THESIS

CLEAVAGE OF EXOSOMAL-ASSOCIATED TRANSFERRIN RECEPTOR IN DOGS, CATS, AND HORSES: PROGRESS TOWARDS A SOLUBLE TRANSFERRIN RECEPTOR ASSAY

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ABSTRACT

CLEAVAGE OF EXOSOMAL-ASSOCIATED TRANSFERRIN RECEPTOR IN DOGS, CATS, AND HORSES: PROGRESS TOWARDS A SOLUBLE TRANSFERRIN RECEPTOR ASSAY

Iron deficiency anemia and anemia of chronic disease are two complications that patients in human medicine as well as veterinary medicine often encounter. These two diseases usually occur secondary to other primary diseases and are associated with increased morbidity and a decline in prognosis. The diagnosis and differentiation of these diseases is complicated by the fact that many of the parameters used to characterize iron deficiency are also influenced by inflammatory cytokines. Consequently, the detection of iron deficiency in the presence of inflammation or the detection of combined iron deficiency anemia and anemia of chronic disease is difficult. Differentiation of these two diseases is important as treatment for each disease is different and potentially harmful if utilized on a patient who has been misdiagnosed with one disease or the other. In human medicine, soluble transferrin receptor 1 (sTfR) has shown promise as a marker, alone or in ratio with serum ferritin, that can differentiate iron deficiency anemia, anemia of chronic disease, and combined disease.

sTfR is the product of cleavage of transferrin receptor 1 (TfR1) from the surface of exosomes which are released into circulation from maturing reticulocytes. Humans cleave the majority of their exosomal-associated TfR1 yielding substantial levels of circulating sTfR for detection and quantification by clinical assays. However, the level of cleavage in many of our veterinary species, including dogs, cats, and horses, remains

unknown. Additionally, no currently developed sTfR clinical assays have been found to successfully detect sTfR in our veterinary species.

The purpose of this study was to first confirm the presence of exosomes and exosomal-associated TfR1 in the serum of dogs, cats, and horses. Secondly, the level of cleavage of exosomal-associated TfR1 in healthy dogs, cats, and horses was explored to indirectly characterize the anticipated levels of circulating sTfR in these species. Lastly, the level of cleavage of exosomal-associated TfR1 was compared between healthy and diseased dogs and cats to investigate any potential effect of inflammation and chronic disease on the cleavage of exosomal-associated TfR1 and thus on the anticipated levels of circulating sTfR.

The results of this study demonstrated significant evidence indicating the successful isolation of exosomes and identification of exosomal-associated TfR1 from the serum of dogs, cats, and horses. The level of cleavage of exosomal-associated TfR1 in dogs was found to be greater than 50% on average with significant between-individual variation. There was also no significant difference in the means of the proportion of cleavage between healthy and diseased dogs. The level of cleavage of exosomal-associated TfR1 in cats was found to be very low at about 11% without substantial variation between individuals. However, a small but significant difference between healthy and diseased cats was detected. Healthy horses do not appear to cleave exosomal-associated TfR1. These results together would suggest that development of a clinical assay for the detection and quantification of sTfR in these veterinary species may not be successful and consequently may not be worth the time, effort, and expense.

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CHAPTER 1: Literature Review

Introduction

Iron is an essential element to normal mammalian physiology, particularly oxygen transport through the blood. When dysregulated, iron deficiency or restriction can contribute to a variety of well-characterized disease states, notably anemia. Dysregulation can be attributed to assorted pathology but cannot always be well-defined within individuals, as described in more detail below. In human medicine, soluble transferrin receptor (sTfR) holds promise as a marker that can help distinguish between different types of iron restriction.[1,2]

Iron Regulation

Iron is closely regulated in the body as unbound iron is very toxic to cells. Recent reviews of iron regulation in humans and veterinary species discuss homeostasis in greater detail.[3,4] Briefly: Iron enters the body through the diet. Daily dietary iron requirements for veterinary species are not well-established but healthy adult cats and dogs appear to maintain adequate iron levels on commercial feeds. Iron is absorbed from the diet by enterocytes as either heme iron or non-heme iron. Iron is exported out of enterocytes by the transport protein, ferroportin.

Iron released into circulation is then bound predominantly by the protein transferrin. Transferrin is called apotransferrin when it is not bound to iron. It is produced by hepatocytes and is a negative acute phase protein (its concentration in circulation decreases in the presence of inflammatory cytokines such as interleukin-6

and tumor necrosis factor). In health, approximately 0.1% of bodily iron is found in circulating blood as transferrin-bound iron.[5] Transferrin-bound iron is taken into cells when it binds transferrin receptor 1 (TfR1), a transmembrane glycoprotein on the surface of the cells. The iron-transferrin-TfR1 complex is endocytosed into the cell. Iron is released from the complex with acidification of the endosome. The transferrin-TfR1 complex is recycled to the surface of the cell and transferrin is released back into circulation as apotransferrin.

Depending on the type of cell, the iron is used for a variety of physiologic purposes. Importantly, erythroid progenitor cells utilize iron for the production of hemoglobin. In health, 60-70% of bodily iron is found in the hemoglobin of erythroid cells (mature and immature).[5] Macrophages also express TfR1 for iron uptake but additionally remove senescent or diseased erythrocytes from circulation through phagocytosis. Once phagocytosed, the erythrocyte hemoglobin is broken down and the iron can be recycled by the macrophage. Iron within macrophages, hepatocytes, and enterocytes can be stored within the cytoplasm in association with ferritin. Ferritin is an iron-storage protein produced by hepatocytes and is a positive acute phase protein (it is upregulated in the presence of inflammatory cytokines). In health, approximately 20-30% of bodily iron is found in storage forms in macrophages and hepatocytes.[5] Iron can be released into circulation from macrophages, hepatocytes, and enterocytes via ferroportin for utilization by other cells with iron requirements.

Hepcidin is a protein produced predominantly by hepatocytes and is upregulated in the presence of inflammatory cytokines but also by other physiologic and pathophysiologic processes. Hepcidin binds to, inhibits, and causes the degradation of

ferroportin. Accordingly, hepcidin prevents the transfer of iron out of cells such as macrophages, hepatocytes, and enterocytes, thus sequestering iron away from cells such as erythroid progenitor cells.[3–6] Figure 1.1 summarizes the components of iron regulation discussed above.

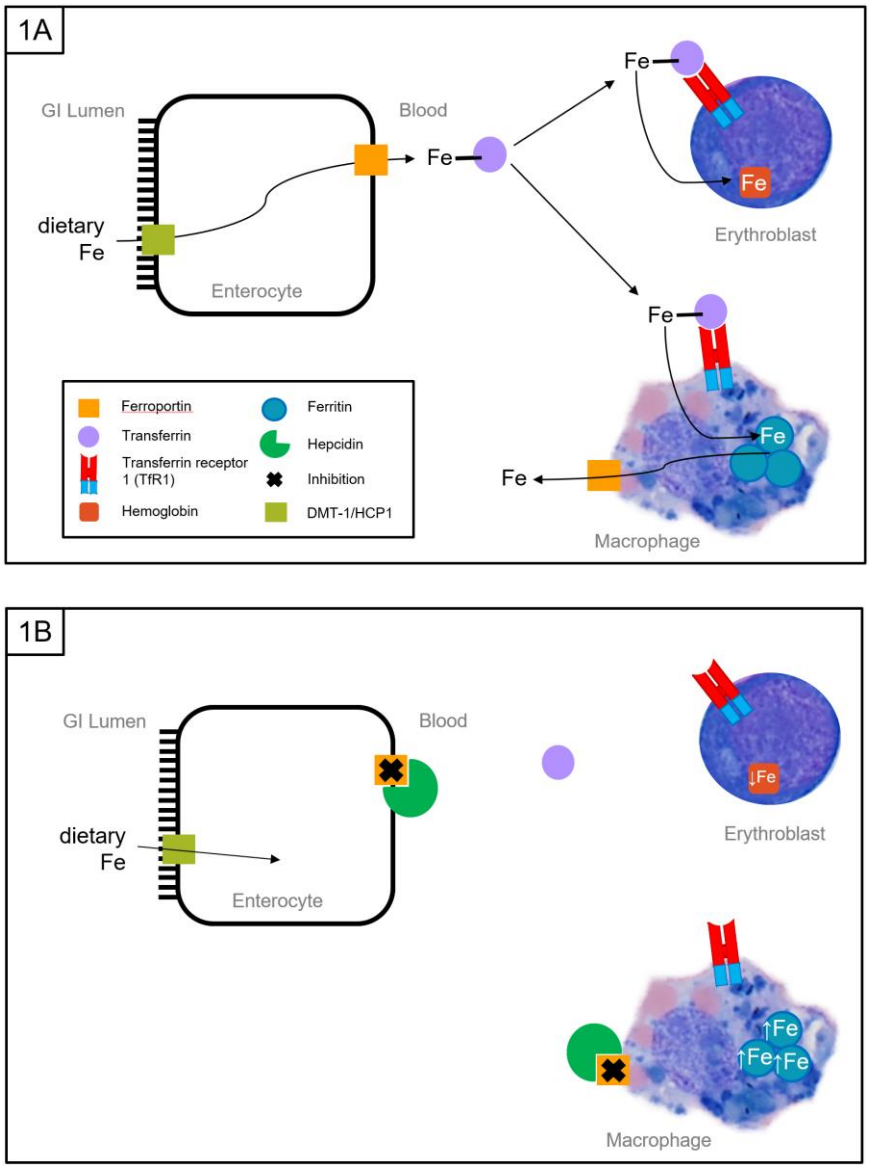

Figure 1.1: Iron regulation in the body (1A) Iron (Fe) is absorbed through the gastrointestinal (GI) tract via divalent metal transporter-1 (DMT-1) or heme carrier protein (HCP1), exported out of GI enterocytes by ferroportin, transported through blood by transferrin, and taken into cells by transferrin receptor 1. In erythroid progenitor cells iron is used for the synthesis of hemoglobin. In macrophages, iron can be stored in

association with ferritin. (1B) When upregulated, hepcidin inhibits ferroportin, sequestering iron within cells. Consequently, iron associated with ferritin will increase while hemoglobin synthesis will be restricted.

Erythropoiesis

Circulating mature red blood cells (erythrocytes) in mammalian blood are derived from bone marrow through a process called erythropoiesis. During erythropoiesis, erythroid progenitor cells mature through a predictable sequence as described in Figure 1.2. This maturation is influenced by several cytokine growth factors, most importantly, erythropoietin. Erythropoietin is primarily produced by renal peritubular interstitial cells. As erythroid progenitor cells mature they produce and accumulate hemoglobin, necessary for their chief task of oxygen transport.

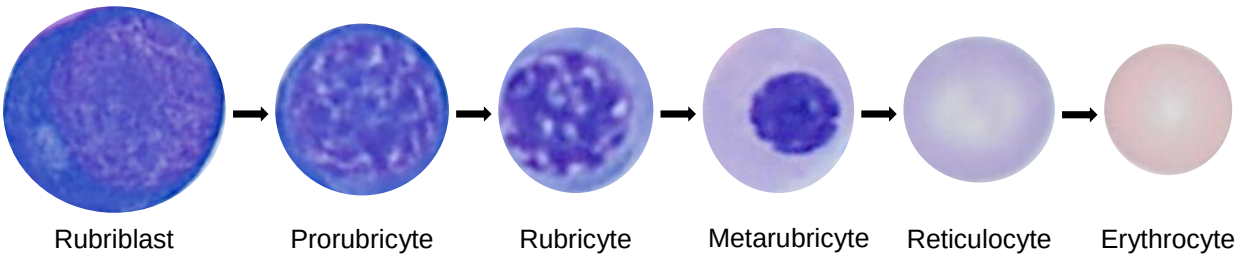

Figure 1.2: Erythroid progenitor cell maturation sequence: common myeloid progenitor → megakaryocyte-erythrocyte progenitor → burst-forming-unit erythrocyte → colony-forming-unit erythrocyte → rubriblast (earliest identifiable progenitor) → prorubricyte → rubricyte → metarubricyte → reticulocyte → erythrocyte[6,7]

Hemoglobin synthesis importantly requires iron. Consequently, erythroid progenitor cells have a higher iron demand than all other mammalian cells.[3,4,6,7] Erythroid progenitor cells obtain iron predominantly through TfR1. As erythroid progenitor cells mature and accumulate the physiologic levels of hemoglobin vital to

mature erythrocyte function, their requirements for TfR1 as well as other membrane proteins and cellular organelles become obsolete. These proteins are subsequently lost through several selective mechanisms. In particular, loss of TfR1 from the surface of reticulocytes occurs predominantly through release of exosomes possessing concentrated numbers of TfR1 on their surface.[6–14] This process was originally described in an in vitro model of sheep reticulocyte maturation.[14] Mature erythrocytes, of the mammalian species that have been investigated, cease to express TfR1 on their surface.[8]

Exosomes

The best characterized and most common pathway of exosome biogenesis begins with the endocytosis of sections of a cell's plasma membrane that possess concentrated numbers of obsolete proteins. Endocytosis yields a cytoplasmic endosome which subsequently becomes a multivesicular endosome when the endosomal membrane buds into its lumen forming small, 50-100 nm vesicles. The multivesicular endosome then fuses with the cell's plasma membrane releasing the intralumenal vesicles into the extracellular space. These vesicles are exosomes (see Figure 1.3).[8,10,15–18]

