

Pregnancy-dependent Growth of Mammary Tumors Is Associated with Overexpression of Insulin-like Growth Factor II¹

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Abstract

We demonstrate that although *IGF-II* gene expression is approximately 3-fold higher in 9,10-dimethyl-1,2-benzanthracene (DMBA)-induced rat mammary tumors (MTs) than in nonneoplastic breast tissue, *IGF-II* mRNA abundance in DMBA-induced MTs is approximately 130-fold higher in pregnant as compared to nonpregnant hosts. This correlated with accelerated tumor growth in pregnant hosts. Immunohistochemical studies of DMBA-induced MTs with an anti-*IGF-II* antibody showed an intense staining of tumor cells for *IGF-II*, whereas a very low staining signal was observed for normal epithelial cells in the lobules. A similar immunostaining pattern was observed in three of three human ductal cancers and adjacent normal breast tissue obtained during pregnancy. DMBA-induced MTs expressed high levels of type I receptor for IGFs as determined by Northern blots. *In vitro* studies confirmed that *IGF-II* is a mitogen for neoplastic epithelial cells derived from DMBA-induced MTs. These results demonstrate that hormonal changes associated with pregnancy accelerate breast cancer cell proliferation in the DMBA-induced MT model and suggest that this acceleration is mediated by up-regulation of *IGF-II* expression within neoplasms.

Introduction

IGFs³ I and II are potent mitogens and inhibitors of apoptosis for many normal and neoplastic cell types, including normal and transformed breast epithelial cells (1-4). The mitogenic effects of *IGF-II* are mediated by the *IGF-I* receptor (5-7). Overexpression of the *IGF-II* gene has been detected in many human tumors of different origins (8-15) as well as in fibroblasts adjacent to malignant tissue (16, 17). Many neoplastic cells are capable of synthesizing *IGF-II* (6, 18-20). Loss of imprinting of the *IGF-II* gene has been implicated in some cases of its overexpression in neoplasms (21, 22). In estrogen receptor-positive cell lines, *IGF-II* mRNA is induced by estradiol (6), suggesting that autocrine expression of *IGF-II* may mediate estrogen-regulated cell growth. Transfection of an *IGF-II* expression vector into a previously estrogen-dependent cell line resulted in hormone-independent growth (23, 24). *IGF-II* transgenic mice develop a diverse spectrum of tumors at a higher frequency than control mice (25). These data suggest that *IGF-II* expression is important in both autocrine and paracrine regulation of cell growth in breast cancer as well as in the pathophysiology of other tumors.

Here, we report an association between pregnancy and overexpression of the *IGF-II* gene in DMBA-induced breast tumors of rats and in human breast tumors.

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³ The abbreviations used are: IGF, insulin-like growth factor; DMBA, 9,10-dimethyl-1,2-benzanthracene; MEM, modified Eagle's medium; MT, mammary tumor; ATCC, American Type Culture Collection.

Materials and Methods

Human Breast Cancer Specimens. Breast cancer and normal secretory breast tissues from three pregnant women were obtained from surgical biopsies. Tissues were fixed in 10% formalin and embedded in paraffin. Immunolocalization of *IGF-II* on 5- μ m-thick sections was performed as described previously (26) using antihuman *IGF-II* polyclonal antibody (Austral, San Ramon, CA).

DMBA-induced MTs. We used the standard DMBA-induced MT experimental model (27) after approval of this study by the McGill Animal Care Committee. Rats ($n = 10-12$) bearing two to three tumors were mated and pregnancy was allowed to proceed until day 16. Tumor volume was determined every 2 days as described previously (28). Normal mammary glands and individual tumors were collected for RNA isolation and immunolocalization of *IGF-II* as described (26, 29).

Cell Culture. Primary breast tumor cells were isolated from surgically excised fresh DMBA-induced tumors as described (30). Primary rat breast cancer cells were maintained as monolayer cultures in α -MEM (Life Technologies, Inc., Grand Island, NY) supplemented with 5 μ g/ml bovine insulin (Sigma Chemical Co., St. Louis, MO) and 10% fetal calf serum (Life Technologies, Inc.). For stimulation experiments, confluent cultures were trypsinized and plated at 2.5×10^4 cells in 24-multiwell plates (Becton Dickinson, Lincoln Park, NJ) in 2.5% fetal calf serum. After 48 h, cell monolayers were rinsed twice with serum-free α -MEM (serum free phenol red-free) medium. *IGF-II* (10 ng/ml; GroPep, Adelaide, Australia) alone or in conjunction with rabbit anti-human *IGF-II* antibody (Astral; 1:800 dilution) was added in triplicate in serum free phenol red-free medium for 48 h. For control, rabbit serum was added at the same dilution. [³H]thymidine incorporation was determined as described previously (31). Statistical analysis was performed using the Mann-Whitney *U* test.

