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*Original article*

# Density of tumor-associated macrophages (TAMs) and expression of their growth factor receptor MCSF-R and CD14 in canine mammary adenocarcinomas of various grade of malignancy and metastasis

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## Abstract

Several years ago, the presence of macrophages in the tumor microenvironment was thought to be an inflammatory response to kill the cancer cells. Now, this is clear that the inflammatory cells that exit blood vessels and migrate to the tumor tissue play an important role in cancer progression. Various cells present in the tumor microenvironment enhance cancer growth and invasiveness by secretion of tumor-enhancing products. That is why tumors should not be treated as only aggregates of cancer cells but as separate structures. Macrophages form a major component of the inflammatory infiltration in tumors, where they are termed tumor-associated macrophages (TAMs).

To the best of our knowledge, up-to-date there were no studies on tumor associated macrophages and the role of the tumor microenvironment in tumor invasion/metastasis in dogs. This is the first study performed to assess if the number of TAMs and expression of MCSF-R (macrophages colony stimulating factor receptor) and CD14 (LPS co-receptor) are associated with the grade of tumor malignancy and its ability to metastasize. We have performed immunohistochemical analysis of 50 canine mammary adenocarcinomas of various grade of malignancy (1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup>) and tumors that gave local or distant metastases.

The results indicate that in dogs, similarly to humans and mice, the number of tumor associated macrophages is related to the cancer ability to metastasize. Our results also indicate that the expression of MCSF-R and, what is particularly new finding, CD14 is associated with tumor malignancy and its ability to metastasize. Hence, these molecules play a role in tumor progression, metastasis and microenvironment interactions. These results show that in dogs we should treat the tumor as a whole organ rather than just try to eliminate the cancer cells.

**Key words:** tumor associated macrophages, CSF-1R, MCSF-R, CD14, mammary adenocarcinoma, tumor microenvironment, immunology, dog

## Introduction

Extensive studies over the last few decades have led to understanding how a normal cell acquires unlimited potential to proliferate, avoids apoptosis and acquires ability to invasion and metastasis. However, it has become apparent that these mutations are not sufficient for cells to gain a really invasive phenotype, so investigators have focused on the tumor microenvironment (for review, see Hanahan et al. 2000) to explain its all interactions.

Several years ago, research in the field of the tumor microenvironment has focused on cytotoxic properties of inflammatory cells. The presence of macrophages in malignant tumors has been well documented, but the significance of this phenomenon was still an open question (Hauptmann et al. 1993). Now this is clear that inflammatory cells that exit blood vessels and migrate to the tumor tissue play an important role in cancerogenesis and cancer progression. This allows to explain why so many cancers arise from the sites of chronic irritation and inflammation. However, the influence of these cells on modulation of cancer cell biology has not been yet entirely recognized (Martins-Green et al. 1994). The microenvironment is composed of various cells depending on the tumor type. For example, in breast cancer there are adipocytes of the mammary fat, fibroblasts, hematopoietic cells, as well as newly formed blood vessels. All these cells probably enhance cancer growth and invasiveness by the secretion of tumor-enhancing products. That is why tumors should not be treated as only cancer cells but as separate structures or even organisms, in which the malignant cells “corrupt” normal cells to promote their survival, growth and invasion. This is termed “immunoeediting” or “immunosculturing” (Lewis and Pollard 2006, Pollard 2008).

Macrophages form a major component of the inflammatory infiltration in tumors (Bingle et al. 2002), where they are termed tumor-associated macrophages (TAMs). They probably play a main role in tumor cell invasion into surrounding tissues, survival, intravasation and metastasis (for review, see Pollard 2008). Their recruitment and “corruption” by tumor cells is caused by many tumor-secreted chemoattractants like: colony stimulating factor-1 (CSF-1 alias M-CSF, macrophages colony stimulating factor), vascular endothelial growth factor (VEGF) and chemokines such as: CCL2 – CCL5, and CCL8. The onset and maintenance of tumor angiogenesis is probably also partially driven by macrophages (Pollard 2008). Clinical studies have shown a correlation between the number of TAMs and poor prognosis for many cancers in humans (e.g. breast, prostate, ovarian, cervical, endometrial, oesophageal, urinary bladder). TAMs are also associated with increased angiogenesis and meta-

stasis in cancer tissues (Jadus et al. 1996, Galon et al. 2006, 2007, Bij et al. 2008, Kim et al. 2008).

