High-mobility Group B1 (HMGB1) and Receptor for Advanced Glycation End-products (RAGE) Expression in Canine Lymphoma

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Abstract. Background: Canine lymphoma is a commonly occurring neoplasia similar to human non-Hodgkin’s lymphoma and, thus, is used as a valuable model for human malignancy. HMGB1 and RAGE are strongly associated with tumour progression and vascularisation. Consequently, deregulated RAGE and HMGB1 may play an important role in the mechanisms involved in lymphoma progression. Materials and Methods: Expression patterns of HMGB1 and RAGE were analysed in 22 canine lymphoma and three canine non-neoplastic control samples via real time PCR and canine beta-glucuronidase gene (GUSB) as endogenous control. Results: HMGB1 was up-regulated in the neoplastic samples, while RAGE expression remained inconspicuous. Conclusion: This study demonstrated similar mechanisms in lymphoma progression in humans and dogs due to overexpression of HMGB1, which was described in human lymphomas. RAGE remained stable in terms of expression indicating that the extracellular HMGB1-induced effects are regulated by HMGB1 itself.

Human and canine tumours share many biological similarities including tumour progression, metastatic pattern and histology. Accordingly, these spontaneously occurring canine tumours represent a valuable model for several human neoplasias and may help to elucidate the biology of those neoplasias. Focusing on haematopoietic tumours, canine lymphoma is a commonly occurring neoplasm serving as an appropriate model for human non-Hodgkin’s lymphoma. The frequency of canine lymphoma cases among haematopoietic malignancies is approximately 83%, representing 7% to 24% of all canine neoplasms (1). The response of this malignancy to chemotherapy protocols varies substantially (2). Thus, the identification of factors involved in the formation and progression of lymphomas are of significant value for future development and evaluation of therapeutic approaches, providing benefits for both species.

During tumour progression, hypoxic and necrotic regions develop as a result of fast cell proliferation. To assure a sufficient supply of oxygen, solid and haematopoietic tumours activate cellular angiogenic mechanisms by secretion of pro-angiogenetic factors such as vascular endothelial growth factor (VEGF), tumour necrosis factor alpha (TNF-α) and interleukin-8 (IL-8) (3-5). In human haematopoietic malignancies, increased angiogenesis, measured by high vascular density levels, was observed in the lymph nodes of B-cell non-Hodgkin’s lymphoma and B-cell chronic lymphocytic leukaemia, as well as in bone marrow specimens from patients with childhood acute lymphoid leukaemia, acute myeloid leukaemia, chronic myelocytic leukaemia, myelodysplastic syndromes and idiopathic myelofibrosis (6-12). VEGF plays an important role in this process as a master regulator of the angiogenic switch. Sustained angiogenesis, measured by high vascular density levels, was observed in the lymph nodes of B-cell non-Hodgkin’s lymphoma and B-cell chronic lymphocytic leukaemia, as well as in bone marrow specimens from patients with childhood acute lymphoid leukaemia, acute myeloid leukaemia, chronic myelocytic leukaemia, myelodysplastic syndromes and idiopathic myelofibrosis (6-12).

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angiogenic and neo-vascularising effects (16). In response to angiogenic and inflammatory signals, HMGB1 is passively released by necrotic cells or actively secreted by activated macrophages. HMGB1 signalling itself is mediated via the receptor for advanced glycation end-products (RAGE) and toll-like receptors (TLR) such as TLR-2 and TLR-4. Activation of these receptors results in activation of nuclear factor kappa B (NFκB), among other factors, which itself acts as a transcriptional enhancer for RAGE, pro-inflammatory cytokines and the pro-angiogenic factor VEGF (17). Due to the positive feedback loop caused by activation of NFκB, a sustained inflammation or angiogenic reaction contributes to disease progression and, in the case of tumour development, to uncontrolled growth and metastasis (17). An in vitro study by Sasahira et al. (18) showed that the HMGB1/RAGE complex induces VEGF expression through the activation of NFκB in two human oral squamous cell carcinoma cell lines. The abrogation of the HMGB1-mediated effect was observed by down-regulation of RAGE expression via application of antisense 5'-oligodeoxynucleic acid, resulting in a significantly lower VEGF secretion (18).

