Luteinizing Hormone/Human Chorionic Gonadotropin Receptors in Breast Cancer

Geri Meduri, Nathalie Charnaux, Hugues Loosfelt, André Jolivet, Frédérique Spyrats, Sylvie Brailly, and Edwin Milgrom


ABSTRACT

Recent studies have suggested that human chorionadotropin (hCG), in addition to its function in regulating steroidogenesis, may also play a role as a growth factor. Immunocytochemistry using two different monoclonal antibodies (LHRB9 and LHR1055) raised against the human luteinizing hormone/human chorionic gonadotropin (LH/hCG) receptor allowed us to detect this receptor in breast cancer cell lines (T47D, MCF7, and ZR75) in individual cancer biopsies and in benign breast lesions. The receptor was also present in epithelial cells of normal human and sow breast. In the latter, its concentration increased after ovulation.

The presence of LH/hCG receptor mRNA was confirmed by reverse transcription-PCR using primers extending over exons 2–4, 5–11, and 9–11.

The proportion of LH/hCG-receptor positive cells and the intensity of the immunolabelling varied in individual biopsies, but there was no obvious correlation with the histological type of the cancer.

These results are compatible with previous studies suggesting that during pregnancy, hCG is involved in the differentiation of breast glandular epithelium and that this hormone may play an inhibitory role in mammary carcinogenesis and in the growth of breast tumors.

INTRODUCTION

LHβ is a pituitary hormone that regulates ovarian and testicular steroidogenesis. hCG is secreted by the placenta and also stimulates ovarian steroid secretion. Both hormones act through the same specific receptor, which is coupled to proteins G and thus displays the characteristic pattern of 7 transmembrane spans (reviews in Refs. 1 and 2). The LH/hCG, follicle-stimulating hormone, and thyroid-stimulating hormone receptors are characterized by a large NH2-terminal extracellular domain, which is the site of hormone binding (3–6). However, recent evidence shows that in addition to their classical endocrine effects, hCG and LH may also be ectopically synthesized and exert paracrine effects regulating the growth of various cell types. The role of these hormones in the occurrence and development of various cancers has been extensively discussed (7–16).

Crystallization and X-ray diffraction studies have established the three-dimensional structure of hCG (17). This structure shows this hormone to be a member of the structural superfamily of cystine knot growth factors, which also includes nerve growth factor, transforming growth factor-β, and platelet-derived growth factor-β.

Synthesis of hCGβ by a variety of cancer cell lines of various origin has been demonstrated (18–20). Concomitant expression of LHβ has often been observed suggesting an activation of the whole hCGβ-LHβ gene cluster (21). hCG thus belongs to the group of embryonically related cancer markers that also includes carciinoembryonic antigen and α-fetoprotein.

A stimulation by hCG of the growth of several tumor cell lines has been reported (11, 12, 16, 22). Kumar et al. (12) have described the secretion of hCG by human lung cancer cells. Incubation with anti-hCG antibodies or with specific antisense RNAs caused these cells to lose their transformed phenotype. Furthermore, when transplanted into nude mice, the tumors underwent necrosis following injection of anti-hCG antibodies. These observations have led to Phase I clinical trials of an anti-hCG vaccine in cancer patients (23). Very recently, Lunardi-Iskandar et al. (24) showed that AIDS Kaposi syndrome-derived cells could be killed by hCGβ. The same authors also reported cases of remission of Kaposi sarcoma in pregnant women.

Pregnancy and, especially, early pregnancy have been shown to be protective for breast cancer (25, 26). This observation led Russo et al. (13, 14) to establish corresponding animal models. They have shown that in the rat, the susceptibility to breast neoplastic transformation by chemical carcinogenesis or radiation is decreased by the differentiation of the gland in parous animals. The same result could be obtained by treatment with hCG (15). Because all of these observations were made in noncastrated animals, the mechanism of action of hCG remained unclear. However, Alvarado et al. (27) showed that hCG could exert a direct antiproliferative effect on human breast epithelial cells and on breast cancer cells in culture. This effect was possibly mediated through secretion of inhibin (28, 29). Because hCG prevented both initiation and progression of mammary cancers, Russo suggested that this hormone might be used clinically to prevent the occurrence of this cancer in high-risk patients (30).

