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IMPACT OF OBESITY ON DEVELOPMENT AND PROGRESSION OF MAMMARY TUMORS IN PRECLINICAL MODELS OF BREAST CANCER

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Abstract

Overweight and/or obesity are known risk factors for postmenopausal breast cancer. More recently increased body weight has also been associated with poor prognosis for both pre- and postmenopausal breast cancer. This relationship has primarily been identified through epidemiological studies. Additional information from *in vitro* studies has also been produced in attempts to delineate mechanisms of action for the association of obesity and body weight and breast cancer. This approach has identified potential growth factors such as insulin, leptin, estrogen and IGF-I which are reported to be modulated by body weight changes. However, *in vitro* studies are limited in scope and frequently use non-physiological concentrations of growth factors, while long follow-up is needed for human studies. Preclinical animal models provide an intermediary approach to investigate the impact of body weight and potential growth factors on mammary/breast tumor development and progression. Here results of a number of studies addressing this issue are presented. In the majority of the studies either genetically-obese or diet-induced obese rodent models have been used to investigate spontaneous, transgenic and carcinogen-induced mammary tumor development. To study tumor progression the major focus has been allograft studies in mice with either genetic or dietary-induced obesity. In general, obesity has been demonstrated to shorten mammary tumor latency and to impact tumor pathology. However, in rodents with defects in leptin and other growth factors the impact of obesity is not as straightforward. Future studies using more physiologically relevant obesity models and clearly distinguishing diet composition from body weight effects will be important in continuing to understand the factors associated with body weight's impact on the mammary/breast cancer development and progression.

Introduction

Overweight and/or obesity are widely recognized as a risk factor the development of postmenopausal breast cancer (WCRF/AICR) [1-3]. Further a recent paper suggests that obesity may be a risk factor for triple-negative breast cancer in premenopausal women [4]. The risk has been assessed as both Hazards Ratio as well as the percentage of cases that may be due to overweight/obesity. Further evidence indicates that weight gain is an independent risk factor [5]. In addition, obesity at the time of breast cancer diagnosis is associated with advanced state of the disease [6] and has been reported to influence progression of the disease, responses to therapy, disease free survival and/or death regardless of menopausal

status [6]. For example, in a recently published study from Greece women with a body mass index ($BMI = \text{weight (kg)} \div \text{height (m}^2\text{)}$) of greater than 30 had a 27% increase in mortality compared to those with a BMI of less than 25 who would be considered to be normal weight [7]. In another recent paper obesity was associated with a worse prognosis but only for hormone-receptor positive breast cancer [8]. If this effect of obesity on prognosis occurs regardless of whether breast cancer is detected in premenopausal or postmenopausal women this suggests a different etiology of body weight for breast cancer development versus progression.

These data have primarily been obtained from epidemiological studies of cross-sectional, retrospective and prospective design. Also in many of the studies height and weight have been obtained by recall not by actual measurements potentially leading to errors in values used to calculate BMI and possibly to some of the discrepancies in the findings. Therefore there are still many unknown aspects of the effect of body weight on breast cancer development and progression.

The major explanations for how obesity influences breast cancer risk factor has focused on identification of serum growth factors associated with elevated body weight. In particular, for hormone dependent breast cancer there have been determinations that growth factors such as estrogen, leptin, insulin, and insulin-like growth factor-I (IGF-I) in particular may mediate the effect of obesity on breast cancer. Numerous *in vitro* studies highlighting the impact of these individual growth factors on cell proliferation, angiogenesis and apoptosis on human breast cancer cell lines have supported roles for these proteins as growth factors in breast cancer. Numerous review papers have addressed many of these issues. Recently, the role of chronic inflammation associated with obesity has also been under consideration for providing an environment conducive for tumorigenesis including breast cancer [9-12].

To evaluate the complexities of obesity's impact on breast cancer studies using preclinical animal models should bridge the gap to connect the human observations with the *in vitro* experimental studies. Animal models provide the opportunity to undertake long term investigations which can be carried out in a much more reasonable time frame than those in humans. Further such an approach could allow for the evaluation of the effects of obesity at specific time points and also evaluate the impact of interventions on the disease process. Highlights of publications that address the issue of obesity and mammary tumorigenesis will be presented here. Further suggestions as to what types of studies may be considered in the future to address this important interrelation as the obesity epidemic affects populations worldwide.

Genetic Obesity and Mammary Tumorigenesis

Several of the early studies published on the effects of obesity and mammary tumorigenesis were done using the A^{vy} yellow obese mouse. In this strain, mutations in the *agouti* gene cause ubiquitous expression of the agouti protein that has appetite stimulating effects; and the defect is associated with hyperinsulinemia [13].

