Obesity increases the incidence of 7,12-dimethylbenz(a)anthracene-induced mammary tumors in an ovariectomized Zucker rat model

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Abstract. Obesity is associated with increased risk for postmenopausal, but not premenopausal breast cancer. Recently, we reported that intact obese Zucker rats had increased susceptibility to DMBA-induced mammary tumors compared to lean Zucker rats. In the present study, we investigated whether excessive adipose tissue would promote mammary tumor induction in the absence of ovarian estrogen. Lean and obese rats were sham-operated or ovariectomized at 40 days old and were gavaged at 50 days old with 65 mg/kg DMBA. Rats were weighed and palpated twice weekly for detection of mammary tumors and sacrificed 135 days post-DMBA treatment. Obese sham-operated (O/S) rats had a shorter latency period (102 days) compared to lean sham-operated (L/S) (134 days) and obese ovariectomized (O/O) rats (123 days). At the end of the experiment, 36% of the O/O rats developed mammary tumors while lean ovariectomized (L/O) rats developed no mammary tumors (P<0.001), and 59% of the O/S rats developed mammary tumors compared to 30% of the L/S rats (P<0.05). In summary, obesity increases the susceptibility of ovariectomized Zucker rats to DMBA-induced mammary tumors, suggesting that adipose tissue-derived estrogen in obese animals may be sufficient to promote DMBA-induced tumors in this model. These results suggest that obesity in postmenopausal women may increase breast cancer risk due to increased breast tissue exposure to adipose tissue-derived estrogen. In conclusion, we have developed an animal model to further investigate the role of obesity in breast cancer development in postmenopausal women.

Introduction

The rate of obesity has doubled in the United States in the past two decades, and obesity is now an epidemic. In addition to the relationship to Type 2 diabetes mellitus, obesity is also associated with development of several other serious health conditions, including cardiovascular diseases, hypertension, stroke, and certain types of cancer including breast cancer (1-3). The relationship between obesity and the development of specific cancers, including renal cell carcinoma and esophageal and colorectal cancers, has been well documented (4).

Breast cancer is the second leading cause of death from cancer in women in the US. The American Cancer Society estimated that during 2006, 212,920 women will be diagnosed with new cases of breast cancer and that 40,970 women will die from this disease (5). Increasing body weight has been associated with increased risk for postmenopausal, but not premenopausal breast cancer (6). Many clinical and pathologic characteristics of breast cancer place certain women at increased risk of poorer outcome, varying among ethnic groups. Possible explanations for the differential prognoses, such as differences in body weight, treatment, diet, genetics, estrogen level or estrogen receptor and hormonal status, continue as avenues of exploration (7,8).

In the Nurses Health Study, women gaining more than 9 kg from 18 years of age to midlife doubled their risk for breast cancer, compared with women who maintained a stable weight (9). For example, the risk increases by 3% for each kg/m² increase in BMI (10). A recent large population-based cohort study of more than 145,000 Austrian women that examined the relationship between overweight, obesity and certain types of cancer including breast cancer (1-3). The relationship between obesity and the development of specific cancers, including renal cell carcinoma and esophageal and colorectal cancers, has been well documented (4).

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containing progestin is associated with increased risk of breast cancer in postmenopausal women. Other studies have implicated elevated circulating estrogen levels to be responsible for this increased risk (13,14).

Several serum growth factors that are associated with increased body weight are also implicated in breast cancer risk. Higher insulin levels are associated with obesity and may enhance cellular proliferation leading to the development of breast cancer (15). Leptin is another serum growth factor that is positively associated with body weight or body fat (16,17). Leptin has been shown to upregulate aromatase activity in breast cancer cells which results in increased estrogen levels, thereby stimulating proliferation (18). A positive association has been suggested between high serum insulin levels and poor breast cancer outcome due to increased leptin production by adipose tissue (19).

In animal models, higher body weight has been associated with increased incidence of both spontaneous and chemically induced mammary tumors (20-23). The obese Zucker (fa/fa) rat (24,25) is an ideal animal model to investigate the role of obesity on chronic disease development. Obesity in the Zucker rat is caused by a mutation in the leptin receptor gene (26,27). Obese Zucker (fa/fa) rats are known to develop hyperinsulinemia and become insulin resistant. They are an ideal model to investigate the effects of genetic obesity and non-insulin-dependent diabetes mellitus on other chronic disease development such as cancer (28-30). The most valuable contribution of the Zucker rat has been its utility as a model of human early-onset, hyperplastic-hypertrophic obesity. In contrast to obese Zucker (fa/fa) rats, lean Zucker rats have normal metabolic function and are ideal controls.

