Abstract. Cyclo-oxygenase (Cox-2) plays an important role in mammary carcinogenesis, nevertheless, its role in canine mammary tumors, and particularly in inflammatory mammary carcinoma (IMC), is unknown. Tumor Cox-2 levels were analyzed by enzyme immunoassay, in post-surgical tumor homogenates of 129 mammary tumors (62 dysplasias and benign tumors, 57 malignant non-IMC and 10 IMC) from 57 female dogs. The highest Cox-2 values were detected in the IMC group. In non-IMC malignant tumors, high values of Cox-2 were related to skin ulceration (p<0.001) and tumor size (p<0.001). The follow-up study revealed that high Cox-2 levels were related with recurrence (p=0.002), metastases (p<0.001), disease-free survival (p<0.001) and overall survival (p<0.001). This study demonstrates an association between intra-tumor Cox-2 levels and poor prognosis. The high levels found in IMC cases could indicate a special role of Cox-2 in the inflammatory phenotype and open the possibility of additional new therapeutic approaches in this special type of mammary cancer in humans and dogs.

Canine mammary tumors (CMT) are the most frequent neoplasms in the female dog. They affect females almost exclusively, with an increasing incidence from 6-7 years of age onwards (1). The proportion of malignant tumors is about 50%. The prediction of clinical behavior in malignant mammary tumors is difficult because they are very heterogeneous from their pathological aspects to their clinical behavior (2).

In the last decade, several researchers have been involved in the study of prognostic factors in canine mammary cancer (3-7), however the establishment of new parameters potentially useful for prognosis and treatment of mammary cancer in the dog is still relevant.

Spontaneous canine mammary tumors have been suggested as a comparative model for the study of human breast cancer (8, 9). Recently, canine inflammatory mammary carcinoma (IMC) has been proposed, by our group, as a natural model for human inflammatory breast carcinoma (IBC) (10).

IBC is the most aggressive and lethal form of human breast cancer (11, 12). Multimodality therapy has improved its very poor prognosis (13), but overall survival is still significantly low in IBC human patients when compared to non-inflammatory forms of breast carcinoma (11). To date, research on IBC has been conducted to elucidate its distinctive genetic characteristics and mechanisms of lymphatic invasion (14-17), in order to offer future effective therapies. IMC is also a poorly known, very aggressive mammary cancer with histological and clinical characteristics similar to IBC (18). IBC and IMC are diagnosed on the basis of a rapid progression of signs, such as localized or generalized induration, redness, edema and pain of the mammary gland; the unique histopathological hallmark is the presence of dermal lymphatic invasion (18, 19). The search for new therapeutic modalities in order to improve survival is necessary in both species.

Some studies suggest the potential role of NSAID (non-steroid anti-inflammatory drugs) blocking cyclo-oxygenases (-1 and -2) in the prevention and treatment of malignant tumors in humans (20-22). Cyclo-oxygenase-2 (Cox-2) belongs to the cyclo-oxygenase family that catalyzes the conversion of arachidonic acid into prostaglandins and other eicosanoids.
Two isofoms of prostaglandin synthetase have been identified (Cox-1 and Cox-2). Cox-1 is constitutively expressed, while Cox-2 is inducible by growth factors, inflammatory stimuli, tumor promoters and oncogenes (24, 25). Since the first study by Hwang and colleagues in 1998 (26), the overexpression of Cox-2 in human breast cancer has been detected by several researchers (27-30). However, there are no enzyme-immunoassay (EIA) studies that have determined the Cox-2 content in tumor tissue homogenates of the mammary gland. Canine Cox-1 and Cox-2 proteins present more than 90% homology with the human counterparts, as attested by the cloning of canine Cox-1 and Cox-2 (31). Cox-2 immunorepression has been detected in several canine tumors (32-36), suggesting a similar role of Cox-2 in dogs and in humans; however, the prognostic value of Cox-2 in canine mammary tumors remains unknown. The potential prognostic value of Cox-2 in human breast cancer remains controversial: overexpression of Cox-2 has been linked to poor prognosis in some studies (37-39), while a relationship between Cox-2 expression and prognosis has not been found in others (40, 41). No studies have determined Cox-2 levels in inflammatory mammary carcinoma in both humans and dogs.

The aim of this study was to determine the tissue content of Cox-2 in canine mammary tumors, including IMC, and to establish its relationship with clinicopathological parameters and prognostic value.

