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Insulin-like growth factor (IGF)-I obliterates the pregnancy-associated protection against mammary carcinogenesis in rats: evidence that IGF-I enhances cancer progression through estrogen receptor- α activation via the mitogen-activated protein kinase pathway

Gudmundur Thordarson¹, Nicole Slusher¹, Harriet Leong¹, Dafne Ochoa¹, Lakshmanaswamy Rajkumar², Raphael Guzman², Satyabrata Nandi² and Frank Talamantes¹

¹Department of Molecular, Cell, and Developmental Biology, University of California, Santa Cruz, California, USA

²Cancer Research Laboratory, University of California, Berkeley, California, USA

Corresponding author: Gudmundur Thordarson, gummi@biology.ucsc.edu

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Abstract

Introduction Pregnancy protects against breast cancer development in humans and rats. Parous rats have persistently reduced circulating levels of growth hormone, which may affect the activity of the growth hormone/insulin-like growth factor (IGF)-I axis. We investigated the effects of IGF-I on parity-associated protection against mammary cancer.

Methods Three groups of rats were evaluated in the present study: IGF-I-treated parous rats; parous rats that did not receive IGF-I treatment; and age-matched virgin animals, which also did not receive IGF-I treatment. Approximately 60 days after *N*-methyl-*N*-nitrosourea injection, IGF-I treatment was discontinued and all of the animal groups were implanted with a silastic capsule containing 17 β -estradiol and progesterone. The 17 β -estradiol plus progesterone treatment continued for 135 days, after which the animals were killed.

Results IGF-I treatment of parous rats increased mammary tumor incidence to 83%, as compared with 16% in parous rats treated with 17 β -estradiol plus progesterone only. Tumor incidence and average number of tumors per animal did not differ between IGF-I-treated parous rats and age-matched virgin rats. At the time of *N*-methyl-*N*-nitrosourea exposure, DNA

content was lowest but the α -lactalbumin concentration highest in the mammary glands of untreated parous rats in comparison with age-matched virgin and IGF-I-treated parous rats. The protein levels of estrogen receptor- α in the mammary gland was significantly higher in the age-matched virgin animals than in untreated parous and IGF-I-treated parous rats. Phosphorylation (activation) of the extracellular signal-regulated kinase-1/2 (ERK1/2) and expression of the progesterone receptor were both increased in IGF-I-treated parous rats, as compared with those in untreated parous and age-matched virgin rats. Expressions of cyclin D₁ and transforming growth factor- β_3 in the mammary gland were lower in the age-matched virgin rats than in the untreated parous and IGF-I-treated parous rats.

Conclusion We argue that tumor initiation (transformation and fixation of mutations) may be similar in parous and age-matched virgin animals, suggesting that the main differences in tumor formation lie in differences in tumor progression caused by the altered hormonal environment associated with parity. Furthermore, we provide evidence supporting the notion that tumor growth promotion seen in IGF-I-treated parous rats is caused by activation of estrogen receptor- α via the Raf/Ras/mitogen-activated protein kinase cascade.

Keywords: estrogen receptor- α , growth hormone/insulin-like growth factor-I, mammary carcinogenesis, mitogen-activated protein kinase progesterone receptor

Introduction

A first full-term pregnancy early in life confers effective nat-

ural protection against breast cancer in women [1]. Rats exhibit a similar phenomenon, in that parity prevents

ER = estrogen receptor; ERK1/2 = extracellular signal-regulated kinase-1/2; GH = growth hormone; IGF = insulin-like growth factor; MAPK = mitogen-activated protein kinase; MNU = *N*-methyl-*N*-nitrosourea; PI3-K = phosphatidylinositol 3-kinase; PR = progesterone receptor; TEB = terminal end bud; TGF = transforming growth factor.

chemically induced mammary carcinogenesis [2]. The causes of this pregnancy-associated protection against mammary carcinogenesis are still being investigated. Changes in the mammary epithelia, such as high degree of differentiation, low level of proliferation, increase in cell cycle length, reduction in carcinogen binding to epithelial cells, and increased capacity for DNA repair have been associated with parity in rats [3-7]. More recently it was shown that gene expression is altered in the mammary gland of parous mice and rats as compared with virgin animals [8]; similarly, rats that have been made refractory to mammary tumorigenesis by estrogen and progesterone treatments also exhibit differences in the mammary gland gene expression as compared with untreated rats [9]. However, the functional significance of these alterations in gene expression in relation to the susceptibility of the mammary gland to carcinogenesis has not been demonstrated.

Parity also causes changes in the circulating levels of hormones that regulate mammary gland development and may affect the susceptibility of the mammary gland to tumorigenesis. For example, parity in women causes a persistent reduction in the concentration of prolactin in serum [10,11], and similarly parous rats have a significantly reduced circulating concentration of growth hormone (GH) as compared with nulliparous, age-matched animals [2]. Furthermore, in our previous studies [12] we demonstrated that treatment of parous rats with low doses of 17 β -estradiol and progesterone abolishes the protective effects induced by pregnancy. Therefore, the pregnancy-associated protection against mammary cancer can be nullified by changing the hormonal environment of the animal.

These findings cast doubts upon whether the changes in the mammary epithelia of parous animals are permanent phenotypical alterations or a reflection of altered hormonal environment. As mentioned above, we found a reduction in the circulating concentration of GH associated with parity in rats. An increasing body of evidence now indicates that the GH/insulin-like growth factor (IGF)-I axis is a determining factor in the susceptibility of the breast to cancer development. Early studies showed that administration of GH together with estrogen and progesterone restores chemically induced mammary tumorigenesis in hypophysectomized rats [13]. Later studies showed that inhibition of GH secretion reduces chemically induced mammary carcinogenesis in rats [14,15]. Similarly, mice carrying a transgene expressing GH antagonist (modified bovine GH) are at reduced risk for developing mammary cancer as compared with littermates not carrying the transgene [16]; in contrast, mice overexpressing GH exhibit an increase in mammary cancer development as compared with mice with normal GH levels [17].

Direct effects of IGF-I on normal mammary gland development and mammary carcinogenesis are also evident. IGF-I deficient (knockout) mice exhibit very limited mammary epithelial development [18]; this defect was remedied by IGF-I and 17 β -estradiol treatment, whereas 17 β -estradiol treatment alone was ineffective. Overexpression of IGF-I transgene targeted to the mammary gland by placing it under the control of whey acidic protein promoter inhibits involution of the mammary epithelia after lactation, indicating that IGF-I acts as a survival factor for the mammary epithelia [19,20]. This inhibition of involution is acquired, at least partly, through reduction in apoptosis of mammary cells [19,21]. Furthermore, continuous breeding of mice carrying the whey acidic protein promoter-regulated IGF-I transgene results in mammary cancer development, albeit after a long latency period [21].

