Quantitative PCR and Immunohistochemical Analyses of HMGB1 and RAGE Expression in Canine Disseminated Histiocytic Sarcoma (Malignant Histiocytosis)

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Abstract. Background: Disorders of histiocytic origin affecting humans and dogs share various similarities. Canine disseminated histiocytic sarcoma (DHS) (formerly known as malignant histiocytosis) is an aggressive neoplasm of interstitial dendritic cells (DCs). The receptor for glycation end products (RAGE) and the high mobility group box 1 protein (HMGB1) have been shown to be required for the maturation and migration of DCs. Thus, deregulation of the expression of these genes could have a major effect on the progression of histiocytic disorders. Materials and Methods: Neoplastic canine DHS samples and non-neoplastic control samples were analysed immunohistochemically and via real-time PCR. Results: Significant down-regulation of RAGE in the lung tumour samples and down-regulation of HMGB1 in the lung, lymph node and spleen tumour samples were detected compared to their non-neoplastic counterparts. Conclusion: RAGE and HMGB1 expression down-regulation in canine DHS points to a role in the progression of histiocytic disorders.

Several well-documented neoplasias of histiocytic origin ranging from localized, reactive and benign lesions to systemic syndromes with rapid clinical progression leading to death, show similar biological behaviour in both humans and dogs (1, 2).

The term “histiocyte” including macrophages and dendritic cells (DCs) represents a subset of leukocytes which are able to migrate into tissues. These cells play an important role in the immune system by presenting antigens to naive T-cells (dendritic cells) and by phagocytosing cellular debris and pathogens (macrophages) (1, 2). Disseminated histiocytic sarcoma (DHS) is a rare and very aggressive histiocytic neoplasm of interstitial DCs and usually occurs in multiple sites, mainly the lymph nodes, lungs, spleen and liver (1-4). Bernese mountain dogs show a high breed-specific predisposition for DHS (approximately 25% affected, mean age of onset 6.5 years) but the mode of inheritance is still not well understood and multigenic involvement is suggested (5-7). The progression of the disease is very rapid and prognosis is poor with a median survival time of 128 days and a one year survival rate of 0%-30% (2). Human malignant histiocytic-cell related proliferations including histiocytic sarcomas and Langerhans cell histiocytosis (LCH) also show very aggressive progression resulting in the death of most patients with widespread disease involving e.g. lymph nodes, intestine, liver, spleen, lung and bone marrow (2, 8) showing either macrophage or dendritic origin (8, 9).

High mobility group box 1 (HMGB1) (amphoterin or HMG-1) acts in the nucleus as a DNA-binding nuclear protein, while in the extracellular environment as a proinflammatory cytokine and also as a dose-dependent mediator of angiogenic and neo-vascularising processes (10). HMGB1 expression varies in different tissues and can be either developmentally regulated or respond to signals from the environment (11). HMGB1 induced signalling is mediated via receptor for advanced glycation end products (RAGE) and toll-like receptors (TLR) (12). RAGE expression is high during embryonic development while in adult tissues RAGE expression is widespread but relatively low except in the lung, where the expression levels remain high throughout life. RAGE expression increases in inflammatory-related pathological states (13-17). DCs are able to secrete HMGB1 in response to inflammatory stimuli for proliferation, survival and polarization of naive CD4+ T cells (18) and for canine T-cell proliferation (19). The maturation and migration of DCs into lymphoid organs was described to depend on HMGB1.
and its receptor RAGE due to HMGB1-RAGE induced cellular pathways (20-22). Taken together, the RAGE-HMGB1 complex thus represents a checkpoint in DC maturation, migration and immune response. The deregulation of the expression of HMGB1 and RAGE could thus have an effect on dendritic cell biology. In histiocytic disorders such as LCH, dendritic Langerhans cells (LCs) are described to be arrested in an immature, partially activated stage and show a different proliferation pattern than “normal” dendritic LCs leading to an accumulation in the affected tissues and disease progression (23). In canine DHS the pivotal role of HMGB1-RAGE on DCs could thus play an important role in the progression and severity of disease. Since canine RAGE and HMGB1 genes correspond closely to their human counterparts (14, 24, 25) characterisation of the biology of canine neoplastic histiocytic disorders could benefit both dogs and human patients.

