Expression of Vascular Endothelial Growth Factor in Canine Inflammatory and Non-inflammatory Mammary Carcinoma

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Summary

Inflammatory mammary carcinoma (IMC) is the most aggressive type of mammary tumour in the dog and has been proposed as a model for human inflammatory breast cancer. The aim of this study was to investigate angiogenesis in canine IMC by immunohistochemical assessment of the expression of vascular endothelial growth factor (VEGF). Tissues from 19 cases of IMC were compared with tissues from 27 cases of invasive mammary carcinoma without inflammation (non-IMC). Immunohistochemical expression of oestrogen receptor (ER), progesterone receptor (PR) and HER-2 receptor was also assessed. VEGF was strongly expressed in all IMCs and the percentage of VEGF-immunoreactive tumour cells was significantly higher in IMC than in non-IMC ($P = 0.02$). There was no difference in HER-2 receptor expression between IMC and non-IMC, and no IMC expressed ER or PR. These results suggest that VEGF may contribute to the high angiogenic phenotype of canine IMC and that this expression may underlie the tendency towards local and systemic metastasis of these tumours.

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Introduction

Human inflammatory breast carcinoma (IBC) is a type of mammary cancer that is associated with particularly aggressive biological behaviour and a poor prognosis (Chevallier et al., 1987; Tavassoli and Devilee, 2003). The staging of cancer published by the American Joint Committee on Cancer, classifies IBC as a T4d stage IIIb breast carcinoma (Tavassoli and Devilee, 2003). IBC is a clinicopathological entity that is distinct from other T4 lesions and is characterized by a diffuse, brown coloured thickening of the breast skin and erysipelic edge, with or without an underlying detectable mass. Despite its name, IBC is not associated with any significant degree of inflammatory infiltration, and is not an inflammatory condition (Tavassoli and Devilee, 2003). IBC is not a specific histological type of cancer and any of the microscopical variants of breast carcinoma can be present (Robbins et al., 1974).

IBC is characterized microscopically by invasion of dermal lymphatics. This dermatotropism is believed to be responsible for the clinical signs of the tumour and likely enables effective dissemination to distant sites (Kleer et al., 2004). The survival of patients with IBC has significantly improved with the use of combined modality therapy (Kleer et al., 2000); however, the significant number of relapses indicates a need to identify potential prognostic markers for this aggressive disease that may result in more targeted therapeutic approaches (McCarthy et al., 2002).

Inflammatory mammary carcinoma (IMC) has been described in the dog (Pérez-Alenza et al., 2001) and cat (Pérez-Alenza et al., 2004). The clinical and pathological features of canine IMC are similar to those of human patients and canine IMC has been proposed as an animal model for the human counterpart. Canine IMC has aggressive behaviour and a poor prognosis, and has higher prevalence (17.7%) among canine malignant mammary tumours (Peña et al., 2003b) than the prevalence of
human IBC (5%) among human mammary tumours (Wedam et al., 2006). Canine IMC can be subclassified as primary (without a previous mammary tumour) or secondary (after the excision of a previous mammary tumour) (Pérez-Alenza et al., 2001).

There is increasing evidence that angiogenesis is essential for the growth, invasion and metastasis of tumour cells (Fox et al., 2001). The vascular endothelial growth factor (VEGF) family, which includes VEGF-A–VEGF-D growth factors and placental growth factor, plays a role in the induction of angiogenesis in many human tumour types (Nicosia, 1998). VEGF-A and VEGF-B are important in regulating angiogenesis, while VEGF-C and VEGF-D are specifically involved in lymphangiogenesis (Van Der Auwera et al., 2005). All major isoforms of VEGF have been identified in the dog and the receptor binding regions of the human and canine molecules share amino acid sequence. VEGF receptors are also identical in the dogs and man. VEGF expression in canine tumours is similar to that observed in human malignancies and the VEGF signalling system is similar in both species in physiological and pathological processes (Scheidegger et al., 1999).

VEGF-C expression has recently been quantified by real-time reverse transcriptase polymerase chain reaction in canine mammary neoplasms, and its expression is reported to be significantly higher in malignant tumours and those that have metastasized to lymph nodes (Qiu et al., 2008). Since human IBCs tend to be highly vascular tumours due to their angiogenic and angioinvasive potential (Kleer et al., 2000), the aim of the present study was to evaluate VEGF expression in canine IMC.