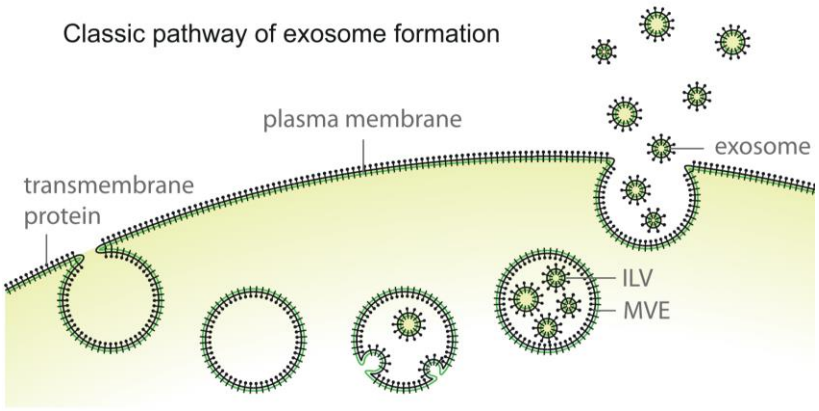

Figure 1.3: Classic pathway of exosome formation. Endocytosis of a section of plasma membrane is followed by the creation of intraluminal vesicles (ILVs) within a multivesicular endosome (MVE). Next, the MVE fuses with the plasma membrane, allowing the expulsion of ILVs into the extracellular space as exosomes.[15]

Exosomes are produced by reticulocytes as well as many other cell types; some examples include enterocytes, kidney tubule epithelium, epididymis epithelium, dendritic cells, and cancer cells.[19–24] Moreover, individual cells may release exosomes with varying protein profiles.[25] Regardless of origin, exosomes usually express proteins such as HSP 70, TSG101, CD63, and other tetraspanins, which are commonly used as markers to identify exosomes.[8,15–18] Pertaining to veterinary species, exosomes have been successfully isolated from the blood of sheep, pigs, rats, rabbits, chickens, and horses.[12,13,26]

The role of exosomes in physiologic homeostasis and the development, prevention, and progression of disease is being extensively studied and full review of the literature pertaining to this topic is beyond the scope of this paper. Several recent reviews of exosomes contain additional information into the variety of cells that produce exosomes and some of their suspected physiologic and pathophysiologic roles.[8,15–18]

Transferrin Receptor

To better understand the potential utility of sTfR it is important to first discuss TfR1, from which it is derived. As previously discussed, TfR1 is a transmembrane glycoprotein that functions in the cellular procurement of iron through the endocytosis of the iron-transferrin-TfR1 complex.[3–6] TfR1 is present on all cells to varying degrees with erythroid progenitor cells among those exhibiting the highest levels of TfR1 expression.[4] As previously mentioned, TfR1 is selectively shed from the surface of maturing reticulocytes via exosome formation and release.[6–14]

In veterinary medicine, the utility of TfR1 has been evaluated as a marker in canine brain tumors and canine lymphoma.[27,28] Additionally, previous research performed in this laboratory has found that increased levels of serum exosomal-associated TfR1 may serve as an indicator of a regenerative effort in equine anemia.[26]

In humans, the exosomal-associated TfR1 appears to be largely cleaved from the surface of exosomes to form a soluble extracellular domain of TfR1 free in the serum, referred to as sTfR. This is corroborated by the finding that greater than 80% of the TfR1 detected in human serum is of a molecular weight consistent with sTfR (approximately 80-85 kDa).[29] Conversely, an in vitro model of sheep reticulocyte maturation demonstrated that only approximately 25% of the exosomal-associated TfR1 is cleaved yielding a smaller proportion of sTfR relative to that observed in human serum.[11] Circulating granulocytes are suspected to be responsible for this cleavage as demonstrated in an in vitro model. In this model, human granulocytes exhibited greater proteolytic activity than the other species under investigation.[30] The truncated cytoplasmic domain of TfR1 remains associated with the exosome following cleavage

and will henceforth be referred to as cTfR (see Figure 1.4). Cleavage appears to occur exclusively on the surface of exosomes as the truncated cytoplasmic domain has not been detected in association with reticulocyte or erythrocyte membranes.[11,30] In fact, TfR1 is detected predominantly as a whole monomer or dimer on the plasma membranes of reticulocytes.[30]

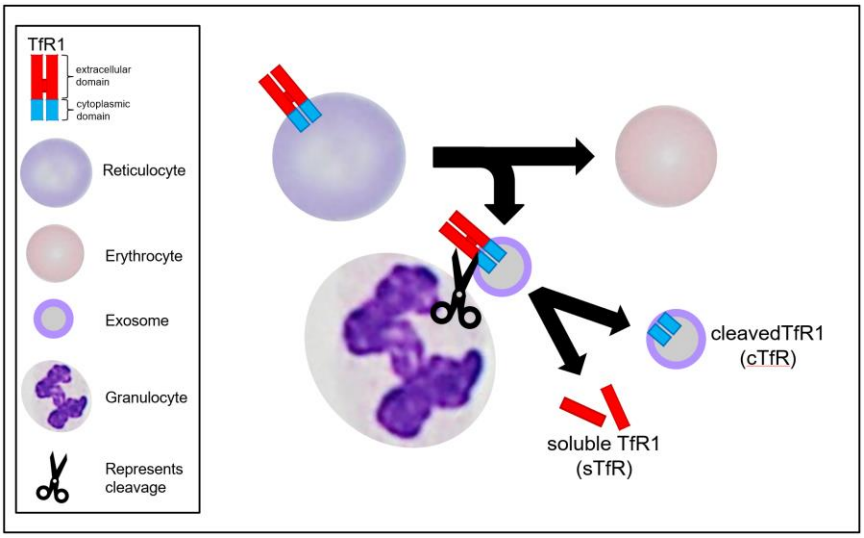

Figure 1.4: Cleavage of TfR1 from the surface of exosomes. As reticulocytes mature into erythrocytes they shed TfR1 in exosomes. Granulocytes have been demonstrated to cleave TfR1 from the surface of exosomes into a sTfR form and an exosomal-associated cTfR form.

Iron Deficiency Anemia

The importance of the potential that sTfR holds as a diagnostic marker is highlighted by the complexity of the diseases it may help to characterize. Iron deficiency anemia is one of these diseases and occurs in response to a total body deficiency of iron. When the body lacks adequate iron, iron stores are depleted and erythropoiesis continues but is iron-restricted. Consequently, the level of erythropoiesis is insufficient to maintain physiologic erythrocyte concentrations leading to anemia.

Iron deficiency can occur due to chronic loss of blood outside of the body, malabsorption of iron, or intake of an iron deficient diet. Iron deficient diets leading to iron deficiency in our veterinary patients are very rare and most often occur in neonates.[4] Foals, in particular, can be pre-disposed to iron deficiency anemia due to decreased dietary iron. This is especially true when the dam has low milk iron content or the foal is not provided iron-sufficient forage or pasture access.[31,32] Iron deficiency secondary to wide-spread gastrointestinal disease, such as inflammatory bowel disease, has been reported and may be due to a combination of gastrointestinal blood loss and malabsorption.[33] Ongoing external loss of blood is the most common cause of iron deficiency in our veterinary species and may commonly be due to gastrointestinal or ectoparasites, bleeding gastrointestinal or urinary tract neoplasms, ulceration, or other disease, and inherited or acquired coagulation disorders.[5] Interestingly, when iron deficiency is present, the intestine increases iron absorption and transfer into the blood in an attempt to compensate.[34]

Initially, iron deficiency anemia can be regenerative as erythropoiesis and hemoglobin production preferentially utilize the iron that is still available. However, it often is non-regenerative or becomes non-regenerative with progression of iron deficiency.[4] Iron-restricted erythropoiesis leads to the formation of progressively microcytic (decreased mean cell volume [MCV]) and hypochromic (decreased mean cell hemoglobin concentration [MCHC]) erythrocytes as cellular production attempts to maintain a physiologically appropriate concentration of hemoglobin within individual erythrocytes. Decreased MCV and MCHC may not be detected by automated

hematologic instruments until late in the course of disease, largely due to the extended life span of erythrocytes in circulation (see table 1.1).[5,6,35]

Treatment of iron deficiency anemia is aimed at iron supplementation and correcting any underlying cause of blood loss or malabsorption.[4,5]

Table 1.1: Erythrocyte life spans in circulation for selected mammals.[35]

Species	Erythrocyte life span (days)
Dog	100
Cat	72
Horse	143
Human	120

Anemia of Chronic Disease

Anemia of chronic disease is often difficult to differentiate from iron deficiency anemia and can additionally cause disease in combination with iron deficiency anemia. When independent of true iron deficiency, the anemia associated with chronic disease is typically mild to moderate, non-regenerative, and not usually microcytic or hypochromic. However, cases of microcytosis and hypochromasia associated with anemia of chronic disease in canine and feline patients have been reported.[36–39]

In veterinary species, anemia of chronic disease is observed secondary to a wide range of diseases including neoplasia, infection, autoimmunity, and others and is classically associated with a poorer prognosis.[4,39–41] The underlying pathogenesis regarding the development of anemia secondary to such a wide range of diseases is complex and multifactorial. It involves the sequestration of iron away from erythroid progenitors, thus hampering erythropoiesis and causing a functional iron deficiency. This iron-restricted erythropoiesis contributes to the development of anemia. From an

evolutionary stand point, this iron sequestration is suspected to represent a shield against infectious microbes that also have physiologic iron requirements. This occurs under the influence of inflammatory cytokines such as interleukin-1, interleukin-6, interferon-gamma, and tumor necrosis factor which, among other activities, cause the upregulation of hepcidin and ferritin and the downregulation of transferrin. Recall, ferritin stores iron within cells and hepcidin inhibits ferroportin, blocking the export of iron out of cells such as enterocytes, macrophages, and hepatocytes for utilization by other cells such as erythroid progenitor cells.[3–6,39,40] Other factors appearing to contribute to the development of anemia of chronic disease include a diminished erythrocyte life span, modifications of erythroid progenitor cell proliferation and maturation, and variable erythropoietin levels.[39,40]

Therapeutic intervention for anemia of chronic disease is important as anemia is usually associated with a poorer prognosis in a wide variety of diseases. Intervention is typically aimed at resolution of the primary disease process. Iron supplementation is typically contraindicated. Therapy can also involve erythropoietin administration and blood transfusion.[39,40]

Anemia associated with chronic kidney disease or congenital or acquired portosystemic shunt can also appear similar to iron deficiency anemia and anemia of chronic disease.[38,42–45] Chronic kidney disease can result in decreased functional renal parenchyma and a consequent diminishment of erythropoietin production as well as toxic inhibition of proliferation of erythroid progenitor cells by uremic toxins. This pathology results in a non-regenerative anemia that is typically normocytic and normochromic.[42,44,45] Portosystemic shunt is often associated with a microcytic,

hypochromic, non-regenerative anemia that is suspected to be due to dysregulation of iron homeostasis secondary to impaired liver function.[38,43] Although not typically anemic or hypochromic, breed associated microcytosis in the Akita, Chow Chow, Shar Pei, and Shiba Inu canine breeds is not thought to be pathologic but should be kept in mind when evaluating patients for underlying causes of microcytosis.[38,46–49] Lastly, there is evidence supporting the development of iron-restricted erythropoiesis associated with aging in dogs which is suspected to occur secondary to both chronic disease and iron deficiency. However, there is an ongoing struggle to disambiguate the underlying causes of this iron-restricted erythropoiesis, as demonstrated by the following discussion.[50]

Diagnostic Approaches to Body Iron Status

The diagnosis and differentiation of iron deficiency anemia, anemia of chronic disease, or combined disease is not straightforward in our veterinary species. The diagnostic tests available to veterinarians are often influenced by other physiologic and pathophysiologic processes. The common tests and expected alterations associated with these different diseases are described below.

Evidence of anemia is usually first detected on a complete blood count (CBC). Erythrocyte and reticulocyte parameters are variably deranged with both iron deficiency anemia and anemia of chronic disease. Changes in the erythrocyte indices often occur later in the disease process due to the long life span of erythrocytes and preferential utilization of iron for erythropoiesis. Consequently, erythrocyte parameters are not always useful indications of disease.[4,38,51] Alterations in reticulocyte indices can be detected earlier in the disease course because reticulocytes mature within 1 to 2 days in

circulation.[52] Accordingly, alterations in reticulocyte parameters may be better early indications of disease than erythrocyte parameters.[38,51,53,54] In addition to the decreased hematocrit and hemoglobin characteristic of anemia, iron deficiency anemia may exhibit increased reticulocytes (evidence of regenerative response), decreased MCV, decreased MCHC, increased red cell distribution width, increased percentage of hypochromic erythrocytes, decreased reticulocyte hemoglobin content, decreased reticulocyte mean cell volume, increased percent hypochromic reticulocytes, increased percent microcytic reticulocytes, decreased percent macrocytic reticulocytes, increased percent reticulocytes with low hemoglobin content, and decreased percent reticulocytes with high hemoglobin content.[4,38,51,53–55] Erythrocyte morphology can also be deranged with iron deficiency, with poikilocytes indicative of oxidative damage visualized on blood smears. These include keratocytes, acanthocytes, and schistocytes among others. However, other disease processes and causes of oxidative injury can also present with similar poikilocytosis so these changes are not specific for iron deficiency.[31,49] Anemia of chronic disease will classically present with a decreased hematocrit and hemoglobin, low to normal reticulocyte count, normal MCV, and normal MCHC without significant alterations in reticulocyte parameters.[39,40] However, most of the alterations in erythrocyte and reticulocyte parameters observed with iron deficiency can frequently be associated with anemia of chronic disease as well.[38,56] In dogs, average cell hemoglobin concentration of reticulocytes, reticulocyte hemoglobin content, percent hypochromic reticulocytes, and percent reticulocytes with low hemoglobin content show utility for the differentiation of iron deficiency anemia from anemia of chronic disease, portosystemic shunt, and breed associated microcytosis when specific cutoff values are used.

