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Obesity, expression of adipocytokines, and macrophage infiltration in canine mammary tumors

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ABSTRACT

Obesity influences the development, progression and prognosis of human breast cancer and canine mammary cancer (MC) but the precise underlying mechanism is not well-documented in the fields of either human or veterinary oncology. In the present study, the expression of major adipocytokines, including leptin, adiponectin, and leptin receptor (ObR) in benign ($n = 28$) and malignant ($n = 70$) canine mammary tumors was investigated by immunohistochemistry and on the basis of the subject's body condition score (BCS). To evaluate the relationship between obesity and chronic inflammation of the mammary gland, macrophages infiltrating within and around tumoral areas were counted.

The mean age of MC development was lower in overweight or obese dogs (9.0 ± 1.8 years) than in lean dogs or optimal bodyweight (10.2 ± 2.9 years), and the evidence of lymphatic invasion of carcinoma cells was found more frequently in overweight or obese group than in lean or optimal groups. Decreased adiponectin expression and increased macrophage numbers in overweight or obese subjects were significantly correlated with factors related to a poor prognosis, such as high histological grade and lymphatic invasion. Leptin expression was correlated with progesterone receptor status, and ObR expression was correlated with estrogen receptor status of MCs, regardless of BCS. Macrophage infiltration within and around the tumor may play an important role in tumor progression and metastasis in obese female dogs and may represent a prognostic factor for canine MCs.

Introduction

Obesity is a serious health problem that influences the development and prognosis of breast cancer in post-menopausal women (Key et al., 2001). In humans, a high body mass index (BMI) is a risk factor for mammary inflammatory carcinoma (Chang et al., 1998), which has a worse prognosis than non-inflammatory carcinoma both in women (Hance et al., 2005) and in female dogs (Marconato et al., 2009). The exact mechanism by which obesity influences the development and prognosis of human breast cancer remains unknown, although various factors secreted by adipocytes, including aromatase, leptin, adiponectin, estrogens, and insulin-like growth factor-1, have been implicated (Lorincz and Sukumar, 2006).

Aromatase concentrations are raised in obesity and this is considered to increase the risk of breast cancer by elevating circulating and local estrogen levels in obese post-menopausal women (Bulun et al., 2012). A case–control study revealed the association between obesity in young age and development of mammary gland tumors in dogs (Alenza et al., 1998). According to a recent research (Sorenmo et al., 2011), obesity in young dogs may increase the risk of mammary tumors through increased estrogen production, thus exposing the mammary tissue to high estrogen levels and consequent carcinogenesis.

Although adiponectin is produced and secreted by adipose tissue, its circulating level strongly decreases with the excessive accumulation of adipose tissue in the body in both humans (Arita et al., 1999) and dogs (Ishioka et al., 2006). Numerous reports have documented the anti-proliferative and apoptotic effects of adiponectin in human breast cancer cell lines (Takahata et al., 2007; Nakayama et al., 2008; Jardé et al., 2009). A decreased plasma adiponectin level is significantly correlated with breast cancer risk in obese post-menopausal women (Mantzoros et al., 2004).

Leptin, a major protein that increases in concentration in obesity, may promote carcinogenesis of the mammary tissue through its interaction with the leptin receptor (ObR) (Laud et al., 2002; Jardé et al., 2008, 2011). Leptin affects breast cancer by stimulating growth of normal mammary epithelial cells and tumor cells, tumor invasion, angiogenesis, and aromatase activity (Rose et al., 2002; Lorincz and Sukumar, 2006).

Several studies have reported that chronic inflammation and macrophage recruitment mediated by monocyte chemotactic protein-1 (MCP-1, also known as CCL2) are closely related with mammary

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gland tumors both in obese women (Morris et al., 2011) and in obese mouse models (Arendt et al., 2013). Obesity-related macrophage infiltration of the mammary gland was reversed with caloric restriction in mice (Bhardwaj et al., 2013).