Materials and Methods

Animals and tumors. One hundred and twenty-nine (129) spontaneous canine mammary tumors (CMT) from 57 female dogs presented at the Veterinary Teaching Hospital of the University Complutense of Madrid, Spain, were prospectively included in the study. The animals ranged in age from 6 to 14 years and were of different breeds. Thirteen (13) normal mammary glands (incisional biopsies by “tru-cut” needle) from 8 beagle female dogs without a history of mammary neoplasia (aged 6-10 years) were used as controls.

Clinical procedures. At the first visit, a complete history and physical examination was done in all animals. Age, breed, reproductive status (spayed or not spayed) and history of prevention of estrus with hormonal treatment were recorded. After a complete physical examination, all mammary glands and regional lymph nodes (axillary and inguinal) were evaluated. The size of the tumor, adherence to skin and/or underlying tissues, and the presence of skin ulceration of each tumor were evaluated. The rate of growth was determined based upon owner information (defined as slow, medium or fast). Animals with at least 1 malignant tumor were categorized by clinical staging using a modified TNM WHO’s system (42). The tumors were classified as T1 (<3 cm), T2 (≥3 and <5 cm) or T3 (≥5 cm). The enlarged lymph nodes were cytotologically examined to define their involvement. The presence of distant metastases was assessed by radiological evaluation of the thorax. Using this system, 4 clinical stages were established: local (without lymph node involvement), locally advanced (advanced local invasion, including inflammatory mammary carcinoma), regional (lymph node affectionation) and distant (presence of distant metastases).

Surgical excision of the tumors was done in all animals with local or regional stages. In locally advanced stage (inflammatory mammary carcinomas - IMC), surgery was not recommended, and only palliative therapy with antibiotics and corticosteroids was applied. In these cases, samples were obtained by “tru-cut” biopsies or at necropsy.

None of the animals included in this study presented distant metastases at first presentation.

Follow-up study. After surgical excision, all the female dogs with at least one malignant mammary tumor (n=27) were followed-up for a period of 30 months or until death (from the disease or other cause). Each animal was clinically evaluated every 3-4 months in order to detect the presence of local recurrences and/or distant metastases. Disease-free survival (DFS, time from surgical excision to the occurrence of metastases or recurrences) and overall survival (OS, time from surgery to death by tumor) were determined in each case. In those cases bearing more than one malignant tumor, the neoplasia with the most aggressive histological features was chosen in order to perform the statistical associations with the follow-up variables and with the survival.

Only animals with local or regional stages were included in the prognostic study. Female dogs with IMC were excluded from the follow-up study because of the short survival time after diagnosis (less than 30 days). All the animals that died from others causes distinct of mammary neoplasia were eliminated from the follow-up study.

Statistical analysis. The statistical software SPSS 12.0 was used for statistical analysis. ANOVA test and the Student’s t-test were used for continuous variables. The Chi-square test was used for studying categorical variables. Analyses of variance (F-test, pooled t-tests if variances are assumed to be equal or Welch test or separate t-test if variances are not equal) were used to study the differences in means of continuous variables. The Kaplan-Meier method was used for survival analysis, and the differences were studied by the log-rank test. The Cox-2 cut-off point was calculated by the mean of values from malignant tumors non-IMC considered to the
follow-up study. All values were expressed as mean±SEM. In all statistical comparisons, \( p<0.05 \) was accepted as denoting significant differences.

**Results**

**Animals.** Twenty female dogs presented only benign tumors and/or dysplasias, and 37 presented at least one malignant tumor (10 with IMC and 27 with malignant tumors non-IMC).

In the group of dogs with malignant tumors, 18 were in local stage of disease, 10 dogs were in clinically advanced local stage (female dogs with IMC) and 9 in regional clinical stage with metastasis at regional lymph nodes.

**Tumors.** A total of 129 canine mammary lesions (22 dysplasias, 40 benign tumors, 57 non-IMC and 10 IMC) were histopathologically diagnosed. The group of dysplasias consisted of lobular \( n=19 \) and ductal hyperplasias \( n=3 \). Benign tumors included simple \( n=12 \) and complex adenomas \( n=10 \), and mixed benign mammary tumors \( n=18 \). Malignant tumors included carcinoma in situ \( n=6 \), simple \( n=18 \) and complex carcinomas \( n=19 \), solid carcinomas \( n=8 \), carcinosarcomas \( n=6 \) and 10 IMC. Samples of mammary gland taken from the healthy control beagles \( n=13 \) were absent of any histological alteration.

**Cox-2 levels in tissue homogenates.** The mean Cox-2 values among the groups were significantly different \( (p<0.001) \) (Table I), except between dysplasias and benign tumors \( (p=0.65) \). In non-IMC malignant tumors, Cox-2 detection was higher in ulcerated compared with non-ulcerated neoplasms \( (p<0.001) \), as well as in larger tumors compared with smaller ones \( (p<0.001) \). Likewise, Cox-2 levels were significantly elevated in tumors with fast rate of growth, in tumors adhered to skin, and in tumors with adherence to underlying tissues (Table II).