Lifetime estrogen exposure is a major risk factor in human breast cancer development. In postmenopausal women, the most important source of estrogen is from precursors synthesized in adipose tissue. Recently, we reported that obesity increased the rate of DMBA-induced mammary tumor development in intact female Zucker rats (31). In order to determine whether estrogens derived from adipose tissue are sufficient to promote mammary tumor development in the DMBA-induced rat model, we used ovariectomized Zucker rats as a model to compare the rate of mammary tumor development in the presence and absence of ovarian estrogen in obese and lean Zucker rats.

**Materials and methods**

**Experimental design.** All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas for Medical Sciences. Female Zucker rats were sham-operated (lean, n=30; obese fa/fa, n=27) or ovariectomized (lean, n=31; obese fa/fa, n=25) at the age of 40 days by Harlan Industries (Indianapolis, IN). The animals were housed at animal facilities at the Arkansas Children’s Hospital Research Institute, two per cage in polycarbonate cages and allowed ad libitum access to water and a semi-purified AIN-93G diet (Teklad, Madison, WI), as was reported previously (32). At the age of 50 days, all rats received via gavage 65 mg/kg DMBA (Sigma Chemical Co., St. Louis, MO) as previously reported (31). Rats were weighed twice weekly and were palpated for mammary tumor detection (twice weekly) beginning 2 weeks after DMBA treatment. Rats with tumor masses exceeding 2.5 cm in diameter were sacrificed early for humane reasons according to our IACUC-approved animal protocol. Rats were sacrificed 135 days post-DMBA treatment. All mammary tumors were excised, counted and weighed. Tumor sections were placed in 10% neutral buffered formalin and sections (5 µm) of the paraffin embedded tumors were stained with hematoxylin and eosin for histological analysis.

**Pathology.** All tumors were evaluated as previously reported (31) in a blinded protocol and were classified as benign or intraductal proliferation (IDP) as shown by multiple papillomas or with ductal hyperplasia, ductal carcinoma in situ (DCIS) or invasive ductal and lobular carcinoma (IDC).

**Statistical analysis.** A Kaplan-Meier analysis of tumor latency was performed (33,34). A generalized Wilcoxon log-rank
test (35) was used to compare median tumor-free times. A Chi-square test (36) was used to compare percentage of animals with tumors in each treatment group for each experiment. Where the sample was small, a Fisher’s exact test (36) was performed. The median numbers of tumors per tumor-bearing rat (multiplicity) for each group were compared using the non-parametric Kruskal-Wallis test (37). The total tumor volume and weight for each rat were calculated and compared for each group using also the Kruskal-Wallis test. Statistical significance was set at P<0.05, and all P values were unadjusted for multiple comparisons. For the few rats that were sacrificed early due to tumor burden, we assumed that the number of tumors remained constant until the end of the study. Data analyses were generated and plots were constructed using SPSS(c) version 13.0 for Windows (SPSS Inc., Chicago, IL).

Results

Body and organ weights. The average body weights (mean ± standard error) are shown in Table I and Fig. 1a. The L/O and O/O rats gained significantly more weight (P<0.001) than L/S and O/S rats. Obesity was associated with a significant (P<0.001) increase in absolute liver weights as well as relative liver weights, expressed as a percentage of body weight in both sham-operated and ovariectomized rats. However, ovariectomy was associated with a significant decrease in both absolute and relative liver weight compared to intact rats (P<0.001) (Table I).

Time course for tumor formation, latency and multiplicity. The time course of palpable mammary tumor detection is shown in Fig. 1b, and data are presented in Table II. The latency (the number of days after DMBA treatment until detection of the first mammary tumor) in the O/S group was 62 days, compared to 73 days in the L/S group and 101 days in O/O rats. No mammary tumors were detected in the L/S group during the course of this experiment. Tumor latency was shorter in the O/S group by 11 days when compared to the L/S group. Moreover, in obese rats, ovariectomy caused a delay of 39 days in tumor latency compared to in O/S rats (Table II). Using the day at which 25% of the rats developed mammary tumors as another marker of mammary tumor latency, O/S rats had a shorter latency period of 102 days compared to 134 days for L/S rats and 123 days for O/O rats.

