Cutaneous mucinosis in shar-pei dogs is due to hyaluronic acid deposition and is associated with high levels of hyaluronic acid in serum

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Abstract
Cutaneous mucinosis affects primarily shar-pei dogs. Hyaluronic acid (HA) is considered to be the main component of mucin and CD44 is the major cell surface receptor of HA, necessary for its uptake and catabolism. The aims of this study were to identify the composition of the mucin in cutaneous mucinosis of shar-pei dogs, investigate the correlation between the deposition of HA and the expression of CD44, and determine whether shar-pei dogs with cutaneous mucinosis presented with elevated levels of serum HA. In skin biopsies, the mucinous material was stained intensely with Alcian blue and bound strongly by the hyaluronan-binding protein. No correlation was found between the degree of HA deposition in the dermis and the expression of CD44 in the skin of shar-pei dogs affected or unaffected by cutaneous mucinosis. A clear positive correlation was found between the existence of clinical mucinosis and the serum HA concentration. In control dogs, serum HA ranged from 155.53 to 301.62 µg L⁻¹ in shar-pei dogs; without mucinosis it ranged from 106.72 to 1251.76 µg L⁻¹ and in shar-pei dogs with severe mucinosis it ranged between 843.51 to 2330.03 µg L⁻¹. Altogether, the results reported here suggest that mucinosis of shar-pei dogs is probably the consequence of a genetic defect in the metabolism of HA.

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Introduction
Cutaneous mucinosis refers to an excessive deposition of mucinous substance in the dermis that clinically is manifested as a thickening of the skin or as a vesicular appearance. Histologically, cutaneous mucinosis appears as a lightly basophilic ground substance that displaces dermal collagen fibres. A generalized cutaneous mucinosis, probably of genetic origin, occurs primarily in the Chinese shar-pei dogs, giving them its characteristic appearance (Fig. 1). Severe mucinosis is associated with secondary diseases such as intertrigo, bacterial infections and entropion, all of which can be serious and can lead to severe deterioration in the health of the animal. Although cutaneous mucinosis is a common condition in shar-pei dogs, little is known regarding its aetopathogenesis, prevention and treatment.

One histochemical investigation demonstrated that the mucinous material deposited in the skin of hypothyroid dogs was Alcian blue (AB)/Schiff’s periodic acid (PAS)-positive corresponding to acid glycosaminoglycans (GAGs), concluding that probably the only polysaccharide compound involved in canine dermal mucinosis was hyaluronic acid (HA). Another study demonstrated that in the dermis of shar-pei dogs with cutaneous mucinosis, the predominant mast cells were chymase-positive, instead of the more common tryptase-positive, suggesting that these cells might play a role in the pathogenesis of this disease.

Mucin is a histological term, used to define a jelly-like, clear, viscid substance. This term includes several biochemical entities, among them sulphated and carboxylated GAGs, such as dermatan sulphate and chondroitin-6-sulphate, and nonsulphated GAGs, including HA, which is the main component in most human and animal mucinosis. HA is a straight-chain GAG polymer of the extracellular matrix. It is synthesized as a large, negatively charged, unbranched polymer that is composed of repeated disaccharides of glucuronic acid and N-acetylgalactosamine. HA differs from other GAGs in many ways. Other GAGs

Figure 1. Shar-pei dog affected by mucinosis. Note the marked skin folds on the head.
are composed of proteoglycans synthesized and assembled in the endoplasmic reticulum and Golgi apparatus and are secreted in a similar way to other glycoproteins. HA is synthesized as an unmodified polysaccharide by one of the three different but related HA synthases (HAS-1, HAS-2 and HAS-3). HA has remarkable hydrodynamic characteristics, especially in terms of its viscosity and ability to retain water and also forms a multivalent template for interactions with proteoglycans and other extracellular molecules. A comparative study of a case of cutaneous mucinosis in a child and the mucinosis of the shar-pei dogs detected high levels of HA in the blood of shar-pei dogs affected by mucinosis and suggested that the disease could be due to a defect in the metabolism of HA. Therefore, we assumed as a first working hypothesis that mucinosis in the shar-pei dog is due to an accumulation of HA in the dermis.