Northern Blot Analysis. Total RNA from cell, normal mammary tissue, and DMBA-induced MTs were isolated as described previously (29). The blots were prehybridized and hybridized with either ³²P-labeled *IGF-II* (ATCC) or *IGF-I* receptor (ATCC) probes as indicated in the figure legends. Integrity and equal loading of RNA were verified by hybridizing the blots to a rat 18S rRNA probe (ATCC). Slot blot hybridization was performed to quantitate *IGF-II* mRNA. Serial dilutions of pooled total RNA (5-50 μ g) appropriate to yield a linear densitometric signal were immobilized on Zeta probe paper using a filtration vacuum Bio-dot[®] SF (Bio-Rad). Prehybridization and hybridization were performed as described (29). Multiple exposures were analyzed using densitometry as described (29).

Results

Rats bearing DMBA-induced tumors were mated, and subsequently showed clear acceleration of growth of their tumors during the second half of pregnancy (Fig. 1). Northern blot analysis revealed multiple *IGF-II* transcripts ranging from 4.2 kb to 0.68 kb in 11 of 11 DMBA-induced MTs of pregnant rats, whereas the same transcripts were undetectable from normal mammary tissues of these same rats (Fig. 2A). Northern blots also showed that both nonneoplastic mammary glands and DMBA-induced tumors sampled during pregnancy had similar levels of *IGF-I* receptor transcripts (Fig. 2B). Subsequent hybridization of the blots with an 18S rRNA probe showed comparable amounts of total RNA loaded per lane (Fig. 2C).

Densitometric scanning of the slot blots revealed that *IGF-II*

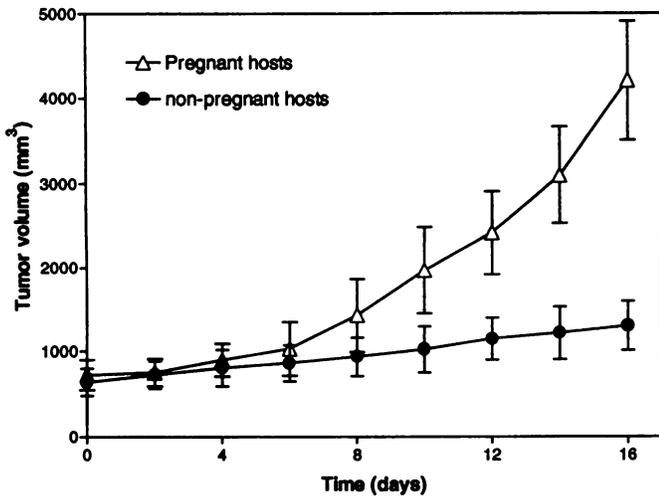


Fig. 1. Growth rate of DMBA-induced breast cancers in pregnant and nonpregnant rats. Rats ($n = 10$) bearing two to three tumors were mated, and tumor growth was measured with calipers as described previously (28). Points, means; bars, SD.

cent normal breast tissue using IGF-II antibody showed that IGF-II peptide was clearly localized to all neoplastic cells with a faint or no signal in normal secretory epithelial cells in the physiologically expanded terminal ductules (Fig. 4A), indicating that IGF-II was either not normally expressed or had ceased to be expressed in nonneoplastic rat breast epithelial cells during pregnancy. A similar IGF-II staining pattern was also observed in three of three examples of benign and malignant human breast tissues excised during pregnancy (Fig. 4B).

To test the hypothesis that IGF-II plays a role in mediating tumor cell growth, primary cells from DMBA-induced MTs were treated *in vitro* with IGF-II. Fig. 5 shows a 2.5-fold induction of the basal proliferation by 10 ng/ml IGF-II. This induction was significantly attenuated by IGF-II antibody, whereas preimmune serum was without significant effect. These results suggest that the rapid proliferation rate of DMBA-induced MTs during pregnancy may at least, in part, be a consequence of autocrine growth stimulation mediated by IGF-II expression.