Materials and Methods

Animals

Cases of IMC were identified retrospectively from the Tumour Registry of the Department of Animal Pathology of the University of Pisa. Nineteen entire bitches with IMC were recorded between 2000 and 2006. These animals all had clinical and histological features consistent with IMC. The group included seven dogs of mixed breed, two Dalmatians, two Dobermanns, two standard poodles, two English setters, two boxers, one West Highland white terrier and one Breton. The mean age at diagnosis was 11.2 ± 2.2 years. In all cases there was involvement of multiple mammary glands. The main clinical signs recorded were a rapid onset, firmness, warmth, oedema and erythema of the skin (Fig. 1). Underlying mammary nodules were often not clinically appreciable. In all cases a primary IMC was diagnosed. Thirteen of the bitches (68%) were humanely destroyed after the histological diagnosis was made, but were not available for necropsy examination. The mean survival time for bitches that were not destroyed was 1 month. A control group of mammary tumours from 27 bitches with non-IMC involving a single mammary gland was also selected from the Tumour Registry.

Histology and Immunohistochemistry

Affected mammary tissues and overlying skin were fixed in 10% neutral-buffered formalin and embedded in paraffin wax. Sections (4 μm) were stained with haematoxylin and eosin (HE). Mammary tumours were classified according to the criteria of the World Health Organization (WHO; Misdorp et al., 1999). The diagnosis of IMC was confirmed by the observation of tumour emboli within dermal lymphatics. Tumours were graded according to the criteria of Elston and Ellis (1991) as well-differentiated (WDC), moderately differentiated (MDC) or poorly differentiated (PDC) carcinomas.

The expression of VEGF, HER-2, oestrogen receptor-α (ER) and progesterone receptor (PR) was assessed by immunohistochemistry (IHC) with the Envision + © system horseradish peroxidase (HRP) labelled polymer (DakoCytomation, Milan, Italy). VEGF expression was evaluated with rabbit polyclonal anti-human VEGF (A-20; Santa Cruz Biotechnology, CA, USA) diluted 1 in 200 in Tris-buffered saline (TBS; pH 7.4) including 0.05% Tween 20. Positive controls consisted of a section of a VEGF-positive human breast carcinoma (courtesy of G. Bevilacqua, Department of Oncology, University of Pisa). Primary antisera was substituted with an unrelated rabbit polyclonal antibody as negative control. 3,3′-diaminobenzidine was employed as chromogen.
VEGF expression was assessed as described by Lewis et al. (2000) and Millanta et al. (2002) by selecting five non-adjacent, non-overlapping fields and counting the percentage of immunoreactive neoplastic cells per field at a magnification of ×400. VEGF expression was recorded as the mean of these counts. The percentage of immunolabelled cells in tumour emboli of IMCs was also recorded.

To define the steroid receptor status of the tumours, a primary rabbit polyclonal anti-human ER-α (Zymed Laboratories, CA, USA) diluted 1 in 50 and a primary mouse monoclonal anti-human PR (Clone PR 4–12; Oncogene Research Products, MA, USA) diluted 1 in 100 were employed. The steroid receptor status was scored by counting the percentage of positive nuclei/1000 cells in 10 selected fields as described by Millanta et al. (2005a). A negative cut-off was defined at 5%.

HER-2 receptor expression was evaluated by use of a primary rabbit anti-human HER-2/neu polyclonal antibody (DakoCytomation, Milan, Italy) diluted 1 in 50. The scoring of HER-2 labelling was assessed according to established guidelines (Koeppen et al., 2001; Martin de las Mulas et al., 2003; Millanta et al., 2005b) as 0 (no labelling), + (weak, incomplete membrane labelling), + + (moderate, complete membrane labelling of at least 10% of neoplastic cells) and + + + (strong, membrane labelling of at least 10% of neoplastic cells). Tumours scoring + + or + + + were considered to have overexpression of HER-2.

**Statistical Analysis**

The SPSS Advanced Statistics 13.0 software (SPS Inc., Chicago, USA) was used for statistical analysis. Analysis of variance was performed by ANOVA. Differences between groups were evaluated using the Bonferroni test. The Chi-square test was used to assess associations between the category variables, namely the values 0 to + + + as defined above. Statistical significance was set at 5%.

**Results**

Ten of the 19 IMCs (52.6%) were simple tubulopapillary carcinomas and nine tumours (47.4%) were simple carcinomas of solid type. Of these latter, a comedocarcinoma pattern was detected in four cases. Dermal invasion was observed in 15/19 tumours (78.9%), but in all cases marked invasion of dermal lymphatic vessels by tumour emboli was evident (Fig. 2A). Those lymphatic vessels without emboli were markedly dilated in all cases. Mild infiltration of lymphocytes and plasma cells was noted in non-ulcerated areas of each tumour. In addition to dermal invasion, there was also infiltration of underlying adipose and muscular tissues.

Fifteen (55.5%) of the non-IMCs were complex carcinomas and 12 (44.5%) were simple carcinomas (eight tubulopapillary and four solid carcinomas). Fourteen (51.8%) were classified as WDCs, nine (33.3%) as MDCs and four (14.8%) were PDCs. Lymphatic invasion was detected in 11/27 (40.7%) carcinomas. In the remaining 16 tumours only a local stromal invasion could be observed.