The A^{vy} mouse model of obesity was used to assess the development of both spontaneous and chemically-induced mammary tumors. For example, spontaneous mammary tumor

incidence (murine mammary tumor virus (MMTV)) reached almost 100% for both breeding lean and virgin yellow obese female mice at 8 months of age while virgin lean mice did not reach 100% incidence until 16 months of age [14;15]. It was also reported that hyperplastic alveolar nodules (HAN) were detected at a younger age in obese mice, *i.e.*, 64% of virgin A^{vy} yellow obese mice by 6-7 months of age compared to only 34% of lean mice [16]. Further at this age there were no detectable mammary tumors in lean mice compared to a 20% incidence in the yellow obese mice. Note that these obese mice are infertile and thus the effects of pregnancy were not examined.

Carcinogen-induced mammary tumorigenesis was also investigated in A^{vy} mice (balb/c strain) treated with one of two doses of 7,12-dimethylbenz[a]anthracene (DMBA) (1.5 or 6.0 mg) which was administered at 8 weeks of age and repeated weekly for six weeks [17]. After over a year of follow-up mammary tumor incidence was somewhat higher in the A^{vy} yellow obese compared to the lean agouti mice, 43% versus 33% at the 1.5 mg dose and 86% versus 71% at the higher dose of 6.0 mg (note, the authors did not indicate whether these differences were significant). However, latency defined as “time to tumor” was significantly shorter for A^{vy} yellow obese mice than for the lean agouti mice at both the lower ($p<0.0021$) and higher ($p<0.0007$) DMBA doses. In this case the effect of obesity was stronger on tumor latency than on tumor incidence.

Additional investigations of the effect of genetic obesity on the development of mammary tumors have been done using the *obob/Lep^{ob}Lep^{ob}* mouse strain. This is a homozygous recessive trait associated with hyperinsulinemia and infertility which became a widely used obesity model following its introduction in 1950[18]. In 1994 the metabolic defect of *Lep^{ob}Lep^{ob}* mice was reported to be a defect in the production of, leptin [19]. The first study using this mouse strain to investigate mammary tumor development was published in 1962 and indicated that *Lep^{ob}Lep^{ob}* female obese mice had reduced spontaneous mammary tumor development compared to lean mice although the age of mammary tumor detection was shorter, 10.7 versus 17.6 months of age [20].

No further studies were done with this mouse model for over 30 years when it was crossed with a transgenic mouse model of mammary tumorigenesis to provide a model of postmenopausal breast cancer. In this case the *Lep^{ob}Lep^{ob}* mice were selected not because of their genetic defect *per se* but to avoid potential complications of interpreting results of feeding a high fat diet to induce obesity. The transgenic mouse strain, MMTV-TGF- α which over-expresses human transforming growth factor-alpha (TGF- α), was chosen as the breast cancer model because it was reported to have mammary tumor incidence of 30% at 16 months of age [21]. This characteristic would allow detection of changes in latency as well as incidence over time. Lean and obese mice were followed for two years and interestingly no mammary tumors were detected on the *Lep^{ob}Lep^{ob}*-TGF- α mice [22]. In contrast, lean wild-type TGF- α mice or those lean mice with one copy of the obese gene had mammary tumor incidence rates of 50% and 67% respectively. A second similar study using *Lepr^{db}Lepr^{db}* mice (reported in 1995 to have a defect in the leptin receptor (OB-R) but with high circulating levels of leptin [23] also found that these genetically obese mice did not develop mammary tumors while the wild-type and heterozygous mice had incidence rates of 69% and 82%, respectively [24]

While these studies with *Lep^{ob}Lep^{ob}* and *Lep^{db}Lep^{db}* mice were underway increasing information about leptin was being published. Although, most of the work was focused on the direct relationship of leptin to its roles in obesity, body weight regulation and food intake there were some indications that leptin had additional physiological actions. In particular, the leptin receptor was detected in several human breast cancer cell lines and the addition of leptin promoted breast cancer cell proliferation [25-27]. These studies led to the hypothesis that leptin could be a growth factor associated with obesity due to elevated levels in the enlarged fat depots.