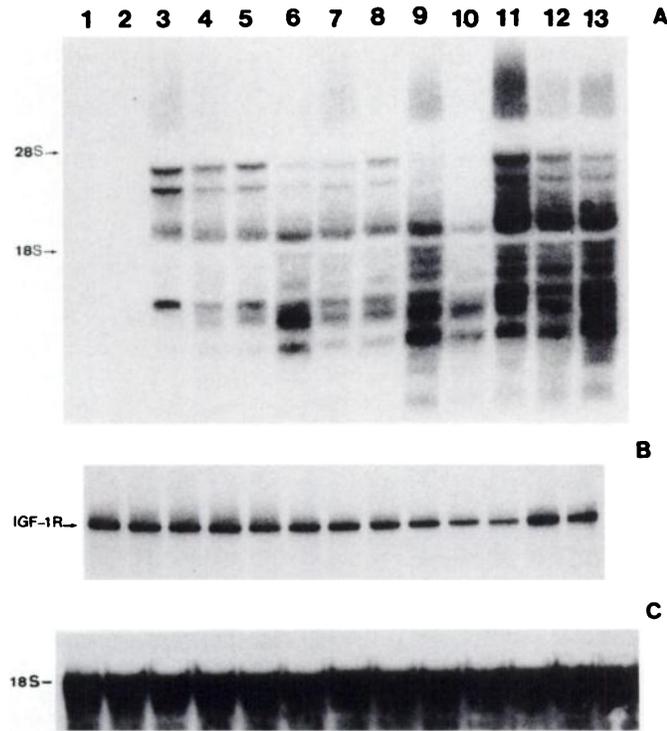


Fig. 2. Representative Northern blot analysis of normal mammary tissue and DMBA-induced breast tumors of pregnant rats. Blots were performed using pooled total RNA from normal mammary tissues ($n = 4$) of pregnant rats (Lane 1), nonneoplastic mammary tissues ($n = 4$) of 14-day pregnant rats with DMBA-induced tumors (Lane 2), and DMBA-induced MTs ($n = 3$) of 14-day pregnant rats (Lanes 3-13). Blots were hybridized with rat IGF-II cDNA (A), human IGF-I receptor cDNA (B), and rat 18S rRNA cDNA (C).

mRNA levels were about 130-fold higher in DMBA-induced MTs of pregnant rats than in DMBA-induced MTs of nonpregnant rats. IGF-II mRNA levels were about 500-fold higher in DMBA-induced MTs in pregnant hosts than in nonneoplastic mammary tissues of pregnant or nonpregnant hosts (Fig. 3). IGF-I transcripts in DMBA-induced tumors were very low and pregnancy independent (data not shown), suggesting that IGF-I was unlikely to be involved in tumor growth during pregnancy.