In this context, a study by Wolfsberger et al. (19) examined the expression pattern of VEGF in canine lymphomas, demonstrating a high expression of VEGF in 60% of the analysed tumours. However, studies in human neoplasias focusing on RAGE and HMGB1 expression levels showed a significant up-regulation of RAGE and HMGB1 in pancreatic cancer, prostate cancer and colon cancer (20). In summary, the interaction of HMGB1, RAGE and VEGF is strongly associated with vascularisation and plays a key role in tumour progression. Consequently, deregulation of RAGE and HMGB1 expression may play a key role in lymphoma progression. To elucidate this, the present study analysed the expression patterns of the previously characterized canine HMGB1 and RAGE genes (21-23) in a set of canine lymphomas and controls.

Materials and Methods

Tissue samples. Fine-needle aspirates of enlarged lymph nodes of 22 lymphoma-bearing dogs (19 multicentric and three intestinal lymphomas) and three inconspicuous lymph node were examined. The control lymph node samples were derived from dogs diagnosed with diseases other than haematopoietic neoplasia and with clinically unaltered peripheral lymph nodes. All diagnoses were cytoglogically or histologically confirmed. Clinical staging was performed according to the World Health Organization (WHO) clinical staging system (24) and determination of the immunophenotype of the 19 multicentric lymphomas was performed by flow cytometry (25). Among the 19 multicentric lymphomas, 15 samples showed B-cell origin, two samples were of T-cell origin and two samples could not be determined. The dogs represented 13 different breeds, namely Beagle, Dogo Argentino, German Shepherd, Golden Retriever, Hovawart, Jack Russell Terrier, Jagdterrier, Mixed-breed, Munsterlander, Pitbull, Rottweiler, Teckel and West Highland. After collection, the samples were immediately frozen in liquid nitrogen and stored at −80°C until RNA isolation. All samples were provided by the Small Animal Clinic, University of Veterinary Medicine, Hannover, Germany.

RNA isolation and cDNA synthesis for transcript characterisation. The tissue samples were homogenised using the iron-beads QIAshredder homogeniser method (Qiagen, Hilden, Germany). Following, total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. To avoid genomic DNA contaminations, on-column DNase digestion with the RNase-Free DNase set (Qiagen) was performed.

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

Quantitative real-time RT-PCR. Relative real-time PCRs were performed with the Applied Biosystems 7300 real-time PCR System (Applied Biosystems, Darmstadt, Germany). The canine Glucuronidase Beta transcript was chosen as endogenous control (26). Two μl of each cDNA corresponding to 25 ng of total RNA was amplified in a total volume of 25 μl using universal PCR Mastermix (Applied Biosystems) with 600 nM of each primer and 200 nM fluorogenic probe. The following PCR conditions were applied: 2 min at 50°C and 10 min at 95°C, followed by 45 cycles with 15 s at 95°C and 1 min at 60°C. Primer and probe sequences were as follows: HMGB1 forward primer: 5’AAATGGAGGCCAGACGGG3’, HMGB1 reverse primer: 5’TCCTTTGCCCATGTCTATTTAAATATTTTC3’, HMGB1 probe: 5’6-FAM-CTGGGCGACTCTGTGCCTCGCT-TAMRA3’, RAGE forward primer: 5’GTCTGTGGGGAGCAGTAGTAGG3’, RAGE reverse primer: 5’TTCATCACTGCGACAGTGCAG3’, RAGE probe: 5’GGTGGCGCTTCTCACAAGTGA-TAMRA3’, GUSB forward primer: 5’TGCATCTCAGTTGTATCTC3’, GUSB reverse primer: 5’CTGCCACATGGACCCCATCC3’, GUSB probe: 5’6-FAM-CCGCCACTACTATGCCATCGTGTG-TAMRA3’.