VEGF expression was observed in all IMCs, invariably with strong, diffuse to granular, intracytoplasmic immunoreactivity (Fig. 2B). The same labelling pattern was observed in the neoplastic emboli within the lymphatic vessels (Fig. 2C). The percentage of VEGF-immunoreactive tumour cells was 80 ± 13.8. The percentage of VEGF-positive neoplastic cells in tumour emboli did not significantly differ from the VEGF values of the primary tumour (P > 0.05). In non-IMC, VEGF expression was also observed in all the cases examined, with a percentage of positive neoplastic epithelial cells 33 ± 23 (Fig. 2D). VEGF expression was significantly higher in IMCs than in non-IMCs (P < 0.01; Fig. 3). In non-IMCs, VEGF values were 28.2 ± 5.2 in locally invasive carcinomas and 40 ± 7.5 in carcinomas with lymphatic vessel invasion. VEGF expression did not significantly differ between these two groups (P = 0.2). The comparison between VEGF values in IMC and non-IMCs is presented in Fig. 3.

HER-2 protein overexpression was detected in 19/19 (100%) IMCs and in 7/27 (26%) non-IMCs. HER-2 overexpression was not significantly associated with increased VEGF expression in IMCs (P = 0.9).

Steroid receptor expression was very low in IMCs. In the rare cases where ER and PR positivity were observed (1/19 tumours for ER and 2/19 for PR), the percentage of positive nuclei was under the established 5% cut-off, so that all the cases were considered as negative. On the other hand, ER expression was observed in 25/27 (92.6%) non-IMCs (mean percentage of positive nuclei: 69 ± 27) and PR expression was detected in 14/27 (52%) tumours (mean percentage of positive nuclei: 17 ± 22). ER expression was significantly higher in non-IMCs than in IMCs (P < 0.01). The number of PR-positive IMCs and non-IMCs was not significantly different (P < 0.1).

**Discussion**

The term IBC was initially used in human medicine to describe a type of locally advanced mammary carcinoma that stimulates an inflammatory process of the skin resembling dermatitis or mastitis (Chambler et al., 2000). Later, in order to define IMCs, a large number of cases were analyzed, including clinical and pathological data. In this study, we have focused on the specific features of IMCs, such as their immunophenotype, receptor status, and VEGF expression. The results showed that IMCs have a higher expression of HER-2 than non-IMCs, which is consistent with previous studies (Koeppen et al., 2001; Martin de las Mulas et al., 2003; Millanta et al., 2005b). This may suggest a role for HER-2 in the pathogenesis of IMCs, as it has been associated with aggressive behaviour in other types of cancer (Chambler et al., 2000).

VEGF expression was also observed in IMCs, with a significantly higher expression than in non-IMCs. This finding is consistent with the idea that VEGF plays a role in the angiogenesis of IMCs (Koeppen et al., 2001; Martin de las Mulas et al., 2003; Millanta et al., 2005b). The high expression of VEGF in IMCs may contribute to the development of a more aggressive phenotype, as it has been associated with increased tumour cell proliferation and decreased apoptosis (Koeppen et al., 2001; Martin de las Mulas et al., 2003; Millanta et al., 2005b).

Steroid receptor expression was very low in IMCs, with only a few cases showing positive expression. This finding is consistent with previous studies (Koeppen et al., 2001; Martin de las Mulas et al., 2003; Millanta et al., 2005b). The low expression of steroid receptors may contribute to the aggressive behaviour of IMCs, as they are involved in the regulation of cell proliferation and apoptosis (Koeppen et al., 2001; Martin de las Mulas et al., 2003; Millanta et al., 2005b).
et al., 1995). In veterinary medicine, IMCs are primarily recognized in the dog and have been reported in three cats (Pérez-Alenza et al., 2004). Canine IMC was first described in 10 bitches with similar clinical and pathological characteristics to those observed in women with IBC (Susaneck et al., 1983). In dogs, the presence of primary and secondary post-surgical IMCs is reported (Pérez-Alenza et al., 2001) and the histopathological features, Ki67 proliferation index, steroid hormone profile and the expression of p53 tumour suppressor protein have been characterized in these lesions (Peña et al., 2003a,b). The prevalence of canine IMCs ranges between 4.4 and 7.6% of all mammary tumours (Pérez-Alenza et al., 2001) and this is relatively higher than that described in man (Wedam et al., 2006). In both the canine and human tumours there is often extensive lymphatic and vascular invasion with tumour emboli. This biological behaviour leads to a poor prognosis in both species.