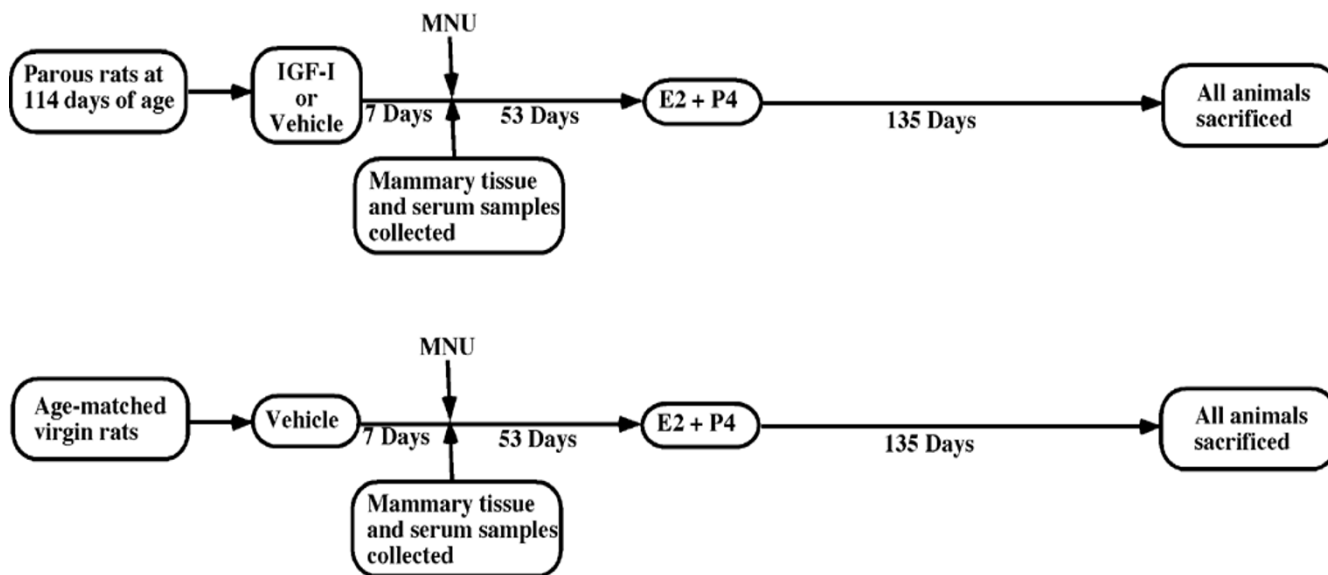
Epidemiologic studies have shown that a high circulating concentration of IGF-I is correlated with increased risk for breast cancer development [22], and GH and IGF-I concentrations in serum are elevated in breast cancer patients [23,24]. How the GH/IGF-I axis affects mammary tumorigenesis is not well established. The mitogenic activity of IGF-I in normal mammary epithelia is well known [25], and GH has been shown to regulate estrogen receptor (ER) expression in the mammary gland [26]. Indeed, we have found a significant reduction in levels of ER in mammary gland of parous rats as compared with age-matched virgin animals [2]. In the present study we investigated the effects of IGF-I treatment on the parity-associated protection against mammary cancer.

Methods

Animals

Female Sprague-Dawley rats, 6–15 per group, were kept in a 14-hour light/10-hour dark lighting schedule, and were given free access to food and water. To generate parous animals, virgin rats were mated at 50–55 days of age. After parturition, the pups were removed and the mammary glands of the mothers were allowed to involute for 40 days. At that time, the parous rats were used for experimentation. *N*-methyl-*N*-nitrosourea (MNU) was purchased from Ashe Stevens (Detroit, MI, USA) and administered in a single intraperitoneal injection [27]. Three groups of rats were used in the experiments. In one group, parous rats were treated with recombinant human IGF-I (Genentech Inc., South San Francisco, CA, USA) at a dose of 0.660 mg/kg body weight per day, administered via an Alzet osmotic pump (Durect, Cupertino, CA, USA), for 60 days commencing 7 days before MNU injection and continued for an additional 53 days. The second group included parous rats that did not receive any IGF-I treatment. The third group included age-matched virgin control animals, which also did not receive any IGF-I treatment. Approximately 60 days after the MNU injection, 1 week after the IGF-I treatment

Figure 1



Schematic representation of the animal treatments used in the present study. E2, 17 β -estradiol; IGF, insulin-like growth factor; MNU, *N*-methyl-*N*-nitrosourea; P4, progesterone.

was terminated, all animals were implanted with a silastic capsule [28] containing 20 μ g 17 β -estradiol (Sigma, St Louis, MO, USA) and 20 mg progesterone (Sigma). The capsules were changed every 2 months. The 17 β -estradiol plus progesterone treatment was continued for 135 days, after which all animals were killed. To assess the development of the mammary gland and the serum concentrations of IGF-I at the time of carcinogen administration, animals from each of the three groups were killed 7 days after commencement of treatment. Normal mammary tissues and serum were collected and stored at -80°C until they were analyzed (Fig. 1).

Wholemounts were prepared from the right second and third glands from the animals and used for assessment of mammary development. The frozen mammary tissues were used for Western blot analyses as described below and to assess total DNA, α -lactalbumin, and IGF-I contents of the mammary gland.

The MNU-treated animals were palpated weekly for detection of mammary tumors and tumors were removed from the animals under anesthesia when they had grown to 1.5 cm in diameter. At the time of tumor collection, a small sample was excised from each tumor for histologic classification. The serum samples were used to measure the circulating concentrations of IGF-I at the time of MNU injection.

The care and use of animals in the study was approved by the Chancellor's Animal Care Committee at the University of California at Santa Cruz.

Assessment of α -lactalbumin in mammary tissues

The content of α -lactalbumin in the mammary tissues was used as an indicator of differentiation of the mammary epithelia at the time of carcinogen exposure. The tissues were ground to a fine powder in liquid nitrogen with pestle and mortar and then homogenized on ice with a Polytron homogenizer (Brinkmann Instruments Inc., Burlingame, CA, USA) in 2 volumes (weight/vol) of 50 mmol/l Tris-HCl, 5 mmol/l MgCl₂ buffer (pH 7.5) containing 1 mmol/l Pefabloc (Roche Molecular Biochemicals, Indianapolis, IN, USA) and 1 μ mol/l Pepstatin A (Sigma). After the tissues had been homogenized, 20 μ l samples were obtained and used for measuring the total DNA content of the preparation, and the remainder of the samples were extracted for 1 hour and then centrifuged at 20,000 *g* for 30 min; both steps were conducted at 4°C. The supernatant was collected and used to measure total protein and α -lactalbumin concentration. A radioimmunoassay, specific for rat α -lactalbumin, was developed. Rat α -lactalbumin was purified from rat milk in accordance with a previously established method [29]. Antiserum generated against rat α -lactalbumin was generously provided by Dr Kurt E Ebner at the University of Kansas Medical Center. The within and between coefficient variations of the assay were 3.0% and 18.4%, respectively.

Wholemount preparation and tumor histology

Mammary gland wholemounts were prepared as described previously [30]. Briefly, animals were killed and pinned down on a corkboard, ventral side up. A skin incision was made between the nipples and down both the hind legs,

creating a cut in the shape of an inverted 'Y'. The mammary glands were exposed, and the right second and third glands removed and fixed in 10% buffered formalin. After de-fattening the tissue in acetone, the mammary epithelia was stained with iron/hematoxylin and inspected under a microscope for assessment of overall development. All tumors were classified microscopically. For that, a small specimen was obtained, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. The sections were then stained with hematoxylin and eosin and classified.

Measurement of IGF-I concentration in mammary tissues

IGF-I was extracted from the mammary glands as has previously been described [31]. Briefly, the tissues were pulverized as described above, homogenized in 1 N acetic acid (1 g tissue/5 ml acetic acid), and extracted on ice for 2 hours. After centrifugation at 20,000 *g* for 6 min, the supernatant was collected and the tissues extracted again as before, and the two extractions for each sample were combined and lyophilized. The lyophilized material was reconstituted in 50 mmol/l Tris/HCl (pH 7.8) at 1 g original wet weight of tissue per 2 ml buffer and assayed using radioimmunoassays for rat and, for those animals treated with human IGF-I, for human IGF-I (Diagnostic Systems Laboratories, Webster, TX, USA) either undiluted or diluted in assay buffer.