However, other studies using genetically obese rat models also with defects in the leptin receptor have not reported consistent results for the development of chemically-induced mammary tumors. In fact two different strains, LA/N-cp Corpulent rats [28] and *fa/fa* Zucker rats [29] DMBA-induced mammary tumor incidence was increased and latency was reduced significantly compared to lean rats. In the Corpulent rats tumor size and tumor burden were reported to be increased while in the Zucker rats there was not an effects of obesity on either of these factors, but there was a significant increase in the number of obese rats with at least one invasive and lobular carcinoma compared to lean rats. In a second study using the Zucker rat ovariectomies were performed at 40 days of age [30]. In intact rats results were similar to this groups' first study with the obese rats having a shorter latency and increased incidence of mammary tumors compared to corresponding lean rats. However, although ovariectomy resulted in no mammary tumors to be detected in lean rats in obese rats this surgical intervention resulted in 36% incidence of mammary tumors in the obese rats compared to 30% for intact lean and 59% for intact obese rats. In an additional study lean and obese Zucker rats given DMBA were also given hormones to mimic pregnancy and some of each genotype were also fed a high fat diet (45% fat calories versus 16% in control diet) to further induce excessive weight gain [31]. Obese rats on the control diet had a 26% mammary tumor incidence which was similar to that of lean Sprague-Dawley rats, 22%, but higher than that of lean Zucker rats with only a 3% incidence. When fed the high fat diet the Obese Zucker rats had a 50% incidence compared to 39% for the corresponding Sprague-Dawley and 8% for the lean Zucker rats. In contrast to these reports in another case Zucker rats did not form DMBA-induced mammary tumors (Cleary, M.P. and Morton, R. unpublished). In an additional study a different carcinogen, *N*-methyl-*N*-nitrosourea (MNU) was administered to lean and obese Zucker rats [32]. Palpable tumor incidence rates were similar for both lean and obese rats but malignant carcinomas were found in only 10% of the obese rats compared to 50% of the lean ones. On the other hand colon cancer was detected in 13% of obese rats but not in any lean rats.

The fact that in some cases mammary tumors developed despite defects in the leptin receptor may be a consequence of permissiveness of the leptin receptor in different colonies of rats in conjunction of the very high serum leptin levels characteristic of these models which may override receptor defects. A summary of the results of the effects of genetic obesity on mammary tumor development are presented in Table 1.

Diet-induced obesity and mammary tumorigenesis

Following interest in the potential effects of high fat diets as causative in breast cancer many rodent studies were conducted prior to the 1990's addressing this issue. A number of reviews have been published focused on results of these types of studies which were primarily focused on carcinogen-induced tumors in Sprague-Dawley rats and spontaneous tumors in different strains of mice [33-37]. Effects on body weight were not the primary focus of these investigations. More recently diets with levels of fat 30% or greater up to 60% of total caloric intake have been fed to rodents to induce weight gain to study the effects of diet-induced obesity on mammary tumor development. The response of the animals to weight gain can be dependent upon the amount of dietary fat as well as strain and sex. Here, we focus on studies where body weight increased and obesity resulted from the consumption of the high-fat diets.

With respect to the effect of diet-induced obesity on the development of mammary tumors studies have been published using several different mouse models for breast cancer comparing animals all fed the same moderately high fat diet and then divided into Obesity-Prone and Obesity-Resistant groups based on weight gain allowing for comparison of body weight independent of diet composition. Two studies have been reported using the MMTV-TGF- α mice as a model of hormone response breast cancer as well as a study in MMTV-neu mice as a model of estrogen receptor negative breast cancer with HER2/neu overexpression [38-40]. MMTV-TGF- α mice on the C57BL6 background designated as Obesity-Prone had mammary tumors detected at an earlier age compared to the non-obese, Obesity-Resistant counterparts and/or to Low-Fat lean mice [38;40]. Also the Obesity-Prone mice had some tumors which were classified as high-grade adenocarcinomas. Serum leptin levels were significantly higher in Obesity-Prone mice compared to Obesity-Resistant and Low-Fat mice [40]. However mammary fat pad and mammary tumor analyses for leptin related proteins by either mRNA or protein expression, *i.e.*, leptin, OB-R, OB-Rb and Stat3 only detected differences between high fat diet fed mice and those fed the low-fat diet. These were either not affected or were significantly reduced in the high fat diet fed mice. Reduction in PARP and its cleaved products and also caspase-3 were also found in mice fed the high fat diet compared to the low fat diet.

This same approach was applied to MMTV-neu mice (FVB/N background) but there was not a significant effect of body weight on mammary tumor development [39]. Although in retrospect mammary tumor incidence was highest in Obesity-Prone mice, 67%, compared to 45% in Low-Fat mice with Obesity-Resistant and Overweight mice having values of 59% and 56% respectively suggesting an effect. It is also worth noting that in comparison to MMTV-TGF- α mice (C57BL6 background) where Obesity-Prone mice reached body weights in excess of 40 grams [38;40] the Obesity-Prone MMTV-neu mice on the FVB/N background mice had an mean body weight of only 33 grams [39]. Further highest leptin levels in the MMTV-neu mice were 5-6 ng/ml versus 15 ng/ml in the MMTV-TGF- α mice. In a recent study where the focus was more on the high fat diet *per se* MMTV-neu mice were fed a 45% fat calorie diet in comparison to 10% fat diet. at 30 weeks of age there was a slight but significant increase in body weight of the High Fat Diet mice 27.1 ± 1.3 grams compared to 24.4 ± 0.6 grams for the Low-Fat Diet mice (mean $\pm$ sem $p < 0.05$) [41].

Mammary tumor development was followed until 400 days of age. Median age at which the first tumor was detected was similar in both groups of mice, however, almost twice as many High-Fat Diet mice developed a second tumor, 61% as did the Low Fat Diet mice, 36%, although the age of second tumor detection was not different. In general these findings agree with human studies indicating that obesity is implicated in the development of hormonally responsive breast cancers with the effect on hormone negative tumors less consistent.