HA is produced by many cutaneous cells, such as fibroblasts and keratinocytes, and remains in the extracellular matrix, bound to other glycoproteins and GAGs. CD44 is the major cell surface receptor for HA, as well as being responsible for its uptake and catabolism. Studies in transgenic mice and in several human diseases including lichen sclerosus and atrophicus, and myxoid dermatofibromas, have inversely correlated the expression of CD44 in cells and the accumulation of HA: a low expression of CD44 in epithelial cells is associated with depositions of HA in the surrounding dermis. On the other hand, in other cutaneous diseases such as follicular mucinosis, this correlation could not be established.

Given this background, the aim of this investigation was to establish a better understanding of the aetiopathogenesis of cutaneous mucinosis of shar-pei dogs and, first, to identify the major component of the mucin. Secondly, if HA was found to be the major component of mucin, the investigation sought to correlate the deposition of HA with the expression of CD44 and to know if the serum of shar-pei dogs with mucinosis had elevated levels of HA.

Materials and methods

Skin biopsies

In this retrospective study, skin samples from 14 dogs were selected from the archives of the Pathology Service of the Veterinary School of the Universitat Autònoma de Barcelona (2004–2006) to be used for the histological and immunohistochemical studies. Ten dogs were shar-peis with a clinical and histological diagnosis of mucinosis. These dogs clinically presented marked wrinkles on head, neck and extremities and/or mucinous vesicles. Histologically, deposits of mucinous material were evident in the dermis. From each dog, between two and four skin biopsies were sampled from the head and the extremities, using 4- to 6-mm punch biopsy instruments. The remaining four dogs belonged to other breeds and were free of skin diseases. All samples were fixed in 10% buffered formalin and embedded in paraffin.

Serum samples

Serum samples were obtained from 21 dogs brought to the Veterinary Teaching Hospital of the Universitat Autònoma de Barcelona. Eight were shar-pei dogs (five intact males, three females; age range 6 months to 3 years) affected by severe cutaneous mucinosis, as evaluated clinically by two of the authors (MB and LF). These eight shar-pei dogs without clinical evidence of mucinosis (four males, of which one neutered, and four females; age range 1–5 years) and five were healthy adult dogs of other breeds which served as normal controls. Blood samples were collected, allowed to coagulate at room temperature and centrifuged, and the serum separated and frozen at −20 °C until use.

Histological staining

All skin specimens were cut into 4-μm paraffin sections and stained with haematoxylin and eosin (H&E) and with AB at pH 2.5 plus PAS. To demonstrate that the stained material was HA, negative controls were digested with 40 μg mL−1 hyaluronate lyase from Streptomyces hyalurolyticus (Sigma-Aldrich, St Louis, MO, USA) in 50 mM sodium acetate, 0.15 mM NaOH, pH 6.0, at 37 °C, for 2 h. Sections were incubated in parallel with the same solution without the enzyme.

Histochemical and immunohistochemical methods

The slides were dewaxed and rehydrated through a series of xylool and graded alcohols. Endogenous peroxidase was blocked by immersing the slides in 0.75% H2O2 in methanol for 30 min. For the histochemical detection of HA, a biotinylated hyaluronan-binding protein (bHABP) derived from bovine cartilage (Seikagaku Ltd, Tokyo, Japan) was used. Negative control sections were previously treated with 40 μg mL−1 hyaluronate lyase from Streptomyces hyalurolyticus (Sigma-Aldrich, St Louis, MO, USA), as described previously by Miquel-Serra et al., to remove hyaluronan from the tissue. All sections were incubated overnight at 4 °C with bHABP (5 μg mL−1 in phosphate-buffered saline–0.1% bovine serum albumin PBS-BSA). After washing with PBS, all samples were incubated for 1 h with goat serum (20%) to block nonspecific binding sites. After washing in PBS, sections were incubated for 1 h at room temperature with avidin–biotin–peroxidase complex (Immunopure ABC peroxidase staining kit, Pierce, Rockford, IL, USA). The reaction was visualized using 0.05% 3,3′-diaminobenzidine (DAB, Sigma-Aldrich) and 0.03% hydrogen peroxide in PBS, for 10 min at room temperature. The slides were counterstained with Mayer’s haematoxylin for 30 s, washed, dehydrated and mounted.