**Follow-up study.** A follow-up study was performed in 25 animals from the 27 with malignant non-IMC tumors (2 animals were missing during follow-up). The mean follow-up time was 18 months, with a minimum of 3 months and a maximum of 30 months.

Cox-2 values were higher in animals with malignant tumors in regional clinical stage. Cox-2 values were also higher in tumors that recurred compared with non-recurring tumors, and in tumors that metastasized compared with

### Table I. Cox-2 concentrations in canine mammary tumor homogenates and normal mammary glands.

<table>
<thead>
<tr>
<th>Normal mammary glands ((n=13))</th>
<th>Dysplasias and benign tumors</th>
<th>Malignant tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cox-2 (ng/g) ( n=22)</td>
<td>4.98±0.6(^a)</td>
<td>10.69±1.65(^b)</td>
</tr>
</tbody>
</table>

**IMC-Canine inflammatory mammary carcinoma.**

\(^*\)ANOVA test \((p<0.05)\). Groups with different superscripts letters denoting statistical differences, Duncan test \((p<0.05)\)

### Table II. Association of Cox-2 tissue concentrations with clinicopathological parameters in all canine non-IMC mammary tumors \((n=57)\).

<table>
<thead>
<tr>
<th>Clinicopathological parameters</th>
<th>Number of samples</th>
<th>Cox-2 (ng/g)</th>
<th>( P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of growth</td>
<td>Slow</td>
<td>17</td>
<td>38.42±7.46</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>24</td>
<td>36.78±6.94</td>
</tr>
<tr>
<td></td>
<td>Fast</td>
<td>16</td>
<td>91.19±10.53</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>T1 &lt;3 cm</td>
<td>28</td>
<td>31.54±7.42</td>
</tr>
<tr>
<td></td>
<td>T2 ≥3 cm and &lt;5 cm</td>
<td>16</td>
<td>60.66±11.37</td>
</tr>
<tr>
<td></td>
<td>T3 ≥5 cm</td>
<td>13</td>
<td>82.39±12.25</td>
</tr>
<tr>
<td>Adherence to skin</td>
<td>No</td>
<td>28</td>
<td>37.97±6.73</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>29</td>
<td>66.65±8.19</td>
</tr>
<tr>
<td>Adherence to underlying tissues</td>
<td>No</td>
<td>40</td>
<td>41.39±5.95</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>17</td>
<td>78.85±10.22</td>
</tr>
<tr>
<td>Skin ulceration</td>
<td>No</td>
<td>49</td>
<td>43.89±5.52</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>8</td>
<td>105.7±6.71</td>
</tr>
<tr>
<td>Histological diagnosis</td>
<td>Ductal carcinoma (&quot;in situ)&quot;</td>
<td>7</td>
<td>14.89±2.91</td>
</tr>
<tr>
<td></td>
<td>Complex carcinoma</td>
<td>19</td>
<td>34.00±5.17</td>
</tr>
<tr>
<td></td>
<td>Simple carcinoma</td>
<td>18</td>
<td>47.62±9.30</td>
</tr>
<tr>
<td></td>
<td>Solid carcinoma</td>
<td>8</td>
<td>95.04±14.61</td>
</tr>
<tr>
<td></td>
<td>Carcinosarcoma</td>
<td>6</td>
<td>107.23±12.66</td>
</tr>
</tbody>
</table>

4271
those that did not metastasize during the follow-up (Table III). Longer disease-free survival \((p<0.001)\) and overall survival \((p<0.001)\) were statistically associated to high levels of Cox-2 (Figures 1 and 2).

Discussion

The EIA technique is not currently used to determine Cox-2 content in canine or human mammary tumors. In canine mammary tumors, the expression has been established by immunohistochemistry (9, 36). In humans, the expression has been determined by immunohistochemistry (27), PCR and immunoblotting (26, 28). In the present study, levels of Cox-2 were detected in all the samples analyzed (normal and neoplastic canine mammary glands). The highest values were found in IMC specimens, and the lower values in normal mammary gland.

Detection of Cox-2 by immunohistochemistry in normal tissues from dogs (mammary gland and nasal mucosa) and mouse (lungs) has been previously reported (9, 35, 44), which might be evidence of constitutive Cox-2 expression. However, more studies are necessary to clarify its role in normal canine tissues.