Protein and DNA assays

The serum concentrations of endogenous IGF-I and exogenous IGF-I (human IGF-I in treated rats) were assessed using radioimmunoassay kits from Diagnostic Systems Laboratories, as described above. The protein concentrations of extracted mammary samples were measured with BCA protein assay kit (Pierce, Rockford, IL, USA) using bovine serum albumin (Sigma) as the reference standard. The total DNA content of the mammary gland homogenates was assessed by fluorometric DNA assay [29] using calf thymus DNA (Sigma) as reference standard.

Western blot analyses

Western blot analyses were carried out as recently described [32]. Briefly, mammary gland tissues were homogenized and extracted, and solubilized protein electrophorized on 7.5–20% SDS-PAGE, depending on the size of the protein being analyzed, at 50–100 μ g total protein/lane, as determined by BCA protein assay (Pierce). Proteins were transferred to a PVDF membrane and the specific protein bands detected using chemiluminescence reagents and CL-X Posure™ Film (Pierce). Western blot analyses were carried out on cyclin D₁ using antibody sc-450 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); total ER- α using antibody Ab-15 (NeoMarkers Inc., Fremont, CA, USA); progesterone receptor (PR) using antibody no. A 0098 (DakoCytomation Inc., Carpinteria, CA,

USA); total and phosphorylated extracellular signal-regulated kinase-1/2 (ERK1/2) using antibodies #9102 and #9106, respectively (Cell Signaling Technology Inc., Beverly, MA, USA); and transforming growth factor (TGF)- β_3 using antibody GF16 (Oncogene Research Product, San Diego, CA, USA). Protein bands, detected with chemiluminescence, were quantified using the ImageJ (version 1.24o) image analysis program (National Institutes of Health, Bethesda, MD, USA).

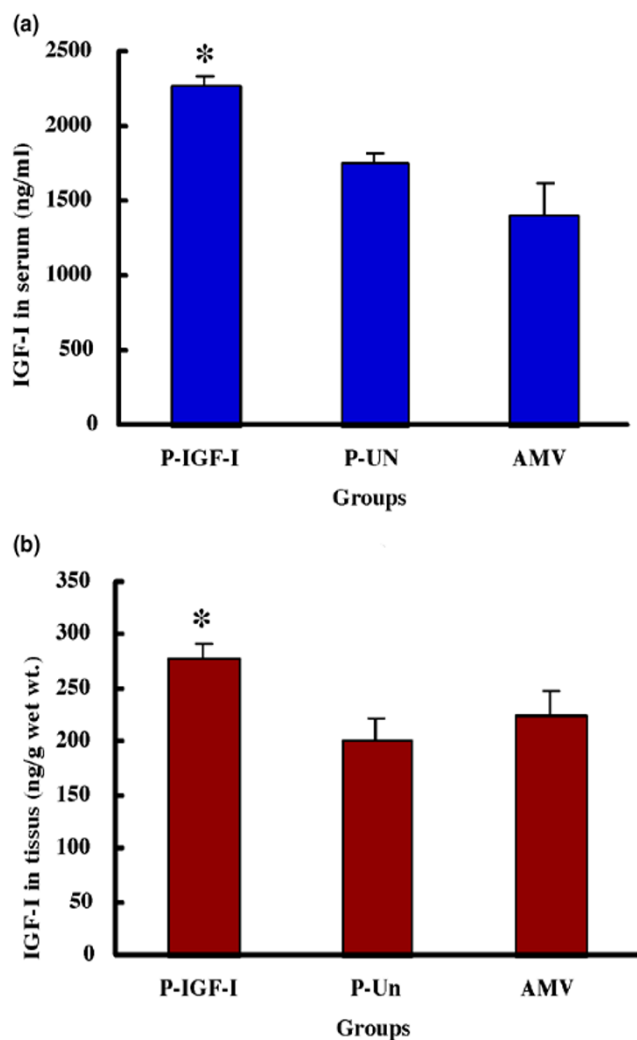
Statistics

Incidence of mammary cancer was analyzed using 2 \times 2 contingency tables and χ^2 tests. All other statistical analyses were carried out using analysis of variance and Fisher's protected least significant difference test. $P < 0.05$ was considered statistically significant.

Results

Treatment of parous rats with 0.66 mg IGF-I/kg body weight per day resulted in an increase in the circulating concentration of total IGF-I to 2289.2 ± 68.8 ng/ml (mean \pm standard error), which was a significantly higher concentration than that found in intact parous and age-matched virgin rats (Fig. 2a). The IGF-I concentration in the mammary tissues at the time of carcinogen exposure was also elevated in the IGF-I-treated parous animals as compared with untreated parous rats, but did not differ significantly between IGF-I-treated parous and age-matched virgin animals (Fig. 2b). Body weight gain did not differ between the animal groups, when assessed at the time of MNU injection and at the termination of the experiment (Table 1). However, the elevation of IGF-I in parous rats caused a significant increase in mammary tumor incidence as compared with parous rats treated only with 17 β -estradiol plus progesterone, beginning 60 days after the MNU injection. Tumor incidence in IGF-I-treated parous rats and age-matched virgin rats that also received 17 β -estradiol and progesterone treatment 60 days after MNU injection did not differ significantly, and neither did the average number of tumors per animal in these two groups (Table 2). All the animals that did carry mammary tumors developed at least one carcinoma (mostly ductal, papillary, and cribriform carcinomas). Development of fibroadenoma was rare (only 6–7%) and did not differ between IGF-I-treated parous and age-matched virgin rats.

The total DNA content of the mammary glands was significantly lower in the parous rats that did not receive IGF-I treatment, as compared with IGF-I-treated parous rats and age-matched virgin animals (Fig. 3). The stage of differentiation, as measured using the content of α -lactalbumin in the mammary gland, was significantly higher in mammary tissues from untreated parous rats compared with IGF-I-treated parous and age-matched virgin rats (Fig. 4). Inspection of the whole mounts revealed that the epithelial

Figure 2

Insulin-like growth factor (IGF)-I concentration in (a) serum and (b) mammary tissues of parous rats treated with insulin-like growth factor (IGF)-I (P-IGF-I), untreated parous rats (P-Un), and age-matched virgin rats (AMV). IGF-I treatment (0.66 mg/kg body weight/day) was continued for 7 days before samples were collected. IGF-I concentrations for P-IGF-I rats are combined endogenous (rat IGF-I) and exogenous (human IGF-I) values. Values are expressed as mean \pm standard error. * $P < 0.05$: versus AMV and P-Un in panel a, and versus P-Un in panel b.

structures were less dense in the parous untreated rats than in the parous IGF-I-treated and particularly in the age-matched virgin rats. Also apparent from the inspection of the whole mounts was that terminal end-buds (TEBs) were present in all of the animal groups and did not appear to be less abundant in the parous untreated animals than in age-matched virgin and IGF-I-treated parous rats (Fig. 5). These structures are traditionally associated with a high rate of epithelial proliferation and high susceptibility to cancer development.

The total protein level of ER- α in the mammary gland at the time of carcinogen injection was significantly higher in age-matched virgin animals than in untreated parous rats, and treatment of parous rats with IGF-I further lowered the concentration of ER- α (Fig. 6). Phosphorylation of ERK1/2 was significantly increased in mammary tissues of IGF-I-treated parous rats, whereas the lowest level of phosphorylation of ERK1/2 was found in the mammary tissues from untreated parous rats (Fig. 7). No difference was found in the levels of total ERK1/2 expression in mammary glands from the three groups of rats (Fig. 8). Like phosphorylation of ERK1/2, the expression of PR in mammary tissues was significantly elevated in animals treated with IGF-I, but PR expression was lowest in untreated parous animals (Fig. 9).