Instead of BMI or waist-to-hip ratio in human medicine, the body condition score (BCS) is used to estimate the nutritional condition of dogs in veterinary medicine. Although BCS is a subjective measurement made by a veterinarian's visual assessment of the animal, circulating leptin levels (as a marker of obesity in dogs) were elevated in dogs with a higher BCS (Ishioka et al., 2007; Ricci and Bevilacqua, 2012).

In the present study, we analyzed clinical, histopathological, and immunohistochemical prognostic factors on the basis of BCS in female dogs with mammary tumors. Leptin, ObR, and adiponectin expression in the canine mammary tumors (CMTs) were analyzed by immunohistochemistry (IHC) and correlated with the clinical and histopathological prognostic factors and estrogen receptor (ER) and progesterone receptor (PR) expression. We hypothesized that obesity affects the development, progression, and metastasis of canine mammary carcinoma (MC) by recruitment of macrophages. To evaluate the relationship between obesity and chronic inflammation of the mammary gland, we counted the number of macrophages infiltrating within and around tumoral areas on the basis of BCS.

Materials and methods

Study population and samples

Formalin-fixed and paraffin-embedded tissue specimens from primary CMTs were examined. Ninety-eight CMT specimens were randomly selected for the study, including 28 benign and 70 malignant mammary tumors. The subjects were divided into two groups on the basis of their BCS: group 1 (BCS = 2 or 3), lean or optimal bodyweight; and group 2 (BCS = 4 or 5), overweight or obese. All specimens were obtained from the Veterinary Medical Teaching Hospital of Konkuk University and private local animal hospitals and were submitted to the Department of Veterinary Pathology, Konkuk University, Seoul, Korea 2011–2013.

Histopathology

Sections (4 μm) were stained with hematoxylin and eosin (HE) for histological evaluation. Histological typing of CMTs was based on the Proposed Histologic Classification by Goldschmidt et al. (2011). Histological grade was assessed as well-differentiated (grade I), moderately-differentiated (grade II), and poorly-differentiated (grade III) according to the grading system proposed by Peña et al. (2013). The presence of central necrosis in the tumor and lymphatic invasion of tumor cells were evaluated.

Immunohistochemistry

Tissue sections (5 μm) were deparaffinized in xylene, rehydrated in graded ethanol and washed in phosphate buffered saline (PBS). Sections were incubated in 3% H_2O_2 for 20 min at room temperature (RT), followed by three washes in PBS. Antigen retrieval was performed by boiling in either Tris–EDTA (pH 9.0; ER, PR, and CD3) for 15 min or in citric acid (pH 6.0; Ob, ObR, adiponectin, and myeloid/histiocyte antigen) for 20 min in a microwave (750 W, high power).

After washing three times in PBS, the slides were treated with primary antibodies (see Appendix: Supplementary Table S1). For the anti-ER and anti-adiponectin antibodies, 5% normal goat serum was applied first as a blocking agent. As isotype controls, mouse IgG_1 (eBioscience), mouse IgG_{2a} (Biolegend), and rabbit immunoglobulin fraction (Dakocytomation) were used. Slides were incubated with horseradish peroxidase conjugated secondary antibodies (REAL Envision kit, DAKO) for 20 min at RT after washing in PBS four times. The slides were then washed four times in PBS, and treated with development reagents. Development reactions were stopped by washing in distilled water, followed by counterstaining with Gill's hematoxylin.