However, this study evaluated dogs with significant iron deficiency and consequently suggested that the overlap observed in the reticulocyte parameters between iron deficiency anemia and anemia of chronic disease may hinder the detection of early iron deficiency using these parameters.[38]

Serum iron is often available on veterinary biochemical panels. It can be decreased in either disease but is also influenced by a wide variety of physiologic and pathophysiologic mechanisms including simple diurnal variation.[4–6,40,49,57,58] However, a decrease in serum iron in addition to CBC results that may suggest iron deficiency anemia or anemia of chronic disease may prompt ancillary diagnostics to better characterize a patient's body iron status.

Bone marrow iron stores are classically considered the gold standard for evaluation of body iron status in dogs. However, stainable bone marrow iron is not usually apparent even in healthy cats and consequently holds no utility in feline patients. Visual assessment of bone marrow iron levels is decidedly subjective and is of low sensitivity, even with stains such as Prussian blue to highlight the iron. Additionally, bone marrow collection is a far more invasive procedure than venipuncture, consequently, serum tests would be preferred if available. Classically, bone marrow iron stores are expected to be decreased to absent with iron deficiency and normal to increased with anemia of chronic disease, although other pathologic and physiologic causes can also alter levels.[4,5,49,53,59–61]

Transferrin is not routinely measured but, as a negative acute phase protein, is expected to decrease with inflammation. Typically, total iron binding capacity (TIBC) is measured as an indirect reflection of transferrin levels. TIBC is the sum of serum iron

and the unbound serum capacity for iron-binding. Classically, TIBC is expected to be low normal to decreased with inflammation and thus anemia of chronic disease. With iron deficiency, TIBC is characteristically normal to elevated.[4,5,39,40,49,53] A ratio can be calculated from the serum iron level to TIBC to give the percent transferrin saturation. The percent transferrin saturation is expected to decrease with iron deficiency and anemia of chronic disease but is also subject to high variability due to the influences on serum iron.[4,40,51,53]

Serum ferritin levels are reflective of body iron stores in healthy individuals. Consequently, serum ferritin levels are expected to be decreased with iron deficiency. Conversely, ferritin levels are expected to increase with a variety of disease processes including inflammation (and thus anemia of chronic disease) as ferritin is a positive acute phase protein.[4,5,39,40,49,53,62–64]

Hepcidin levels are not commonly measured to evaluate body iron status. However, hepcidin is expected to increase with anemia of chronic disease secondary to inflammatory cytokines and decrease with iron deficiency secondary to the anemic state.[3–6]

TfR1 expression on cells is also not commonly measured to evaluate body iron status. However, TfR1 expression is expected to be upregulated with iron deficiency and possibly normal to downregulated in the presence of inflammatory cytokines (and thus anemia of chronic disease).[5,65,66] Table 1.2 demonstrates the expected alterations in the parameters discussed with iron deficiency anemia and anemia of chronic disease.

Table 1.2: Classical changes expected in several iron-related parameters with iron deficiency anemia and anemia of chronic disease. ↓ = decreased; ↑ = increased; N = within normal limits

	Serum Iron	TIBC (transferrin)	% transferrin saturation	Ferritin	Hepcidin	TfR1	Bone Marrow Iron
Iron Deficiency Anemia	↓	N to ↑	↓	↓	↓	↑	↓
Anemia of Chronic Disease	↓	N to ↓	↓	N to ↑	↑	N to ↓	↑

The problem with these diagnostic tests arises when there is evidence of chronic disease (chronic inflammation, infection, neoplasia) and a concurrent anemia with characteristics that could be consistent with iron deficiency or anemia of chronic disease. As discussed, in the presence of inflammation, many of these diagnostic parameters will be altered regardless of the true underlying body iron status. Consequently, the anemia may be due to true iron deficiency or anemia of chronic disease or a combination of both diseases. Treatment of the anemia requires an accurate understanding of the underlying disease pathogenesis. As such, there is a need for a clinical assay which can more clearly delineate iron deficiency anemia, anemia of chronic disease, and combined disease.

Serum sTfR

Serum sTfR has been utilized to measure the intensity of erythropoiesis in humans and rats as it directly reflects the magnitude of erythroid mass.[30,67,68] Profound increases in sTfR have been documented with both iron deficiency and hemolytic anemia.[29,30,68] Accordingly, sTfR also appears to decrease with iron overload.[69] In

humans, sTfR is not significantly impacted by inflammation and consequently allows for the detection of underlying iron deficiency even when an inflammatory disease is present. The utility of sTfR was found to be greater than measurement of ferritin alone. However, sensitivity and specificity were increased by pairing it with logarithmically transformed ferritin measurement.[1,58] Additionally, increases in sTfR were found to indicate iron deficiency during early or mild stages of disease before other parameters were altered.[2] Table 1.3 demonstrates the changes in sTfR and ferritin expected with the diseases discussed.

Table 1.3: Expected changes in serum sTfR and ferritin concentrations with iron deficiency anemia, anemia of chronic disease, and combined disease.

	sTfR	**Ferritin**
Iron Deficiency Anemia	↑	↓
Anemia of Chronic Disease	≈	N to ↑
Combined Disease	↑	↓ to ↑

A clinical assay for the detection and quantification of sTfR in veterinary species would be a desirable and valuable tool for the detection of iron deficiency in the presence of inflammation or anemia of chronic disease in veterinary patients. Available sTfR assays and anti-sTfR antibodies have been previously evaluated and failed to detect sTfR in the veterinary species tested. Additionally, the degree of cleavage, and thus the level of sTfR relative to whole TfR1 in these species is currently unknown. This study aimed to characterize the degree of TfR1 cleavage as well as the influence of inflammation/disease on the level of cleavage in dogs, cats, and horses with the overarching goal of development of a clinical sTfR assay.

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CHAPTER 2: Isolation and Verification of the Presence of Exosomes in Canine, Feline, and Equine Serum

Introduction

Exosome isolation and characterization has not been extensively studied in most veterinary species. To characterize the degree of exosomal-associated TfR1 cleavage in dogs, cats, and horses, exosomes first had to be isolated and their presence verified in canine, feline, and equine serum. A common technique for the isolation of exosomes from serum, plasma, or cell culture is ultracentrifugation. This technique has been used to successfully isolate exosomes from the blood of humans, horses, sheep, rabbits, rats, pigs, and chickens.[1–3] The ultraprecipitate obtained from this technique should have concentrated, cell-free exosomes that can be positively identified through different procedures.

One such procedure is the examination of the ultraprecipitate using transmission electron microscopy (TEM) for the presence of extracellular vesicles consistent with exosomes. Exosomes have previously been described by TEM as 50 to 100 nm membrane-bound vesicles.[1,3,4]

Exosomes can also be demonstrated through western blot for the detection of common exosome markers such as TfR1, HSP 70, TSG101, CD63, and other tetraspanins.[5–9] Using these markers, another procedure used to confirm the presence of exosomes in a sample is discontinuous density gradient fractionation. The goal of this procedure is to detect the presence of exosomes, by common exosome markers, at

densities appropriate for exosomes. Different types of gradients are available. OptiPrep discontinuous density gradients have been documented to yield serum derived exosomes in gradient fractions of densities 1.09 to 1.11 g/ml[10] and 1.06 to 1.24 g/ml.[11]

As previously discussed, during maturation reticulocytes shed TfR1 from their surface through the release of exosomes. Consequently, reticulocytes are expected to express TfR1 while erythrocytes should lose expression of TfR1. Additionally, as cleavage of TfR1 is suspected to occur only on the surface of exosomes, reticulocytes and erythrocytes should not express the cleaved cytoplasmic domain of TfR1.[2–5,12–17] Demonstration of these phenomena on canine and feline reticulocytes and erythrocytes would support the existence of exosomes with associated TfR1 in the serum of these species.

The focus of this study was to verify the successful isolation of exosomes from the serum of dogs, cats, and horses. We aim to demonstrate the presence of exosomes in canine, feline, and equine serum isolated via a common technique and verified through a variety of previously utilized methods. Importantly, a previous study performed by this laboratory has utilized TEM and OptiPrep discontinuous density gradient to verify the presence of exosomes in ultraprecipitates obtained from equine serum.[1] Consequently these procedures were not repeated on equine samples for this study.

Material and Methods

Exosome Isolation Using Ultracentrifugation

Serum samples were collected from left-over canine, feline, and equine patient samples at the Colorado State University Veterinary Teaching Hospital Clinical Pathology Laboratory and processed as previously described.[1] Briefly, samples were thawed and lipids and cellular debris were removed by centrifugation at 8000 × g for 20 minutes at 4°C. Phosphate buffered saline (PBS; pH 7.4) was used to dilute the serum supernatant prior to ultracentrifugation at 100,000 × g for 1.5 hours at 4°C (Rotor TH-641 [Thermo Scientific, Waltham, Massachusetts, USA]). Most of the supernatant was removed. Approximately 1 ml of remaining supernatant was used to resuspend the ultraprecipitate obtained. The resuspended pellet was centrifuged at 13,000 × g for 20 minutes at 4°C. The supernatant was removed and the ultraprecipitate was resuspended in 5 µl of 100:1 PBS:protease inhibitor cocktail (Halt Protease Inhibitor Cocktail [Thermo Scientific, Waltham, Massachusetts, USA]) pH 7.4 and stored at -80°C until use in the procedures described below.

Transmission Electron Microscopy

Ultraprecipitates from pooled canine and feline serum were obtained following the above procedure and prepared for electron microscopy as previously described.[1] Briefly, the ultraprecipitate pellet was resuspended in 2.5% glutaraldehyde in PBS and prepared for electron microscopy by washing 3 times (10 min each) with 0.1 mol/l sodium phosphate buffer, pH 7.3 and post-fixing for one hour with 1% osmium tetroxide in 0.1 mol/l sodium phosphate buffer. The samples were washed with buffer and

embedded in 1.5% agarose. The agarose-embedded ultraprecipitate was dehydrated in a graded ethanol series, 15 min each in 50%, 70%, 80%, 90% and 2 × 100%. After the second 100% step, the pellet was incubated for 15 min in 1:1 ethanol:propylene oxide and twice for 15 min each in 100% propylene oxide. The sample was infiltrated with Epon resin over several days, and the resin was then polymerised for 24 h at 65°C. Ultrathin sections (60–90 nm) were cut from the resin-embedded samples using a Diatome diamond knife and a Reichert Ultra-cut E ultramicrotome, mounted on grids and post stained with uranyl acetate and lead citrate. The sections were examined and photographed using a JEOL JEM-1400 electron microscope operated at 100 kV.

Western Blot for CD63

Ultraprecipitates from pooled canine, feline, and equine serum were obtained following the above procedure. The ultraprecipitate was separated on a readymade 10% Bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, California, USA) using MOPS buffer (Invitrogen, Carlsbad, California, USA) under reducing conditions. The iBlot Dry Blotting System (Invitrogen, Carlsbad, California, USA) was used to transfer the gel to a polyvinylidene difluoride membrane (Invitrogen, Carlsbad, California, USA). Platelets isolated from the whole blood of each respective species served as positive and negative (secondary antibody only) controls. Membranes were blocked with 5% non-fat dry milk in 1× Tris-buffered saline with Tween for one hour, incubated with monoclonal goat anti-CD63 IgG antibody (antibodies-online.com, Atlanta, Georgia, USA) overnight, followed by rabbit HRP-labelled anti-goat IgG antibody (Santa Cruz Biotechnology, Dallas, Texas, USA) for one hour. Peroxidase activity was detected with Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, United

Kingdom) and the bands were imaged on a ChemiDoc-It^{TS2} Imager (UVP, Upland, California, USA).

OptiPrep Discontinuous Density Gradient Fractionation

Ultraprecipitates from pooled canine and feline serum were obtained following the above procedure. The ultraprecipitates were ultracentrifuged on a discontinuous density gradient as previously described.[1] Briefly, ultraprecipitates were resuspended in 10 µl of 20 mmol/l HEPES buffer (Invitrogen, Carlsbad, California, USA) and placed at the top of a previously prepared, discontinuous 0–60% iodixanol (step-wise, 10%; OptiPrep [Sigma-Aldrich, St Louis, Missouri, USA]) gradient in 20 mmol/l HEPES buffer. The preparations were then ultracentrifuged at 100,000 × g for 24 hours. 15 and 16 fractions were collected from the canine and feline preparations respectively. The density of each fraction was determined. Proteins were isolated from each fraction by trichloroacetic acid (100%) (Fisher Scientific, Waltham, Massachusetts, USA) precipitation. Protein pellets were resuspended in LDS sample buffer (Invitrogen, Carlsbad, California, USA) and probed for the presence of TfR1 by western blot.