Expression of cyclin D₁ was lowest in mammary gland from age-matched virgin animals but was similar in mammary tissues from the two parous groups (Fig. 10). Similarly, the levels of TGF- β_3 were found to be lowest in mammary glands from age-matched virgin rats, but TGF- β_3 did not differ in mammary tissues obtained from the untreated and IGF-I-treated parous animals (Fig. 11).

Discussion

Several years ago we found that the circulating levels of GH and, to a lesser extent, of prolactin are significantly reduced in parous rats, and we speculated that this reduction in serum concentration of GH might be a determining factor in the reduced susceptibility of the mammary gland to cancer development associated with parity [2]. Much other evidence links GH and/or IGF-I (the GH/IGF-I axis) with both normal mammary gland development [25,33] and possible involvement in carcinogenesis of the breast [34,35]. In the present study we demonstrated that IGF-I treatment increases mammary tumorigenesis in parous rats to a level similar to that in age-matched virgin animals. We also previously showed that long-term treatments of parous rats with low doses of 17 β -estradiol and progesterone obliterate the parity-associated protection against mammary cancer [12]. In addition, it has been shown that dissociated mammary epithelial cells obtained from MNU-treated young virgin rats develop fewer tumors and exhibit a longer latency period when transplanted into parous syngeneic hosts as compared with cells transplanted into virgin syngeneic hosts [36].

The prevailing hypothesis regarding how parity protects the breast against cancer development has been that the mammary epithelia consist of undifferentiated, fast-growing TEB and terminal duct structures that are highly susceptible to carcinogenesis, and of differentiated, slow-growing alveolar structures that exhibit refractoriness to cancer development. Extensive differentiation of the mammary gland seen at parturition rids the gland of the fast-growing susceptible TEBs and terminal ducts, replacing them with

Table 1**Body weight**

Groups	Body weight (g) at MNU injection (mean \pm SEM)	Body Wt. (g) at Sacrifice (mean \pm SEM)
P-Un	286 \pm 3.2 (<i>n</i> = 7)	303 \pm 3.5 (<i>n</i> = 6)
P-IGF-I	286 \pm 6.2 (<i>n</i> = 7)	317 \pm 4.5 (<i>n</i> = 6)
AMV	293 \pm 5.4 (<i>n</i> = 7)	309 \pm 9.6 (<i>n</i> = 6)

Body weight at the time of *N*-methyl-*N*-nitrosourea (MNU) injection and just before the animals were killed at the termination of the experiment for untreated parous rats (P-Un), parous rats treated with insulin-like growth factor-I (P-IGF-I), and untreated virgin rats that were age matched with the parous animals (AMV).

Table 2**Mammary carcinogenesis in N-methyl-N-nitrosourea injected rats**

Groups	Cancer incidence (%)	Cancer load number/rat (mean \pm SEM)	Latency range (days)
P-UN (<i>n</i> = 6)	16*	0.167	121
P-IGF-I (<i>n</i> = 6)	83	2.17 \pm 0.75	57–176
AMV (<i>n</i> = 6)	100	2.20 \pm 0.45	92–217

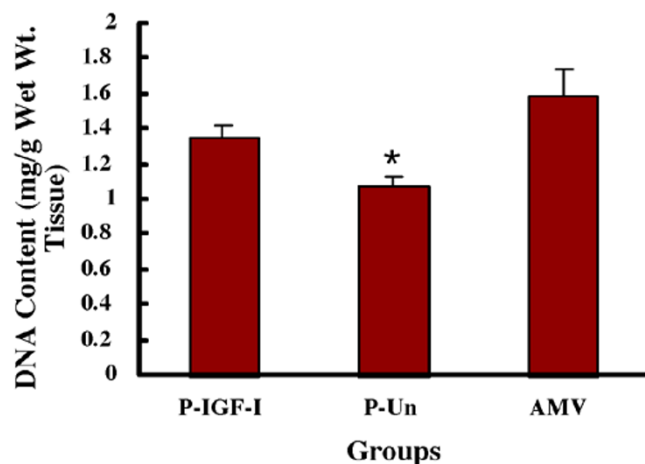
Mammary tumor incidence, latency, and load in untreated parous rats (P-Un), parous rats treated with insulin-like growth factor -I, and in virgin rats that were age matched with the parous animals (AMV). **P* < 0.05 versus P-IGF-I and AMV.

the differentiated, refractory alveolar structures. Accompanying the reduction in the proliferation rate of the mammary epithelia of the parous animals are other changes, such as increased capacity for DNA repair, decreased binding of the carcinogen to the DNA, and increased length of cell cycle.

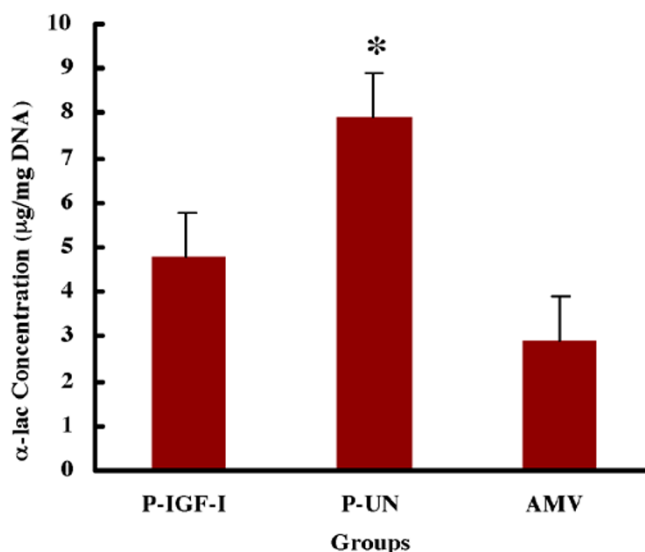
According to this hypothesis, this condition of the mammary gland is retained after involution of the gland; that is, differentiation of the mammary gland acquired during pregnancy is a permanent state [3-7]. It is now clear, based on a number of studies, that this hypothesis inadequately explains the differences in susceptibility of mammary gland to tumorigenesis between virgin and parous animals. First, it has long been questioned whether there is much difference in the proliferative activity of the mammary gland of virgin as compared with parous rats. In terms of structure, TEBs are as abundant in the mammary gland of parous as they are in virgin rats [12,37], which we confirmed in the present study. Assessment of proliferation confirms findings from the structural studies. Using thymidine incorporation, Sinha and coworkers [37] did not find any difference in labeling index between mammary glands from parous and age-matched virgin animals at the time of carcinogen exposure.

However, this study and previous reports support the notion that the mammary gland maintains higher levels of differentiation, at least in terms of milk-specific protein expression, after pregnancy or hormonal treatment that causes pregnancy-like development of the gland, as

compared with mammary glands of virgin, intact animals [8,9,12]. Nevertheless, it has been difficult to demonstrate an association between a previous differentiated state of the mammary gland and its subsequent susceptibility to tumorigenesis. For example, stimulating the development of the mammary gland almost to a lactational state by increasing the circulating levels of prolactin and progesterone without changing the 17 β -estradiol concentration in serum does not confer protection of the gland against MNU-induced carcinogenesis, whereas treatment of the animals with 17 β -estradiol either alone or with progesterone does provide protection [38]. Also, Rajkumar and coworkers [39] were unable to establish a good correlation between mammary differentiation and 17 β -estradiol-induced protection against tumorigenesis, in that full protection was conferred after a short-term estrogen treatment without full lobule–alveolar development. Similarly, Medina and coworkers [40] found a discrepancy between mammary development and the level of protection using low doses of estrogen and progesterone, and termination of pregnancy before any significant differentiation of the mammary gland has taken place confers partial protection against mammary tumorigenesis [37]. Further refuting the notion that differentiation of the mammary gland is a prerequisite for refractoriness to tumorigenesis, and supporting the claim that the hormonal environment is the determining factor, is the finding that virgin Sprague–Dawley dwarf rats lacking functional GH, caused by a point mutation of the *gh* gene [41], exhibit the same refractoriness to mammary tumorigenesis as normal, parous Sprague–Dawley rats [42,43].