All samples were measured in triplicate and non-template controls and non-reverse transcriptase control reactions were included for each run.

A precedent absolute real-time PCR reaction was carried out with all PCR assays using the same templates and dilution steps in order to ensure the comparability between the PCR reactions showing similar amplification efficiencies appropriate for relative quantification PCRs.

For the analysis based on the ΔΔCT method, the sample within the control group with the most stable CT values for target and endogenous control was defined as the calibrator for the analysis of RAGE/GUSB as well as HMGB1/GUSB relative real-time PCR.

Statistical analysis. Statistical analysis of the relative real-time PCR results applying various hypothesis test was performed with the software REST 2008, version 2.0.7 (27). REST determines whether there is a significant difference between samples and controls taking into account reaction efficiencies and using randomisation techniques. Regarding clinical parameters, a Mann-Whitney-test was performed using SPSS 15.0 statistic software (SPSS Inc., Chicago, IL, USA). The HMGB1 and RAGE expression levels were evaluated for statistical significance regarding WHO substage (substage a vs. substage b), WHO stage (clinical stage III and IV vs. V), and, additionally, for the comparison of multicentric lymphomas vs. control dogs and intestinal lymphomas. A p-value <0.05 was considered to be statistically significant.
Table I. Hypothesis tests of relative HMGB1/GUSB and RAGE/GUSB real-time PCR results in canine lymphoma using the REST software. A total of 25 samples with 3 control and 22 tumour samples (3 intestinal lymphomas, 15 B-cell lymphomas, 2 T-cell lymphomas and 2 of unknown origin) were analysed. A p-value <0.05 was considered statistically significant.

<table>
<thead>
<tr>
<th>Hypothesis test:</th>
<th>Expression pattern of HMGB1 compared to control group (p-value)</th>
<th>Expression pattern of RAGE compared to control group (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group vs. tumour group</td>
<td></td>
<td></td>
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<tr>
<td>All tumour samples (n=22)</td>
<td>Up-regulation (p=0.001)</td>
<td>Not significantly different (p=0.155)</td>
</tr>
<tr>
<td>Intestinal lymphoma (n=3)</td>
<td>Up-regulation (p=0.030)</td>
<td>Not significantly different (p=0.096)</td>
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<td>B-cell lymphoma (n=15)</td>
<td>Up-regulation (p=0.001)</td>
<td>Not significantly different (p=0.07)</td>
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<tr>
<td>T-cell lymphoma (n=2)</td>
<td>Up-regulation (p=0.033)</td>
<td>Not significantly different (p=0.792)</td>
</tr>
<tr>
<td>Unknown multicentric lymphoma (n=2)</td>
<td>Not significantly different (p=0.135)</td>
<td>Not significantly different (p=0.303)</td>
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</table>

Results

Expression analyses of HMGB1 and RAGE in canine lymphomas were performed using relative real-time PCR on lymph node aspirates from 22 lymphoma-bearing dogs and three dogs without haematopoietic neoplasias. Both real-time reactions were analysed using the ΔΔCT method and the results are shown in Figures 1 and 2. In both graphs, the same samples were put in the same position to compare the tumour samples directly for both candidate genes. The HMGB1 expression (Figure 1) quotient in the tumour samples varied from 1.587 (sample 7) to 8.549 (sample 3) relative to the defined calibrator. The median expression levels were 1.33 for the control group (samples 1-3), 4.22 for the intestinal lymphoma group (samples 4-6), 4.26 for the B-cell lymphoma group (samples 7-21), 3.83 for the T-cell lymphoma group (samples 22-23) and 3.13 for the unknown lymphoma group (samples 24-25). In the case of RAGE (Figure 2), the relative quotients varied from 0.36 (sample 10) to 18.282 (sample 3). The median expression levels were 0.55 for the control group (samples 1-3), 6.69 for the intestinal lymphoma group (samples 4-6), 1.73 for the B-cell lymphoma group (samples 7-21) and 0.56 for the T-cell lymphoma group (samples 22-23) and 1.61 for the unknown lymphoma group (samples 24-25).