To the best of our knowledge, up-to-date there were no studies on tumor associated macrophages and the role of the tumor microenvironment in cancer invasion and metastasis in dogs. In our previous studies on gene expression in canine mammary cancer cell lines, we have documented that cell lines with metastatic potential show higher expression of chemokines involved in tumor microenvironment modulation and macrophages migration like SEMA or IL-8 (Król et al. 2010). Therefore, we decided to investigate if the interactions within tumors in dogs are similar to those reported in humans and mice. This is the first study in this field performed to asses if the number of TAMs and expression of MCSF-R and CD14 is associated with grade of tumor malignancy and tumors ability to metastasize. We have performed immunohistochemical analysis of 50 canine mammary adenocarcinomas of various grade of malignancy (1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup>) and tumors that gave local or distant metastases.

## Materials and Methods

### Tissue samples

Tissue sections from canine mammary tumors were obtained from the archives of the Department of Pathology and Veterinary Diagnostics, Faculty of Veterinary Medicine, Warsaw University of Life Sciences – WULS (Poland). The samples were surgically obtained during the mastectomy from 50 female dogs of different breeds. Each tumor sample was fixed in 8% neutral buffered formalin and routinely embedded in paraffin. The 5 micrometer sticks were fixed on the slides and stained with hematoxylin and eosin (HE) and subjected to the histological evaluation. The wide history of the patients is known including the information about the presence/absence of metastases. The tumors that gave metastases were surgically removed together with the metastatic site and the presence of neoplastic cells in this site was histologically confirmed. The immunohistochemical examination of cytokeratin, vimentin, actin, s100 protein and p63 protein expression was assessed (data not shown). The tumor types of specimens were classified based on the World Health Organization (WHO) Histological Classification and Mammary Tumors of the Dog and Cat classification (Misdorp et al. 1999). Histological tumor grading was performed on HE-stained sections using a Misdorp (2002) classification. All the tumors examined were classified as adenocarcinomas. The mammary carcinoma grading was assessed considering tubule formation, degree of differentiation and mitotic index as: the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> grade of malignancy. Among 50 mammary tumors, 19 were con-

sidered as the 1<sup>st</sup> grade of malignancy, 9 tumors as the 2<sup>nd</sup> grade of malignancy, 11 tumors as the 3<sup>rd</sup> grade of malignancy; and 11 of the tumors gave local or distant metastases (these tumors were of the 2<sup>nd</sup> and 3<sup>rd</sup> grade of malignancy).

### Immunohistochemistry

Five  $\mu\text{m}$  sections from paraffin blocks containing tumor tissue were baked in 37°C overnight. After dewaxing in xylene and rehydration in ethanol, for antigen retrieval, the slides were placed in 0.02 M citrate buffer, pH 6.0 and boiled in the decloaking chamber. The samples were incubated in the Peroxidase Blocking Reagent (Dako, Denmark) for 10 min at room temperature prior to the antibody incubation. After 30 min incubation in 5% bovine serum albumin (Sigma Aldrich, Germany), the following primary antibodies were used (diluted in 1% bovine serum): mouse monoclonal MAC387 (Dako), rabbit polyclonal MCSF Receptor antibody (other designations: MCSF-R or CSF-1R) and rabbit polyclonal CD14 antibody (both obtained from Abcam, United Kingdom). According to the manufacturer's instructions the slides were incubated for 1 hour with the primary MAC387 antibodies at room temperature and at +4°C with MCSF-R and CD14 antibodies overnight. For the staining the EnVision kit (Dako) was used (Labelled Polymers consist of secondary anti-mouse or anti-rabbit antibodies conjugated with the Horseradish peroxidase HRP enzyme complex). To develop the colored product, the 3,3'-Diaminobenzidine (DAB) substrate was used. Finally, the hematoxyline was used for nuclei counterstaining.

For each immunohistochemical experiment, two controls (negative and positive) were performed: as a positive control the tissues of canine lymph nodes and tonsils were used (all antibodies were specific to the macrophages only), whereas the negative control constituted the staining without use of primary antibodies.