Immunohistochemical staining of DMBA-induced MTs and adja-

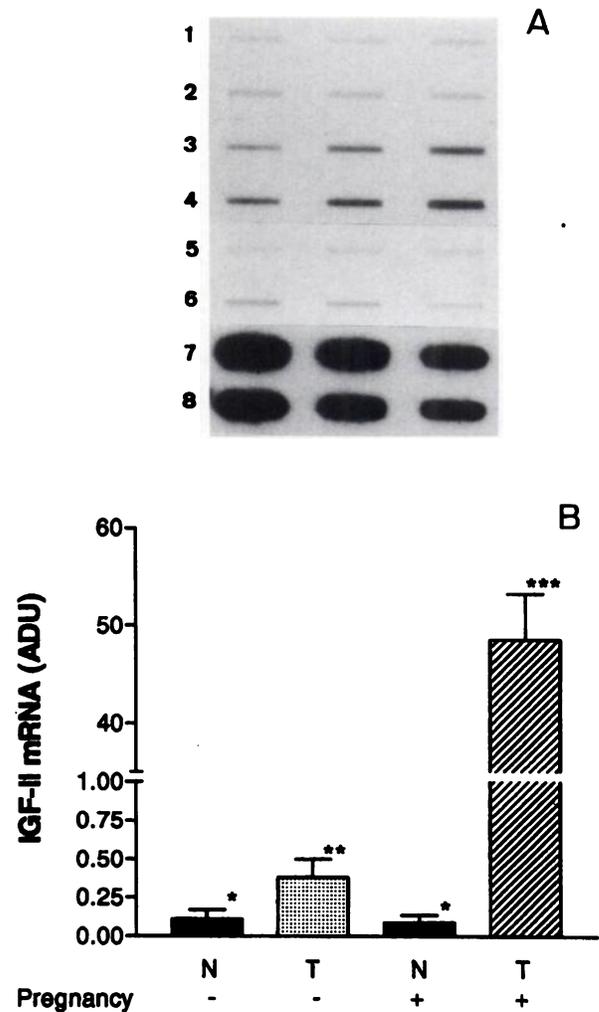


Fig. 3. Representative slot blot hybridization of IGF-II mRNA in DMBA-induced tumors and nonneoplastic mammary tissue. A, representative autoradiogram of a slot blot of pooled total RNA extracted from normal nonpregnant mammary tissues ($n = 3$; Rows 1 and 2), DMBA-induced MTs ($n = 3$) of nonpregnant rats (Rows 3 and 4), nonneoplastic mammary tissues ($n = 3$) of pregnant rats (Rows 5 and 6), and DMBA-induced MTs ($n = 3$) of pregnant rats (Rows 7 and 8). Blots were hybridized with the rat IGF-II cDNA probe as described in "Materials and Methods." B, mean change in IGF-II mRNA. The difference between normal breast, tumors of nonpregnant rats, and tumors of pregnant rats was statistically significant ($P < 0.001$, Mann-Whitney U test). Bars, SE.

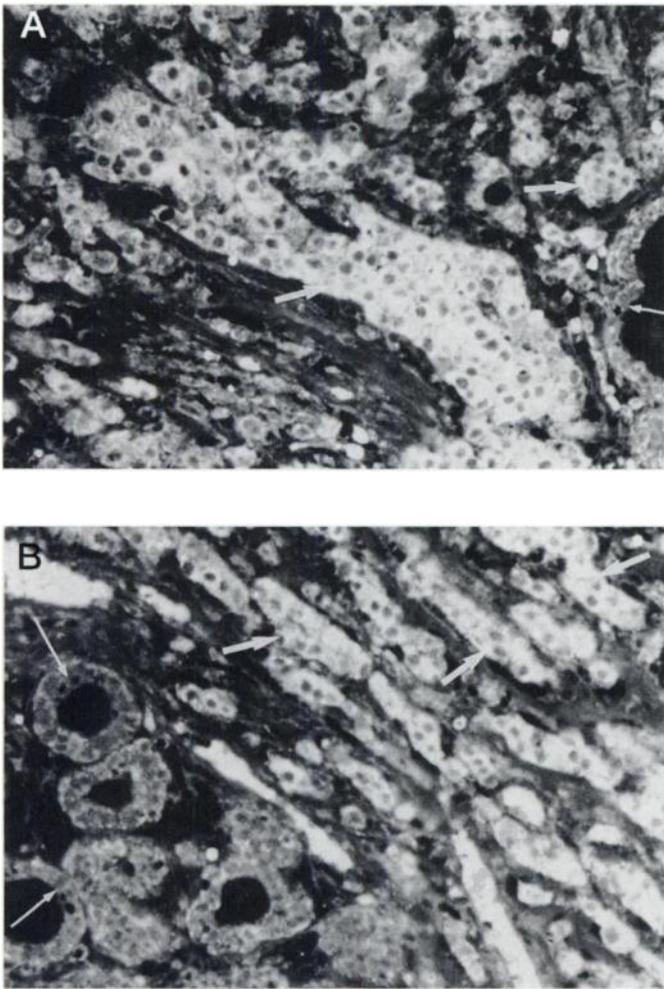


Fig. 4. Immunostaining of normal and neoplastic rat and human breast tissues for IGF-II. A, representative DMBA-induced breast tumor of 14-day pregnant rat showing intense staining of tumor cells for IGF-II (thick arrows), whereas a very low staining signal was observed in normal epithelial cells in the lobules (thin arrow). B, representative invasive ductal carcinoma from pregnant woman showing intense staining of the morphologically disorganized carcinoma cells (thick arrows) in contrast to weak staining in normal terminal ductules (thin arrows). $\times 400$.

Discussion

Our results suggest that marked *IGF-II* gene overexpression relative to normal breast tissue occurs in experimental DMBA-induced MTs of rats and in human breast cancer tissues during pregnancy. These data reveal a previously undescribed marked effect of pregnancy on IGF-II mRNA abundance in the DMBA-induced MT model. Although DMBA-induced MTs show about a 3-fold increased IGF-II expression relative to normal breast in nonpregnant hosts, in pregnant hosts IGF-II expression is two orders of magnitude higher than that seen in normal breast of pregnant or nonpregnant rats, or in DMBA-induced MTs of nonpregnant hosts. This correlates with accelerated tumor growth in pregnant hosts in the DMBA-induced tumor model.

