Cloning and Characterization of a Novel Pregnancy-Induced Growth Inhibitor in Mammary Gland

H. Huynh, C. Y. Ng, C. K. Ong, K. B. Lim, and T. W. Mark Chan

Growth factors and growth inhibitors play crucial roles in the growth regulation and differentiation of mammary epithelial cells. Studies have shown that during pregnancy, with the onset of terminal differentiation, there is a dramatic decrease in the proliferation of the mammary epithelial cells. Here we report the cloning and characterization of a novel pregnancy-induced cDNA, OKL38, from a human ovarian cDNA library. This cDNA encodes for a protein of approximately 34.5 kDa. Tissue distribution studies through Northern analyses revealed the ubiquitous nature of OKL38 transcripts in most tissues, with the highest levels observed in the ovary, kidney, and liver. The onset and advancement of pregnancy also gave rise to a concomitant increase in OKL38 gene expression. In situ hybridization revealed that OKL38 mRNA was further detected in mammary secretory epithelial cells. However, low levels of OKL38 transcripts were observed in the various human breast cancer cell lines studied and were barely detectable in all dimethylbenz(A)anthracene-induced mammary tumors examined. Transfection studies with OKL38 cDNA with MCF-7 cells resulted in growth inhibition in vitro and reduction in tumor formation in vivo. These observations led to speculation that OKL38 may play a vital role in the growth regulation and differentiation of breast epithelial cells during pregnancy and its implications in tumorigenesis.

Breast cancer is the most common cancer and the second cause of cancer death in women. Globally, the incidence of breast cancer appears to be increasing, and an annual worldwide incidence of over one million is predicted by the turn of this century (1).

Epidemiological studies have demonstrated that for women early age at menarche, late age at first pregnancy, and late age at menopause tend to have an increased risk for breast cancer (2, 3). The lifetime risk of breast cancer is 2–5 times higher in women who have a first pregnancy after age 30 yr than in women whose first pregnancy is at an age younger than 20 yr (2, 3). It has been hypothesized that first pregnancy at a young age may differentiate breast cells early in life, after which they would become less susceptible to carcinogens (4–6). This hypothesis was supported by the observation that in animal models, mammary tumorigenesis is facilitated when the administration of carcinogen precedes pregnancy; however, it decreases when the carcinogen exposure occurs during pregnancy (7). Normal and prolonged lactation in mice and rats is also recognized to result in a decrease in the incidence of spontaneous or carcinogen-induced mammary tumors and an increase in tumor age compared with forced breeding without lactation (7). Accordingly, mammary DNA synthesis is also at a very low level during lactation in mice and rats. These observations in experimental animals show the protection properties of pregnancy and lactation against mammary tumorigenesis.

Peptide growth factors and inhibitors tend to play key roles in regulating the proliferation of normal breast epithelium (8). The importance of peptide growth factors in the pathogenesis and behavior of breast neoplasms is evident in the enormous amount of literature that has accumulated in the past decade concerning the roles of epidermal growth factor, IGFs, TGFα, TGFβ, and fibroblast growth factor (8, 9). To date, the best characterized inhibitor is TGFβ (10). IGF-binding protein-3 (11–14), and mammary-derived growth inhibitor (15) have also been reported to possess a negative regulatory function. Abnormal expression of the various growth factors and growth inhibitors has been implicated in tumorigenesis (15–17). Taken together these observations would suggest that the interruption of growth factor action (or production) or enhancement of growth inhibitor production by breast cancer cells would represent new strategies for the arrest of tumor growth.

We report the cloning and characterization of a novel cDNA, OKL38, which was localized in breast epithelial cells with increased gene expression during pregnancy and lactation. OKL38 expression was low in breast cancer cell lines and was barely detectable in dimethylbenz(A)anthracene (DMBA)-induced breast tumors. Transfection of human MCF-7 breast cancer cells with OKL38 cDNA leads to a reduction in proliferation and tumor formation in nude mice. The results suggest that OKL38 may play an important role in the differentiation and growth of breast epithelial cells.

Materials and Methods

mRNA differential display

Total RNA was isolated either from pooled mammary tissues of 60-d-old nonpregnant (n = 4) or 18-d pregnant rats (n = 4) as described previously (18). Differential display was performed using the RNAmap kit according to the manufacturer’s protocol (GeneHunter Corp., Nashville, TN). Briefly, 5 μg deoxyribonuclease I-treated total RNA were reverse transcribed with T<sub>4</sub>M<sub>100</sub> (where n may be G, A, T, or C), followed by PCR amplification in the presence of [α-33P]deoxy-ATP (NEN Life Science Products, Boston, MA) using the corresponding T<sub>4</sub>M<sub>100</sub> primer, downstream, and one arbitrary primer supplied with the kit, AP<sub>1</sub>-AP<sub>p</sub>,

Abbreviations: DMBA, Dimethylbenz(A)anthracene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Detection were performed according to the manufacturer’s protocol. Lipidized for 5 min in chloroform and allowed to dry at room temperature.