### Data analysis

Three consecutive tissue sections were stained and used to the analysis. The 10-20 pictures of each slide were taken (depending on the sample size) using Olympus microscopy BX60. To avoid analysis of inflammatory cells due to necrosis, we examined only cancer cells and stroma where residue tumor-associated macrophages. The colorimetric intensity of the IHC-stained antigen spots (brown color) was counted by a computer-assisted image analyzer (Olympus Microimage™ Image Analysis, software version 4.0

for Windows, USA). The antigen spot color intensity is expressed as a mean pixel optical density on a 1-256 scale.

The statistical analysis was performed using Prism version 3.00 software (GraphPad Software, USA). The ANOVA and Tukey HSD (Honestly Significant Difference) post-hoc test were applied.  $P \leq 0.05$  was regarded as significant whereas  $P \leq 0.01$  and  $P \leq 0.001$  as highly significant.

## Results

### TAMs density demonstrated as MAC387 expression is higher in the metastatic tumors

The MAC387 antigen is specific for macrophages. In the slides examined, the expression of MAC387 was specifically found only in the macrophages, but not in the cancer tissue. Analysis of MAC387 expression (Fig. 1a) showed its higher expression in the group consisted of 11 canine mammary adenocarcinomas that gave local or distant metastases. The mean optical density measured in these tumor samples (reflecting the number of macrophages in the slides examined) was 111.46 (SD = 7.24) what differed highly significantly ( $p < 0.001$ ) from the samples of other tumor groups (Fig. 1b). The mean optical density of the other tumor groups: adenocarcinomas of the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> grade of malignancy were: 76.35 (SD = 13.23), 75.08 (SD = 11.15) and 76.31 (SD = 11.09), respectively. The significant differences between these three groups were not found (Fig. 1b).

### MCSF-R expression is related to the tumor grade of malignancy and its ability to metastasize

The expression of MCSF-R was found in macrophages as well as in the cancer tissue (Fig. 2a). The highest expression of this antigen was found in the tumors of the 3<sup>rd</sup> grade of malignancy (140.79; SD = 6.13) and in the metastatic group (137.24; SD = 6.11). The lowest expression of MCSF-R was found in the tumors of the 1<sup>st</sup> grade of malignancy (117.44; SD = 8.4). The mean optical density related to MCSF-R expression of the tumors of the 2<sup>nd</sup> grade of malignancy was 126.33 (SD = 7.69). The significant difference was found between MCSF-R expression in tumors of the 1<sup>st</sup> and 2<sup>nd</sup> grade of malignancy ( $p < 0.05$ ). The mean optical density related to MCSF-R expression in tumors of the 1<sup>st</sup> and 2<sup>nd</sup> grade of malignancy differed from the metastatic/3<sup>rd</sup> grade of malignancy groups ( $p < 0.001$  and  $p < 0.01$ , respectively) (Fig. 2b).

