Differential Expression of Human Wnt Genes 2, 3, 4, and 7B in Human Breast Cell Lines and Normal and Disease States of Human Breast Tissue¹

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Abstract

Wnt gene expression was investigated by ribonuclease protection analysis in human breast cancer, nontumorous breast tissue, and a variety of human breast cell lines. We report the expression of Wnt3, Wnt4, and Wnt7b in human breast cell lines and Wnt2, Wnt3, Wnt4, and Wnt7b in human breast tissues. Wnt3a and Wnt7a were absent in the cell lines and tissues tested. The level of expression of Wnt2 and Wnt4 was 10- to 20-fold higher in fibroadenomas than it was in normal or malignant breast tissue, and in 10% of tumors Wnt7b expression was 30-fold higher than in normal or benign breast tissues. In contrast to the mouse, in which Wnt1 and Wnt3 are involved in tumorigenesis, our results suggest that Wnt2, Wnt4, and Wnt7b may be associated with abnormal proliferation in human breast tissue.

Introduction

Wnt genes (reviewed in Refs. 1 and 2) form a large family, the first member of which was discovered as a result of its role in mouse mammary cancer (3). In murine breast tumors, mouse mammary tumor virus was found consistently at preferential loci in the tumor DNA. The gene activated at the commonest of these integration sites (initially called int 1) was later shown to be homologous to the Drosophila "wingless" gene (4), and the combination of these two terms gave rise to the name "Wnt" (5).

Numerous new Wnt genes have subsequently been identified in many organisms including not only Drosophila (6), Xenopus (7), mice (8), and humans (9) but also a variety of fish, birds, and reptiles (2). Of the above, the profile of Wnt expression and function has been studied in most detail in the mouse. Ten murine genes have been identified, which show homology ranging from 50 to 90% in amino acid content (8).

The factors which control Wnt gene expression either as individual genes or as an interacting group have not yet been identified. Wnt proteins contain 350–380 amino acids, including 24 highly conserved cysteines and a number of glycosylation sites which, with one exception, are poorly conserved. In Wnt1, with the exception of some temperature-sensitive mutants, mutations affecting cysteine residues abolish biological activity, whereas those affecting glycosylation sites do not (10, 11). When in the cell, the protein is bound to the chaperonin-binding protein (12) and is associated with the endoplasmic reticulum and the Golgi apparatus. The N terminus of the protein carries a signal peptide as well as an adjacent signal peptidase recognition site. This, together with the observations that mutants lacking the signal peptide are inactive (10) and that there are no apparent transmembrane domains, suggests that Wnt protein is secreted. Indeed, Papkoff and Schryver (13) have shown that Wnt1 is present on the cell surface. However, relatively little is known about the precise molecular structure and function of the Wnts because the strong association of the proteins with the extracellular matrix has to date prevented isolation of active protein.

At a cellular level, Wnt genes show specific spatiotemporal expression in both embryonic and adult tissues of species in which they have been investigated. Studies in the mouse (14), Xenopus (15), and other systems all suggest a role for Wnt genes as signal transducers functioning locally to produce biological effects in cells adjacent to the source of secretion. Thus, Wnt genes play a part in tissue organization events such as central nervous system development in the mouse and segmentation in Drosophila.

Interest in the role of Wnt genes in breast biology stems from studies in the mouse. Gavin and McMahon (16) observed that mouse breast tissue showed differential expression of Wnt4, Wnt5a, Wnt5b, Wnt6, and Wnt7b which correlated with mammary changes associated with pregnancy and lactation. This and the work of Buhler et al. (17), demonstrating the expression of Wnt2 in the mouse mammary gland, suggest a role for Wnt genes in normal breast development. In addition, a number of lines of evidence show that some Wnt genes have growth-promoting properties in breast epithelium. Thus, Wnt1 and Wnt3 are two of the genes, which, when activated by mouse mammary tumor virus, cause mouse mammary tumors. Also, Wnt1 (18) and Wnt2 (19) transform some mouse mammary epithelial cell lines in vitro. Wnt1 transgenic mice (20) and mice bearing reconstituted mammary glands transfected with Wnt1 (21) show hormone-independent hyperplasia and an increased incidence of breast tumors. Wnt1 may produce these effects in both autocrine and paracrine mechanisms (22). It is pertinent that those Wnt genes which are associated with growth deregulation are those which are absent in the normal adult breast. Thus, an overall hypothesis consistent with the above data is that, in the mouse, Wnt genes are involved in the developmental processes of normal breast but aberrant Wnt gene expression can contribute to the development of mammary cancers.