Immunohistochemical scoring

Nuclear ER and PR expression >10% of tumor cells was considered as positive (Gama et al., 2008). For leptin, ObR, and adiponectin, the percentage of stained cells on each slide was calculated from five representative fields at a magnification of ×40. Evaluation of leptin and ObR expression was performed as previously described (Ressel et al., 2012). Leptin staining was considered as negative when the staining intensity of tumor cells was weaker than that of adipocytes in each slide or when <5% tumor cells were strongly stained. Samples with >5% tumor cells that were strongly stained were categorized as positive. With regard to ObR expression, tumor samples with >5% positive cells were classified as positive. For adiponectin expression, tumor samples with no staining, staining of <10% tumor cells, or weak staining of >10% tumor cells were classified as negative, while those with weak to strong staining of >10% tumor cells were classified as positive. Cytoplasmic expression represented positivity for leptin and adiponectin, while both cytoplasmic and membrane expression represented positivity for ObR.

The number of myeloid/histiocyte antigen-positive macrophages and CD3-positive T lymphocytes was analyzed in a 2.4-mm^2 area per tumor section using automated image analysis software (Image Pro Plus 5.1, Media Cybernetics). Images of the tumor sections were acquired at ×400 magnification and five fields of hotspot images were obtained within and around tumoral areas.

Imaging and statistical analyses

Digital images of HE and IHC slides were acquired using a BX41 microscope (Olympus) and digital image transfer software (Leica Application suite 2.7, Leica).

Associations between clinical and histopathological features and expression of leptin, ObR, and adiponectin were performed using the Pearson's χ^2-test or Fisher's exact test. A log-linear model was applied for detailed analysis. Associations of the mean number of macrophages or T lymphocytes according to BCS with histological grade, lymphatic invasion of tumor cells, and central necrosis were evaluated using Student's *t* test or analysis of variance (ANOVA). Statistical significance was established at $P < 0.05$. IBM SPSS statistics software program version 20 (IBM) was used for all statistical analyses.

Results

Clinical and histopathological data

A total of 64 intact and 34 neutered female dogs were included in the study. The mean age of the subjects was 9.8 ± 2.5 years (range, 5–17 years). Dogs with a BCS of 2 (lean, $n = 7$) or 3 (optimal bodyweight, $n = 49$) were assigned to group 1 ($n = 56$), while those with a BCS of 4 (overweight, $n = 37$) or 5 (obese, $n = 5$) were assigned to group 2 ($n = 42$). Following microscopic examination, the samples were categorized as 28 benign and 70 malignant tumors. Appendix: Supplementary Table S2 summarizes the clinical (including BCS and histopathological) data of total 98 samples and presents further details. Histological grade was classified as I ($n = 44$), II ($n = 14$), and III ($n = 12$). There were seven and 26 MC samples with evidence of lymphatic invasion and central necrosis, respectively.

Analyses of leptin, leptin receptor, and adiponectin expression

Both benign and malignant tumors expressed leptin ($n = 46$, Fig. 1A), ObR ($n = 36$, Fig. 1B), and adiponectin ($n = 72$, Fig. 1C and D); the results are described in Table 1. Of the 70 MCs, 50% ($n = 35$) were leptin-positive, 42.9% ($n = 30$) were ObR-positive, and 51.4% ($n = 36$) were adiponectin-positive. Expression of ObR tended to be higher in MCs (30/70, 42.9%) than in benign tumors (6/28, 21.4%; $P = 0.047$).

Effects of body condition score on and immunohistochemical features

Table 2 summarizes the features of the 70 MCs according to BCS; 38/56 tumors in group 1 and 32/42 tumors in group 2 were malignant. The mean age of dogs in group 2 was relatively lower than that of dogs in group 1 ($P = 0.013$). No differences in histological classification, histological grade, central necrosis, and T cell infiltration were observed between groups. However, the number of dogs with evidence of lymphatic invasion was greater in group 2 (6/32, 18.8%) than in group 1 (1/38, 2.6%; $P = 0.042$). Of all MCs, 52.9% (37/70) were ER-positive and 58.6% (41/70) were PR-positive. There were no significant BCS-related differences in ER and PR expression.

Adiponectin-positive MCs were observed more frequently in group 1 (27/38, 71.1%) than in group 2 (9/32, 28.1%; $P < 0.001$).