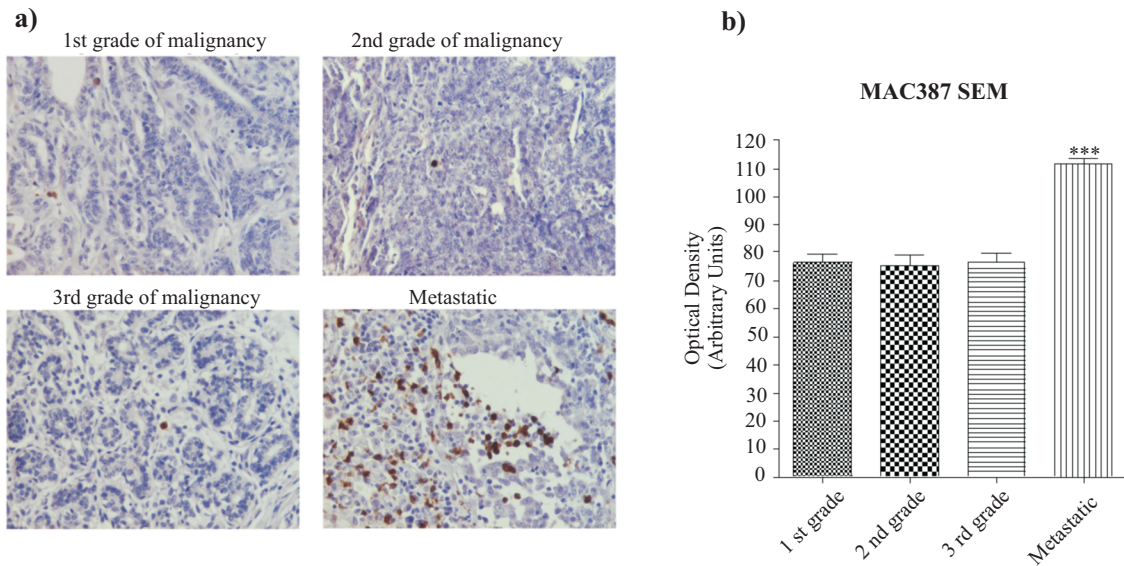


Fig. 1. a) Pictures of tumor associated macrophages in canine mammary adenocarcinomas of the 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup> grade of malignancy and tumors that gave local/distal metastases (n = 50) obtained with Olympus BX60 microscope (x200). The macrophages specific MAC387 antigen is represented by brown colored structures. b) The graph of mean optical density (and SEM) of MAC387 antigen in canine mammary adenocarcinomas of the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> grade of malignancy and in the group of tumors that gave local or distal metastases (n = 50). Ten to 20 pictures in each slide were analyzed. The colorimetric intensity of the IHC-stained antigen spots was counted by a computer-assisted image analyzer (Olympus Microimage, Image Analysis, software version 4.0 for Windows, USA) and the antigen spot color intensity is expressed as mean pixel optical density on a 1-256 scale. The statistical analysis was performed using Prism version 3.00 software (GraphPad Software, USA). The ANOVA and Tukey HSD (Honestly Significant Difference) post-hoc tests were applied.  $P \leq 0.001$  was regarded as highly significant and marked as \*\*\*.

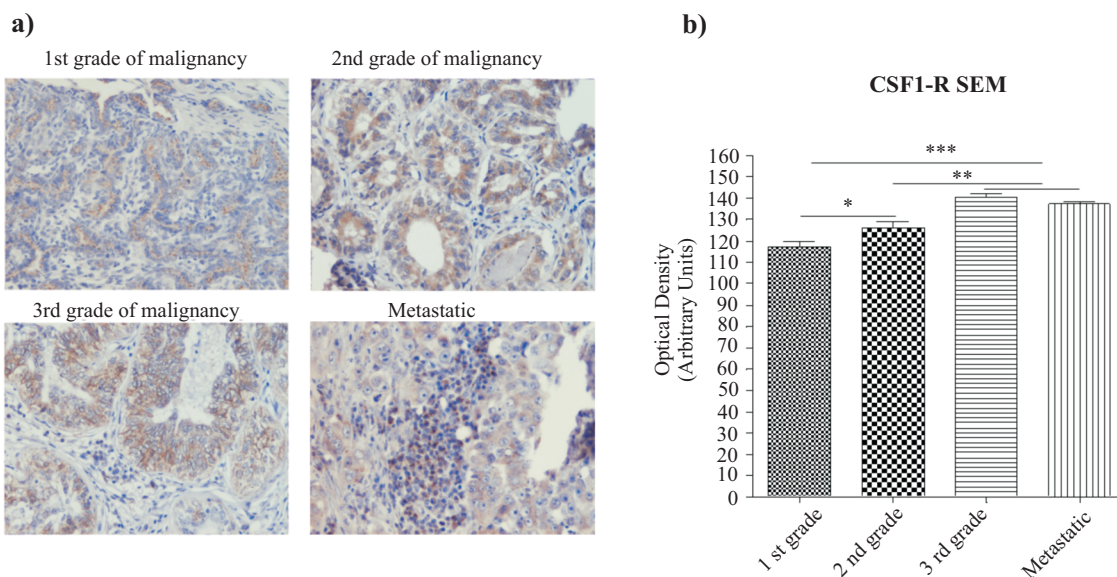


Fig. 2. a) Pictures of M-CSF-R in canine mammary adenocarcinomas of the 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup> grade of malignancy and tumors that gave local/distal metastases (n = 50) obtained using Olympus BX60 microscope (x200). The M-CSF-R antigen is specific for macrophages and cancer tissue and it is reflected as brown color. b) The graphs of mean optical density (and SEM) of M-CSF-R antigen in canine mammary adenocarcinomas of the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> grade of malignancy and in the group of tumors that gave local or distal metastases (n = 50). Ten to 20 pictures of each slide were analyzed. The colorimetric intensity of the IHC-stained antigen spots was counted by a computer-assisted image analyzer (Olympus Microimage™ Image Analysis, software version 4.0 for Windows, USA) and the antigen spot color intensity is expressed as mean pixel optical density on a 1-256 scale. The statistical analysis was performed using Prism version 3.00 software (GraphPad Software, USA). The ANOVA and Tukey HSD (Honestly Significant Difference) post-hoc tests were applied.  $P \leq 0.05$  was regarded as significant and marked as \*; whereas  $P \leq 0.01$  and  $P \leq 0.001$  as highly significant and marked as \*\* and \*\*\*, respectively.