Figure 1. Body weights and mammary tumor incidence and multiplicity in DMBA-treated rats. (a) Body weights of L/S (n=30), L/O (n=31), O/S (n=27) and O/O (n=25) female rats following oral gavage of 65 mg/kg DMBA at 50 days of age (study day 0). (b) Mammary tumor incidence (percentage of rats with tumors) in female rats. Dashed lines indicate the post-DMBA days at which 25% of the O/S, O/O, L/S and L/O rats developed at least one mammary tumor. (c) Mammary tumor multiplicity in O/S, O/O, L/S and L/O rats at 73, 104 and 135 days post-DMBA treatment.

Figure 2. Mammary tumor histology. (a) Intraductal proliferation (intraductal papilloma). Original magnification, x40. (b) Higher magnification of panel a. Original magnification, x200. (c) Ductal carcinoma in situ. Original magnification, x40. (d) Higher magnification shows a proliferation of uniform neoplastic cells with high N/C ratio within ductal structures. Original magnification, x200. (e) Invasive ductal carcinoma, arrow shows tumor necrosis in lower left corner. Original magnification, x200. (f) Higher magnification shows neoplastic cells diffusely infiltrating the stroma. Original magnification, x200.
At the end of the experiment, 59% of O/S rats developed mammary tumors compared to 30% of the L/S rats ($P<0.05$, Fig. 1b). However, 36% of the O/O rats developed mammary tumors while no tumors were detected in L/S rats ($P<0.001$). The O/S rats had a higher incidence of mammary tumor development than O/O rats (59% versus 36%) ($P=0.086$). The tumor multiplicity (median number of mammary tumors per tumor-bearing rat) was not significantly affected in either O/S or L/S rats (median, 1 tumor/rat; range, 1-4 tumors/rat) (Table II). Also, we evaluated tumor multiplicity at three time points (73, 104 and 135 days post-DMBA treatment) and the results are shown in Fig. 1c. At each of these time points, the O/S group consistently had more mammary tumors and more rats with at least one tumor compared with other groups. However, tumor multiplicity for the L/S and O/O groups was similar during these time points. These results indicate for the first time that mammary tumors can be induced by DMBA in obese Zucker rats in the absence of ovarian estrogens in contrast to the lack of mammary tumor development in L/O Zucker rats.

**Mammary tumor histology.** Mammary tumor histology is presented in Table II. Nine rats in the L/S group, 13 rats in the O/S group and six rats in the O/O group developed mammary tumors. Within the L/S group, one rat had at least one tumor graded as intraductal proliferation (IDP) while no other groups developed IDP graded tumors. One rat in the L/S group, seven rats in the O/S group and four in the O/O group had at least one tumor graded as ductal carcinoma in situ (DCIS), or invasive ductal and lobular carcinoma (IDC) as described in the Materials and methods. Obesity was associated with a non-significant increase in tumor weight.

**Discussion**

Obesity has been recognized as a risk factor for breast cancer development in postmenopausal women. The increase in risk for obese postmenopausal women has been attributed to increased estrogen exposure due to the high levels of aromatase expression in peripheral adipose tissue. In order to determine whether other sources of estrogen such as adipose tissue, may promote the development of mammary tumors in the DMBA model, we compared the rate of mammary tumor induction in...
overweight women. Studies by MacDonald androstenedione to estrone (46,47), especially in obese and produced in peripheral adipose tissue by aromatization of have shown that most estrogen in postmenopausal women is higher circulating estrogen levels than lean post-menopausal breast cancer risk (13,14). Obese postmenopausal women have shown that lifetime exposure to estrogen can increase development and growth (38-43). Estrogen is a necessary factor for the induction of mammary tumors by DMBA (41,43-45). The fact that O/O, but not L/O rats are susceptible to DMBA-induction of mammary tumors strongly implicates adipose tissue as the source for the estrogen necessary for tumor induction in the absence of ovarian estrogen. These data demonstrate for the first time the development of mammary tumors in O/O Zucker rats in the absence of ovarian estrogen. Our present study confirms that obesity is a major risk factor for mammary tumor development in the DMBA-induced Zucker rat model of mammary carcinogenesis. Furthermore, we have developed a new animal model to study the induction of mammary tumors in O/O rats. We believe that mammary tumorigenesis in O/O Zucker rats mimics the conditions that are present in obese postmenopausal women. These conditions include a lack of gonadal estrogen and aromatization of androgens to estrogen in adipose tissue as the major source of estrogen.