Samples 6 (intestinal lymphoma), 15, 20 and 21 (B-cell lymphoma) showed the highest RAGE expression levels and, accordingly, in samples 6, 20 and 21 high HMGB1 expression levels were also detected. Samples 20 and 21 had the highest HMGB1 expression within the group of B-cell lymphomas and sample 6 showed the highest expression of HMGB1 in the intestinal lymphoma group. Sample 15 showed an average HMGB1 expression level.

Hypothesis tests of the relative real-time PCR results were performed using the REST software. Analysis of expression of control samples vs. intestinal lymphoma showed a highly significant up-regulation of HMGB1 (p=0.001), while RAGE showed no significant expression value (p=0.155). The test was also performed for all subgroups within the sample collective (intestinal lymphoma, B-cell lymphoma, T-cell lymphoma and unknown multicentric lymphoma). Comparison of the control samples vs. intestinal lymphomas revealed a significant difference for HMGB1 (p=0.03) but not for RAGE (p=0.096). B-Cell lymphomas were significantly different to the control samples for HMGB1 (p=0.001) but not different for RAGE (p=0.07). Compared to the control samples, T-cell lymphomas showed significant up-regulation of HMGB1 expression (p=0.033) but no significant difference of RAGE expression (p=0.792). The group of multicentric lymphoma samples, which were unable to be determined by flow cytometry, showed no statistically significant difference in the expression of HMGB1 (p=0.135) nor RAGE (p=0.303) in comparison to the control group. The results of the statistical analysis using the REST programme are displayed in Table I.

The Mann-Whitney test regarding the difference in the expression levels between the multicentric lymphomas and the control samples revealed significant differences for HMGB1 (p=0.003) but not for RAGE (p=0.078). The expression of HMGB1 and RAGE also showed no significant differences between multicentric and intestinal lymphomas (p=0.907 for HMGB1 and p=0.702 for RAGE). WHO stage and substage of the samples were not significantly associated with HMGB1 and RAGE expression values (results not shown).

Discussion

Vascularisation under normal conditions is strongly regulated by a delicate balance of pro- and anti-angiogenic molecules. In the case of tumour progression, sustained secretion of pro-angiogenic factors such as HMGB1, RAGE and VEGF is triggered by tumour microenvironmental hypoxic and necrotic areas leading to neo-vascularisation. Regarding haematological malignancies, increased vascularity was observed in B-cell non-Hodgkin’s lymphoma (9) and high serum levels of VEGF were associated with poor outcome (14). In this context, a study targeting canine lymphomas showed high levels of VEGF in the analysed tumour samples, indicating an involvement of pro-angiogenic factors in lymphoma progression (19, 28).
Figure 1. Relative HMGB1/GUSB expression in canine lymphoma. A total of 25 samples were analysed. The set of samples consisted of 3 control and 22 tumour samples of the following subgroups: 3 intestinal lymphomas, 15 B-cell lymphomas, 2 T-cell lymphomas and 2 of unknown origin. Green bars: lymph node control; orange bars: intestinal lymphoma; red bars: B-cell lymphoma; brown bars: T-cell lymphoma and grey bars: lymphomas of unknown origin.