Recently a similar approach of inducing dietary-induced obesity was used in rats to assess the effect of body weight on the development of carcinogen-induced mammary tumors [42]. Dietary-induced obesity was implemented in female Wistar rats using a slightly higher high fat diet with 46% fat by calories than that used in the mice studies described above and rats were classified as Obese, Mid-weight and Lean. Mammary tumor were a result of MNU injected at ~52 days of age. Within each body weight category most were ovariectomized (OVX) at 19 weeks of age with the remaining rats designated as SHAM (9 rats with representatives from the different body weight groups, *i.e.*, 3 Obese, 2 Mid-weight and 4 Lean). Tumor numbers was similar in all body weight groups before OVX but afterwards Obese-OVX and SHAM (regardless of body weight status) rats almost doubled their mammary tumor numbers compared to pre-OVX values, while tumor numbers remained relatively stable in Lean-OVX and Mid-Weight-OVX rats. In addition, Obese-OVX rats had 70% of their mammary tumors expressing high ER- α levels which was similar to the value for SHAM rats while Nonobese-OVX (combined Lean-OVX and Mid-weight-OVX) rats had only 30% of mammary tumors classified as expressing high ER- α . Pre-surgery estradiol levels were similar in all body weight groups and post-surgery were reduced substantially, therefore the results suggest that some other factor in the Obese-OVX rats may be able to substitute for this hormone.

A second paper from MacLean's laboratory presented additional results from the rats in the study just described but with the rats now classified as Obesity-Prone (Obese) and Obesity-Resistant (Lean), *i.e.*, heaviest and light groups [43]. These two body weight groups into further divided into Low and High Energy Excess subgroups based on weight gain in the 48 hour period prior to euthanasia. A dual tracer approach was used to assess trafficking of glucose and dietary fat in liver, tumor, mammary gland, muscle, and retroperitoneal fat pads. Glucose uptake in tumors from Obese-High Energy-Excess rats was statistically greater than that of the Obese-Low Energy Excess rats and also higher than that of the two Lean groups although not statistically different. In contrast, for mammary gland the Lean-High Energy Excess group had the highest value and for liver the lowest. There were no differences among the groups for the other two tissues (liver and muscle). With respect to dietary fat retention the only significant difference noted was for mammary gland which tended to have higher values in Lean and Obese High-Energy Excess groups. Another aspect of this study was the observation that obesity was associated with high progesterone receptor expression in mammary tumors. Further analyses indicated that progesterone receptor expression in Obese-High Energy Excess tumors was associated with increased glucose uptake, reduced dietary fat retention and high fatty acid synthetase expression.

An alternative approach for comparing mammary tumor development in obese vs. non-obese rats has been developed in the laboratory of Dr. Henry Thompson. In this case

Sprague-Dawley rats were bred from the commercially available Levin strains (Taconic Farms), which were originally selectively bred for >20 generations for resistance (Diet-Resistant) or susceptibility (Diet-Susceptible) to obesity when fed a diet containing 32% by kcal [44-47]. These two rats were used to evaluate the effects of body weight on mammary tumors induced by their rapid emergence induction method. In this approach female Sprague-Dawley rats are given MNU at 21 days of age [48] and mammary tumors begin to emerge after sexual maturity and recapitulate the process of breast carcinogenesis in women [49;50]. At the end of the 9 week study, Diet-Susceptible rats weighed 15% more than Diet-Resistant rats, with a 6-fold elevation in circulating leptin indicating expanded adiposity in Diet-Susceptible rats [51]. All cancer endpoints evaluated, including incidence (26% increase), multiplicity (2.5-fold increase), and burden (5.4-fold increase), support a role for excess weight gain and adiposity in the accelerated mammary tumor development observed in Diet-Susceptible rats compared to Diet-Resistant rats. For example, Diet-Susceptible rats reached a 50% incidence of mammary tumors at 39 days post-carcinogen compared to 49 days post-carcinogen for the Diet-Resistant rats, a 16% faster detection of palpable tumors, and at study termination only 9% of Diet-Susceptible rats were tumor-free compared to 35% of Diet-Resistant rats. Since these were rats were ovary-intact, this is considered a model of premenopausal breast cancer and suggests that body weight impacts tumor development in a population with elevated breast cancer risk.