**Materials and Methods**

**Canine tissue samples.** HMGB1 and RAGE gene expression was assessed by real-time PCR in 20 DHS tumour samples obtained during clinical examination (7 liver, 3 lung, 2 lymph node and 8 spleen) from 15 dogs (4 female and 11 male; 1 Australian Shepherd and 14 Bernese mountain dogs) aged between four and nine years. Eight non-neoplastic surgically removed control samples (3 liver, 1 lung, 2 lymph node and 2 spleen tissues) from six dogs (1 female and 5 male; 2 Bernese mountain dog, 2 German Shepherd, 1 Mixed breed and 1 Newfoundland) were also assessed. These dogs were aged between six and eleven years and euthanized due to DHS unrelated diseases (anal fissure, ascites, interstitial pneumonia, lung sarcoma, mastocytoma and peritonitis). The non-neoplastic samples were collected from clinically unaltered lung, liver, lymph node and spleen tissues. After collection, the samples were immediately frozen in liquid nitrogen and stored at –80°C until RNA isolation.

The immunohistochemical study of the HMGB1 and RAGE proteins included 13 neoplastic DHS samples (5 liver, 2 lung, 3 lymph node and 3 spleen) from six dogs (1 female and 5 male; 1 Australian Shepherd and 5 Bernese mountain dog). The tumour samples from five of these dogs were also analyzed in the gene expression study. As non-neoplastic control an additional 20 samples (5 liver, 5 lung, 5 lymph node and 5 spleen) from five dogs (4 female and 1 male; 1 Labrador retriever, 2 Mixed breed, 1 Rottweiler and 1 Schnauzer) were included. These dogs were euthanized due to DHS unrelated diseases (arthritis, epilepsy, kidney dysplasia, meningocerebralitis and tibial fracture). After collection the samples were fixed in 10% neutral buffered formalin and embedded in paraffin. All the DHS diagnoses were cytologically and histologically confirmed accordingly to the WHO nomenclature. All the samples were provided by the Small Animal Clinic, University of Veterinary Medicine, Hannover, Germany.

**RNA isolation and cDNA synthesis for transcript characterization.** The tissue samples were homogenized using stainless steel-beads and a Qiagen-TissueLyser II homogenizer (Qiagen, Hilden, Germany). The total RNA was isolated using an RNeasy Mini Kit according to the manufacturer’s instructions (Qiagen). To avoid genomic DNA contaminations, on-column DNase digestion with an RNase-Free DNase set (Qiagen) was performed.

The respective cDNA syntheses were performed using 250 ng total RNA of each sample and a QuantiTect Reverse Transcription Kit following the manufacturer’s protocol (Qiagen).