Figure 3

The total DNA content of mammary glands of parous rats treated with insulin-like growth factor (IGF)-I (P-IGF-I), untreated parous rats (P-Un), and age-matched virgin rats (AMV). The IGF-I treatment (0.66 mg/kg body weight/day) was continued for 7 days before samples were collected. Values are expressed as mean \pm standard error. * $P < 0.05$ versus AMV and P-IGF-I.

Figure 4

The concentration of α -lactalbumin (α -lac) in mammary tissues obtained from parous rats treated with insulin-like growth factor (IGF)-I (P-IGF-I), untreated parous rats (P-Un), and age-matched virgin rats (AMV). The IGF-I treatment (0.66 mg/kg body weight/day) was continued for 7 days before samples were collected. Values are expressed as mean \pm standard error. * $P < 0.05$ versus AMV and P-IGF-I.

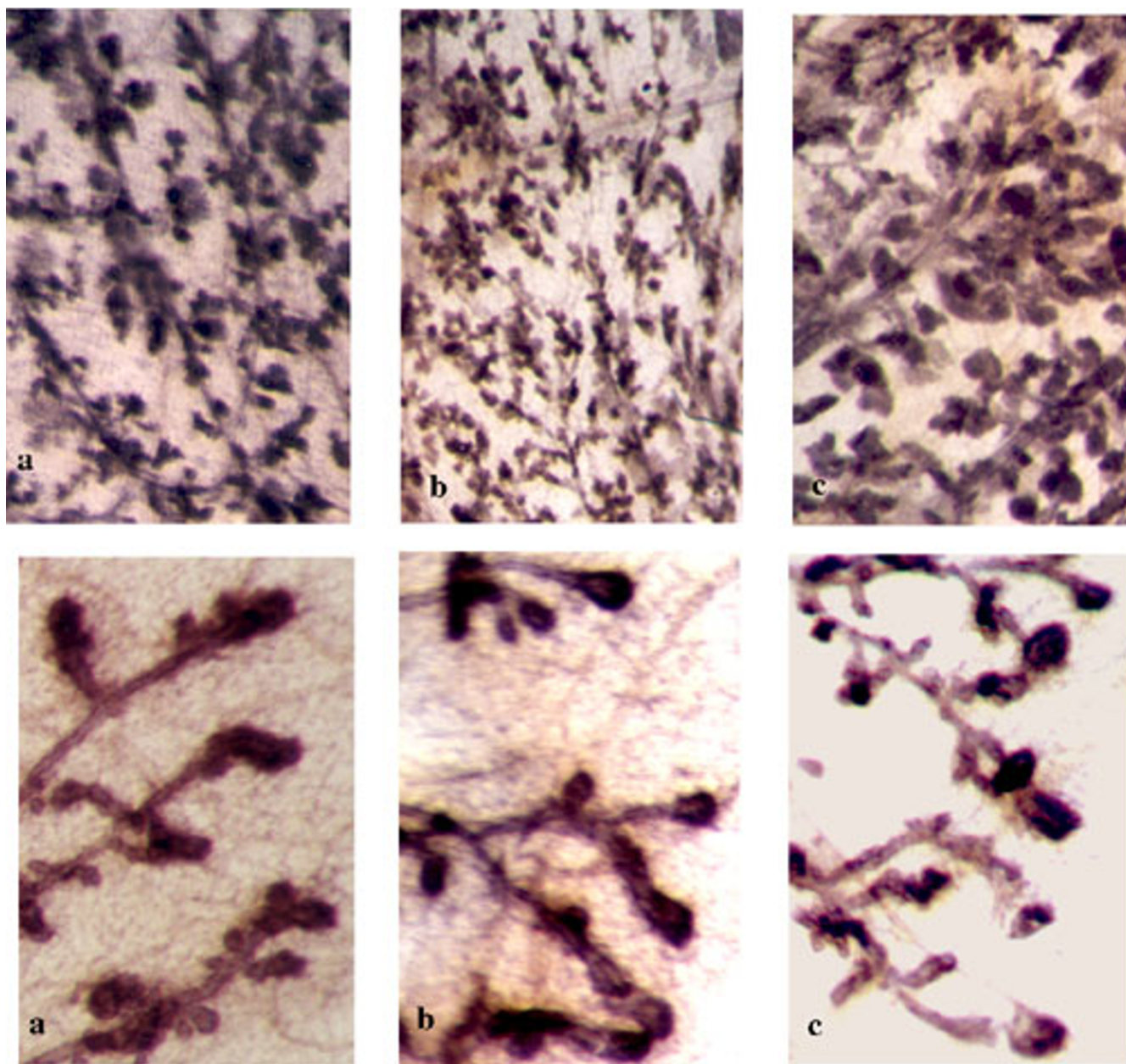
As mentioned above, we reported earlier [2] that the circulating concentrations of both GH and prolactin are reduced in parous rats as compared with age-matched virgin rats. Importantly, it has now been demonstrated that short-term treatment of virgin rats with 17β -estradiol alone or in combination with progesterone, to achieve circulating lev-

els of these hormones comparable to those seen in late pregnant animals, confers mammary tumor refractoriness and significant reduction in the circulating concentrations of GH and prolactin [44]. Furthermore, GH-deficient Sprague–Dawley dwarf rats exhibit the same refractoriness to mammary tumorigenesis that is seen in parous rats [42,43], but when treated with GH the dwarf rats acquire the same high susceptibility seen in normal virgin Sprague–Dawley rats [43]. Also, in the present study we show that the same increase in mammary tumorigenesis can be achieved by treating the parous rats with IGF-I. Therefore, it is unequivocal that the activity of the GH/IGF-I axis is fundamental in determining the level of mammary carcinogenesis. However, the question remains as to how the hormonal environment affects the mammary gland to increase or decrease its tumorigenesis. Will the answer be found in a difference in expression of specific genes in the mammary gland at the time of carcinogen exposure, causing an increase/decrease in transformation upon carcinogen exposure?

Using DNA microarrays, D'Cruz and coworkers [8] identified a number of genes that were differentially expressed in mammary glands from parous and virgin rats and mice. Similarly, Ginger and coworkers [9] used a subtractive suppressive hybridization method to analyze the differences in gene expression of the mammary gland from susceptible (intact virgin) and refractory (estrogen and progesterone treated) Wistar–Furth rats. Again, a number of genes were found to be differentially expressed in the susceptible and refractory glands, but it remains to be seen whether any of these differentially expressed genes are involved in determining the susceptibility of the mammary gland to tumor development.