Immunohistochemical analysis of both rat and human breast tumors showed that the breast cancer cells produced high levels of IGF-II peptide compared to normal breast epithelial cells. These observations suggest an autocrine role of IGF-II in the growth behavior of breast cancer cells during pregnancy. This suggestion was reinforced by *in vitro* experiments demonstrating that a treatment of cells from DMBA-induced MTs with IGF-II led to increased DNA synthesis and that this effect was attenuated by coincubation of IGF-II with IGF-II antibody.

A paracrine role for IGF-II in the regulation of breast tumor growth was proposed based on previous studies (16, 17) which showed that IGF-II is produced predominantly in breast stromal elements. In our present study, we showed that the *IGF-II* gene expression in rat DMBA-induced tumors and human breast tumors during pregnancy appeared to be epithelial cell specific since IGF-II peptide was found at very high levels in neoplastic epithelial cells but not in normal epithelial cells. These observations suggest that secretion of IGF-II by breast tumor cells might act in an autocrine fashion to stimulate their growth, and is consistent with tissue culture results showing that estrogen increases autocrine IGF-II by MCF-7 and T47D breast cancer cells (6).

Analysis of total RNA from DMBA-induced tumors revealed a multiple molecular weight IGF-II RNA species ranging from 4.2 kb to 0.68 kb. Ethidium bromide staining of the gels showed discrete 28S and 18S rRNAs with the 28S band being about double in intensity, making it unlikely that degradation occurred in these samples. In rat, multiple transcripts ranging from 6.0 kb to 1.2 kb have been detected. Several of these species can be accounted for by the use of alternate promoters and by variable RNA processing or polyadenylation at the 3' end of the gene (32). Enhanced levels of promoter 3- and 4-driven IGF-II mRNA have been detected in many human tumors of different origins (8–15) and cancer cell lines (18, 19), which suggest that differential activation of multiple promoters could very well play a crucial role in *IGF-II* gene regulation. The molecular mechanisms by which the *IGF-II* gene is activated in breast cancer cells during pregnancy are not known. Several reports have demonstrated the loss of imprinting of the *IGF-II* gene in Wilms' tumors (21, 22). In breast tumor cells, growth inhibitors or tumor suppressors may be lost or inactivated. The absence of those growth regulators might permit pregnancy-associated hormones to abnormally activate the *IGF-II* gene in neoplastic cells instead of inducing expression of genes that lead to differentiation as occurs in nonneoplastic breast epithelial cells (33). Our results motivate further research to characterize host influences on IGF-II expression by neoplasms. Furthermore, our observations may be relevant to clinical observations concerning transiently increased rates of breast cancer detection following pregnancy (34) and worse prognosis of breast cancer detected during pregnancy (35),

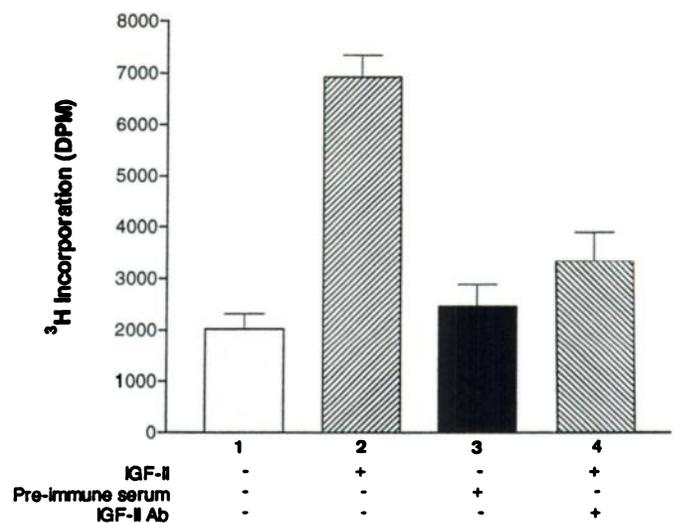


Fig. 5. Effect of IGF-II on primary DMBA-induced breast cancer cell DNA synthesis. Cells were cultured as described in "Materials and Methods." Cells were incubated with serum-free α -MEM (Lane 1), 10 ng/ml IGF-II (Lane 2), rabbit serum (1:800 dilution; Lane 3), and 10 ng/ml IGF-II in combination with rabbit anti-human IGF-II (1:800 dilution; Lane 4). Means of triplicate experiments were plotted. IGF-II-induced proliferation rates were significantly decreased in the presence of anti-IGF-II antibody ($P < 0.001$, Mann-Whitney *U* test). Bars, SEM.

although the latter observations have not been confirmed in all studies (36).

Acknowledgments

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