**Real-time PCR.** For relative quantification of the HMGB1 and RAGE transcript levels in the samples the beta-glucuronidase (GUSB) and hypoxanthine-guanine phosphoribosyltransferase (HPRT) genes were used as endogenous controls. The real-time PCRs were analysed according to the delta delta CT (ΔΔCT) method. All the PCR amplifications were carried out using the Eppendorf Mastercycler ep realplex real-time PCR System (Eppendorf AG, Hamburg, Germany). Two μl of each cDNA corresponding to 25 ng of total RNA were amplified in a total volume of 25 μl using universal PCR Mastermix (Applied Biosystems, Darmstadt, Germany) with 600 nM of each primer and 200 nM fluorogenic probe. The sequence data for the PCR assays used in this study were: HMGB1: forward primer: 5’-AAGTGAGAGCCAGACGGG-3’, reverse primer: 5’-TTCTTTGCCCCATGTTTAATTATTTTC-3’, probe: 5’-6-FAM-CTGGGGCGACTCTGTCGCTCCTGCT-TAMRA-3’. RAGE: forward primer: 5’-GGTGTTGCGGGGACGAGTAGTGGAG-3’, reverse primer: 5’-TCTGTTGCCAGCCTTGTTCAAGCTT-3’, probe: 5’-6-FAM-AAGCCGCTGGTGGCTAACTGTG-TAMRA-3’. GUSB: forward primer: 5’-TTGGTCTGAGGATGGGCA-3’, reverse primer: 5’-CTGGCAATGGAACCCATATTC-3’, probe: 5’-6-FAM-CGCCCACTACTATGCTCCATCGTGTTG-TAMRA-3’. HPRT: forward primer: 5’-CTCTTCTGAGCAGGAAACCT-3’, reverse primer: 5’-TCTACTCTAATTCACAGGACGT-3’, probe: 5’-6-FAM-CCTGGTTGGGCTCGTGAGTCGCGTA-TAMRA-3’. The PCR conditions were: 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 15 sec at 95°C and 1 min at 60°C.

All the samples were measured in triplicate and for each run non-template controls and non-reverse transcriptase control reactions were included. A precedent efficiency analysis of all the PCR assays used in this study was performed by applying the same template and dilution steps. The PCR reactions of the HMGB1, RAGE, GUSB and HPRT assays showed comparable efficiencies ensuring an appropriate real-time PCR analysis.

For the ΔΔCT analysis the calibrator was defined after performing relative real-time PCR with HMGB1 as the target gene. The sample within the control group that showed the most stable CT values for the target and endogenous control was defined as the calibrator. For analysis of the relative real-time PCR targeting RAGE expression, the same sample was used as the calibrator to assure comparability between the analyses of HMGB1 and RAGE relative expression patterns.

**Histological and immunohistochemical procedures.** All the formalin-fixed and paraffin-embedded samples were cut (2-4 μm) and stained with haematoxylin and eosin (HE) by standard histochemical procedures. Serial sections were dewaxed and antigen retrieval was performed by treatment with either microwave with citrate-buffer (Quartett, Berlin, Germany) (HMGB1; 20 min) or demasking solution (Quartett, Berlin, Germany) (RAGE; 20 min). The inhibition of endogenous peroxidase activity was achieved by immersion in 0.5% H2O2 (v/v) in methanol for 20 min. Non-specific binding was blocked with inactivated goat or horse serum diluted 1:5 in phosphate-buffered saline (PBS; pH 7.1). The blocking serum was drained and replaced by the first antibody rabbit polyclonal to HMGB1 (1:15,000; Abcam, Cambridge, UK) or goat polyclonal to RAGE (1:1200; Abcam). After washing, the tissue sections were incubated with a biotin-conjugated goat-anti rabbit IgG or horse-anti goat IgG (Vector Laboratories, Burlingame, CA, USA). The avidin-biotin-peroxidase

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reagent (Vector Laboratories) was used according to the manufacturer’s instructions including a tyramine amplification technique (26). The chromogen used was 3,3'-diaminobenzidine-tetrahydrochloride (Sigma Aldrich, Munich, Germany) 0.05% (w/v) with 0.03% H2O2 (v/v) as substrate in 0.1 M Tris-buffered saline (Tris-hydroxymethyl-aminomethane; Merck, Darmstadt, Germany) pH 7.6. The tissue sections were counterstained with Mayer’s haematoxylin and mounted.

Statistical analysis. Statistical analysis of the relative real-time PCR results applying the hypothesis test was performed with the relative expression software tool (REST) 2008, version 2.0.7 (27). REST determined the significance of differences in gene expression between the control and the neoplastic samples taking into account real-time PCR reaction efficiencies and using randomization techniques. The statistical analysis was performed separately for HMGB1 and RAGE within the four analysed tissue groups (liver, lymph node, lung, spleen) and the two different reference genes (GUSB, HPRT). A p-value of <0.05 was considered to be statistically significant.