An additional study examined early effects of obesity induced by feeding a 60% fat calorie diet to Sprague-Dawley rats on mammary tissue [52]. The high fat diet feeding was initiated at 8 weeks of age and after 5 weeks the rats were treated with DMBA (10 mg) at which time the low-fat fed rats designated as Normal Weight averaged 272 grams and the Obese 322 grams. No attempt was made to divide Obese rats into obesity-prone versus obesity resistant groups nor was this addressed but given the small number of rats per group used this would have been difficult (n=6 from Normal Weight and Obese groups and n=3 for respective control rats without DMBA). The rats were followed for 7 additional weeks and prior to termination were injected with 5-bromo-2-desoxyuridine (BrdU) to determine *in vivo* cell proliferation at an early point in mammary tumor development verified by the fact that no Normal Weight rats had developed palpable tumors and only one Obese rat did. Cell proliferation in mammary glands of the rats not given DMBA were similar in Normal Weight and Obese rats while both groups of DMBA treated rats had higher levels than those of non-treated rats but the Obese-DMBA had the highest value a four-fold increase above the non-DMBA treated rats while the Normal Weight-DMBA rats had two-fold increase.

A summary of the studies of diet-induced obesity and its effects on different mammary tumors of several different etiologies is presented in Table 2. In general results are supportive of a role of elevated body weight and shortened latency and tumor burden and in some cases there are effects on tumor latency.

Chemically-induced obesity and mammary tumorigenesis

Another approach to inducing obesity in rodents is to damage the hypothalamus. In mice this has been done using goldthioglucose (GTG). Following a single injection of GTG independent of age and sex mice rapidly gain weight and become obese over a range of

GTG doses [53-57]. Several studies have used this approach to study mammary tumorigenesis. For example, C3H mice were injected at 2-3 months of age with GTG (10 mg) which resulted in 80% of the survivors developing obesity [58]. The 50% incidence rate for development of spontaneous mammary tumors for the GTG obese mice was reached at 295 days of age at which time the incidence rate was only 19% for the lean mice. The lean group did not reach the 50% incidence level until they were 354 days of age. In another study C3H mice that were ovariectomized at 12 weeks of age and two weeks later given GTG (7.5 mg) and spontaneous mammary tumor development was reduced to 16% compared to 44% for obese intact mice while it was 34% for lean intact mice but no tumors developed in the control OVX mice [59].

In another study ovariectomized GTG (0.5 mg/kg) obese mice were implanted with T47-D human breast cancer cells [60], an estrogen receptor positive (ER⁺) cell line. This cell line was chosen because previously it was reported to increase proliferation with exposure to leptin [25;27]. GTG-obese mice reached on average 50 grams compared to 33 grams for lean mice. Mice were not only inoculated with the cells but also implanted with estradiol pellets. Interestingly, mice did not exhibit any tumor growth. This was followed by a second experiment which included mice implanted with placebo pellets rather than estradiol. In this environment the GTG (0.3 mg/kg) obese mice without estradiol had 100% incidence of mammary tumors as did 50% of the non-obese mice while GTG-obese mice with estradiol again had 0% incidence. In both experiments serum leptin levels of the GTG-obese mice were elevated six-fold or more above levels of lean mice.

Diet-Induced Obesity and Tumor Progression-

The last study described above that used implanted cells may be considered an example of the effect of body weight on tumor progression rather than tumor development. Although many rodent studies have focused on examining the role of body weight on the development of mammary tumors there are an increasing number of reports evaluating the effects of body weight on tumor progression. These experiments have utilized both xenografts from implanting human breast cancer cell lines into immunocompromised mice and allografts from implanting syngeneic mouse derived mammary cancer cell lines.

In one case the xenograft approach was used to try to assess the effects of body weight on the development of ER⁺ versus estrogen receptor negative (ER⁻) human breast cancer cell lines in mice on the same background strain in response to diet-induced obesity [61]. This was a follow-up to the results presented above for MMTV-TGF- α and MMTV-neu mice where findings suggested that body weight influenced development of ER⁺ tumors to a greater extent than it did ER⁻ tumors. However, background strains of the mice were different which complicated data interpretation. In particular the FVB/N mice were not as responsive to high fat feeding compared to C57BL6 mice resulting in their weight gain being quite a bit less and serum leptin levels were lower too. To address this MCF-7 (ER⁺) and MDA-MB-231 (ER⁻) human breast cancer cell lines were implanted into Obesity-Prone and Obesity-Resistant C57BL6 immunosuppressed RAG-1 mice [62]. Mice were started on the high fat diet at 8 weeks of age and inoculated with the cell lines at 10 weeks of age at which time those receiving the MCF-7 cells also were implanted with estrogen pellet and

those receiving MDA-MB-231 cells received a placebo pellet. Mice were followed for 20 weeks. At the termination of the study tumor growth was quite limited in mice with MCF-7 cells with ~50% implantation rate regardless of body weight status or having been fed a low versus high-fat diet while for the MDA-MB-231 mice feeding the high fat diet resulted in 90% tumor take compared to 60% for the low-fat group. In general serum leptin and IGF-I levels were not related to tumor factors such as tumor size or tumor grade. Due to the small size of the MCF-7 tumors further analyses of tumor proteins was not done. For the MDA-MB-231 there were no consistent patterns for protein measurements associated with cell proliferation/apoptosis or leptin and IGF-I actions (OB-Rb, OB-R, JAK2, STAT3, pSTAT3, PCNA, Cyclin D1, Bcl-2, Bax, IGF-1R and IGBP3).