In our study, Cox-2 concentrations were significantly increased in malignant tumors (IMC and non-IMC) compared with benign and dysplastic lesions. Cox-2 levels were also correlated with several clinical parameter characteristics of increased malignancy, such as large tumor size, skin ulceration, adherence to skin, adherence to underlying tissues and more aggressive histological types (solid carcinoma and carcinosarcoma).

In our series, the Cox-2 levels were significantly increased in dogs with lymph node invasion and/or distant metastasis during follow-up. This might reflect the ability of Cox-2 to promote metastasis by inducing production and activation of matrix metalloproteinases (25, 45) or by stimulating angiogenesis (46-48). In this study, the Cox-2 levels were related to a reduced disease-free survival and overall survival in dogs with non-IMC malignant mammary tumors. This is the first study to report the association between Cox-2 levels and a poor prognosis in canine mammary cancer, although some studies have related Cox-2 immunoeexpression to a worse prognosis in human breast cancer (37-39). The mechanisms by which Cox-2 contributes to the poor prognosis in breast cancer have not been elucidated. It is likely that Cox-2 overexpression represents part of a complement of malignant characteristics of the carcinomatous state, as has been proposed by others (49).

<table>
<thead>
<tr>
<th>Clinical stage</th>
<th>Number of animals</th>
<th>Cox-2 (ng/g)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local</td>
<td>18</td>
<td>36.39±3.73</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Regional</td>
<td>9</td>
<td>98.1±15.57</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Recurrences</th>
<th>Number of animals</th>
<th>Cox-2 (ng/g)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>20</td>
<td>48.12±7.01</td>
<td>0.002</td>
</tr>
<tr>
<td>Yes</td>
<td>5</td>
<td>108.64±19.49</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metastases</th>
<th>Number of animals</th>
<th>Cox-2 (ng/g)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>18</td>
<td>40.51±4.91</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Yes</td>
<td>7</td>
<td>110.90±14.26</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Relationship between Cox-2 tissue levels and disease-free survival (DFS).

Figure 2. Relationship between Cox-2 tissue levels and overall survival (OS).
Treatment with Cox-2 inhibitors reduces the incidence and the multiplicity of experimentally-induced breast tumors in animal experimental models (50). In the rat mammary carcinogenesis model, the selective anti-Cox-2 celecoxib reduced mammary tumor development and the volume of pre-existing tumors (51). There are no previous studies on the benefit of Cox-2 inhibitors in canine mammary tumor prevention or therapy. In humans, therapeutic studies have shown promising results in bladder, prostate and breast cancer (22, 52). In the dog, piroxicam administration induced tumor apoptosis and reduced tumor angiogenesis of invasive bladder tumors (53). According to our results, Cox-2 inhibitors should be tested as an adjuvant therapy in dogs with malignant mammary neoplasias.

Inflammatory mammary carcinoma (named IBC in humans and IMC in dogs) is a rare and aggressive tumor with an extremely poor prognosis in both canine and human species (19, 54). Its incidence has increased in the last decade (11), and the prevalence in our Institution is relatively high since we are a referral center in Spain (18). Clinical signs (edema, erythema, firmness and warmth of the mammary glands), histopathological features (dermal lymphatic invasion), and behavior (very aggressive condition) (55) are similar to those described in human inflammatory breast carcinoma (56). In IBC, multimodal treatment (chemotherapy and radiotherapy) has improved survival, however the prognosis of this neoplasm is still very poor (57). Inflammatory mammary cancer cells have been useful in the study of metastatic phenomena, since they are highly aggressive and typically angioinvasive (15, 16). In the present study, Cox-2 concentrations were higher in the IMC group with respect to the non-IMC malignant tumors (Table 1), suggesting a special role for Cox-2 in the inflammatory type of cancer, probably related to the previously indicated functions of extracellular matrix degradation (25, 45) and stimulation of angiogenesis (48). Our results advocate further studies to confirm the participation of Cox-2 in the inflammatory phenotype and to open the possibility of new therapeutic approaches.

This study demonstrates the association between intra-tumor Cox-2 levels and clinicopathological features of malignancy and poor prognosis. The high levels of Cox-2 found in cases of poor prognosis, including IMC, open the possibility of additional new therapeutic approaches in this special type of mammary cancer in humans and dogs.

Acknowledgements

A Ph.D. Grant (SFRH/BD/10883/2002) from the Foundation of Science and Technology (FCT), Portugal supported this study. We thank Dr. Pedro Cuesta, of the processing Data Center of the Complutense University, Madrid, Spain, for his assistance with the statistical work.

References


4274


Received May 18, 2005
Accepted July 26, 2005