Animals

Animal experiments were approved by local animal care committee. Female Sprague Dawley rats, 50 g old at the beginning of the experiments, were obtained from Charles River Laboratories, Inc. (Quebec, Canada). To study changes in OKL38 expression during pregnancy and lactation of normal rats, pregnant rats were killed on d 0, 4, 10, 16, and 21 of pregnancy and d 3 of lactation. Matings dates were established from the appearance of vaginal plugs. Day 1 of pregnancy was determined by the presence of a vaginal plug. The animals were killed, and the mammary gland was collected as described above.

In situ hybridization

For mRNA in situ hybridization, recombinant plasmid pcDNA3.0 containing a 650-bp OKL38 fragment (nucleotides 1–650 of the OKL38 sequence, GenBank accession no. AF191740) was linearized to generate sense and antisense digoxigenin-labeled RNA probes using the digoxigenin RNA labeling kit (Roche Molecular Biochemicals, Indianapolis, IN). Fresh mammary tissues from 60-d-old nonpregnant and 18-day pregnant rats were treated with PBS containing 0.5% active diethyl pyrocarbonate for 10 min, embedded in OCT (Miles Laboratories, Elkhart, IN), and stored at −80°C. Serial 7- to 8-μm OCT-frozen sections were heated for 2 min at 50°C and dried for 30 min. To minimize nonspecific background caused by lipid vesicles, the sections were de-lipidized for 5 min in chloroform and allowed to dry at room temperature. Sections were fixed in PBS containing 4% paraformaldehyde. Prehybridization, hybridization, posthybridization, and immunological detection were performed according to the manufacturer’s protocol.

Induction of mammary tumors by DMBA

We used the standard DMBA-induced mammary tumor experimental model (20) to study the expression of the OKL38 gene during pregnancy. Mammary carcinomas were induced by a single intragastric administration of 20 mg DMBA (Sigma, St. Louis, MO) dissolved in 1 ml peanut oil at 50–52 d of age. This standard procedure yields palpable (>0.5 cm) tumors in about 75% of animals by d 80 after carcinogen administration. Rats bearing DMBA-induced breast tumors were mated. Matings dates were established from the appearance of vaginal plugs. Pregnant rats were killed on d 16 of pregnancy, and tumors were collected.

To determine OKL38 gene expression in human breast cancer cell lines, MCF-7, T47D, ZR75, MDA-231, Hs578T, and HBL-100 were grown to 90% confluence. Poly(A)RNA was extracted, and Northern blotting to verify their differential expression in mammary tissues. cDNAs representing differential expressed mRNAs were excised from the above differential gel (C) or GAPDH cDNA (B). The band representing mRNA that is induced during pregnancy is marked by an arrowhead.

Statistical analysis

Differences in OKL38 gene expression were analyzed by t test. Differences in cell number and tumor number between parental lines and transfectants were tested using the Mann-Whitney U test.

Western analysis

To detect OKL38 protein, controls and OKL38-transfected MCF7 cells were grown to 90% confluence, harvested, and lysed with a buffer containing 1 mM CaCl₂, 1 mM MgCl₂, 1% Nonidet P-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μM phenylmethylsulfonylfluoride, and 100 mM NaVO₄. Cell lysate was used to determine changes in the levels of OKL38 by Western blotting as previously described (21). Differentially in situ hybridization was performed using 4- to 8-wk-old athymic nude mice (CD1 nu/nu, Charles River Laboratories, Inc.). Each cell line was assayed in four mice, and each mouse received an injection of 5 × 10⁶ cells into an inframammary fat pad and another identical injection of the same cell line into a contralateral fat pad. Estrogen supplementation was given by a surgically placed (lower back) sc 0.25-cm SILASTIC brand tube (Dow Corning Corp., Midland, MI; inner diameter, 0.0635 cm; outer diameter, 0.12 cm) containing 17β-estradiol (rate of release, 0.6 μg/day) on the day of injection of tumor cells. Tumors started to appear in the fourth week. Animals were inspected once every 3 d until the tumors in controls reached about 1–1.4 cm³. The experiments were repeated a total of three times.