In a syngeneic model, Met-1, an ER-mammary tumor cell line, was subcutaneously implanted into ovariectomized FVB/N mice which were fed either a low fat (10% fat calories) or high fat (60% fat calories) diet from 6 weeks of age [63]. The high fat diet mice weighed ~30% more than low fat diet mice and they had ~80% more body fat. After 19 weeks on the diets the tumor cells were injected subcutaneously and followed for 35 days at which time the tumors from the mice on the high fat diet were significantly bigger 1450 mm³ versus ~1000mm³ (extrapolated from figures). Another component of this study was to treat some mice with estradiol which reduced tumor growth while alcohol included in the drinking water enhanced tumor growth in both low and high fat fed mice. High fat diet/obese mice had higher leptin levels than low fat diet mice and alcohol consumption dramatically reduced this with little effect on insulin.

Another approach to obtain mice with different body weights was to feed diets with differing fat contents [64]. Female C57BL6 mice were fed three different diets with those fed a high fat diet (5.2 kcal/g, 60% fat calories) were designated as Obese. A second group was classified as Overweight group were fed a normal low fat control diet (3.8 kcal/g, 10% fat calories) and a third group, Lean, were 30% calorie restricted (3.78 kcal/g 14% fat calories). Further to mimic premenopausal versus postmenopausal status some mice were ovariectomized at five weeks of age. After 25 weeks on the diets mice were inoculated with mammary tumor cells (ER+) obtained from syngeneic Wnt-1 transgenic mice. After 44 days tumor growth was the greatest in the non-OVX obese and Overweight mice compared to the Lean intact mice while for the castrated mice the Obese-OVX mice had the biggest tumors followed by the Overweight-OVX and then the Lean OVX. In general tumor growth of the ovariectomized mice were reduced compared to intact corresponding groups. Serum insulin, tissue plasminogen activator inhibitor (t-PAI) and IGF-I levels were elevated in response to the body weight increases while there was little effect on adiponectin and resistin levels.

Another study was recently published using this approach which included the addition of treatment with an mTOR inhibitor, RAD001 to some of the mice two weeks after cell inoculation [65]. Additionally, the Obese mice were switched from the high fat to the control diet at 17 weeks of age and cells were injected at 20 weeks of age. Tumor weight of the Formerly Obese mice was higher than that of the Control and Calorie Restricted groups and the addition of RAD001 to all groups resulted in reduced tumor growth. Unfortunately an Obese group was not included for the treatment component.

In an attempt to more closely mimic postmenopausal breast cancer C57BL6 were ovariectomized sy 60 weeks old and then they were fed either high fat 60% or low fat 4% diets for 8 weeks [66]. The mice were then inoculated with syngeneic E0771 cells and tumor growth followed for four additional weeks. After 5 weeks consumption of the high fat diet body weights were significantly heavier in the OVX-high fat mice compared to the OVX-low fat. At study termination OVX-high fat mice were 33% heavier than OVX-low fat mice and tumor weight was increased 200% and tumor weight was positively correlated with visceral fat pad weight. One focus of this study was angiogenesis which was found to be increased in tumors from the OVX-high fat mice as assessed by CD-131 staining and VEGF protein levels and VEGF was also higher in plasma.

In a different approach Wnt-1 cells dissociated from MMTV-Wnt-1 mammary tumors were transplanted into *Lep^{ob}Lep^{ob}*, *Lep^{db}Lep^{db}* or wildtype female mice at six weeks of age and growth followed until a tumor size of 1500 mm³ or 45 days post-inoculation was reached [67]. Body weights of the two obese strains were similar but there was little tumor growth in the leptin deficient *Lep^{ob}Lep^{ob}* mice and the tumors that did form which much smaller than those in wildtype mice. In contrast *Lep^{db}Lep^{db}* mice reached the tumor cut off size of 1500mm³ at which time the tumors from the wildtype mice averaged 280 mm³. In another aspect of this study it was determined that the leptin-deficient *Lep^{ob}Lep^{ob}* mice had fewer tumor stem cells in the tumors that did for and further leptin addition enhanced tumorsphere formation in CD29+CD24- cells which express the leptin receptor.

Effects of Diet-Induced Obesity on Mammary Tissue

There are additional published papers that although tumor development/progression is not an endpoint do provide interesting insights into the quest as to how body weight impacts mammary tumorigenesis. Highlights of some of these investigations will be presented.