We studied here the expression of a few specific genes that we considered likely to be important for the susceptibility of the mammary gland to carcinogenesis. However, we found it difficult to relate an increase or a decrease in gene expression to an increase or a decrease in tumorigenesis of the mammary gland at the time of carcinogen exposure. For example, we found the lowest expression of the ER- α in mammary tissues from IGF-I-treated parous rats, but mammary tumorigenesis in these animals was the same as in age-matched virgin rats. The same difficulties are encountered when interpreting the results for expression levels of cyclin D₁ and TGF- β ₃. Cyclin D₁, which is a cell cycle regulator [45] and causes mammary cancer when overexpressed, as evident from studies in the cyclin D₁ transgenic mouse model [46], did not correlate well with tumorigenesis, with the lowest expression occurring in animals with the highest tumorigenesis (age-matched virgin rats). D'Cruz and coworkers [8] previously showed that cyclin D₁ mRNA levels are increased in the mammary gland of parous rats as compared with age-matched virgin ani-

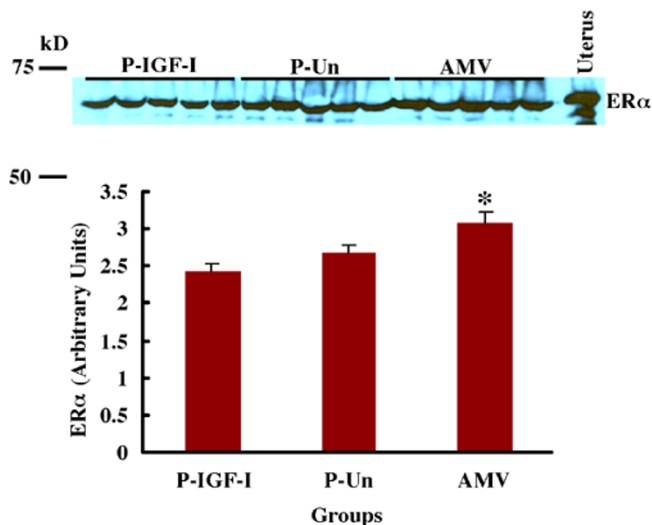
Figure 5



Photomicrographs showing mammary gland wholemounts from parous rats treated with insulin-like growth factor (IGF)-I (upper row, panel a; lower row, panel a), untreated parous rats (upper row, panel b; lower row, panel b), and age-matched virgin rats (upper row, panel c; lower row, panel c). The IGF-I treatment (0.66 mg/kg body weight/day) was continued for 7 days before the animals were killed and the wholemounts were prepared. Note the smaller alveolar structures of the mammary epithelia from parous animals, particularly untreated parous rats, as compared with the age-matched virgin rats (upper row of panels) and the presence of terminal end-buds in the mammary glands from all groups (lower row of panels). Magnifications: 6.25 \times (upper row) and 10 \times (lower row).

mals. Also, as D'Cruz and coworkers reported, we found the lowest level of TGF- β_3 expression in mammary tissues of age-matched virgin rats, which is a possible indication of a high proliferation rate [47], but no difference was found in mammary tissues obtained from untreated and IGF-I-treated parous animals, although tumorigenesis in these two parous groups differs significantly.

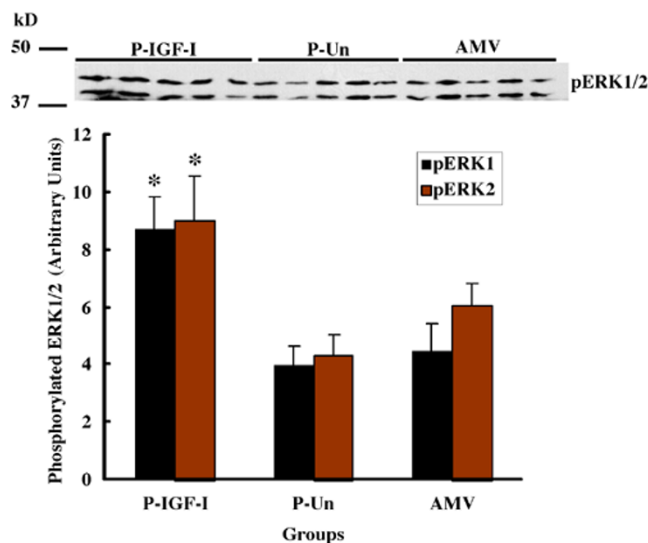
It should also be considered in this context that there might actually not be much, if any, difference in susceptibility to tumor initiation (transformation and fixation of mutation) between mammary glands of parous and virgin animals. It was recently demonstrated that tumor formation does occur in the mammary gland of parous rats, with incidence rate and multiplicity similar to that in age-matched virgin

Figure 6

Western blot analysis showing estrogen receptor (ER)- α expression in mammary gland extracts from parous rats treated with insulin-like growth factor (IGF)-I (P-IGF-I), untreated parous rats (P-Un), and age-matched virgin rats (AMV). The IGF-I treatment (0.66 mg/kg body weight/day) was continued for 7 days before samples were collected. The protein samples (100 μ g/lane) were electrophorized on 7.5% SDS-PAGE, transferred to PVDF membrane, and probed with antibody specific to ER- α (Ab-15; NeoMarkers Inc., Fremont, CA, USA). Protein bands were detected using enhanced chemiluminescence reagents (upper image) and quantified using the ImageJ (version 1.24o; National Institutes of Health, Bethesda, MD, USA) image analysis program (bar chart). Values are expressed as mean \pm standard error. * $P < 0.05$ versus P-IGF-I and P-Un.

rats [48]. However, these tumors stay largely latent at microscopic size and grow only to a palpable size upon hormonal stimulation, as we demonstrated here and showed in previous studies [12]. That is, tumor formation does occur in the mammary epithelia of parous rats, but the hormonal environment of these animals is altered to the extent that it is not capable of promoting tumor progression. Supporting the notion that the difference in palpable mammary tumor development between parous and virgin animals lies primarily in the promotion of growth, but not in transformation, is the fact that the protection – conferred either by pregnancy or by hormonal treatment – is equally effective regardless of whether it takes place before or after exposure to the carcinogen [38,39,48]. Importantly, pregnancy-associated protection, when applied after carcinogen exposure, does not rid the gland of the transformed cells during the differentiation and involution processes as evident by the fact that microtumors are present in the mammary gland of parous animals regardless of whether the carcinogen exposure takes place before or after pregnancy occurs [48].

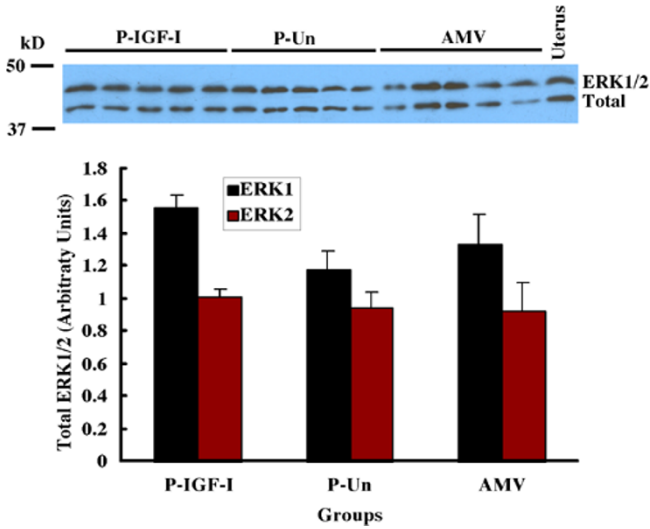
Therefore, tumor formation does take place in the mammary gland of parous rats with frequency almost equal to that in