MCF-7 cell stable transfectant cell lines

The entire coding region of OKL38 cDNA was cloned into the mamalian expression vector pCDNA3.1/His (Invitrogen, Carlsbad, CA). The OKL38-pcDNA3.1/His sequence was confirmed by sequencing. MCF-7 cells were seeded at 2 × 10⁴ in 100-mm culture dishes in 90% αMEM (Life Technologies, Inc., Grand Island, NY) containing 10% FCS with garamicin 24 h before transfection. Cells were transfected with 5 μg full-length OKL38 cDNA (pcDNA3.1/His-OKL38) or pDNA3.1/His control plasmid DNA and 28 μl Lipofectamine reagent (Life Technol-
Fig. 2. Nucleotide and deduced amino acid sequences of human OKL38 cDNA. Nucleotides are numbered on the left, and amino acids are numbered on the right. The polyadenylation signal in the 3' untranslated region is in bold.
For nonpregnant mammary tissue, there was approximately 12–15% mammary epithelial cells. For pregnant mammary gland, the percentage of epithelial varied from 25–75% depending on the stages of pregnancy and lactation. The remaining cells were adipocytes, fibroblasts, and myoepithelial cells. There was no separation of adipose and stromal cells from epithelial cells before RNA extraction. Poly(A) RNA was isolated from the indicated tissues of female rats using the Oligotex mRNA kit as described by the manufacturer (QIAGEN, GmbH, Hilden, Germany). Northern blots were performed on poly(A) RNA or total RNA, and blots were hybridized with human OKL38 or human glyceraldehyde-3-phospho-
phate dehydrogenase (GAPDH; American Type Culture Collection, Manassas, VA) cDNAs as previously described (18). mRNA levels were determined by densitometric scanning of autoradiographs.

**Results**

Employing the method of differential display using RNA derived from the mammary tissues of nonpregnant and 18-day pregnant rats yielded 18 differentially expressed bands. The majority of differentially expressed bands were milk protein cDNAs. Further sequencing analyses established a novel 450-bp DNA fragment (Fig. 1A, lane 2). Northern analyses with the 450-bp probe confirmed the differential expression of this particular 1.6-kb cDNA in the 18-day pregnant mammary sample (Fig. 1C). Higher magnification clearly shows that stromal cells (arrow) were negative for OKL38 (D). Magnification, ×400 (A–C) and ×1600 (D). Note that due to the delipidization of the tissues in chloroform, the secretory epithelial cells appeared as if they were poorly preserved.

Fig. 5. **In situ** hybridization with antisense RNA probe for OKL38 expression in rat mammary gland. Mammary tissue sections from 60-d-old and 18-day pregnant rats were subjected to **in situ** hybridization as described in **Materials and Methods**. Sense control OKL38 probe showed no background staining in mammary secretory epithelial cells (A). Low OKL38 mRNA was detected in alveolar epithelial cells, but not in stromal cells or blood vessel (arrow; B). The OKL38 signal was increased in secretory epithelial cells of mammary tissue derived from 18-day pregnant rats (C). Higher magnification clearly shows that stromal cells (arrow) were negative for OKL38 (D). Magnification, ×400 (A–C) and ×1600 (D).

To directly clone the human cDNA, the 450-bp rat cDNA probe was used for the screening of a full-length human cDNA from a human ovarian library. This gave rise to the isolation of eight positive clones, of which one clone harboring the longest insert of about 1.6 kb was further studied and sequenced to its entirety using automated cycle sequencing.

Blast search with the nonredundant nucleotide database (GenBank) revealed no significant homologies with any known nucleotide sequence. Figure 2 shows the nucleotide and deduced amino acid sequences of human OKL38 cDNA. The full-length 1607-bp cDNA (GenBank accession no. AF191740) possesses an initiator ATG start codon (position 127 bp) followed by a single open reading frame of 317 amino acids with a calculated molecular mass of 34.5 kDa. The stop codon terminates at position 1078 bp, followed by a 3′-untranslated region of 529 bp. No putative transmembrane domains or nuclear signal peptide were localized in OKL38 using the publicly available Tmpred program (22). The hydrophobicity average of OKL38 was 0.067193, and thus it was predicted to be a soluble protein using the SOSUI program.
stages of pregnancy and analyzed via Northern blotting. The levels of OKL38 mRNA were very low in mammary tissues of nonpregnant rats as evident in Fig. 4. With the onset and progression of pregnancy, a rapid increase in OKL38 mRNA and maximal levels of OKL38 gene expression was observed during the period of lactation, as shown in Fig. 4.