In view of this we have investigated whether a similar mechanism might operate in some human breast cancers. The only human Wnt genes so far described are Wnt1, Wnt2 and Wnt3. Using PCR³ techniques, we have generated fragments specific to several human Wnt genes for use as templates in ribonuclease protection analysis. We have examined Wnt gene expression in benign and malignant diseases of the breast and compared their expression profile to that of normal breast tissues. An immortalized human breast epithelial cell line and human breast cancer cell lines were examined as a pure epithelial population. Our results show that, as in the mouse (16), only a subset of Wnt gene expression is normal in human breast tissue. Interestingly, quantitative differences in expression are seen between normal tissue and benign and malignant tumors of the human breast.

Received 11/16/93; accepted 3/15/94.

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This work was funded by the Imperial Cancer Research Fund, the Oxford District Research Committee [E. L. H., R. B., A. L. H.], and Hoffman La Roche J. A. M., A. P. M.] contributed equally to this study.

1. To whom requests for reprints should be addressed, at Imperial Cancer Research Fund, University of Oxford, John Radcliffe Hospital, Headington, Oxford, OX3 9DU, United Kingdom; ER, estrogen receptor.

2. The abbreviations used are: PCR, polymerase chain reaction; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EGFR, epidermal growth factor receptor; MCF7adr, Adryami-resistant MCF7; ER, estrogen receptor.

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Wnt GENE EXPRESSION IN HUMAN BREAST TISSUE

Cell Culture. Cell lines used in this study were obtained from the following sources: MTSV1–7 (23) from Dr Joyce Taylor, Imperial Cancer Research Fund, London; T47D, MDA231, MDA361, BT20, MDA415, MDA453, and MDA157 from the American Type Culture Collection, Bethesda, MD; ZR9BI1 and ZR75 from Dr. Eva Valverius, University Hospital, S-75185, Uppsala, Sweden; MCF7 from Dr. Barbara Durkacz, Cancer Research Unit, University of Newcastle upon Tyne; and MCF7adr from Dr. Ken Cowen, National Institutes of Health. MTSV17 were cultured in DMEM-10% FCS, with 5 µg/ml hydrocortisone (Sigma) and 20 µg/ml bovine insulin (Sigma). MDAMB157 was cultured in RPMI medium-10% FCS. All other cell lines were cultured in DMEM-10% FCS. All cells were cultured on plastic culture plates (Becton Dickinson) at 37°C, 5% CO2, 95% air, in a humidified incubator. All cultures were free of Mycoplasma. DMEM and RPMI were obtained from the Imperial Cancer Research Fund Clare Hall laboratories. FCS was obtained from Globepharm.

Tissue Selection. Human breast tissue samples were selected as follows. Wnt expression was examined in normal, benign disease, and malignant breast tissue. Details of the samples of breast tissue from which RNA was extracted are given in the legend to Fig. 2. Normal breast RNA was obtained either from breast reduction tissue samples (n = 4) or from normal breast tissue adjacent to diseased breast excised at operation (n = 4). For normal tissues, the age range of the patients was 18–78 years (mean, 42 years; median, 46 years). The tissues representing benign breast disease consisted of fibroadenomas (n = 5), benign phylloides tumors (n = 2), and fibrocystic disease (n = 2). For benign tissues, the age range of the patients was 25–52 years (mean, 39 years; median, 38 years). The tumor tissues were obtained postoperatively and were chosen to represent different subgroups (patient age range, 32–86 years; mean, 56 years; median, 57 years). By receptor status the tumors consisted of 40% ER positive, EGFR positive; 30% ER positive, EGFR negative; 30% ER negative, EGFR positive. The size of the tumors at the time of resection ranged from 8–40 mm, 40% of the tumors were lymph node positive, 19 of the tumors were ductal and one was lobular in type. Receptor analysis and tumor tissue handling was performed as previously described by Lejeune et al. (24). Tumors were considered ER positive if analysis showed at least 10 fmol

**Fig. 1.** Amino acid sequences of PCR-generated fragments of human Wnt3a, Wnt4, Wnt7a, and Wnt7b, aligned with the corresponding mouse sequences. H, human sequences; M, mouse sequences.
Table 1 Summary of Wnt expression profile in human breast cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Wnt2</th>
<th>Wnt4</th>
<th>Wnt7b</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTSV1-7</td>
<td>+a</td>
<td>-</td>
<td>+ (50)</td>
</tr>
<tr>
<td>MCF7</td>
<td>-</td>
<td>-</td>
<td>+ (30)</td>
</tr>
<tr>
<td>ZR75</td>
<td>-</td>
<td>-</td>
<td>+ (50)</td>
</tr>
<tr>
<td>T47D</td>
<td>+</td>
<td>-</td>
<td>+ (50)</td>
</tr>
<tr>
<td>MCFadr</td>
<td>+ (10)</td>
<td>-</td>
<td>+ (1)</td>
</tr>
<tr>
<td>MDA231</td>
<td>-</td>
<td>-</td>
<td>+ (100)</td>
</tr>
<tr>
<td>MDA361</td>
<td>-</td>
<td>-</td>
<td>+ (50)</td>
</tr>
<tr>
<td>BT20</td>
<td>+ (1)</td>
<td>-</td>
<td>+ (160)</td>
</tr>
<tr>
<td>MDA415</td>
<td>-</td>
<td>+ (1)</td>
<td>+ (20)</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>+ (45)</td>
</tr>
<tr>
<td>MDAMB157</td>
<td>-</td>
<td>-</td>
<td>+ (4)</td>
</tr>
<tr>
<td>ZR9B11</td>
<td>-</td>
<td>-</td>
<td>+ (50)</td>
</tr>
</tbody>
</table>