Figure 2. Relative RAGE/GUSB expression in canine lymphoma. A total of 25 samples were analysed. The set of samples consisted of 3 control and 22 tumour samples of the following subgroups: 3 intestinal lymphomas, 15 B-cell lymphomas, 2 T-cell lymphomas and 2 of unknown origin. Green bars: lymph node control; orange bars: intestinal lymphoma; red bars: B-cell lymphoma; brown bars: T-cell lymphoma and grey bars: lymphomas of unknown origin.
These findings raise the question whether RAGE and HMGB1 are also deregulated in lymphomas. For several other neoplasias such as pancreatic, prostate and colon cancer deregulation of the expression of HMGB1 and RAGE has been described (20).

Therefore, the present study examined the expression of HMGB1 and RAGE in lymph node samples of canine lymphomas in comparison to lymph nodes from dogs without haematopoietic neoplasias. The tumour samples were cytologically and histologically confirmed and flow cytometry determined the immunophenotype of the multicentric lymphomas. The present study showed a significant up-regulation in HMGB1 expression in the analysed lymphoma samples, while the detected RAGE expression did not change significantly when compared to the control samples. Furthermore, statistical analyses were performed not only with all control and all tumour samples together as groups, but also for the different subgroups of the sample collective. The sample numbers of intestinal, T-cell and unknown lymphomas were too small for an appropriate powerful statistical analysis (n=3, 2 and 2, respectively) and, consequently, more samples of both non-neoplastic and tumour types would be necessary for further analyses. However, the results of the present study indicated a trend of the expression pattern of HMGB1 and RAGE in this lymphoma types.

Meyer et al. (29) examined the expression of HMGB1 in human non-Hodgkin lymphoma using real-time PCR and showed high levels of HMGB1 expression, while RAGE expression was not analysed. The HMGB1 overexpression detected in the canine lymphoma samples strongly emphasises that similar HMGB1-related mechanisms exist in canine and human lymphoma progression. Furthermore, in canine lymphomas, elevated HMGB1 serum levels were detected and the prognostic value of initial and sequential serum level was suggested (30).

The RAGE expression data determined herein indicated that the HMGB1-induced effects in all 22 analysed lymphoma samples are regulated by the overexpression of HMGB1 itself, while RAGE, as a receptor, remains stable in terms of expression. Using tumour tissue microarray slides, a study by Hsieh et al. (31), detected a borderline positive staining for RAGE antibodies in lymphoma tumours, suggesting that only certain cells or vessels on the tissue disc were stained positive, thus, indicating low levels of RAGE in the tumour slides analysed; however, that finding was not discussed further in detail by the authors. Besides the present study, there have been no significant data published concerning RAGE expression in lymphomas. In summary, the data of the present study showed significant deregulation of HMGB1 but not RAGE in canine lymphoma, contradictory to the observations in many solid tumours. However, the TLR receptors 2 and 4 also participate in the complex signalling pathways induced by HMGB1 and, thus, should be considered with regard to lymphoma development and progression. Accordingly, recent studies concerning the expression of TLR receptors showed associations of expression/overexpression of these receptors in gastric carcinoma, colon cancer and breast cancer (32). Additionally, gene polymorphisms in TLR-2 and -4 gene sequences have been hypothesised as possible contributors to follicular lymphoma and mucosa-associated lymphoid tissue lymphoma (33).

The analysed samples were of 13 different dog breeds and some breeds show higher incidences in development of malignant lymphomas; for example, the German Shepherd, Beagle and Golden Retriever breeds (34). In the present study, the two highest values for HMGB1 in B-cell lymphomas were detected in a Beagle and a German Shepherd.

In conclusion, pathways leading to vascularisation and tumour progression are described by the complex interaction of all involved factors including their interactions on DNA and protein level. In the present study, it should be noted that an up-regulated HMGB1 signalling was mediated not only by RAGE, but also by TLR-2 and -4 and the extensive interactions between all factors. Thus, elucidating whether the interaction between HMGB1 and its respective receptors plays a key role in the mechanisms involved in lymphoma development and progression will be of significant value for the development of therapeutic approaches for the dog as patient and as a model system for human non-Hodgkin’s lymphoma.

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