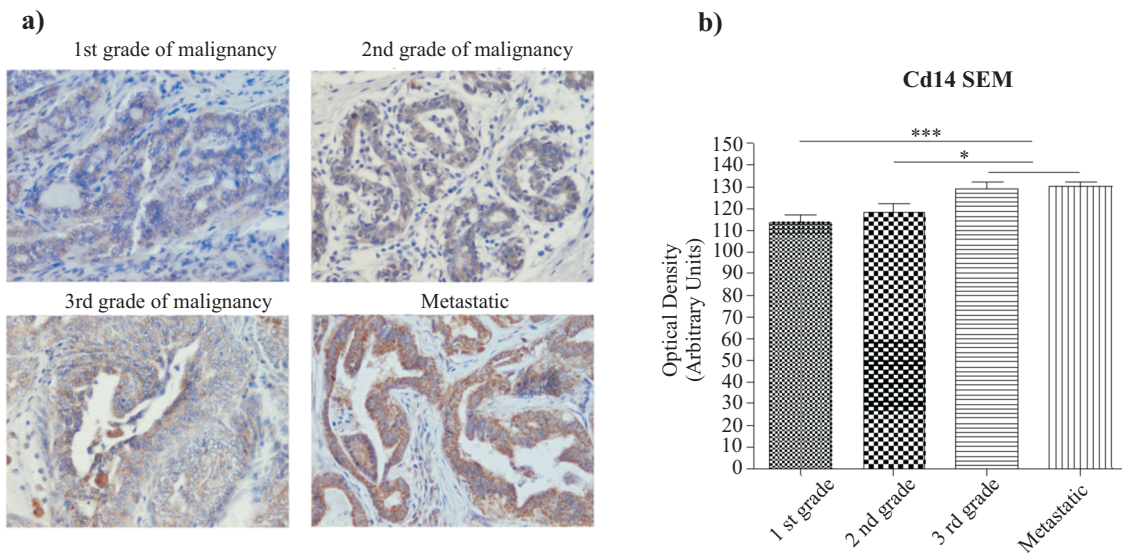


Fig. 3. a) Pictures of CD14 in canine mammary adenocarcinomas of the 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup> grade of malignancy and tumors that gave local/distal metastases (n = 50) obtained using Olympus BX60 microscope (x200). Surprisingly, the CD14 antigen was specific not only for the monocytes/macrophages, but also for the cancer tissue (reflected as brown color). The colorimetric intensity of the IHC-stained antigen spots was counted by a computer-assisted image analyzer (Olympus Microimage™ Image Analysis, software version 4.0 for Windows, USA) and the antigen spot color intensity is expressed as mean pixel optical density on a 1-256 scale. b) The graphs of mean optical density (and SEM) of CD14 antigen in canine mammary adenocarcinomas of the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> grade of malignancy and in the group of tumors that gave local or distal metastases (n = 50). Ten to 20 pictures of each slide were analyzed. The colorimetric intensity of the IHC-stained antigen spots was counted by a computer-assisted image analyzer (Olympus Microimage™ Image Analysis, software version 4.0 for Windows, USA) and the antigen spot color intensity is expressed as mean pixel optical density on a 1-256 scale. The statistical analysis was performed using Prism version 3.00 software (GraphPad Software, USA). The ANOVA and Tukey HSD (Honestly Significant Difference) post-hoc tests were applied.  $P \leq 0.05$  was regarded as significant and marked as \*; whereas  $P \leq 0.01$  as highly significant and marked as \*\*\*.