Figure 7

Western blot analysis showing the level of phosphorylation of extracellular signal-regulated kinase-1/2 (pERK1/2) in mammary tissues from parous rats treated with insulin-like growth factor (IGF)-I (P-IGF-I), untreated parous rats (P-Un), and age-matched virgin rats (AMV). The IGF-I treatment (0.66 mg/kg body weight/day) was continued for 7 days before samples were collected. The protein samples (50 μ g/lane) were electrophorized on 10% SDS-PAGE, transferred to PVDF membrane, and probed with antibody generated to phosphorylated form of human ERK1/2 (#9106; Cell Signaling Technology Inc., Beverly, MA, USA). Protein bands were detected using enhanced chemiluminescence reagents (upper image) and quantified using the ImageJ (version 1.24o; National Institutes of Health, Bethesda, MD, USA) image analysis program (bar chart). Values are expressed as mean \pm standard error. * $P < 0.05$ versus P-Un and AMV.

age-matched virgin animals, but only the tumors in the virgin rats progress to palpable size. We do not yet understand the reason for this, but the differences here must be subtle and may only become apparent after the transformation of the mammary tissue. That is, parity would have little effects on growth of normal mammary tissues, but after transformation the environment of the mammary tumors will not stimulate growth of the cancerous cells. It is not an unknown phenomenon that mammary tumors require different environmental factors for growth from those of normal mammary epithelia. For example, some 40 years ago Huggins and coworkers [49] demonstrated that treatment of rats carrying chemically induced mammary cancers with 17β -estradiol and progesterone destroyed over 50% of their tumors, whereas the same hormonal treatment caused an ample development of the normal mammary epithelia. They also pointed out that hormonal deprivation, such as ovariectomy, kills a large percentage of hormone dependent mammary tumors, whereas the normal mammary epithelia remain viable, only exhibiting slower growth and metabolic rates [49]. Also, when normal mammary epithelia from virgin rats are transplanted into the cleared fat pad of parous rats, growth of these normal cells

Figure 8

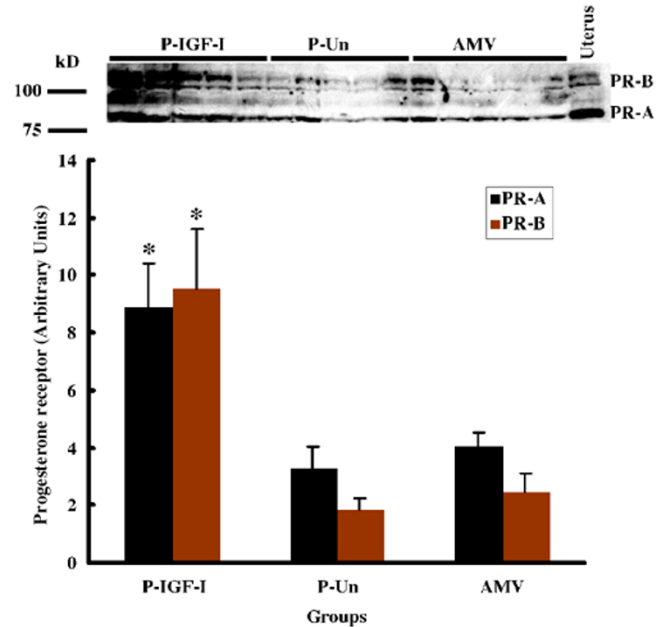


Western blot analysis showing the level of total extracellular signal-regulated kinase-1/2 (ERK1/2) in mammary tissues from parous rats treated with insulin-like growth factor (IGF)-I (P-IGF-I), untreated parous rats (P-Un), and age-matched virgin rats (AMV). The IGF-I treatment (0.66 mg/kg body weight/day) was continued for 7 days before samples were collected. The protein samples (50 µg/lane) were electrophorized on 10% SDS-PAGE, transferred to PVDF membrane, and probed with antibody generated to rat ERK1/2 (#9102; Cell Signaling Technology Inc., Beverly, MA, USA). Protein bands were detected using enhanced chemiluminescence reagents (upper image) and quantified with the ImageJ (version 1.24o; National Institutes of Health, Bethesda, MD, USA) image analysis program (bar chart). Values are expressed as mean ± standard error.

is no different from the growth of normal cells transplanted into virgin host. That is, the hormonal environment of the parous animals is just as capable of stimulating growth of normal mammary epithelia as the environment of the virgin hosts. However, when transformed epithelial cells are transplanted into cleared fat pads of virgin and parous hosts, the hormonal environment of the virgin hosts is significantly more favorable for growth of the transformed cells than that of the parous animals [36].

When we administered IGF-I to the parous animals, the environment of the transformed cells was sufficiently altered for them to progress to palpable tumors. The growth promoting activity of IGF-I on mammary cancer cells is well established. For example, when a number of different mitogens were tested for growth promoting activity in MCF-7 and T47D breast cancer cells, IGF-I was shown to have the highest mitogenic activity [50]. We found in the present study that although the total expression of ERK1/2 did not differ between the animal groups, the activity (phosphorylation) of ERK1/2 was significantly elevated in IGF-I-treated rats as compared with the other groups. This indicates that activity of the Raf/Ras/mitogen-activated pro-

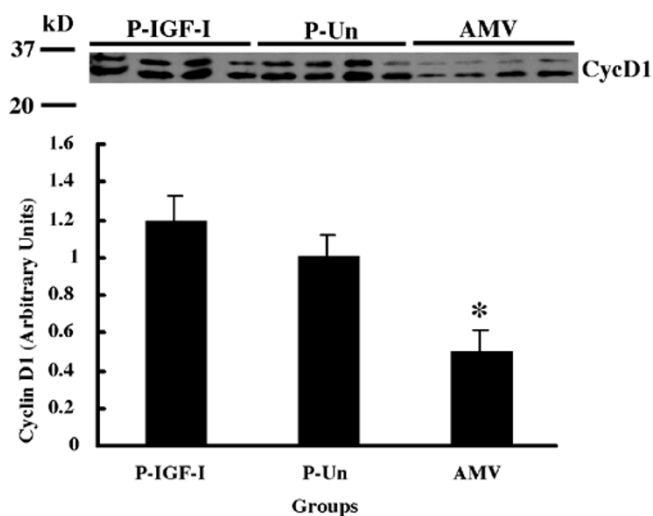
Figure 9



Levels of progesterone receptor (PR) in mammary glands obtained from parous rats treated with insulin-like growth factor (IGF)-I (P-IGF-I), untreated parous rats (P-Un), and age-matched virgin rats (AMV). The IGF-I treatment (0.66 mg/kg body weight/day) was continued for 7 days before samples were collected. Protein samples (50 µg/lane) were electrophorized on a 7.5% SDS-PAGE; Western blot analysis was carried out using a specific anti-PR antibody (no. A 0098; DakoCytomation Inc., Carpinteria, CA, USA) and specific protein bands detected using enhanced chemiluminescence reagents. The upper image shows the results of the Western blot analysis and the bar chart shows the quantitation and statistical analysis of the results. Values are expressed as mean ± standard error. **P* < 0.05 versus P-Un and AMV.

tein kinase (MAPK) cascade was significantly increased in the mammary glands of IGF-I-treated animals. Concomitant with the increased activity of the Raf/Ras/MAPK cascade, we found a significant increase in the expression of the PR in mammary tissues from IGF-I-treated parous rats. Because ERK1/2 phosphorylates (activates) ER-α [51] and because ER-α is an essential regulator of the expression of PR [52], we conclude from these results that the IGF-I treatment caused increased activation of the MAPK cascade, resulting in increased activation of ER-α, which in turn enhanced its transcriptional activity and tumor growth.