For CD44 immunohistochemical detection, the nonspecific sites were blocked with 20% rabbit serum in TBS for 30 min, and sections were incubated with a rabbit monoclonal anti-CD44 antibody (kindly provided by S. Alldinger, Justus-Liebig-Universität, Giessen, Germany) diluted 1:100 at 4 °C overnight, as described previously by Serra et al. After washing several times, the sections were incubated with biotinylated rabbit antirat immunoglobulin (1:200) for 1 h at room temperature, followed by the avidin–biotin complex, and then by DAB visualization and AB plus PAS counterstaining.

Detection of serum HA levels

Serum HA concentration was determined using a competitive ELISA kit (Echelon Biosciences Inc., Salt Lake City, UT, USA), following the manufacturer’s instructions.

Results

Histological, histochemical and immunohistochemical results

With H&E, in sections of the biopsies of shar-pei dogs with clinical mucinosis, the collagen fibres of the dermis were separated by a pink-grey substance. The accumulation of this substance was more marked in the upper dermis and, in severe cases, led to the formation of lakes or superficial dermal vesicles (Fig. 2a). This interstitial material was intense blue in the AB/PAS stain (Fig. 2b). Only rarely did the mucin deposition reach the subcutis. In all sections previously treated with hyaluronidase, this material was absent and in its place empty spaces appeared. This mucinous, AB-positive material also bound strongly to the HABP (Fig. 2c).

Negative controls and controls treated with hyaluronidase were clearly negative in the

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The dermis of control dogs did not show any mucinous deposition.

In the skin of normal control dogs, CD44 was expressed in keratinocytes of the basal and spinous layers of the epidermis and in the outer and inner root sheath of the follicular epithelium. It was expressed more mildly in basal cells of sebaceous glands, in the acini of the sweat glands and in the ducts of both sebaceous and sweat glands. In the dermis, CD44 immunostaining was observed in fibroblasts, mast cells and macrophages (Fig. 2d). In shar-pei dogs, the expression was the same. No correlation could be established between the degree of mucin deposition in the dermis and the expression of CD44 in the different cutaneous structures.

Serum HA concentration

The results of the determination of HA using competitive ELISA can be seen in Table 1. A clear positive correlation was found between the existence of clinical mucinosis and the serum HA concentration. In control dog serum, HA ranged between 155.53 and 301.62 µg L\(^{-1}\) (mean, 234.8 µg mL\(^{-1}\); median, 244.12 µg L\(^{-1}\)). In shar-pei dogs without mucinosis, serum HA levels ranged between 106.72 and 1251.76 µg L\(^{-1}\) (mean, 532.86 µg L\(^{-1}\); median 448.68 µg mL\(^{-1}\)) and in shar-pei dogs with severe mucinosis HA serum concentrations ranged between 843.51 and 2330.03 µg L\(^{-1}\) (mean, 1500.74 µg L\(^{-1}\); median, 1500.98 µg L\(^{-1}\)).

Discussion

The present results of the histochemical and immunohistochemical staining demonstrated unequivocally that cutaneous mucinosis in the shar-pei dog is due to an accumulation of HA, mainly in the upper dermis. These findings confirm our working hypothesis. In severe cases, the deposition reaches the deep dermis and alters, at least morphologically, the skin. The severity of the deposition is highly variable among the dogs clinically affected by mucinosis, being moderate in some cases and very intense, leading to the formation of mucinous lakes, in the most severe cases.

In normal canine skin CD44 is expressed in keratinocytes of the basal and spinous layers in the epidermis, in the outer and inner root sheath of the follicular epithelium, basal cells of sebaceous glands, ductular epithelium of sebaceous glands and in dermal fibroblasts, mast cells and macrophages; this is the same pattern described in human and mouse skin. A correlation between the deposition of mucin and a reduced expression of CD44, at least with the methods used in our investigations, could not be established. This finding suggests that abnormalities in CD44 expression are not the origin of or associated with cutaneous mucinosis of shar-pei dogs and that reduced catabolism of HA due to decreased cell surface receptors seems unlikely. This finding agrees with recent publications on human cutaneous mucinosis, in which the authors could not find a decreased expression of CD44.

In the present study, serum HA concentrations in normal dogs (X = 234.8 µg L\(^{-1}\)) were in agreement with results...
published previously by Ramsden et al.,10 who found HA serum concentrations between 25 and 321 µg L⁻¹ (mean, 88 µg L⁻¹; median, 73 µg L⁻¹). Our results also show that normal dogs have serum levels of HA similar to those published for healthy humans beings, with an average of 120 µg L⁻¹, although the variation in the dog seems to be wider.