In situ hybridization studies were performed to determine the cell type specificity expression of OKL38 in the mammary gland. An OKL38 antisense RNA digoxigenin-labeled probe was used in mammary tissues of nonpregnant and 18-day pregnant rats. Figure 5B shows that low levels of OKL38 mRNA were localized to the undifferentiated mammary epithelial cells of nonpregnant rats. Intense OKL38 signal was observed in the secretory epithelial cells of the 18-day pregnant mammary tissue (Fig. 5, C and D). No OKL38 signal was detected in blood vessels and stromal cells (Fig. 5, B and D). A control in parallel with a sense OKL38 mRNA probe gave no background signals in the mammary tissue (Fig. 5A).

To determine OKL38 gene expression in human breast cancer cell lines, Northern analysis was performed with poly(A) RNA derived from various cell lines. Accordingly, Fig. 6 shows that OKL38 transcripts were present in all breast cancer cell lines examined, but the mRNA levels were much lower than those in human mammary tissue (Fig. 6C).

Due to its high level of expression in breast tissue during pregnancy, OKL38 expression was further examined in neoplastic tissue using the DMBA-induced mammary tumor experimental system (20). Figure 7 shows the result of a typical experiment in which the animals were exposed to DMBA, followed by the appearance of tumors and subsequent pregnancy. In lactating rats bearing DMBA-induced tumors, the OKL38 transcript seen in normal mammary gland was abundant relative to that in most mammary ductal neoplasms.

Transfection studies were performed to assess the implications of the gene encoding for OKL38 in the growth regulation of breast epithelial cells. Human MCF-7 breast cancer cells were transfected with a recombinant expression construct with a full-length OKL38 cDNA (pcDNA3.1/His-OKL38). As shown in Fig. 8, high levels of OKL38 mRNA expression (~1.6 kb) were evident in representative transfected cell lines SQ13 and SQ18, but were absent in untransfected MCF-7 controls and mock-transfected cells. Subsequently, Western blotting with an anti-6-histidine antibody was used to detect OKL38-related proteins, which yielded an approximately 38-kDa protein band found only in positively transfected cells with the recombinant OKL38 DNA construct (Fig. 8D).

The proliferative behavior of OKL38-expressing clones was evaluated by determining cell number on plastic dishes after 5 days of incubation. The number of cells was significantly less (P < 0.05, by Mann-Whitney U test) in OKL38-expressing transfectants than in controls, as shown in Fig. 8E.

To determine whether overexpression of OKL38 leads to a reduction in tumor formation, in vivo tumorigenicity was performed. The rate of tumor formation after the injection of MCF-7 cells was 100% (eight of eight), that of pcDNA3.1–1 mock-transfected cells was 88% (seven of eight), and those of SQ13 and SQ18 transfected cells was 12.5% (one of eight) and 25% (two of eight), respectively. As shown in Fig. 8F, the
growth of OKL38 transfected cells (SQ13 and SQ18) \textit{in vivo} was significantly lower ($P < 0.01$) compared with that of parental and mock-transfected MCF-7 cells (P and V).

**Discussion**

The study of breast cancer protection associated with pregnancy has led us to the isolation of a novel pregnancy-induced growth inhibitor cDNA, OKL38, via the method of differential display. A homology search with GenBank did not reveal any significant similarities with any published nucleotide sequences. The full-length 1607-bp cDNA encodes a reading frame of 317 amino acids with an approximate molecular mass of 34.5 kDa. Computational analyses predicted the soluble and nonsecretory properties of OKL38 (23). No transmembrane domains or nuclear localization signal peptide was localized to the OKL38 protein sequence (22). These probably indicate that OKL38 has an intracellular function.

A survey of the distribution and abundance of the OKL38 transcripts revealed a ubiquitous presence in all examined tissues, but higher levels were apparent in the ovary, kidney, and liver. Two higher molecular mass species of mRNA were detected, but it is unclear whether these transcripts are the precursory molecules of the mature transcript or different transcripts arising from differential splicing.

It is interesting to note here that the gene expression of OKL38 was low in the MCF-7 cells and various human breast cancer cell lines studied. This allows speculation that the reduction of OKL38 production may be required for the accompanying cellular changes from a normal to a malignant state and a tendency to confer a selective growth advantage over normal cells. Increasing levels of OKL38 after the transfection of MCF-7 cells lead to a reduction in cellular growth and tumor formation in nude mice, suggesting an important role in growth regulation and tumorigenesis. This demonstration of tumor suppressor activity in MCF-7 cells is provocative and serves to support the hypothesis that OKL38 is unrelated to well characterized growth inhibitory or tumor suppressor proteins. The observed antitumor properties are comparable to the results of previously well studied assays for Rb, p53, and H19 (25–28).