One of the primary hypothesis as to how obesity is linked to increased breast cancer risk is that elevated aromatase activity in adipose tissue converts circulating androgens to estrogen providing as essential growth factor for breast cancer cell proliferation [68;69]. Recently this aspect of breast cancer etiology was studied in a high fat diet ovariectomized mouse model [70]. Mice were ovariectomized at 5 weeks of age and randomized to receive either a high fat diet (60% fat by calories) or low fat diet (10% fat by calories) and followed for 10 weeks. There was a graded effect on body weight with the high fat ovariectomized mice weighing the most followed by high fat, the low fat ovariectomized and the low fat intact mice were the lightest. It was noted that various measurements associated with inflammation were increased in the heavier mice particularly the high fat ovariectomized group compared to the low fat lighter weight mice. Assessment measurements included determining the number of inflammatory foci in the mammary gland as well as in visceral fat, measuring prostaglandin E2 levels in mammary glands and aromatase activity. Further, it was determined that pro-inflammatory mediators and aromatase activity were affected differently in adipocytes versus stromal vascular fractions of the mammary gland. Although mammary tumor development *per se* was not presented now that the model has been established this is clearly a next step. However given the results of other studies showing that ovariectomy compromises tumor development this may not turn out to be the best approach.

Different strains of mice and rats have been used to assess the effects of dietary-induced obesity on mammary tumor development with diets initially introduced at different ages. It is thus interesting to note a publication which addresses the impact of age of introduction of high fat diet on mammary tissue in two different mouse strains[71]. Balb-c and C57BL6 female mice were fed a 60% fat calorie diet from 3 to 7 weeks of age to study the effects on pubertal mammary pad development. Body and fat pad weights were significantly affected by high fat diet in the C57BL6 mice and serum leptin was increased, while in Balb-c mice these measurements were not impacted by the diet. It was also found that the pubertal C57BL6 mice fed the high fat diet had reduced mammary duct outgrowth compared to low fat diet mice and reduced terminal end buds as well as reduced cell growth. There were no notable differences between the two strains of mice in a number of measurements related to estrogen and progesterone receptor status. Interestingly switching the C57BL6 mice to the low-fat diet for an additional six weeks resulted in body weight 17 % lower than that of mice continued on the high fat diet and fat pad weights remained similar to that measured at the 10 week age when the mice were switched. Although this was described by the authors to be weight loss it appeared that it was weight maintenance. Thus it is unclear to some extent whether the results are due to the high fat diet or to obesity.

In another study 4 week old C57BL6 mice were fed 56.7% fat diet compared to low fat diet 10% fat calories [72]. The n value was 5/group and the mice were followed until 20 weeks of age at which time the high fat fed mice weighed almost 50% more than the low-fat fed mice and all fat depots measured were significantly heavier as was serum insulin and glucose but with no effect on adiponectin in the high fat diet mice. The high-fat diet mice had higher percentage incomplete ducts and reduced branching in mammary fat pads overall indicating impaired mammary ductal development.

Additional Points of Interest-

There are very few studies using other animal models to address the issue of body weight status and mammary tumorigenesis except in dogs. In one study leanness was associated with reduced risk of the disease but obesity did not enhance its development [73]. However, in another report obesity at one year of age was associated with an significantly increased risk of mammary tumors [74]. Dogs with mammary tumors also were obese at the time of diagnosis but the results did not reach statistical significance ($p<0.06$). The presence of obesity at the time of diagnosis of a malignant mammary tumor in dogs was associated with shorter postoperative survival [75].

The effect of obesity on breast/mammary tumor development and progression has primarily been focused on enlarged adipose depots and secretion/production of growth factors such as leptin and estrogens and the impact on whole body states such as insulin resistance, metabolic syndrome and inflammation. However, there is increasing interest in local effects of not only mature adipocytes but also other cell types associated with adipocytes within the tumor environment of the breast. Several reviews have addressed this issue in detail so here a recent study will be presented [76-78] }.

A unique approach to studying the effects of adipocytes directly on mammary tumor growth was to mix mouse 3T3-F442A pre-adipocytes with MCF-7 human breast cancer cells and injected subcutaneously into female SCID mice or to only inject the MCF-7 cells. [79]. Mice were not supplemented with estrogen. Mice injected with the preadipocytes developed mature adipocytes and tumors while no tumors developed in mice without the preadipocyte co-injection or with preadipocytes injected in the other flank. Further experiments suggested a role of leptin inducing aromatase activity at a local level, *i.e.*, in the adipocytes promoting local estrogen production. Interestingly female C57BL6 mice fed a high fat (60% fat calories) had a 45-fold increase in aromatase activity in the parametrial fat depot. In contrast obese *Lep^{ob}Lep^{ob}* (leptin deficient) mice had significantly reduced aromatase activity compared to lean *Lep⁺Lep⁺* mice. Leptin treatment increased aromatase expression in adipose tissue of both lean *Lep⁺Lep⁺* and obese *Lep^{ob}Lep^{ob}* mice. This study highlights the potential importance of the microenvironment in addition to the whole organism for tumor development.