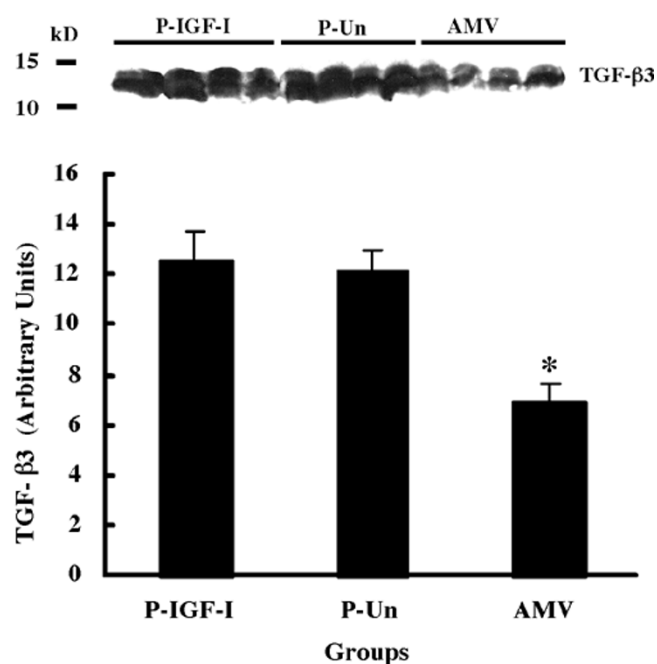
Interactions between the IGF-I and ER-α/17β-estradiol in regulating mammary gland development are unequivocal. For example, very limited mammary epithelial development will take place in the total absence of IGF-I, as demonstrated using the IGF-I^{-/-} mouse model, and administration of 17β-estradiol alone, even in supraphysiological doses, was completely ineffective in restoring mammary epithelial growth in the IGF-I knockout animals [18]. Thus, ER-α is practically inactive in the mammary gland in the absence of IGF-I. IGF-I stimulates the expression of

Figure 10

The expression of cyclin D₁ in the mammary gland of parous rats treated with insulin-like growth factor (IGF)-I (P-IGF-I), untreated parous rats (P-Un), and age-matched virgin rats (AMV). The IGF-I treatment (0.66 mg/kg body weight/day) was continued for 7 days before samples were collected. Protein samples (100 µg/lane) were electrophorized on a 10% SDS-PAGE, transferred to a PVDF membrane, and specific protein bands detected using a specific cyclin D₁ antibody (sc-450; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and enhanced chemiluminescence reagents. The upper image shows the results of the Western blot analysis and the bar chart shows the quantitation and statistical analysis of the results. Values are expressed as mean ± standard error. **P* < 0.05 versus P-Un and P-IGF-I.

17β-estradiol-regulated genes such as the PR, pS2, and LIV-1 in breast cancer cells, apparently through the ER-α, because this IGF-I activity is obliterated by antiestrogens [53,54]. Furthermore, the Raf/Ras/MAPK cascade is one of the major cell signaling systems to mediate the effect of IGF-I on ER-α [51]. Therefore, we demonstrated in the present study that IGF-I acts very similarly in the mammary gland when administered *in vivo* as it does when used for cultured breast cancer cells, in that ER-α is activated via the Raf/Ras/MAPK cascade.

Further delineating the similarity between this rat study and studies carried out on cultured breast cancer cells is the recent finding that IGF-I treatment of MCF-7 cells sharply reduces the total expression of the ER-α, while significantly increasing the expression of the PR and pS2 – an activity that is blocked with antiestrogen [55,56]. Those investigators showed that the phosphatidylinositol 3-kinase (PI3-K)/Act pathway was involved in regulating the enhanced PR expression. However, the effects of the PI3-K/Act pathway on PR expression is complex, because Cue and coworkers [57] found an indication of an IGF-I-induced inhibition of PR expression mediated through the PI3-K/Act cascade – an activity completely independent of ER-α. Therefore, the effects of IGF-I on PR expression may differ depending on

Figure 11

Levels of transforming growth factor (TGF)-β₃ in mammary glands obtained from parous rats treated with insulin-like growth factor (IGF)-I (P-IGF-I), untreated parous rats (P-Un), and age-matched virgin rats (AMV). The IGF-I treatment (0.66 mg/kg body weight/day) was continued for 7 days before samples were collected. Protein samples (100 µg/lane) were electrophorized on a 20% SDS-PAGE; Western blot analysis was carried out using a specific anti-TGF-β₃ antibody (GF16; Oncogene Research Product, San Diego, CA, USA) and specific protein bands detected using enhanced chemiluminescence reagents. The upper image shows the results of the Western blot analysis and the bar chart shows the quantitation and statistical analysis of the results. Values are expressed as mean ± standard error. **P* < 0.05 versus P-Un and P-IGF-I.

whether they are achieved through stimulating ER-α activity or they are mediated independent of ER-α.

How the two major signal transduction system activated by IGF-I receptor/IGF-I, the PI3-K/Act pathway and the Raf/Ras/MAPK cascade interact to coordinate the activity of IGF-I in the mammary epithelia is still largely an unanswered question, but the two signaling pathways appear to have extensive interactions with each other [58,59]. Also, we do not know whether IGF-I is acting here independent of 17β-estradiol to stimulate ER-α, but results from cell culture studies have shown that IGF-I can activate ER-α in the complete absence of estrogen [53,54,60] as well as acting with 17β-estradiol [53,56] to stimulate ER-α. However, we predict that 17β-estradiol is indispensable from the IGF-I activity described here.

The IGF-I stimulated increase in ER-α activity could be of fundamental importance to mammary tumor progression in the parous animals. We know that regulation of ER-α in nor-

mal tissues is under tight control; for example, increased ER- α activity by estrogen administration causes a sharp downregulation in ER- α [52,61]. We have seen that administering low doses of 17 β -estradiol to parous rats, while unequivocally causing a significant stimulation of mammary development and a large increase in mammary tumorigenesis, will cause a significant reduction in mRNA levels of ER- α in the normal mammary gland as compared with those in untreated parous rats that are refractory to tumorigenesis and age-matched virgin animals. However, no difference was found in ER- α levels in tumors from 17 β -estradiol-treated parous rats and age-matched virgin animals (Thordarson G, McCarty M, unpublished data). That is, the tight regulation of ER- α appears to be diminished in mammary tumors compared with normal mammary tissue in that its ligand-mediated down-regulation is lost or at least substantially blunted. This is supported by our previous study, which showed that ER expression is significantly increased in MNU-induced rat mammary tumors as compared with normal mammary tissue [2], and may resemble what has been described in the human breast where a dissociation is found between ER expression and proliferation of normal mammary epithelia, but this dissociation is lost, or is weaker, in breast cancer [62]. We hypothesize that this lax regulation of ER- α in transformed cells is a fundamental step in tumor development, because an increase in ER- α activity, caused by 17 β -estradiol or other agents that are capable of activating the ER- α , no longer provides negative feedback on ER- α expression, leading to uncontrolled growth and ultimately tumor formation.

Conclusions

IGF-I treatment increases the level of mammary tumorigenesis in parous rats to that in age-matched virgin animals. Based on expression analyses of genes that are known to regulate mammary gland development, we found no clear indication of differences in the susceptibility of the mammary tissues from parous and age-matched virgin rats at the time of carcinogen exposure. However, we did find strong evidence for an increase in the growth promoting activity of mammary tissues from IGF-I-treated rats as compared with animals not receiving IGF-I treatment, in that the activity of the MAPK cascade was elevated with a concomitant increase in activation of ER- α . Furthermore, we hypothesize that the cancerous mammary epithelial cells sustain a defect in the ligand-dependent downregulation of ER- α , and that this defect in regulation of ER- α causes uncontrolled growth of the transformed cells in a hormonal environment with stimulatory pressure on the ER- α system, but they remain latent in a hormonal environment lacking this ER- α stimulation.

Competing interests

None declared.

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