DMBA-induced mammary tumor induction has been widely used as a model for human breast cancer. The DMBA-induced mammary tumors are dependent on estrogen for development and growth (38-43). Estrogen is a necessary factor for the induction of mammary tumors by DMBA (41,43-45). The fact that O/O, but not L/O rats are susceptible to DMBA-induction of mammary tumors strongly implicates adipose tissue as the source for the estrogen necessary for tumor induction in the absence of ovarian estrogen. These data demonstrate for the first time the development of mammary tumors in the absence of gonadal estrogen using the obese Zucker rat model.

Several epidemiological and experimental animal studies have shown that lifetime exposure to estrogen can increase breast cancer risk (13,14). Obese postmenopausal women have higher circulating estrogen levels than lean post-menopausal women, which can promote breast cancer development. It has been shown that most estrogen in postmenopausal women is produced in peripheral adipose tissue by aromatization of androstenedione to estrone (46,47), especially in obese and overweight women. Studies by MacDonald et al (47-50) demonstrate that the rate of conversion of androstenedione to estrone is increased by obesity. Another factor associated with obesity is low levels of sex hormone binding globulin which results in increased bioavailable estradiol (51). In postmenopausal women, two sources of estrogens can contribute to breast cancer development. The estrogen synthesized in adipose tissue and skin reaches breast tissue through the circulation by an endocrine mechanism, while estrogen produced locally in breast tissue acts by paracrine or intracrine mechanisms to significantly increase local estrogen concentrations.

Several possible explanations for the increased breast cancer risk in postmenopausal women have been postulated; some mechanisms include the involvement of estrogens and androgens, leptin, insulin, insulin-like growth factor (IGF-1) and the aromatization of androgen to estrogens in adipose tissues.

Circulating leptin increases as body weight and fat mass increase (52,53) which may be another contributing factor for breast cancer development in obese postmenopausal women and obese ovariectomized Zucker rats. Previous data suggest that serum leptin may play an important role in the promotion of breast cancer (18,54,55). Leptin, which is a protein product of the ob gene, is expressed primarily in adipose tissue with activity in other tissues such as breast tissue (56). In other animal models, leptin has been found to increase estrogen levels in mammary tissues which can induce proliferation of normal and malignant mammary cells (57). Obese Zucker rats have increased circulating leptin levels that result from a leptin receptor mutation (58). Increased aromatase activity contributes to increased estrogen synthesis in adipose and breast tissues and may be an important factor in increased mammary tumor susceptibility in O/O Zucker rats.

Higher insulin levels are also associated with obesity and may enhance cellular proliferation leading to development of breast cancer (15). A related protein, IGF-I, functions as a growth factor for mammary epithelial and tumor cells and increases in the circulation as body mass increases. Recently, several epidemiological studies (59-63) have shown that women with elevated blood concentrations of IGF-I have an increased risk of breast cancer.

Animal models have shown an association between serum IGF-I concentrations and mammary cancer risk. For example, LIID mice, which have hepatic IGF-1 gene deletion, have a 75% reduction in serum IGF-I levels but exhibit normal growth and development (64). Also, these mice have shown a delay in onset of chemically and genetically induced mammary tumors (65). We recently reported a significant increase in circulating IGF-1 levels in obese Zucker rats compared to lean littermates (66). This increased level may contribute to the mammary tumor induction that we observed in our obese Zucker rat model.

Other animal models have been used to investigate the association of body weight with increased incidence of both spontaneous and chemically induced mammary tumors in the presence of gonadal estrogen (20,22,23,67). Some studies used obese intact animal models such as the leptin-deficient (MMTV-TGF-α/Leprdb Leprdb) mouse model or the leptin receptor-deficient mouse model (MMTV-TGF-α/Leprdb Leprdb). However, the obese animals in these studies failed to develop mammary tumors (68,69).

The present study used the DMBA-induced mammary tumor model to investigate whether the increased adipose tissue in O/O animals would promote development of mammary tumors. To our knowledge, this study is the first to report that obesity can promote DMBA-induced mammary tumor induction in obese Zucker rats in the absence of ovarian estrogen. We hypothesize that estrogen production in adipose tissue is sufficient to promote mammary tumor development in the DMBA-induced Zucker rat model. The underlying mechanism for this observation is currently under investigation in our laboratory.

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References