Conclusions

Overall it appears that rodent models of cancer can be influenced by body weight status. In general obesity is associated with shortened latency resulting in tumor detection at a younger age and in some cases tumor burden is increased. At the time of study termination elevated incidence can be documented in many publications. However, this can be confusing because terminal endpoints of animal studies are usually predetermined by various guidelines. It is quite possible in many situations that if the experimental period was extended that in fact incidence rates would increase. There does seem to sufficient evidence that estrogen sensitive mammary tumor development is affected by body weight as is the case for human postmenopausal breast cancer as evidenced by results for MMTV-TGF- α mice. Some of the other models are more difficult to interpret because animals are young when studies are undertaken and impact of when used is not the same as attaining normal menopause which occurs over a period of time.

Dietary-induced obesity is most representative of the human situation although genetic obesities have provided interesting data with respect to aspects growth factors. However, feeding extremely high dietary fat levels of 60% is not representative of normal human diets particularly when comparisons are made to animals consuming low levels of dietary fat. Further many of these diets are high in corn oil and/or lard with potential limitations of omega-3 fatty acids and frequently these diets have high sucrose levels. Thus the issue of dietary effects versus body weight effects needs to be considered in future studies.

The fact that young animals as opposed to mature ones are usually used does detract from the possible significance of many of the results obtained since the major impact of body weight in humans appears to be on the development of peri- and postmenopausal breast cancers. Thus, the task in the future will be to utilize physiologically relevant models to focus on mechanisms of action. Overall potential biological mechanisms, which may participate in the association between obesity and breast cancer pathologies, include systemic low-grade inflammation, metabolic and endocrine factors. These findings

demonstrate the complex nature of interactions of obesity with other factors including sex hormones.

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Table 1

Effects of Genetic Obesity on Mammary Tumor Development

Obesity Model	Cancer etiology	Outcome	Reference
Yellow obese A ^{vy}	Spontaneous	Obese mice MTs at younger age than lean mice	[14-16]
Yellow obese A ^{vy}	DMBA	Obese mice shortened latency and increased incidence	[17]
<i>Lep^{ob}Lep^{ob}</i>	Spontaneous	Obese mice shortened latency and reduced incidence	[20]
<i>Lep^{ob}Lep^{ob}</i>	Transgenic-TGF- α	No tumors	[22]
<i>Lep^{db}Lep^{db}</i>	Transgenic-TGF- α	No tumors	[24]
LA/N corpulent	DMBA	Shortened latency 100% latency vs 21% for lean rats Increased tumor number and weight	[28]
Zucker (<i>fa/fa</i>)	MNU	10% carcinomas vs 50% in lean rats	[32]
Zucker (<i>fa/fa</i>)	DMBA	Shortened latency 68% incidence vs 32% in lean rats	[29]
Zucker (<i>fa/fa</i>)	DMBA	Obese intact rats 59% tumor incidence vs 36% for OVX-obese, 30% for lean intact rats and 0% for OVX-lean	[30]
Zucker (<i>fa/fa</i>) (all rats given hormones to mimic pregnancy)	DMBA	Obese rats 26% tumor incidence increased to 50% with high fat (45% fat calories) diet vs 3% and 8% for corresponding lean rats	[31]

Table 2

Effects of Diet-Induced Obesity on Mammary Tumor Development

Obesity Model	Cancer etiology	Outcome	Reference
Diet-induced C57BL6 mice (33% fat by calorie diet from 10 woa mice divided by weight gain)	Transgenic-TGF- α (C57BL6)	Shortened latency More palpable tumors Some high-grade adenocarcinomas in Obesity-Prone mice	[38;40]
Diet-induced C57BL6 mice (33% fat diet- mice divided by weight gain)	Transgenic-neu (FVB/N)	Little effect of obesity on latency or incidence	[39;41]
Diet-induced obesity Wistar rats + OVX @ 18 woa (46% fat diet from 6 woa)	MNU @ 52 days of age	At 18 woa (pre OVX) tumor number similar. Post OVX Obese and SHAM rats increased tumor number while Lean and Midweight no increase OVX-Obese increased ERa positive tumors vs OVX-nonobese	[42]
Diet-induced obesity Wistar rats + OVX @ 18 woa (46% fat diet from 6 woa) - Rats from above study further divided into Low Energy and High Energy Excess Lean and Obese groups	MNU @ 52 days of age	Obesity associated with increased excess nutrient deposit in tumors	[43]
Diet-Sensitive vs Diet-Resistance strains Sprague-Dawley rats ((32% fat diet from 3 woa)	MNU @ 21 days of age	Diet-Sensitive rats had increased tumor incidence and tumor burden	[51]
Diet-induced obesity Sprague-Dawley rats (60% fat from 8 woa vs 4%)	DMBA @ 56 days of age – study terminated after 7 weeks- early stage disease	Obese-DMBA rats highest in vivo cell proliferation	[52]