The seemingly low abundance of OKL38 transcripts in
poly(A) mRNA extracted from the entire mammary gland is significantly increased above baseline at the time of physiological changes associated with pregnancy and lactation (specifically maximal breast epithelial differentiation). In situ hybridization revealed that the majority of the OKL38 mRNA was expressed in the secretory epithelial cells of the mammary gland during the period of lactation. These observations document the existence of a certain degree of hormonal regulation in the expression of OKL38. A strong relationship exists among the onset of differentiation, the inhibition of proliferation, and the concurrent expression of OKL38. Due to its putative hormonal regulation and coupled with the

![Fig. 8. Effects of stable transfection of a breast cancer cell line with OKL38 cDNA. RNA loading amounts were compared by ethidium bromide staining of 18S and 28S rRNA (A). Northern blot hybridization with a [32P]deoxy-CTP-labeled OKL38 probe (B) of total RNA (50 µg/lane) extracted from parental MCF-7 cells (P), pcDNA3.1 vector-transfected cell lines (lines 1, 2, and 34), and OKL38 transfectants (SQ13 and SQ18). Immunodetection of 38-kDa OKL38 (D) and β-tubulin (C) in stably transfected cell lines SQ13 and SQ18 is shown. Proliferative behavior of the clones expressing OKL38 was found by determining cell number on plastic dishes after 5-day incubation (E). The number of cells was significantly less (P < 0.05, by Mann-Whitney U test) in OKL38-expressing transfectants than in controls. The tumor growth curves of stably transfected cell lines SQ13 and SQ18 are shown (F). The rate of tumor growth was significantly slower (P < 0.01, by Mann-Whitney U test) in OKL38-expressing transfectants (SQ13 and SQ18) than in controls (P and V).]
reduction in breast cancer cell growth and inhibition of tumor formation in the animal model, it is tempting to therapeutically employ drugs or hormones to attenuate OKL38 expression to lower the risk of breast cancer. Experiments are currently underway to study the use of pregnancy-associated hormones that are responsible for the induction of OKL38 expression.

The absence of any detectable signal in the neoplastic tissue is of particular interest. Normally in breast tissue, pregnancy-associated hormonal changes are followed by a concomitant up-regulation of OKL38 expression in vivo, but this normal induction of expression was not apparent in the neoplastic breast tissue. There is a possibility that neoplastic progression is associated with a significant reduction in OKL38 expression, although the molecular events leading to the inactivation of OKL38 in DMBA-induced breast tumors are as yet unclear. It is likely that the OKL38 expression may be absent from the mammary tumors due to consequences of genetic alterations such as deletion, mutation, or inappropriate hypermethylation (29–31). Current studies aim to further decipher the mechanisms responsible for the silencing of OKL38 expression in breast tumors.

Presently, the abundance of OKL38 mRNA transcripts and its functional significance in the liver, ovaries, and kidney is unknown, but it is intriguing that the breast seems to be a tissue in which OKL38 expression fluctuates according to the various differentiation state (which varies considerably in this organ according the stages of development and lactation, etc.). This is in contrast to the stable expression of OKL38 in the other organs characterized by relatively low cellular turnover and extensive differentiation (i.e. kidney and liver). Investigations are underway pertaining to the possible role(s) of OKL38 in these tissues.

In summary, it is tempting from our data to speculate that OKL38 possesses putative growth inhibitory and tumor-suppressing properties. Its expression is strongly up-regulated, as seen in the mammary gland during pregnancy and lactation. The expression of OKL38 is also associated with differentiation and a low proliferative rate. Additional knowledge contributing to the growth inhibitory and anti-tumor activity of OKL38 protein will be of relevance to further understanding of the basis of the protective effects of pregnancy on subsequent cancer risk (2, 3). Taken together, the data suggest that the enhancement of OKL38 production by breast cancer cells may present new promising strategies for the arrest of tumor growth.

Acknowledgments

Received December 18, 2000. Accepted April 2, 2001.

Address all correspondence and requests for reprints to: Dr. Hung Huynh, Laboratory of Molecular Endocrinology, Division of Cellular and Molecular Research, National Cancer Center of Singapore, Singapore 169610. E-mail: cmrhth@nccs.com.sg.

References