### Expression of CD14 is related to the grade of tumor malignancy and its ability to metastasize

Surprisingly, the expression of CD14 (LPS co-receptor) which is thought to be a monocyte/macrophage marker was found not only in macrophages but also in the cancer tissue (Fig. 3a). This finding is very interesting, as there is no information in the literature about its expression in cancer cells. Moreover, we found that its expression is related to the grade of tumor malignancy and its ability to metastasize. The highest expression of this antigen was found in metastatic group (130.33; SD = 8.0) and in tumors of the 3<sup>rd</sup> grade of malignancy (129.41; SD = 10.94). The lower expression of CD14 was found in the tumors of the 1<sup>st</sup> grade of malignancy (113.86; SD = 10.78) and in the tumors of the 2<sup>nd</sup> grade of malignancy (118.34; SD = 12.15). The mean optical density of CD14 antigen differed highly significantly ( $p < 0.01$ ) between tumors of the 1<sup>st</sup> grade of malignancy and metastatic/3<sup>rd</sup> grade of malignancy groups. The mean optical density of tumors of the 2<sup>nd</sup> grade of malignancy differed significantly from the metastatic/3<sup>rd</sup> grade of malignancy groups ( $p < 0.05$ ) (Fig. 3b).

### Discussion

The role of tumor associated macrophages in the regulation of metastasis is evident. High number of TAMs in primary tumors is correlated with metastasis of various tumor types (Leek et al. 1996, Hanada et al. 2000). TAMs probably influence the disaggregation of metastatic cells from the primary tumor, intravasation and the development of secondary tumors at distant sites. The escape of tumor cells from the main tumor mass and the intravasation of cancer cells into blood vessels usually occurs in areas of the highest accumulation of macrophages. TAMs promote cancer metastasis through several mechanisms: (1) promotion of angiogenesis (via IL-6, IL-8, MIF, VEGF, TNF- $\alpha$  and CSF-1), (2) induction of tumor growth (via IL-6, EGF, MIF) and (3) enhancement of tumor cell migration and invasion (via proteases; MMP1, TIMP1, ICAM1, Wnt5) (Lewis and Pollard 2006). These findings are complement with those suggesting the presence of correlation between the number of macrophages in the tumor stromal compartment and the metastatic potential of the cancer cells (Ohno et al. 2004). Bingle et al. (2002) revealed that in 80% of

tumor cases examined, an increased number of macrophages was associated with poor patient prognosis. Also other authors found macrophages density as an independent predictor of poor outcome (Dave et al. 2004, Farinha et al. 2005). Dr. Jeffrey Pollard, who is a “father” of the tumor microenvironment field, described microarray results of the follicular lymphoma which indicated that a gene expression signature characteristic for macrophages was an independent predictor of poor outcome (Pollard 2008). This information closely correlates to the results of our experiment. We found that the density of TAMs (showed as a mean optical density of MAC387 antigen) in canine mammary adenocarcinomas is significantly higher in the that gave metastases than in the tumors that did not metastasize (Fig. 1a,b). We did not find any differences in the density of macrophages between the various adenocarcinomas of different grade of malignancy. We assume that macrophages in canine mammary carcinomas may play a role in metastatic process, but not in tumor malignancy. It correlates well with clinical studies showing that increased number of macrophages in the tumor is associated with poor patient survival because of metastases (Oberge et al. 2002). However this relationship has been established in humans and mice, but up-to-date it has not been investigated in dogs.

There are some factors responsible for the migration of macrophages to the tumor mass and their “immunoediting”. The most important factor is CSF (colony stimulating factor, termed also CSF-1 or MCSF), which is a hematopoietic growth factor. CSF binds to its specific receptor (CSF-1R or MCSF-R) and thereby stimulates the proliferation, differentiation and activity of monocytes, macrophages and their bone marrow progenitors (Richardson et al. 2009). CSF-1 is synthesized by various cells including tumor cells (Maher et al. 1998). In some kind of tumors, its production is accompanied to MCSF-Receptor expression. Chambers et al. (1997) have reported that CSF-1R expression on the surface of carcinoma cells may be treated as a strong and independent poor prognostic factor.

Depletion of CSF-1 significantly decreased the tumor infiltration of macrophages and macrophages depletion resulted in slower progression of pre-invasive tumors to malignant lesions and reduced formation of distant metastases (Cecchini 1994).