a Numbers in parentheses, relative level of Wnt expression in each cell line.
Wnt3 Expression Profile

Wnt Gene Expression in Human Breast Tissue

Wnt3 signal

Normal tissue

Benign tissue

Malignant tissue

GAPDH signal

A 120-base pair fragment of the human GAPDH gene cloned into the plasmid P Bluescript SK+ (28) was used to generate antisense GAPDH probes.

RNase Protection Analysis. RNase protection analysis was performed using the described constructs by standard protocols (26). In all assays 10 μg of RNA was hybridized to a cocktail containing 10^7 cpm of each of Wnt and GAPDH probes. Protected fragment signals for both Wnt and GAPDH were quantified by laser densitometry using a Bio Image analyzer (Millipore). In all assays the GAPDH signal was used as a loading control.

Statistical Analysis. P values were derived from Mann-Whitney U test calculations, using the “statview” version 4 program (Abacus Concepts Inc.)

RESULTS

Expression of Wnt RNA was examined by RNase protection using probes for Wnt2, Wnt3, Wnt3a, Wnt4, Wnt7a, and Wnt7b. Expression was assayed in human cell lines, as well as in normal, benign, and malignant human breast tissue.

Wnt Expression in Breast Cell Lines. The panel of cell lines (shown in Table 1 with their Wnt expression profile) consisted of MTSV1–7 (a human immortalized benign mammary epithelial cell line) (23), three ER-positive breast cancer cell lines (MCF7, ZR75, T47D), six ER-negative breast cancer cell lines (Adriamycin-resistant MCF7, MDA231, MDA361, MDA415, MDA457, MDAMB157), and the EGFR-transfected breast cancer cell line ZR9B1. Wnt3, Wnt4, and Wnt7b were expressed in some of the cell lines, but Wnt2, Wnt3a, and Wnt7a were not.

Wnt3 was expressed in 3 cell lines. MTSV1–7 and BT20 showed a similar level of expression, which was 10-fold lower than that found in MCF7adr. Wnt4 was present only in MDA415. Wnt7b was present in all cell lines examined. There was a 100-fold range in the level of expression of Wnt7b between the lowest and highest expressing cell lines. No consistent relationship was found between levels of expression and ER or EGFR status, although the 3 Wnt3-expressing cell lines were all ER negative, EGFR positive.

Wnt Expression in Human Breast Tissue. Figs. 2–5 show the signals obtained for Wnt2, Wnt3, Wnt4, and Wnt7b with the corresponding GAPDH signals and densitometric quantitation of the data.

Wnt2 expression was 10- to 20-fold higher (P = 0.03) in fibroadenomas (n = 5) than it was in normal, malignant, or benign tissues (taken together n = 30). Wnt2 expression was also found in fibroblasts freshly isolated from a breast reduction sample (data not shown).

Wnt3 was expressed at similar levels in normal, benign, and malignant breast tissue. There was a 20-fold range in expression between the lowest and highest expressing samples.

Like Wnt2, Wnt4 expression was higher (P = 0.001) in fibroadenomas (n = 5) than it was in other tissues (taken together n = 31). The increase in expression was approximately 10-fold, with one sample showing a 40-fold elevation.

Wnt7b was expressed at similar levels in normal and benign tissues but showed an elevation of approximately 30-fold in 10% of tumors. Southern analysis of genomic DNA extracted from normal tissue with low Wnt7b expression and from the tumor expressing the highest level of Wnt7b showed no obvious rearrangement of the gene, and slot blot analysis of the same DNA samples showed no genomic amplification (slot blot and Southern data not shown).

DISCUSSION

Our results show that several Wnt genes are expressed in normal human breast tissue. The specific role of Wnt genes as developmental
regulators in all systems in which they have been studied supports the hypothesis that they may play a similar role in human breast tissue. The fact that not all Wnt genes examined are expressed suggests that they have different functions. It is interesting that even the most homologous Wnt genes, such as pairs 3 and 3a and 7a and 7b, show different expression profiles (3 and 7b are expressed, 3a and 7a are not), suggesting a lack of redundancy in Wnt function.

