick PCR Purification Kit, QIAGEN Sciences, Germantown, Md.
p. GenomeLab DTCS-Quick Start Kit, Beckman Coulter, Fullerton, Calif.
q. Beckman Coulter CEQ 8000XL, Beckman Coulter, Fullerton, Calif.
r. Chemicon, Temecula, Calif.
s. RIPA Isolation Buffer, SC-24948, Santa Cruz Biotechnology Inc, Santa Cruz, Calif.
t. BCA, Pierce Biotechnology, Rockford, Ill.
u. Schleicher and Schuell Bioscience Inc, Sanford, Me.
v. Goat-anti-mouse HRP-conjugated antibody No. 185413, Pierce Biotechnology, Rockford, Ill.
w. Goat-anti-rabbit HRP-conjugated antibody No. 1858415, Pierce Biotechnology, Rockford, Ill.
x. SuperSignal West FEMTO Chemiluminescent Substrate, Thermo Scientific, Waltham, Mass.
y. Restore Western Blot Stripping Buffer, Bio-rad Laboratories, Hercules, Calif.
z. Rabbit anti-actin antibody A2066, Sigma-Aldrich Inc, St Louis, Mo.
aa. AlphaEaseFC, Alpha Innotech Corp, San Leandro, Calif.

References

1. Parker JE, Fubini SL, Todhunter RJ. Retrospective evaluation of repeat celiotomy in 53 horses with acute gastrointestinal disease. *Vet Surg* 1989;18:424–431.
2. Hay WP, Mueller PO. Intra-abdominal adhesions. In: White NA, Moore JN, eds. *Current techniques in equine surgery and lameness*. 2nd ed. Philadelphia: WB Saunders Co, 1998;307–310.
3. Baxter GM, Broome TE, Moore JN. Abdominal adhesions after small intestinal surgery in the horse. *Vet Surg* 1989;18:409–414.
4. Eggleston RB, Mueller PO. Prevention and treatment of gastrointestinal adhesions. *Vet Clin North Am Equine Pract* 2003;19:741–763.
5. Bullen TF, Hershman MJ. Therapy for intra-abdominal adhesions. *Hosp Med* 2004;65:340–342.
6. Mutsaers SE. Mesothelial cells: their structure, function and role in serosal repair. *Respirology* 2002;7:171–191.
7. Collatos C, Barton MH, Schleef R, et al. Regulation of equine fibrinolysis in blood and peritoneal fluid based on a study of colic cases and induced endotoxaemia. *Equine Vet J* 1994;26:474–481.
8. Collatos C, Barton MH, Prasse KW, et al. Intravascular and peritoneal coagulation and fibrinolysis in horses with acute gastrointestinal tract diseases. *J Am Vet Med Assoc* 1995;207:465–470.
9. Liakakos T, Thomakos N, Fine PM, et al. Peritoneal adhesions: etiology, pathophysiology, and clinical significance. Recent advances in prevention and management. *Dig Surg* 200;18:260–273.
10. Cheong YC, Laird SM, Shelton JB, et al. Peritoneal healing and adhesion formation/reformation. *Hum Reprod Update* 2001;7:556–566.
11. Eggleston RB, Mueller E, Quandt JE, et al. Use of a hyaluronate membrane for jejunal anastomosis in horses. *Am J Vet Res* 2001;62:1314–1319.
12. Mueller PO, Hay WP, Harmon B, et al. Evaluation of a bioresorbable hyaluronate-carboxymethylcellulose membrane for prevention of experimentally induced adhesions in horses. *Vet Surg* 2000;29:48–53.
13. Prehm P. Hyaluronate is synthesized at plasma membranes. *Biochem J* 1984;220:597–600.
14. Stock AE, Bouchard N, Brown K, et al. Induction of hyaluronan synthase 2 by human chorionic gonadotropin in mural granulosa cells of equine preovulatory follicles. *Endocrinology* 2002;143:4375–4384.
15. Marchal, R, Cailuad M, Martoriati A, et al. Effect of growth hormone (GH) on in vitro nuclear and cytoplasmic oocyte maturation, cumulus expansion, hyaluronan synthases, and connexins 32 and 43 expression, and GH receptor messenger RNA expression in equine and porcine species. *Biol Reprod* 2003;69:1013–1022.
16. Chen WY, Abatangelo G. Functions of hyaluronan in wound repair. *Wound Repair Regen* 1999;7:79–89.
17. Horiuchi T, Miyamoto K, Miyamoto S, et al. Image analysis of remesothelialization following chemical wounding of cultured human peritoneal mesothelial cells: the role of hyaluronan synthesis. *Kidney Int* 2003;64:2280–2290.
18. Yung S, Thomas GJ, Davies M, et al. Induction of hyaluronan metabolism after mechanical injury of human peritoneal mesothelial cells in vitro. *Kidney Int* 2000;58:1953–1962.
19. Edelstam G, Laurent U, Lundkvist O, et al. Concentration and turnover of intraperitoneal hyaluronan during inflammation. *Inflammation* 1992;16:459–469.
20. Yung S, Coles G, Williams J, et al. The source and possible significance of hyaluronan in the peritoneal cavity. *Kidney Int* 1994;46:527–533.
21. Breborowicz A, Korybalska K, Grzybowski A, et al. Synthesis of hyaluronic acid by human peritoneal mesothelial cells: effect of cytokines and dialysate. *Perit Dial Int* 1996;16:274–278.
22. Yung S, Coles G, Davies M. IL-1β, a major stimulator of hyaluronan synthesis in vitro of human peritoneal mesothelial cells: relevance to peritonitis in CAPD. *Kidney Int* 1996;50:1337–1343.
23. Johnston JK, Morris DD. Comparison of duodenitis/proximal jejunitis and small intestinal obstructions in horses: 68 cases (1997–1985) *J Am Vet Med Assoc* 1990;191:183–186.
24. Barton MH, Collatos C. Tumor necrosis factor and interleukin-6 activity and endotoxin concentration in peritoneal fluid and blood of horses with acute abdominal disease. *J Vet Intern Med* 1999;13:457–464.
25. Hunt E, Tennant R, Whitlock RH. Interpretation of peritoneal fluid erythrocyte counts in horses with abdominal disease, in *Proceedings*. 2nd Equine Colic Res Symp 1986;168–174.
26. Parsian H, Rahimipour A, Nouri M, et al. Serum hyaluronic acid and laminin as biomarkers in liver fibrosis. *J Gastrointest Liver Dis* 2010;19:169–174.