Preparation of Reticulocytes and Erythrocytes

Four whole blood samples were collected from left-over patient samples at the Colorado State University Veterinary Teaching Hospital Clinical Pathology Laboratory. These included one each of canine and feline high and low reticulocyte count samples. The whole blood was centrifuged at 13,000 x g for 10 minutes and then the plasma and buffy coats were removed. The packed red blood cells were washed twice in PBS. 0.5 µl of packed red blood cells were resuspended in 10 µl LDS sample buffer and probed

for the presence of TfR1 by western blot. A single canine serum ultraprecipitate sample was also probed to serve as a positive control for the detection of cTfR.

Western Blot for TfR1

Ultraprecipitates from canine and feline serum were obtained following the above procedure. Western blot for TfR1 was performed as previously described.[1] Briefly, the ultraprecipitates were separated on a readymade 10% Bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, California, USA) using MOPS buffer (Invitrogen, Carlsbad, California, USA) under reducing conditions. The iBlot Dry Blotting System (Invitrogen, Carlsbad, California, USA) was used to transfer the gel to a polyvinylidene difluoride membrane (Invitrogen, Carlsbad, California, USA). A canine bone marrow cell lysate was used as a positive control. Pooled left-over sample was used as a negative control with an irrelevant antibody serving as a negative control primary antibody. Membranes were blocked with 5% non-fat dry milk in 1× Tris-buffered saline with Tween for one hour, incubated with monoclonal mouse anti-human TfR1 antibody (Invitrogen, Carlsbad, California, USA) followed by biotin-labelled anti-mouse IgG1 antibody (BD Biosciences, San Jose, California, USA) followed by Streptavidin-HRP conjugated (1:300) (R&D Systems, Minneapolis, Minnesota, USA) for 15 minutes. Peroxidase activity was detected with Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, United Kingdom) and the bands were imaged on a ChemiDoc-It^{TS2} Imager (UVP, Upland, California, USA).

Results

Transmission Electron Microscopy

Membrane bound vesicles of appropriate morphology and size to be consistent with exosomes were detected in the canine and feline ultraprecipitates when examined using TEM (Figure 2.1). These vesicles varied in size from approximately 50 to 100 nm consistent with previous reports of TEM imaged exosomes isolated from horses, sheep, rabbits, rats, and pigs. [1,3,4]

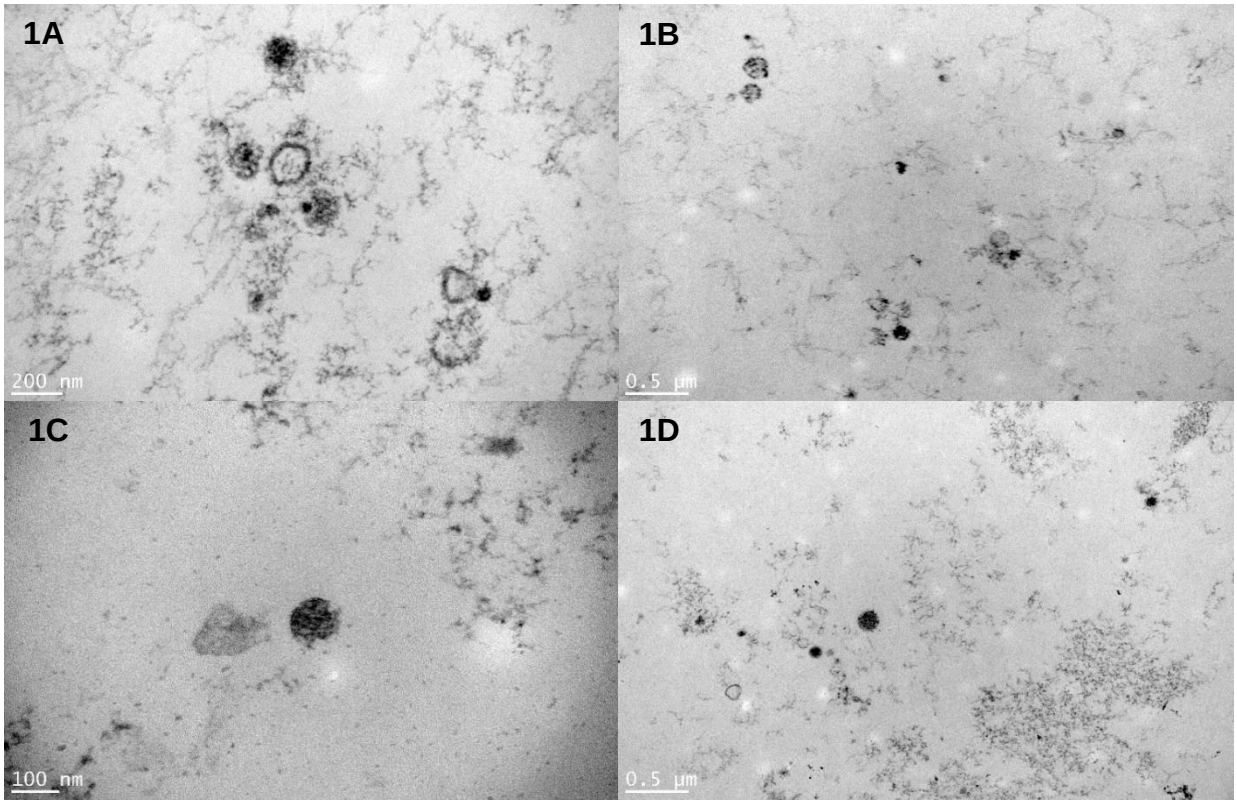

Figure 2.1: TEM images of canine and feline ultraprecipitates. Membrane bound vesicles approximately 50 to 100 nm in diameter are observed, consistent with exosomes. (1A-B) Canine ultraprecipitate. (1C-D) Feline ultraprecipitate.

Western Blot for CD63

CD63 is commonly used as a marker to identify exosomes and can be variably glycosylated with a molecular weight ranging between 40-60 kDa.[5–9,18] Western blot for CD63 was performed on canine, feline, and equine platelets and ultraprecipitates. A band of approximately 40 kDa, consistent with CD63, was detected in all platelet samples and convincingly in the feline and equine ultraprecipitates (Figure 2.2). A faint and broad band was detected in the canine ultraprecipitate and is not considered definitively positive for CD63 (Figure 2.2A and 2.2C).

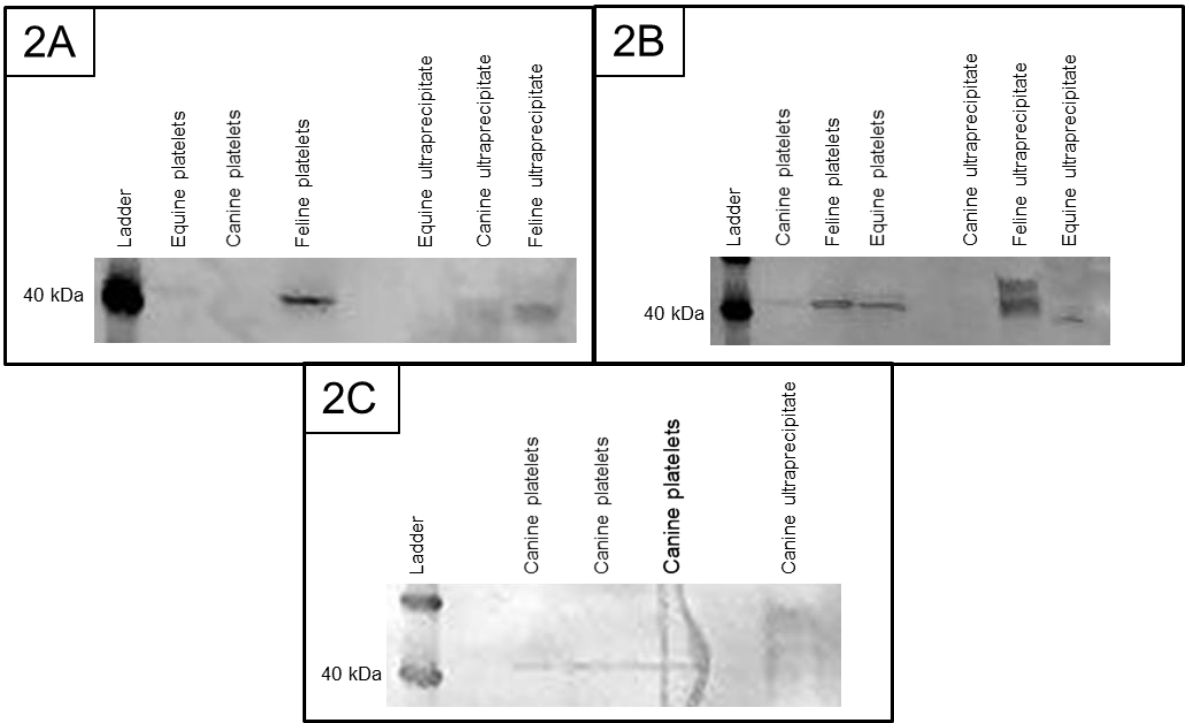

Figure 2.2: Western blot for CD63 on canine, feline, and equine ultraprecipitates (2A) Bands of appropriate molecular weight (40 kDa) are detected in the equine and feline platelet samples and possibly the canine and feline ultraprecipitate samples. (2B) Bands of appropriate molecular weight (40 kDa) are detected in the platelet samples of all species and convincingly in the feline and equine ultraprecipitate samples. (2C) Bands of appropriate molecular weight (40 kDa) are detected in the canine platelet samples and a broad smeared band is detected in the canine ultraprecipitate sample and is of unknown significance.

OptiPrep Discontinuous Density Gradient Fractionation

Western blot for TfR1 performed on fractions obtained from OptiPrep discontinuous density gradient fractionation of canine and feline ultraprecipitates revealed TfR1 in fractions of densities ranging from 1.07-1.12 g/ml for dogs and 1.09-1.10 g/ml for cats (Figure 2.3A and 2.3B, respectively). Previous studies have found exosomes at similar densities.[10,11] The association of TfR1 with exosomes in canine and feline serum is supported by this finding.

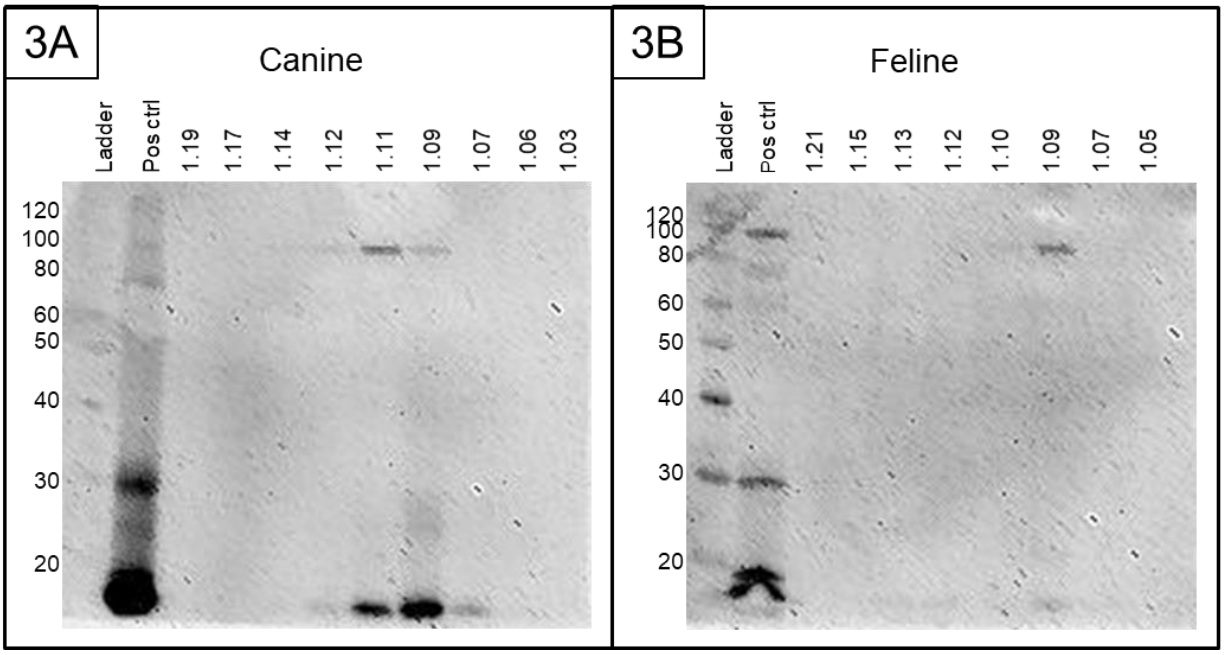

Figure 2.3: OptiPrep discontinuous density gradient fractionation of canine and feline ultraprecipitates (3A) Canine ultraprecipitates: both whole TfR1 (95 kDa) and cTfR (17 kDa) are observed in fractions of density 1.07-1.12 g/ml. (3B) Feline ultraprecipitates: both whole TfR1 (95 kDa) and cTfR (17 kDa) are observed in fractions of density 1.09-1.10 g/ml.[14,19]

Reticulocyte and Erythrocyte Western Blot for TfR1

Western blot for TfR1 was performed on high and low reticulocyte packed red blood cell samples from dogs and cats. Whole TfR1 was detected only in the samples with high reticulocytes. The low reticulocyte samples, consisting predominantly of mature erythrocytes, lacked whole TfR1 and both high and low reticulocyte samples lacked cTfR (Figure 2.4).