Results

HMGB1 and RAGE real-time PCR analysis. Using GUSB as the endogenous control the median HMGB1 expression in liver samples was 0.829 (0.412-1.39) and 0.904 (0.216-1.47); expression in the lung non-neoplastic sample was 1.56 and in the tumour samples the median was 0.727 (0.577-0.995); in the lymph node samples the median was 1.10 (1-1.21) and 1.07 (1.02-1.11) and in the spleen samples median was 1.388 (0.76-2.07) and 0.96 (0.404-1.8) relative to the calibrator (lymph node sample), for the non-neoplastic controls and the tumour tissues, respectively (Figure 1 A). The median RAGE expression was 0.25 (0.08-0.51) and 0.53 (0.04-1.41) in the liver samples; expression in the lung non-neoplastic sample was 30.867 and in the tumour samples the median was 176.82 (0.161-530); the
median values 0.65 (0.3-1) and 0.31 (0.2-0.4) in the lymph node samples and 0.38 (0.09-0.67) and 0.29 (0.03-1.75) in the spleen samples for the non-neoplastic and tumour tissues, respectively (Figure 2A, 3A).

When HPRT was used as the endogenous control the median HMGB1 expression in liver samples was 0.507 (0.22-0.793) and 0.635 (0.22-1.13); expression in the lung non-neoplastic sample was 1.65 and in the tumour samples the median was 0.504 (0.349-0.647); in the lymph node samples median was 1.96 (0.923-1) and 0.582 (0.47-0.69) and in the spleen samples was 1.7 (1.6-1.8) and 0.671 (0.33-1.15) relative to the calibrator (lymph node sample), for the non-neoplastic controls and the tumour tissues, respectively (Figure 1B, 2B, 3B).

The statistical analysis showed a significant down-regulation of RAGE within the lung (p=0.000) tumour samples with both GUSB and HPRT as the endogenous controls. HMGB1 was significantly down-regulated in the lung tumour sample (p=0.000) using GUSB and the lymph node (p=0.000) and spleen (p=0.000) using HPRT compared to the non-neoplastic tissue.

**HMGB1 and RAGE immunohistochemistry.** In all the specimens, the neoplastic cells showed dot-like granular to finely stippled protein expression of HMGB1 predominantly localised within the cytoplasm (Figure 4A). The intensity of the cytoplasmic HMGB1 staining was quite variable within each tumour,
ranging from negative to strongly immunoreactive neoplastic cells. Faint nuclear expression was present in individual neoplastic cells of all the examined tissue samples (Figure 4 A), but the majority of cells had no nuclear expression.

In all non-neoplastic tissue samples, the immunohistochemical staining reaction was restricted to the nucleus (Figure 5).

In all the tumours, all the neoplastic cells showed dot-like granular to finely stippled cytoplasmic RAGE immunoreactivity with sparing of the nucleus (Figure 5A). Multifocally, cytoplasmic signals showed a disk-like formation with a clear central core. The intensity of the cytoplasmic RAGE staining was quite variable within each tumour, ranging from negative to strong. In two dogs, beside the cytoplasmic expression pattern an additional nuclear signal was detected in some tumour cells. In autolytic tumour tissues the RAGE expression was markedly diminished. In the normal tissue, cytoplasmic staining was present in some cell types (Figure 5 B). In the lymph nodes, individual immune cells also exhibited a slight nuclear staining.

### Discussion

RAGE was significantly down-regulated in the lung tumours compared to the non-neoplastic lung tissue independent of the housekeeping control gene while HMGB1 expression was down-regulated in the lung tumours only when using GUSB and in the lymph node and spleen tumour samples with HPRT. Although statistically the HMGB1/GUSB and HMGB1/HPRT results differed, the analysis indicated a trend towards decreased expression of HMGB1 in canine DHS. It is generally recommended to use more than one endogenous control when performing relative real-time PCRs to gain more reliable expression results (28). Furthermore, the number of tissue samples analysed in this study was small for powerful statistical analysis and further analyses of more samples is necessary.