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27. Popot MA, Bonnaire Y, Guechot J, et al. Hyaluronan in horses: physiological production rate, plasma and synovial fluid concentrations in control sample conditions and following sodium hyaluronate administration. *Equine Vet J* 2004;36:482–487.
28. Tulamo RM, Saari H, Konttinen YT. Determination of concentration of hyaluronate in equine serum. *Am J Vet Res* 1990;51;740–742.
29. Greenwald R, Moy W. Effect of oxygen-derived free radicals on hyaluronic acid. *Arthritis Rheum* 1980;223:455–461.
30. Beck-Schimmer G, Oertli B, Pasch T, et al. Hyaluronan induces monocyte chemoattractant protein-1 expression in renal tubular epithelial cells. *J Am Soc Nephrol* 1998;9:2283–2290.
31. Akatsuka M, Yamamoto Y, Tobetto K, et al. Suppressive effects of hyaluronic acid on elastase release from rat peritoneal leukocytes. *J Pharm Pharmacol* 1993;45:110–114.
32. Yung S, Keung Li F, Mao Chan T. Technological advances in peritoneal dialysis research. *Perit Dial Int* 2006;26:162–173.
33. Stylianou E, Jenner LA, Davies M, et al. Isolation, culture and characterization of human peritoneal mesothelial cells. *Kidney Int* 1990;37:1563–1570.
34. Thiollet J, Jaurand MC, Kaplan H, et al. Culture procedure of mesothelial cells from rat parietal pleura. *Biomedicine* 1978;29:69–73.
35. Lewis W. Mesenchyme and mesothelium. *J Exp Med* 1923;38:257–262.
36. Attanoos R, Webb R, Dojcinov S, et al. Value of mesothelial and epithelial antibodies in distinguishing diffuse peritoneal mesothelioma in females from serous papillary carcinoma of the ovary and peritoneum. *Histopathology* 2002;40:237–244.
37. Okamoto S, Ito K, Hironobu S, et al. Ber-EP4 and anti-calretinin antibodies: a useful combination for differential diagnosis of various histological types of ovarian cancer cells and mesothelial cells. *J Exp Med* 2005;206:31–40.
38. Hassan R, Bera T, Pastan I. Mesothelin: a new target for immunotherapy. *Clin Cancer Res* 2004;10:3937–3934.
39. Doglioni C, Dei A, Laurino L, et al. Calretinin: a novel immunocytochemical marker for mesothelioma. *Am J Surg Pathol* 1996;20:1037–1046.

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