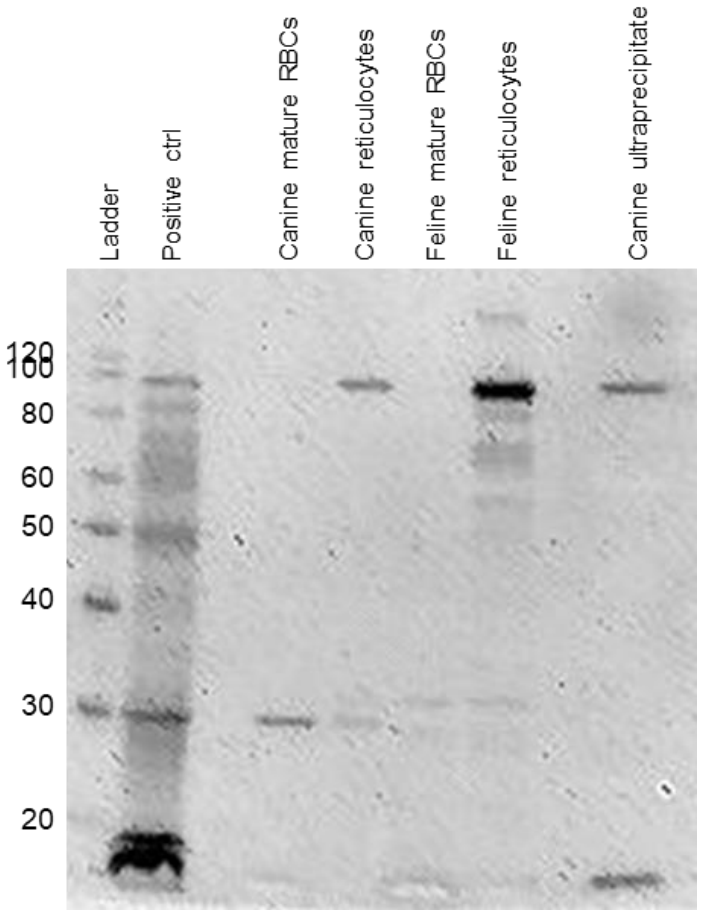

Figure 2.4: Western blot for TfR1 on canine and feline erythrocytes and reticulocytes. Whole monomer TfR1 (95 kDa) is detected in both the canine and feline high reticulocyte samples. Dimer TfR1 (190 kDa) is additionally observed in the feline high reticulocyte sample. Neither monomer nor dimer TfR1 is detected in the canine and feline mature erythrocyte samples. cTfR (17 kDa) is not detected in any erythrocyte or reticulocyte samples but is observed in the canine ultraprecipitate sample.[14,19]

Discussion

Ultraprecipitates obtained from the serum of dogs, cats, and horses convincingly contain exosomes as demonstrated by these experiments. Confirmation of the presence of exosomes in the ultraprecipitates obtained from serum samples was essential prior to pursuing detection and quantification of the proportion of cTfR in these samples.

TEM demonstrated the presence of vesicles morphologically consistent with exosomes within the ultraprecipitates of dogs and cats. Previous work in this laboratory demonstrated the presence of vesicles morphologically consistent with exosomes within the ultraprecipitates of horses.[1] CD63, a common marker of exosomes,[5–9] was identified in the ultraprecipitates of cats and horses. The detection of this marker reinforces the presence of exosomes within the ultraprecipitates. The anti-TfR1 antibody utilized in the western blot protocol is directed against the cytoplasmic domain of TfR1. Consequently, the detection of whole TfR1 (approximately 95 kDa) within the ultraprecipitates corroborates the association of TfR1 with a membrane. Moreover, the identification of whole TfR1 and cTfR in discontinuous density gradient fractions of densities reported to contain exosomes insinuates that the membrane-bound TfR1 is associated with exosomes. Previous work in this laboratory detected TfR1 at densities appropriate for exosomes in the ultraprecipitates of horses on OptiPrep discontinuous density gradient fractionation.[1] The presence of whole TfR1 on reticulocytes and absence of whole TfR1 on mature erythrocytes supports the hypothesis that dogs and cats shed TfR1 from reticulocytes as they mature into erythrocytes, as previously reported in other species. It also supports that cleavage of TfR1 does not occur on the reticulocyte membranes but rather on the surface of exosomes, as previously reported.[2–5,12–17]

In conclusion, our protocol for ultracentrifugation of canine, feline, and equine serum appears to successfully isolate exosomes. We demonstrated findings consistent with the presence of exosomes and association of TfR1 with exosomes using several well-described and relevant techniques.

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CHAPTER 3: Determination of the Proportion of Exosomal-Associated Cleaved TfR1 in Healthy Dogs, Cats, and Horses

Introduction

Recall that TfR1 is shed from maturing reticulocytes through the release of exosomes.[1–9] On the surface of those exosomes, cleavage of TfR1 has been demonstrated, yielding a circulating sTfR and exosomal-associated cTfR. It appears that the vast majority of exosomal-associated TfR1 is cleaved in the human, but cleavage appears to occur to a lesser extent in sheep.[5,10,11] Measurement of sTfR in humans has shown promise at better defining iron deficiency anemia, anemia of chronic disease, and the overlap of these diseases when they occur concurrently in individuals.[10–16]

The premise of sTfR clinical assays in human medicine is the reliable cleavage of exosomal-associated TfR1 to sTfR for detection. In our veterinary species, the development of a clinical assay for the detection of sTfR faces several challenges. Firstly, it is unknown whether dogs, cats, or horses cleave TfR from the surface of exosomes to yield circulating sTfR. Additionally, supposing these species do cleave TfR1, the proportion of cleavage is unknown. Lastly, the reliability and consistency of the proportion of cleavage is also unknown. Characterizing these factors in healthy animals is necessary to understand how the proportion of cleavage affects the expected level of circulating sTfR. For example, if the proportion of cleavage, and thus the level of sTfR, varies greatly between healthy individuals then the generation of narrow reference

intervals will not be possible. Likewise, if the proportion of cleavage, and thus the level of sTfR, is very low, then there would be concern about developing a clinical assay with sufficient lower limits of sTfR detection.

The goal of this study was to characterize the proportion of cTfR relative to whole exosomal-associated TfR1 in the serum of healthy dogs, cats, and horses. We hypothesized that dogs and cats do not cleave all their exosomal-associated TfR1 (as humans do) and horses do not cleave exosomal-associated TfR1 at all. Additionally, we performed a small experiment to confirm that there was no loss of protein during the western blot protocol utilized.

Materials and Methods

Healthy Control Groups (Canine and Feline)

Serum samples were collected from left-over canine and feline patient samples at the Colorado State University Veterinary Teaching Hospital Clinical Pathology Laboratory. Patient records were reviewed retrospectively. Patients had to meet several criteria for inclusion as healthy controls. An unremarkable medical history, physical exam, and hematologic and biochemical results were required. Additionally, the patient needed to have no evidence of subclinical, acute, chronic, or ongoing systemic disease. Canine patients needed to be greater than one year of age. Feline patients as young as 7 months of age were accepted as healthy controls due to the difficulty of obtaining healthy feline controls during the study period.

Healthy Control Group (Equine)

A previous study performed by this laboratory[17] evaluated ultraprecipitates obtained from the serum of 6 healthy horses for the presence of TfR1 on western blot. The protocols used were similar and the same anti-TfR1 antibody was utilized. Despite extensive trouble-shooting and optimization work, we were unable to develop optimal results upon western blotting for TfR1 of equine ultraprecipitates during the current study. Consequently, the western blot images developed for this previous study were re-analyzed as described below, and the proportion of whole monomer TfR1 to cTfR (differentiated based on molecular weight) was assessed for each equine sample.

Exosome Isolation Using Ultracentrifugation

Serum samples were collected from left-over canine, feline, and equine patient samples at the Colorado State University Veterinary Teaching Hospital Clinical Pathology Laboratory as previously described.[17] Briefly, samples were thawed and lipids and cellular debris were removed by centrifugation at 8000 × g for 20 minutes at 4°C. Phosphate buffered saline (PBS; pH 7.4) was used to dilute the serum supernatant prior to ultracentrifugation at 100,000 × g for 1.5 hours at 4°C (Rotor TH-641 [Thermo Scientific, Waltham, Massachusetts, USA]). Most of the supernatant was removed. Approximately 1 ml of remaining supernatant was used to resuspend the ultraprecipitate obtained. The resuspended pellet was centrifuged at 13,000 × g for 20 minutes at 4°C. The supernatant was removed and the ultraprecipitate was resuspended in 5 µl of 100:1 PBS:protease inhibitor cocktail (Halt Protease Inhibitor Cocktail [Thermo Scientific, Waltham, Massachusetts, USA]) pH 7.4 and stored at -80°C until use in the procedures described below.

Western Blot for TfR1

Ultraprecipitates from canine and feline serum were obtained following the above procedure. Western blot for TfR1 was performed as previously described.[17] Briefly, the ultraprecipitates were separated on a readymade 10% Bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, California, USA) using MOPS buffer (Invitrogen, Carlsbad, California, USA) under reducing conditions. The iBlot Dry Blotting System (Invitrogen, Carlsbad, California, USA) was used to transfer the gel to a polyvinylidene difluoride membrane (Invitrogen, Carlsbad, California, USA). A canine bone marrow cell lysate was used as a positive control. Pooled left-over sample was used as a negative control with an irrelevant antibody serving as a negative control primary antibody. Membranes were blocked with 5% non-fat dry milk in 1× Tris-buffered saline with Tween for one hour, incubated with monoclonal mouse anti-human TfR1 antibody (Invitrogen, Carlsbad, California, USA) followed by biotin-labelled anti-mouse IgG1 antibody (BD Biosciences, San Jose, California, USA) followed by Streptavidin-HRP conjugated (1:300) (R&D Systems, Minneapolis, Minnesota, USA) for 15 minutes. Peroxidase activity was detected with Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, United Kingdom) and the bands were imaged on a ChemiDoc-It^{TS2} Imager (UVP, Upland, California, USA). The proportion of whole monomer TfR1 to cTfR (differentiated based on molecular weight) was assessed for each patient by means of freely accessible quantification software (ImageJ, http://rsbweb.nih.gov/ij/).

Statistical Analysis

Descriptive statistics for the proportion of cTfR for both the canine and feline healthy control groups were calculated using R (R Core Team, 2016, R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/).

Loss of Protein Experiment

Ultraprecipitates were obtained from pooled canine and feline serum as previously described. However, a standard amount of serum (2 ml) was used to yield approximately equal amounts of ultraprecipitate. Western blot for TfR1 was performed as previously described. However, the samples were split onto two gels. The transfer for the first gel was shortened to 5 minutes. The transfer for the second gel was performed per the usual protocol (8 minutes).

Results

Canine Healthy Control Group

The healthy canine control group included 52 patients. 12 patients were excluded due to insufficient protein or inadequate membrane image clarity barring assessment of the proportion of cTfR present. Of the 40 remaining patients, there were 14 mixed breed dogs, 7 Labrador Retrievers, 3 Golden Retrievers, 2 French Bulldogs, 2 Anatolian Shepherds, and 1 each of 12 additional dog breeds represented. These patients ranged in age from 1 to 14 years (median = 3 years). There were 20 spayed females, 1 intact female, 16 neutered males, and 3 intact males included.

Feline Healthy Control Group

The healthy feline control group included 31 patients. 10 patients were excluded due to insufficient protein or inadequate membrane image clarity barring assessment of the proportion of cTfR present. Of the 21 remaining patients, there were 17 domestic short hair cats, 2 Siamese, 1 domestic long hair cat, and 1 Russian Blue represented. These patients ranged in age from 7 months to 12 years (median = 5 years). There were 8 spayed females, 1 intact female, and 12 neutered males included.

Measurement of the Proportions of cTfR

Western blot for TfR1 was performed on the canine and feline healthy control ultraprecipitates. The anti-TfR1 antibody used targets the cytoplasmic domain of TfR1. Consequently, exosomal-associated TfR1 is detected as established in chapter 2. Based off the molecular weight of the detected protein bands, whole monomer TfR1 (95 kDa) can be differentiated from cTfR (17 kDa), as demonstrated in dogs (Figures 3.1A) and cats (Figure 3.2A).[5,10] Following imaging, densitometry analysis of the whole monomer TfR1 band and cTfR band for each patient was performed to quantify the proportion of cTfR relative to whole monomer TfR1, as demonstrated for dogs (Figure 3.1B) and cats (Figure 3.2B). These proportions are expressed as a percentage of the total TfR1 detected (whole monomer TfR1 + cTfR).