In the present study, the histological patterns of the tumours were similar to those reported by Peña et al. (2003a). The most common type of tumour was simple carcinoma. The percentage of cases in which dermal invasion by the tumour and/or the dermal vessel
embolization was detected was nearly the same. The comedocarcinoma pattern was observed in 21% of cases in this study and in 35% of bitches in the study of Peña et al. (2003a). In the present study only a tubular-papillary pattern of neoplastic dermal infiltration was observed, while Peña et al. (2003a) also reported a sarcoma-like pattern. This discrepancy appears to be irrelevant, since in the latest WHO classification of human breast tumours it is clearly stated that IBC is not regarded as having specific histological features (Tavassoli and Devilee, 2003). In the present study, most tumours displayed extensive tubule formation and the neoplastic cells had marked nuclear pleomorphism and a high mitotic index. These features are consistent with poorly differentiated carcinoma, and the observations are in agreement with Peña et al. (2003a).

It is well established that the growth and the metastatic spread of breast cancers are related to new blood vessel formation or angiogenesis (Weidner et al., 1991; Folkman and Shing, 1992). Up to now, few studies have investigated the angiogenic phenotype of IBC. It has been shown that angiogenic growth factors and, more recently, lymphangiogenic growth factors are overexpressed in human IBCs in comparison with non-IBCs (Shirakawa et al., 2002; Van Der Auwera et al., 2005). VEGF expression has previously been evaluated in canine and feline spontaneously arising invasive mammary carcinomas (Millanta et al., 2002; Restucci et al., 2002), but the present study represents the first report of VEGF expression in canine IMCs. The expression of this cytokine appears to be correlated with more aggressive behaviour and, in the cat, with a worse prognosis (Millanta et al., 2002; Restucci et al., 2002). In the present study all IMCs were VEGF-positive, with a higher percentage of VEGF-immunoreactive tumour cells than for non-IMCs. The percentage of VEGF-positive neoplastic cells in non-IMCs was similar to that documented by Restucci et al. (2002). Since VEGF is one of the more active promoters of angiogenesis involved in endothelial cell growth and motility and blood vessel permeability (Ferrara, 1999), the results of the present study suggest that VEGF-A may be responsible for the neovascularisation of canine IMC. This is also compatible with findings in studies of human IBC (Kleer et al., 2000). IMC and IBC are both highly vascular lesions and McCarthy et al. (2002) have documented increased microvessel density in IBC compared with breast cancer without an inflammatory phenotype.

The aggressive behaviour of IMC is further suggested by the findings related to steroid receptor expression. The observed high percentage of ER and PR expression in non-IMCs is in agreement with the veterinary literature (Martín de las Mulas et al., 2005; Millanta et al., 2005a), however, no IMCs expressed these receptors. This finding contrasts with the study of Peña et al. (2003a) in which all IMCs were negative for ER expression but almost 70% expressed the PR. In the present study all tumours were scored as negative due to the low percentage of positive nuclei, always less than the defined cut-off level. The discrepancy may be due to different primary antisera employed in each study. However, an ER-negative status appears to be an indicator of worse biological behaviour either in both canine and human mammary tumours (Sartín et al., 1992; Kleer et al., 2000).

No previous study of canine IMC has investigated HER-2 expression but in the present investigation a low percentage of IMCs was defined as having overexpression of this molecule. However, the number of HER-2 overexpressing IMCs and non-IMCs was not significantly different. The observed percentage of IMCs overexpressing HER-2 is similar to that reported for human IBC. Furthermore, HER-2 overexpression is also reported to be similar in human IBCs and non-IBCs (McCarthy et al., 2002) although Parton et al. (2004) described higher HER-2 positivity in IBCs. In the present study, overexpression of HER-2 was not significantly correlated with VEGF expression.

The available archival specimens, including cases with and without clinical evidence of IMC, all collected in a single institution, provided an opportunity to compare biological markers in this specific type of mammary neoplasia. Taken together, our findings confirm the aggressive phenotype of IMC compared with non-IMC. The observed VEGF immunoreactivity supports the hypothesis that the unique histological features of IMC, and the marked tendency to invade and metastasize, may also rely on angiogenic pathways. Further studies of a higher number of cases are required to strengthen these preliminary findings; however, the similarity between determinants of angiogenesis in human and canine inflammatory carcinomas of the mammary gland, suggests that canine IMC may be proposed as a model of the human counterpart. Although the survival of women with IBC has been greatly improved by the use of combined treatment modalities, the survival rates are still very low (Liauw et al., 2004). It has been recently reported that Bevacizumab™, a humanized monoclonal antibody to VEGF, has anti-angiogenic and anti-tumour effects in patients with IBC (Wedam et al., 2006). On this basis, novel insight into the molecular mechanisms responsible for the angiogenic phenotype of human and canine inflammatory carcinomas might also contribute to the development of new treatment strategies.
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References


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