Furthermore, healthy shar-pei dogs seem to have a higher concentration of HA than dogs of other breeds, probably because of the existence of subclinical cases of mucinosis. In this study, HA concentrations in shar-pei dogs with clinically patent mucinosis were between 843.51 and 2330.03 µg L⁻¹ (mean, 1500.74 µg L⁻¹; median, 1500.98 µg L⁻¹), clearly above the normal levels for dogs, and also showing wide variability. This broad dispersion of the values is not unexpected if we consider that, from the clinical point of view, the severity of the disease is also very variable. In fact, mucinosis of the shar-pei dog is probably not a pure qualitative trait (affected/unaffected) but a quantitative trait, with many intermediate cases. Probably most, if not all, shar-pei dogs, are affected in some degree, consequently it is very difficult to draw the line between unaffected and affected animals. This would also explain, at least partially, the absence of data on the prevalence of the disease in the literature. In our study, to avoid this difficulty, we selected extreme cases, with clinically severe mucinosis or clinically normal shar-peis, but even so we detected a broad range of HA serum values. Additional, more extensive investigations are necessary to evaluate if HA can be used in the future as a quantitative, objective marker to assess the severity of the mucinosis in shar-pei dogs and also to establish breeding programmes to avoid the most severe forms of the disease.

Taken together, the results reported here suggest that mucinosis of shar-pei dogs is probably the consequence of a genetic defect in the metabolism of HA, either in the synthesis or in the catabolism. The high serum concentrations of HA could be the consequence of the drainage of HA into the dermal lymphatic vessels. A very similar syndrome has been described in a child born with extreme cutaneous thickening and folding.10 In this case, the authors detected that hyaluronan synthase activity of cultured dermal fibroblasts was increased, whereas hyaluronidase activity in plasma was normal. They concluded therefore that the disease resulted from abnormal control of hyaluronan synthesis. Further investigations of the HA metabolism in shar-pei dogs with mucinosis are needed to advance the molecular aetiopathogenesis of this disease.

References
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lié par la hyaluronan binding protein (HABP). Aucune corrélation n’était observée entre le degré de dépôt d’HA dans le derme et l’expression du CD44 dans la peau des chiens atteints ou non de mucinose cutanée. Une corrélation positive était observée entre la présence de la mucine et la concentration sérique en HA. Pour les chiens contrôles, le taux sérique d’HA variait entre 155.53 µg L⁻¹ et 301.62 µg L⁻¹, chez les Shar-peis sans mucinose entre 106.72 µg L⁻¹ et 1251.76 µg L⁻¹ et chez les Shar-peis avec mucinose entre 843.51 µg L⁻¹ et 2330.03 µg L⁻¹. Pris ensemble, ces résultats suggèrent que la mucinose du Shar-pei est probablement la conséquence d’un défaut génétique du métabolisme de l’HA.

Resumen La mucinosis cutánea afecta preferentemente a perros Shar-pei. El ácido hialurónico (HA) es considerado el componente principal de la mucina y CD44 es el principal receptor de superficie del HA, necesario para su ingestión cellular y catabolismo. Los propósitos de este estudio fueron identificar la composición de la mucina en la mucinosis cutánea de los perros Shar-pei, investigar la correlación entre la deposición de HA y la expresión de CD44, y determinar si los perros Shar-pei con mucinosis cutánea presentaban elevados niveles de HA en el suero. En biopsias de piel el material mucinoso era teñido intensamente con azul de Alcian y se unía fuertemente a la proteína de unión de hialuronan (HABP). No se observó correlación entre el grado de deposición en la dermis y la expresión de CD44 en la piel de perros Shar-pei afectados o no por mucinosis cutánea. Hubo una correlación positiva clara entre la existencia de mucinosis clínica y la concentración de HA en el suero. En perros control el HA en suero osciló entre 155.53 µg L⁻¹ y 301.62 µg L⁻¹, en perros Shar-pei sin mucinosis osciló entre 106.72 µg L⁻¹ y 1251.76 µg L⁻¹ y en perros Shar-pei con mucinosis severa osciló entre 843.51 µg L⁻¹ y 2330.03 µg L⁻¹. En conjunto estos resultados sugieren que la mucinosis de.