The deregulation of RAGE and HMGB1 expression levels has been associated with many pathological states and neoplastic disorders such as pancreatic, prostate and colon cancer (29). In non small cell lung cancer (NSCLC), the expression of RAGE has been found to be strongly reduced...
(30) and correlated with higher tumour stage dependent of histological subtype, while the over expression of RAGE diminished tumour growth, also suggesting that RAGE down-regulation enhances lung cancer progression (31, 32). HMGB1 was also reported to be down-regulated in human NSCLC correlating with the tumour stage (33). Canine DHS might exhibit similar biological mechanisms in RAGE and HMGB1 expression as human NSCLC. However, the different origins of these tumours should be considered.

The present study also showed HMGB1 down-regulation in the lymph node and splenetic tumour samples compared to the controls. In contrast, in human and canine Lymphoma the overexpression of HMGB1 was described (34, 35) suggesting that different biological mechanism of HMGB1 expression might appear in the progression of histiocytic disorders in comparison to haematopoietic neoplasias. No current studies have reported HMGB1 expression patterns in spleen and tumours of the spleen.

In breast carcinoma, melanomas and interestingly also in NSCLC, dendritic cells were also found to be blocked in an immature or partially mature state (36-38) leading to disease progression. As HMGB1 and RAGE play a key role in the maturation of DCs, deregulation could influence the maturation status of DCs within neoplastic tissues. The down-regulation of HMGB1 and RAGE in lung and down-regulation
of HMGB1 in lymph node and spleen neoplastic samples, detected in the present study, could thus have an effect on the maturation of histiocytes in the affected tissues and the progression of canine DHS.

Immunohistochemically cytoplasmatic RAGE was detected in all the tumours and non-neoplastic samples with variable intensity comparable to the RAGE mRNA expression. Unexpectedly, a few tumour samples and individual immune cells in non-neoplastic lymph nodes exhibited slight nuclear immunoreactivity. Additionally, it seemed that the RAGE-antigen is sensitive to autolysis with reduced immunoreactivity. Similarly, high RAGE levels were found in hepatocellular, colorectal and breast carcinoma and the localisation was always cytoplasmatic (39).

The intensity of the cytoplasmic HMGB1 immuno-reactivity was also quite variable within each tumour and again was comparable to the findings for HMGB1 mRNA expression. Generally, in normal cells, HMGB1 shuttles actively between the nucleus and cytoplasm (40). When HMGB1 is underacetylated as it is in most cells, the protein appears predominantly or solely in the nucleus as in the non-neoplastic samples in the present study. Upon activation with lipopolysaccharide (LPS) or cytokines like extracellular HMGB1, macrophages and monocytes acetylate HMGB1, leading to the relocation and accumulation of acetylated-HMGB1 in cytoplasmic lysosomes which upon binding of lysophosphatidylcholine (LPC) secrete the protein into the extracellular environment (40, 41). DC maturation follows a similar path (18) and LCs in LCH correspond to an early activated stage of DC maturation, combining an immature phenotype with high level cytokine expression (23). Similar shuttling of HMGB1 between the nucleus and cytoplasm may occur in canine immune cells and might explain the cytoplasmatic immunoreactivity of the present neoplastic histiocytic cells.

The neoplastic samples in the present study were derived mostly from Bernese mountain dogs while the non-neoplastic samples were from dogs of several different breeds. Additionally, number of the tissue samples was limited, thus any correlation with age, sex or breed was not possible. More dogs should be included in further studies to examine the involvement of genes such as HMGB1 and RAGE in the pathology of DHS.

Conclusion

The down-regulation of RAGE and HMGB1 in lung, lymph node and spleen detected in canine DHS compared to control tissue samples might play a role in the progression of histiocytic disorders.

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