The six previously performed TfR1 western blot images from healthy equine patients were re-analyzed to assess the proportion of cTfR. No protein bands at 17 kDa (the expected location of cTfR) were detected, as demonstrated in Figure 3.3.[5]

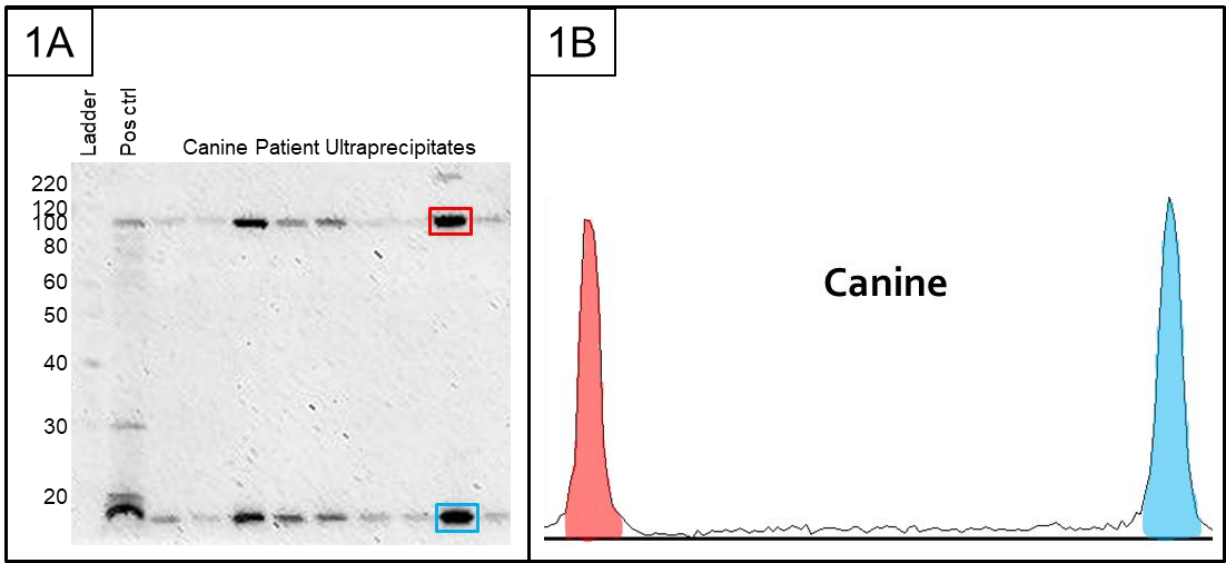

Figure 3.1: Western blot for TfR1 and densitometry tracing of canine ultraprecipitates (1A) Western blot for TfR1 reveals whole monomer TfR1 at approximately 95 kDa (example highlighted by the top red rectangle) and cTfR at approximately 17 kDa (example highlighted by the bottom blue rectangle). (1B) Densitometry analysis of the highlighted bands from 1A yields the tracing displayed and is used to calculate the relative proportions of whole monomer TfR1 (red peak, on the left) and cTfR (blue peak, on the right).

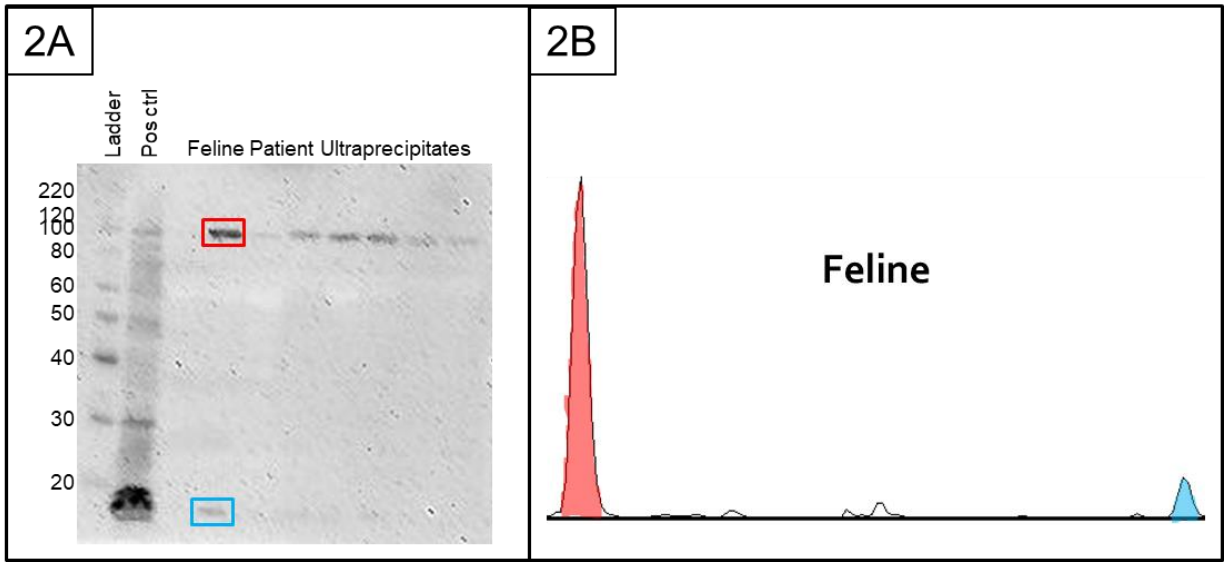

Figure 3.2: Western blot for TfR1 and densitometry tracing of feline ultraprecipitates (2A) Western blot for TfR1 reveals whole monomer TfR1 at approximately 95 kDa (example highlighted by the top red rectangle) and cTfR at approximately 17 kDa (example highlighted by the bottom blue rectangle). (2B) Densitometry analysis of the highlighted bands from 1A yields the tracing displayed and is used to calculate the relative proportions of whole monomer TfR1 (red peak, on the left) and cTfR (blue peak, on the right).

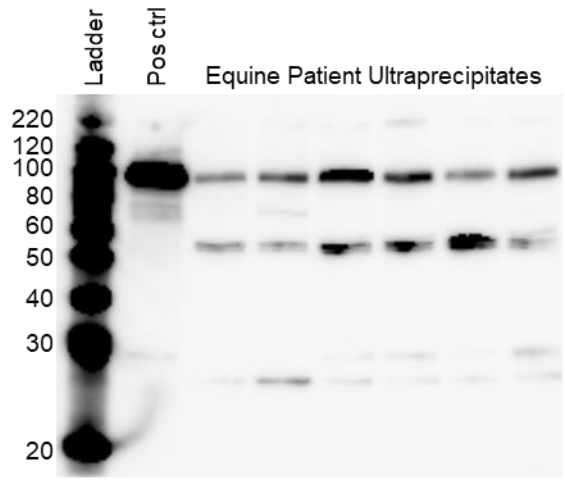

Figure 3.3: Western blot for TfR1 on equine ultraprecipitates, obtained from a previous study performed in the laboratory.[17] Whole monomer TfR1 is found at approximately 95 kDa and a band consistent with cTfR is not observed at approximately 17 kDa.

Proportion of cTfR in Healthy Control Dogs

Following densitometry analysis, the cTfR proportions of all canine healthy control patients were compiled and statistical analysis was performed. The mean percent cTfR was 58.62% with a broad standard deviation of 12.49%. The percent cTfR range was 24.62-82.82%. Graphical representations of these results alongside the feline results can be found in Figures 3.4 and 3.5.

Proportion of cTfR in Healthy Control Cats

Following densitometry analysis, the cTfR proportions of all feline healthy control patients were compiled and statistical analysis was performed. The mean percent cTfR was 11.03% with a standard deviation of 4.54%. The percent cTfR range was 3.3-19.55%. Graphical representations of these results alongside the canine results can be found in Figures 3.4 and 3.5.

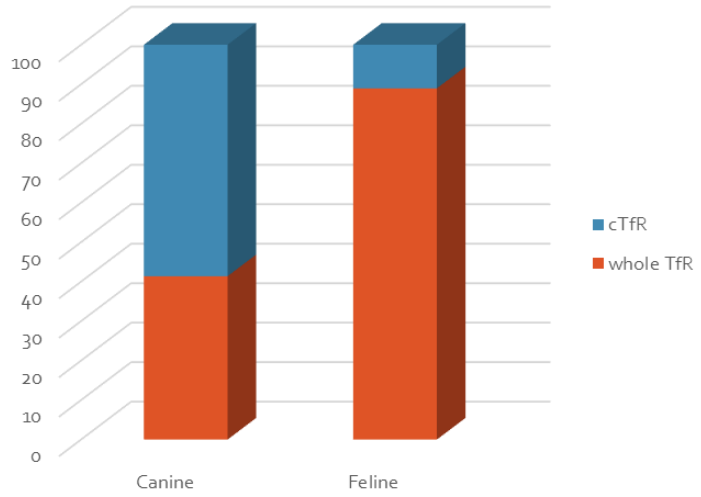

Figure 3.4: Mean proportion of cTfR relative to exosomal-associated whole monomer TfR1. Each bar represents 100% of the TfR1 associated with exosomes. The red section (bottom) of the bar represents the mean proportion of whole monomer TfR while the blue section (top) of the bar represent the mean proportion of cTfR for healthy dogs (left) and cats (right).

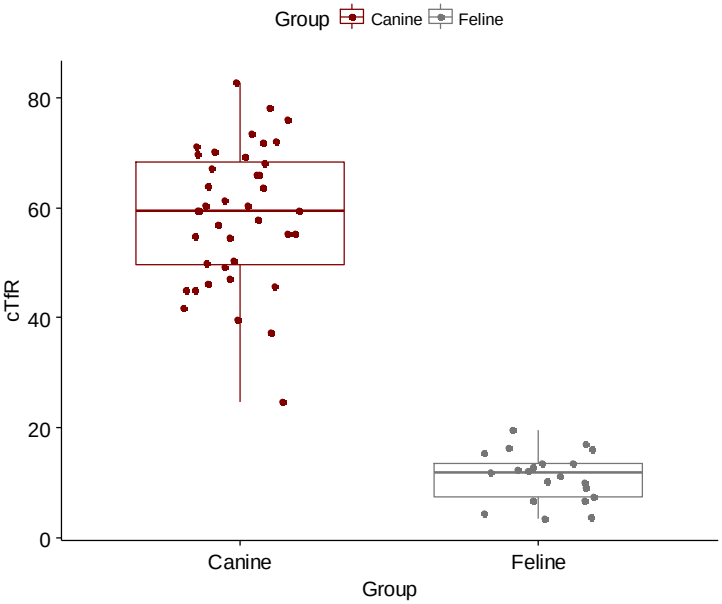

Figure 3.5: Mean and range of the proportion of cTfR relative to exosomal-associated whole monomer TfR1, expressed as a percentage. Healthy canine samples are on the left and healthy feline samples are on the right. The mean proportion of cTfR for dogs is 58.62% and the range is quite wide. The mean proportion of cTfR for cats is fairly low at 11.03% but the range is also much narrower.

Loss of Protein Experiment

To test for a loss of low molecular weight proteins (cTfR) during the transfer step of the western blot protocol, a standardized amount of canine and feline ultraprecipitates were evaluated for the presence of TfR1 following a shortened and standard transfer protocol. Following imaging, densitometry analysis of the whole monomer TfR1 band and cTfR band for each membrane was performed to quantify the proportion of cTfR relative to whole monomer TfR1. The proportion of cTfR was not lower on the standard protocol membrane than on the shortened protocol membrane for either canine or feline ultraprecipitates (Figure 3.6). This indicates that there is no loss of cTfR protein relative to whole monomer TfR1 due to the standard transfer protocol utilized.

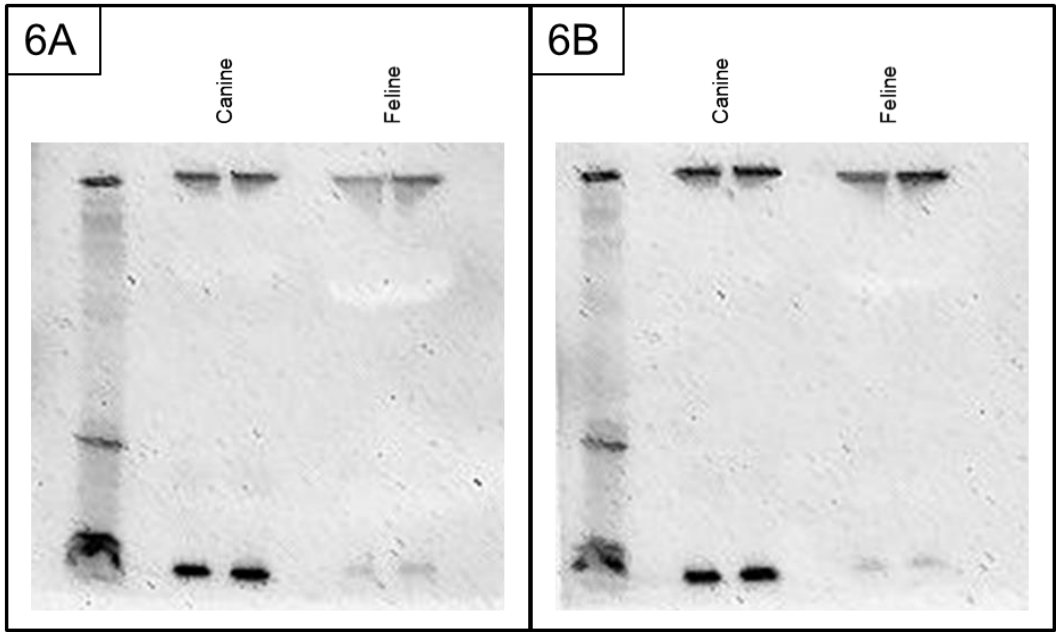

Figure 3.6: Western blots for TfR1, evaluating for protein loss during membrane transfer (6A) Shortened, 5 minute transfer protocol. (6B) Regular, 8 minute transfer protocol. No loss of protein between the shortened and regular transfer protocols was detected when the proportion of cTfR was measured via densitometry analysis.