Our results indicate that in canine mammary adenocarcinomas the CSF-1R is expressed in macrophages as well as in the cancer cells. We found that the expression of MCSF-R is closely related to the grade of malignancy of canine mammary carcinoma, whereas tumors that metastasize have as high expression as the most malignant ones (Fig. 2b). Based on these results, we assume that MCSF may be an important factor not only in macrophages recruitment,

but also in the tumor growth and proliferation. Hence, we suggest MCSF-Receptor should be considered as good marker of malignancy and metastatic ability of canine mammary adenocarcinomas, what is due to its expression in both macrophages and cancer cells. The metastatic group consisted of tumors of the 2<sup>nd</sup> and 3<sup>rd</sup> grade of malignancy. The MCSF-R expression in all tumors within this group was very similar, regardless to their grade of malignancy. This is opposite to non-metastatic tumors where the highest expression of MCSF-R was observed in tumors of the 3<sup>rd</sup> grade of malignancy. Similar results were published by Richardson et al. (2009). They found that in the high grade tumors of soft tissues the expression of MCSF-R was higher than in the low grade tumors. This was observed in both the tumor cell areas and the adjacent stromal areas.

Based on the literature data, CD14 is a specific monocyte/macrophage marker which is a mediator of proinflammatory responses following bacterial lipopolysaccharide binding. There is practically no information in the literature about the expression of this LPS co-receptor in other cells than monocytes, macrophages, their progenitors or leukemic cells however we found its expression in canine mammary tumors. This is the first report describing CD14 expression and correlation with grade of malignancy and metastatic potential in solid tumors. Its expression in canine mammary cancer cells was surprising and requires further investigation.

Four reports describing CD14 expression in mammary cells are available. Stein et al. (2003) revealed a strong increase in CD14 expression in the luminal epithelial cells at day 1<sup>st</sup> of involution caused probably by their involvement in the phagocytosis of neighboring apoptotic mammary epithelial cells. The other group found CD14 and CSF-1R gene expression (using DNA microarrays) in mammary tissue during involution and parturition (Clarkson et al. 2003). They suggested significant role of macrophages expressing CD14 in the regulation of the uninfected mammary gland. However, the authors did not check whether the CD14 and CSF-1R were really expressed in macrophages or also in the mammary tissue. Moreover, Bertucci et al. (2002) using DNA microarrays found “inflammatory cluster” in poor-prognosis primary breast cancers (high expression of CD14 and CSF-1R). The authors interpreted the results as a presence of monocytes/macrophages in the cancer tissue but they did not confirm whether the CD14/CSF-1R expression was really developed in inflammatory cells only or also in cancer cells. The results obtained by McDaniel et al. (2007) who demonstrated that tissue of involuting mammary glands could enhance invasiveness and metastasis of breast cancer cells are also very interesting.

The expression of CD14 in canine cancer cells (Fig. 3a) was very surprising since we expected CD14 expression in TAMs only. Moreover, we found that CD14 expression is related to the grade of tumor malignancy and its ability to metastasize. We found it as a marker that can discriminate the less malignant (the 1<sup>st</sup> and 2<sup>nd</sup> grade of malignancy) tumors from the more malignant (the 3<sup>rd</sup> grade of malignancy and metastatic tumors) tumors. The metastatic group consisted of tumors of the 2<sup>nd</sup> and 3<sup>rd</sup> grade of malignancy. The CD14 expression in all tumors within this group was very similar, regardless to their grade of malignancy.

There is only one available report about the potent role of CD14 in tumorigenesis/tumor microenvironment interactions (Song and Cho 2007). The authors studied the positive effect of CD14 transfection into embryonic kidney cells on endothelial cell migration, an important step of angiogenesis. They suggested that CD14 mediate angiogenesis by inducing bFGF. Theoretically, as a consequence it may enhance the tumor metastasis.

We suppose, that CD14 may play its own role in the tumor microenvironment, possibly in blood vessels formation (Song and Cho 2007) but its function in this field has been not assessed yet. This interesting finding requires further examination.

Cancer metastasis is a complex process. In addition to the cancer cell intrinsic factors, the cancer microenvironment composed of various tumor-associated cells influences the behavior of cancer cells. The results of our study indicate that in dogs, similarly to humans and mice, the number of tumor associated macrophages is related to the cancer ability to metastasize. Our results also indicate that MCSF-R and, what is particularly new finding – CD14, play a role in tumor progression, metastasis and microenvironment interactions. These results also suggest that in dogs, we should treat the tumor as a whole organ rather than just try to eliminate the cancer cells.

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