Our data allow a comparison to be made between the Wnt genes expressed in normal human breast and those Wnt genes that are expressed at some stage of development in the mouse breast (Table 2). Mouse and human breast resemble each other in their expression of Wnt2, Wnt4, and Wnt7b and in the absence of Wnt1, Wnt3a, and Wnt7a. However, they differ in that Wnt3 (absent in normal mouse breast, by Northern analysis at least), is present in the human breast. When studying the mouse, Gavin and McMahon (16) were able to show a clear correlation between breast development during pregnancy and lactation and differential Wnt expression. In our experiments the profile of Wnt expression of postmenopausal breast was no different from that of premenopausal breast. Clearly, the fact that breast tissue from pregnant and lactating women is not readily available will make it difficult to repeat the experiments of Gavin and McMahon in the human. However, the question of Wnt gene regulation may be partially answered by investigating the effect of pregnancy-related and other growth factors on the Wnt expression profile of human mammary epithelial cells in vitro.

Cell line expression of Wnt genes mirrored the tissue expression with the exception of Wnt2, which was absent in cell lines but present in tissues. It is possible that this discrepancy is due to the expression of Wnt2 by stromal cells, which are present in tissues but not in the cell line cultures. This hypothesis is supported by the finding that Wnt2 mRNA is clearly present in fibroblasts freshly isolated from a breast tissue sample, and it is interesting that Wnt2 is elevated in tissues from benign breast disease, where there is a large stromal element. In addition, mesenchymal expression of Wnt2 has been demonstrated in other systems (29, 30). If the in vivo expression of Wnt2 is indeed stromal, this would suggest that the protein can act in paracrine fashion in the human breast and would parallel the in vitro demonstration of Wnt1 paracrine action in coculture assays (22).

If Wnt genes have a role in normal developmental processes of the human breast, then disease states of the breast in which tissue morphology is altered may be associated with changes in Wnt expression. Our results show an increased level of Wnt2 and Wnt4 in fibroadenomas and an elevated level of Wnt7b in approximately 10% of breast tumors. The finding that Wnt2 and Wnt4 are overexpressed in fibroadenomas does not establish an etiological relationship. However, the facts that Wnt1 can cause mammary hyperplasia in the mouse and that fibroadenomas are hyperplastic rather than neoplastic (31) support a causal role for Wnt genes in the pathology of these lesions. It is generally accepted that the term “fibrocystic disease” has no prognostic significance per se but that some of the histological features sometimes exhibited do correlate with an increased risk of breast cancer. These include the different types of epithelial hyperplasia (32). In the mouse, aberrant Wnt expression causes mammary epithelial cell hyperplasia, and it would, therefore, be of great interest to investigate Wnt expression in human breast epithelial hyperplasia, particularly “atypical epithelial hyperplasia” in which a 5-fold increased risk of breast cancer is recognized (32).

Wnt7b was expressed in all tissues examined but was elevated in 10% of tumors. These tumors could not be distinguished from others in the panel on the basis of clinical criteria such as estrogen receptor.
levels, epidermal growth factor receptor levels, differentiation grade, histological type, or nodal status. Our analysis of the tumor expressing the highest levels of Wnt7b would not demonstrate a minor translocation of the gene but makes a major rearrangement of Wnt7b in this tumor unlikely. Furthermore, the gene shows no genomic amplification; therefore, the increased RNA level may be due to increased transcription or decreased degradation.

In all cases in which Wnt signal was detected, the level of expression was nevertheless generally low, in that it was usually necessary to expose the gels for 1 week to obtain signals intense enough for reliable quantitation. This suggests that low quantities of Wnt may be sufficient to produce biological effects and would fit with the observation that, in the appropriate context, low-level Wnt expression is enough to achieve transformation (33).

An association between disease states and differential Wnt expression in the breast does not establish a causal relation between the two. It would be of interest to transfet human Wnt genes into human mammary epithelial cells to demonstrate whether or not altered levels of Wnt expression can be transforming, or at least alter normal mammary epithelial cell biology.

In summary, our results show that several human Wnt genes are expressed in normal breast tissue. Diseased breast shows levels of expression of some Wnt genes different from that found in normal breast. These data establish a role for Wnt genes in human breast biology and justify a further examination of a possible role in breast pathology.

REFERENCES

Table 2 Wnt gene expression in mouse mammary gland and in human breast cell lines and breast tissues

<table>
<thead>
<tr>
<th>Wnt</th>
<th>2</th>
<th>3</th>
<th>3a</th>
<th>4</th>
<th>7a</th>
<th>7b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse breast</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>(Refs. 16 and 17)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human cell lines</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Human tissue</td>
<td>+</td>
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<td>-</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>Normal</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Benign</td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>Malignant</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* +, expression detected; - , expression not detected.


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