Discussion

The level of cleavage, as reflected by the proportion of cTfR relative to whole monomer TfR1, in dogs, cats, and horses is not consistent with the level of cleavage observed in humans and is not consistent between these three species. This suggests that the circulating levels of sTfR in these species will be distinctly different than those of humans as the proportion of cTfR is an indirect reflection of the amount of sTfR.

Dogs appear to cleave, on average, over half of their exosomal-associated TfR1. However, this level of cleavage appears widely variable between individuals. This extensive variation suggests that the circulating levels of sTfR will also be widely variable between healthy individuals. This makes development of a clinical assay for the detection of sTfR difficult as the calculation of reasonably narrow reference intervals seems unlikely given the degree of variation. Without narrow reference intervals, the detection of disease states (such as the expected increase in sTfR with iron deficiency) could be difficult to differentiate from normal, between dog variations.

Cats exhibit a narrower range of cleavage of exosomal-associated TfR1 than dogs, as reflected by their proportion of cTfR. However, on average, the proportion of cTfR in cats appears to be quite low. Therefore, the expected level of circulating sTfR in healthy cats is anticipated to also be low. Consequently, the development of a clinical assay for the detection of sTfR in cats may be largely dependent on the sTfR detection sensitivity of the assay developed.

Horses do not appear to cleave exosomal-associated TfR1 at all. This would suggest that horses do not have circulating sTfR. However, given the small sample size,

evaluation of additional equine serum samples would be desirable to confirm this finding. Additionally, on the western blots for TfR1 performed on equine ultraprecipitates, there were protein bands observed at variable molecular weight. These bands were considered non-specific binding. However, the possibility of TfR1 cleavage at a different location, thus yielding cTfR that is not found at 17 kDa, should also be considered.

In conclusion, while dogs appear to cleave relatively high levels of exosomal-associated TfR1, the wide range of cleavage may hinder development of a clinical assay for sTfR detection. Contrariwise, cats appear to cleave very little of their exosomal-associated TfR1 but the level of cleavage appears more consistent between individuals. On this premise, development of a sTfR clinical assay may be possible. Lastly, horses do not appear to cleave exosomal-associated TfR1 indicating that they may not have significant levels of circulating sTfR. Without circulating sTfR, a clinical assay for the detection of sTfR in horses would not be warranted. Overall, these findings suggest that development of a clinical assay for the detection and quantification of sTfR in these species will be, at the very least, difficult.

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CHAPTER 4: Comparison of the Proportion of Cleaved Tfr Between Healthy and Diseased Canine and Feline Patients

Introduction

In the previous chapter, the proportion of cTfR relative to exosomal-associated whole monomer TfR1 was established in healthy dogs and cats. We found that dogs and cats do not cleave the majority of their exosomal-associated TfR1 as humans reportedly do.[1] Sheep also do not appear to cleave all of their exosomal-associated TfR1 as an in vitro model demonstrated only about 25% cleavage.[2] This differential level of cleavage may be related to the variability in proteolytic activity of granulocytes in different species.[3] The mechanism of cleavage of exosomal-associated TfR1 in dogs and cats has not been investigated but is theorized to similarly involve circulating granulocytes. The proteolytic activity of canine and feline granulocytes against exosomal-associated TfR1 is also unknown.

Granulocytes play an essential role in immunity and disease. Consequently, the roles granulocytes play in chronic disease and potentially in the cleavage of exosomal-associated TfR1 may overlap. This becomes a concern in dogs and cats where all their exosomal-associated TfR1 is not cleaved in healthy individuals. It raises the question of whether increased inflammation, as with significant and chronic disease, may lead to increased cleavage of exosomal-associated TfR1. This could theoretically cause an increase in sTfR even if body TfR1 is not upregulated. This is important as the utility of sTfR in humans is based in the fact that the serum concentration of sTfR does not

appear to be significantly impacted by inflammation but does increase significantly with iron deficiency.[1,3–6] If inflammation were to cause an increase in cleavage of exosomal-associated TfR1 in dogs and cats and thus a subsequent increase in sTfR, then sTfR concentration may not be able to detect an underlying iron deficiency in the face of inflammation.

The focus of this study was to compare the proportion of cTfR between healthy and diseased dogs and cats. The goal was to characterize any possible influence of inflammation or disease on the level of cleavage in the hopes of determining whether the proportion of sTfR to whole TfR1 in the circulation of these veterinary species is consistent regardless of disease state. We hypothesized that the proportion of cTfR does not vary between normal and diseased dogs and cats.

Material and Methods

Disease Groups (Canine and Feline)

Serum samples were collected from left-over canine and feline patient samples at the Colorado State University Veterinary Teaching Hospital Clinical Pathology Laboratory. Patient records were reviewed retrospectively. Patients were included in the diseased group if they exhibited evidence of significant and ongoing systemic disease on physical exam, routine hematologic and biochemical panels, and/or other ancillary diagnostics.

Exosome Isolation Using Ultracentrifugation

Serum samples were collected from left-over canine, feline, and equine patient samples at the Colorado State University Veterinary Teaching Hospital Clinical

Pathology Laboratory as previously described.[7] Briefly, samples were thawed and lipids and cellular debris were removed by centrifugation at 8000 × g for 20 minutes at 4°C. Phosphate buffered saline (PBS; pH 7.4) was used to dilute the serum supernatant prior to ultracentrifugation at 100,000 × g for 1.5 hours at 4°C (Rotor TH-641 [Thermo Scientific, Waltham, Massachusetts, USA]). Most of the supernatant was removed. Approximately 1 ml of remaining supernatant was used to resuspend the ultraprecipitate obtained. The resuspended pellet was centrifuged at 13,000 × g for 20 minutes at 4°C. The supernatant was removed and the ultraprecipitate was resuspended in 5 µl of 100:1 PBS:protease inhibitor cocktail (Halt Protease Inhibitor Cocktail [Thermo Scientific, Waltham, Massachusetts, USA]) pH 7.4 and stored at -80°C until use in the procedures described below.

Western Blot for TfR1

Ultraprecipitates from canine and feline serum were obtained following the above procedure. Western blot for TfR1 was performed as previously described.[7] Briefly, the ultraprecipitates were separated on a readymade 10% Bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, California, USA) using MOPS buffer (Invitrogen, Carlsbad, California, USA) under reducing conditions. The iBlot Dry Blotting System (Invitrogen, Carlsbad, California, USA) was used to transfer the gel to a polyvinylidene difluoride membrane (Invitrogen, Carlsbad, California, USA). A canine bone marrow cell lysate was used as a positive control. Pooled left-over sample was used as a negative control with an irrelevant antibody serving as a negative control primary antibody. Membranes were blocked with 5% non-fat dry milk in 1× Tris-buffered saline with Tween for one hour, incubated with monoclonal mouse anti-human TfR1 antibody (Invitrogen,

Carlsbad, California, USA) followed by biotin-labelled anti-mouse IgG1 antibody (BD Biosciences, San Jose, California, USA) followed by Streptavidin-HRP conjugated (1:300) (R&D Systems, Minneapolis, Minnesota, USA) for 15 minutes. Peroxidase activity was detected with Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, United Kingdom) and the bands were imaged on a ChemiDoc-It^{TS2} Imager (UVP, Upland, California, USA). The proportion of whole monomer TfR1 to cTfR (differentiated based on molecular weight) was assessed for each patient by means of freely accessible quantification software (ImageJ, http://rsbweb.nih.gov/ij/).

Statistical Analysis

Statistical analysis was performed using R (R Core Team, 2016, R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/). A power study was performed on the initial canine data to calculate the sample size necessary to achieve 80% power using Lenth's online power calculator (Lenth, R. V. [2006-9], Java Applets for Power and Sample Size, http://www.stat.uiowa.edu/~rlenth/Power). Data were checked for normalcy. Healthy and diseased group means for the proportion of cTfR were calculated and compared using a two sample t-test. The level of significance was set at $P < 0.05$.

Results

Canine Diseased Group Power Study

The initial diseased and normal canine ultraprecipitate samples (10 per group) were processed and the proportion of cTfR was obtained. This data was used to

perform a power study to calculate the number of canine patients per group necessary to achieve a power of 80% in the two sample t-test when a difference of greater than 5% in the means of the two groups was considered significant. This study yielded a sample size of 35 patients per group. Due to the high number of samples that were thrown out due to insufficient protein or inadequate membrane image clarity, excessive samples were processed, and 40 patients ended up in each group.

Canine Diseased Group

The canine diseased group included 56 patients. 16 patients were excluded due to insufficient protein or inadequate membrane image clarity barring assessment of the proportion of cTfR present. Of the 40 remaining patients, there were 9 mixed breed dogs, 5 Labrador Retrievers, 2 Boxers, 2 Jack Russell Terriers, 2 Basset Hounds, and 1 each of 20 additional dog breeds represented. These patients ranged in age from 2 months to 15 years (median = 10 years). There were 11 spayed females, 4 intact females, and 25 neutered males included. These patients presented with a variety of significant systemic diseases as described in table 4.1.

Feline Diseased Group

The feline diseased group included 53 patients. 33 patients were excluded due to insufficient protein or inadequate membrane image clarity barring assessment of the proportion of cTfR present. Of the 20 remaining patients, there were 9 domestic short hair cats, 8 domestic long hair cats, and 1 each of Balinese, Ragdoll, and Siamese. These patients ranged in age from 1 to 19 years (median = 9 years). There were 7

spayed females, 1 intact female, and 12 neutered males included. These patients presented with a variety of significant systemic diseases as described in table 4.2.

Table 4.1: Distribution of disease in canine diseased group. 17 patients fell into multiple categories. All patients were considered to have significant, often terminal, systemic disease. *Anemia was present in 16 patients in addition to their presenting complaint, but the underlying mechanism of the anemia was not always fully characterized. (n) = number of patients; CNS = central nervous system; GI = gastrointestinal

Disease	**(n)**	**Disease**	**(n)**	**Disease**	**(n)**	**Disease**	**(n)**
Cancer	22	Endocrine	3	Pancreas	1	Autoimmune	4
Cardiovascular	13	GI/Abdominal	7	Kidney	4	Reproductive	1
Pulmonary	4	Liver	1	Urinary tract	1	Infectious	2
CNS (including tumors)	3	Spleen	1	Skin	2	Anemia*	16

Table 4.2: Distribution of disease in feline diseased group. 12 patients fell into multiple categories. All patients were considered to have significant, often terminal, systemic disease. *Anemia was present in 9 patients in addition to their presenting complaint, but the underlying mechanism of the anemia was not always fully characterized. (n) = number of patients; CNS = central nervous system; GI = gastrointestinal

Disease	**(n)**	**Disease**	**(n)**	**Disease**	**(n)**	**Disease**	**(n)**
Cancer	8	CNS	1	Pancreas	1	Autoimmune	1
Cardiovascular	4	GI/Abdominal	4	Kidney	4	Reproductive	1
Pulmonary	2	Liver	2	Urinary tract	1	Infectious	2
Upper Respiratory	3	Endocrine	5	Skin	1	Anemia*	9

Measurement of the Proportions of cTfR

Western blot for TfR1 was performed on the canine and feline diseased group ultraprecipitates. The anti-TfR1 antibody used targets the cytoplasmic domain of TfR1. Consequently, exosomal-associated TfR1 is detected as established in chapter 2. Based off the molecular weight of the detected protein bands, whole monomer TfR1 (95 kDa) can be differentiated from cTfR (17 kDa), as previously demonstrated in dogs (Figures 3.1A) and cats (Figure 3.2A).[1,2] Following imaging, densitometry analysis of the whole monomer TfR1 band and cTfR band for each patient was performed to quantify the proportion of cTfR relative to whole monomer TfR1, as previously demonstrated for dogs (Figure 3.1B) and cats (Figure 3.2B). These proportions are expressed as a percentage of the total TfR1 detected (whole monomer TfR1 + cTfR).

Proportion of cTfR in Diseased Dogs

Following densitometry analysis, the cTfR proportions of all canine diseased group patients were compiled and statistical analysis was performed. The mean percent cTfR was 62.71% with a standard deviation of 16.44%. The percent cTfR range was 19.78-90.55%.

Proportion of cTfR in Diseased Cats

Following densitometry analysis, the cTfR proportions of all feline diseased group patients were compiled and statistical analysis was performed. The mean percent cTfR was 17.11% with a standard deviation of 5.47%. The percent cTfR range was 3.99-25.46%.

Comparison of Canine Healthy to Diseased Mean Proportion of cTfR

The mean proportion of cTfR of both the healthy and diseased canine groups was compared using a two sample t-test. No significant difference between the two means was detected (p-value = 0.2142 > 0.05). Graphical representations of these results can be found in Figure 4.1.

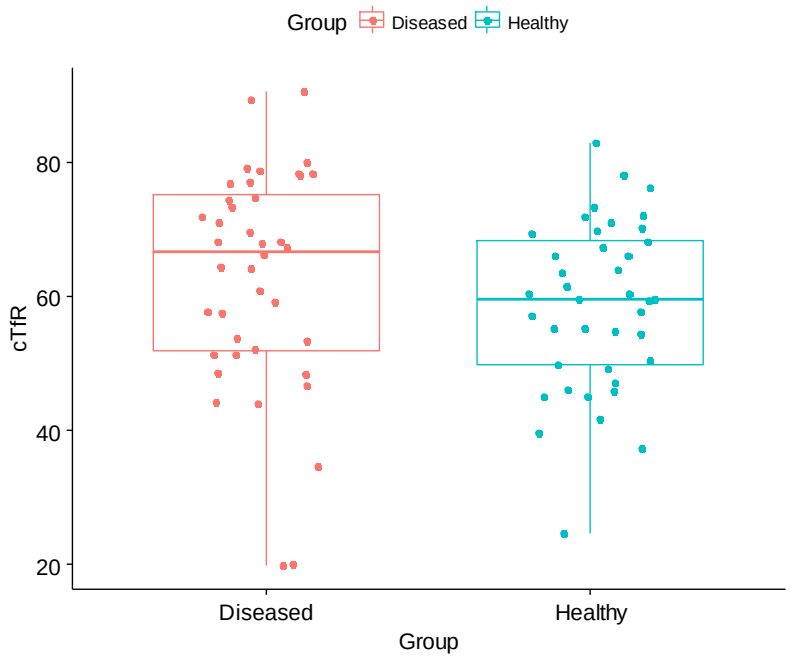

Figure 4.1: Mean and range of the proportion of cTfR relative to exosomal-associated whole monomer TfR1 in healthy and diseased dogs, expressed as a percentage. Diseased canine samples are on the left and healthy canine samples are on the right. The mean proportion of cTfR for diseased dogs is 62.71% and for healthy dogs is 58.62%. No significant difference between the means of the two groups was detected (p-value = 0.2142 > 0.05).

Comparison of Feline Healthy to Diseased Mean Proportion of cTfR

The mean proportion of cTfR of both the healthy and diseased feline groups was compared using a two sample t-test. The diseased group exhibited a significantly higher

mean proportion of cTfR than the healthy group (p-value = 0.0004 < 0.05). The 95% confidence interval for this difference was (2.91%, 9.25%) indicating that the true difference between the means is most likely between 2.91% and 9.25%. Graphical representations of these results can be found in Figure 4.2.

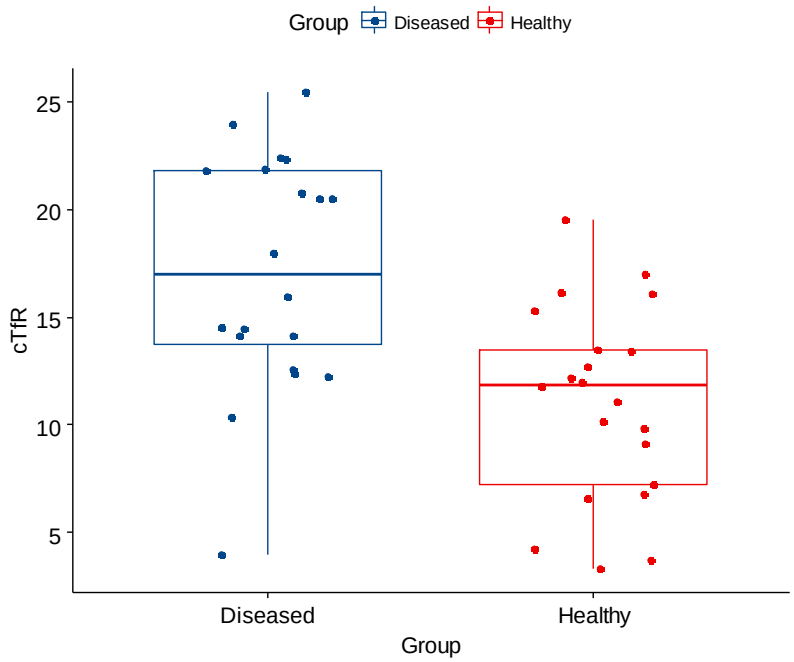

Figure 4.1: Mean and range of the proportion of cTfR relative to exosomal-associated whole monomer TfR1 in healthy and diseased cats, expressed as a percentage. Diseased feline samples are on the left and healthy feline samples are on the right. The mean proportion of cTfR for diseased cats is 17.11% and for healthy cats is 11.03%. A significant difference between the means of the two groups was detected (p-value = 0.0004 < 0.05).

Discussion

The effect of inflammation and chronic disease on the cleavage of TfR1 from the surface of exosomes in canine and feline serum has not previously been studied. Our results indicate that the effect is small to non-existent in both species. Understanding

any potential change in the degree of cleavage with inflammation is important as it would suggest a corresponding change in the circulating levels of sTfR.

No significant difference was detected in the level of cleavage between healthy and diseased dogs. This indicates that inflammation and chronic disease do not significantly alter the cleavage of TfR1 from the surface of exosomes. The caveat to this statement is that a limited variety of diseases were represented in this study. It is possible that a larger pool of diseases or a specific set of diseases would detect an alteration in cleavage that was not demonstrated in this study. Overall, the lack of a significant impact on the level of cleavage due to chronic disease would suggest that inflammation should not significantly impact the level of circulating sTfR as body TfR1 expression has also been shown to not be significantly impacted by chronic disease.[5]

Diseased cats exhibited a statistically significant increase in the proportion of cTfR when compared to healthy cats. However, the 95% confidence interval indicates that the degree of this difference is quite small. Consequently, the biologic and clinical significance of this difference is unknown. It may be that this change in cleavage does significantly impact the concentration of circulating sTfR and would confound the detection of increased sTfR as an indication of iron deficiency in the face of inflammation and chronic disease. On the other hand, the increased level of cleavage may translate to a negligible increase in circulating levels of sTfR, thus allowing for the detection of sTfR increases secondary to iron deficiency even when inflammation and chronic disease are present.

Another potential problem with the results obtained is that the healthy and diseased groups for both the dogs and the cats were not age, gender, and breed

matched. Consequently, age, gender, or breed differences in the level of cleavage in both the healthy and diseased groups could confound the results. Unfortunately, the relatively low number of patients included in the study prevents the characterization of any variations of cleavage associated with these parameters.

In conclusion, the impact of chronic disease on circulating levels of sTfR is suspected to be insignificant in the dog and minimal in the cat based off these results. This finding in the dog would support the development of a clinical assay for the detection and quantification of sTfR. For the cat, additional investigation into the biologic and clinical significance of the difference in cleavage between healthy and diseased individuals would be warranted prior to investing in the development of a clinical assay.

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CHAPTER 5: Concluding Remarks

The first portion of this study established the presence of exosomes in the ultraprecipitates obtained from canine, feline, and equine serum using several recognized techniques. Additionally, the association of TfR1 with the isolated exosomes was verified. Lastly, loss of TfR1 from reticulocytes as they mature to erythrocytes was demonstrated along with the lack of cTfR on reticulocytes and erythrocytes. This initial work was necessary to confirm that exosomal-associated TfR1 was present in the ultraprecipitate samples for the focus of the subsequent two portions of this study.

The second portion of this study characterized the degree of cleavage of exosomal-associated TfR1 in healthy dogs, cats, and horses. Dogs were found to cleave a large fraction of their exosomal-associated TfR1 although the between-individual variation was high. This suggested that dogs might have sufficient levels of sTfR for quantification but that those levels might be too variable for the detection of disease-related alterations in sTfR. Cats were found to cleave very little of their exosomal-associated TfR1 but did demonstrate a more consistent level of cleavage than dogs. The concern with this low level of cleavage is that cats might have too little circulating sTfR for adequate detection and quantification. Horses appear to lack cleavage of their exosomal-associated TfR1. Consequently, it is suspected that horses do not have circulating sTfR making a clinical assay for the detection of sTfR in the horse unwarranted.

The third portion of this study compared the level of cleavage of exosomal-associated TfR1 between healthy and diseased dogs and cats. No significant difference between healthy and diseased dogs was detected. This suggests that chronic disease has no significant impact on the levels of circulating sTfR. Diseased cats exhibited significantly higher levels of cleavage of exosomal-associated TfR1 than did healthy cats. However, this difference was quite small and the biologic and clinical significance of it is unknown.

When taken together, these results suggest that the development of a clinical assay for the detection and quantification of sTfR in dogs, cats, and horses may not be worthwhile. The lack of evidence of cleavage in horses suggests that they do not have circulating sTfR. Alternatively, the presence of some unidentified bands on the equine western blots for TfR1 could suggest that horses actually cleave TfR1 at a different location, thus yielding cTfR of a different molecular weight.

The significant variation in the level of cleavage in dogs suggests that the levels of circulating sTfR would likewise be highly variable. However, the life spans of both exosomes and sTfR are unknown. It has been shown that exosomes can be taken up by macrophages, suggesting one route of clearance that could contribute to differential life spans between exosomes and sTfR.[1] Additionally, it is unknown how or why there is a variation in cleavage. Consequently, it is feasible that, despite the wide variation in the level of cleavage of exosomal-associated TfR1, the level of sTfR in dogs could actually be more consistent across individuals. Lastly, if the level of sTfR is significantly lower in healthy animals than in iron deficient animals then the variation between healthy individuals may not be clinically relevant. One possible means of evaluating this

potential is to look at the actual amount of cTfR between healthy dogs and iron deficient dogs by starting with a standardized amount of serum for exosome isolation.

The same holds true for cats. Although their level of cleavage appears quite low and suggests that sTfR levels may also be low, it is possible that the sTfR may still be sufficiently high for accurate detection and quantification. The significant difference in proportions of cTfR detected between healthy and diseased cats also raises concerns about the influence of inflammation on circulating levels of sTfR. However, this difference is low enough that the biologic and clinical significance of it is questionable. Even if this differential cleavage impacts the levels of sTfR it is suspected that the impact would be minimal. If sTfR were to increase with inflammation (as suggested by the increased proportion of cTfR in diseased cats) then the detection of iron deficiency by an increase of sTfR could be confounded in the presence of inflammation. However, this is largely dependent on how great the impact of inflammation is on sTfR relative to the impact due to iron deficiency.

Some potential problems with this study include that the healthy and diseased groups were not gender, age, or breed matched. Consequently, differences in the proportion of cTfR associated with these parameters could confound the results obtained. Moreover, as the patients in this study were included retrospectively, it cannot be completely ruled out that the healthy animals did not have some sort of underlying systemic illness that was not uncovered on routine exam. Another potential problem is that serum exosomes have different sources besides maturing reticulocytes and could potentially influence the proportion of cTfR detected. Pertaining to the horse samples, only six healthy horses were evaluated. It is possible that there could be more variation

in the cleavage of exosomal-associated TfR1 in equine serum than was detected in these six patients. Lastly, a standardized amount of serum was not used for exosome isolation for each patient. Therefore, the true amount of cTfR between patients could not be compared. However, the variation in starting serum amounts should not have impacted the proportion of cTfR relative to exosomal-associated whole monomer TfR1 detected.

All things considered, the lack of overt exosomal-associated TfR1 cleavage in horses, variability of cleavage in dogs, scant cleavage and variation in cleavage between healthy and diseased cats all suggest that the time and expense required for the development of a clinical assay for the detection and quantification of sTfR may outweigh the potential benefits. Assay development could additionally be complicated by the presence of exosomal-associated whole TfR1 in circulation with sTfR given that none of these species exhibit cleavage of the majority of their exosomal-associated TfR1 as humans do.[2] The potential interferences of significant amounts of circulating exosomal-associated whole TfR1 on sTfR detection and quantification are unknown but are anticipated to impede accurate quantification of sTfR. These results suggest that a sTfR assay may not be reliable or feasible in these veterinary species.

Future areas of study could include determining whether the statistically significant difference in the proportion of cTfR between healthy and diseased cats is biologically or clinically significant. Additionally, characterization of any possible differences in the proportion of cTfR associated with gender, age, or breeds may be warranted. As previously mentioned, investigating the differences in the amount of cTfR between healthy and iron deficient animals by using a standardized amount of serum for

exosome isolation may be insightful. Given that dogs, cats, and horses all appear to have significant levels of exosomal-associated whole TfR1 it may be worthwhile to study how exosomal-associated whole TfR1 can potentially interfere with the detection and quantification of sTfR. Additionally, investigating whether an assay for the detection and quantification of total serum TfR1 (exosomal-associated whole TfR1 in addition to sTfR) is developmentally possible may prove interesting. Quantification of exosomal-associated TfR1 alone as a clinical diagnostic is unlikely to be useful as isolation and processing of serum exosomes is a tedious and cumbersome process that does not lend itself to clinical utility.

In conclusion, at this time and given the results of this study, pursuit of a clinical assay for the detection and quantification of sTfR in dogs, cats, and horses is not considered worthwhile. In the future, pending the results of some of the possible studies described above, development of such an assay may or may not be deemed valuable.

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