A direct action of hCG and LH on normal breast and breast tumors implies the existence of the corresponding receptors in these tissues. Furthermore, because most hormonal markers, including estrogen, progesterone, EGF receptors, and so forth, are present in only a subset of the cancers (31–33), it appeared important to devise a method allowing the detection of tumors containing LH/hCG receptors. We describe here the characterization of such receptors in normal breast and in breast tumors and also report an immunocytochemical method allowing the analysis of individual biopsies.

MATERIALS AND METHODS

Antibodies. The preparation of mouse monoclonal antihuman LH/hCG receptor antibodies will be described in detail elsewhere.4 In summary, mice were given (100 µg/injection) a recombinant protein (human LH/hCG receptor amino acids 75–406 fused to ubiquitin) produced in Escherichia coli. After three s.c. injections at 15-day intervals and an i.p. injection 3 days before the sacrifice, the splenocytes of the mouse were fused to myeloma Sp2 cells. Hybridoma clones were selected, expanded in ascites fluid and antibodies purified on protein A-Sepharose. The specificity of the antibodies was verified by their ability to react with receptor-β-glucuronidase recombinant fusion protein to immunoprecipitate and immunopurify 125I-hCG-receptor complexes prepared from L-cells transfected with an LH/hCG receptor expression vector. The expression vector for LH/hCG receptor was prepared and used for transfection as described for similar studies with the follicle-stimulating hormone-receptor vector (34). The antibodies were shown to immunostain cells trans-
### Table 1. Immunocytochemical study of LH/hCG receptors in patients with breast cancer

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Menopausal status</th>
<th>PT (mm)</th>
<th>Invaded nodes (no.)</th>
<th>Histological type</th>
<th>Grade</th>
<th>ER (fmol/mg protein)</th>
<th>PR (fmol/mg protein)</th>
<th>Stained cells (%)</th>
<th>Staining intensity</th>
<th>ICC score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32</td>
<td>Pre</td>
<td>35</td>
<td>1</td>
<td>Inf. duct.</td>
<td>III</td>
<td>29</td>
<td>40</td>
<td>32</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
<td>Pre</td>
<td>35</td>
<td>0</td>
<td>Inf. duct.</td>
<td>III</td>
<td>2</td>
<td>2</td>
<td>54</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>38</td>
<td>Pre</td>
<td>40</td>
<td>0</td>
<td>Inf. lob.</td>
<td>II</td>
<td>78</td>
<td>100</td>
<td>30</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>Pre</td>
<td>15</td>
<td>0</td>
<td>Inf. lob.</td>
<td>II</td>
<td>483</td>
<td>213</td>
<td>61</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>47</td>
<td>Pre</td>
<td>30</td>
<td>3</td>
<td>Inf. duct.</td>
<td>II</td>
<td>179</td>
<td>129</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>48</td>
<td>Pre</td>
<td>10</td>
<td>0</td>
<td>DIS</td>
<td></td>
<td>0</td>
<td>0</td>
<td>27</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>Pre</td>
<td>60</td>
<td>0</td>
<td>ID papill.</td>
<td></td>
<td>25</td>
<td>28</td>
<td>16</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>59</td>
<td>Post</td>
<td>35</td>
<td>0</td>
<td>Inf. duct.</td>
<td>II</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>61</td>
<td>Post</td>
<td>22</td>
<td>0</td>
<td>Inf. duct.</td>
<td>II</td>
<td>168</td>
<td>214</td>
<td>64</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>67</td>
<td>Post</td>
<td>50</td>
<td>0</td>
<td>Inf. duct.</td>
<td>II</td>
<td>1</td>
<td>2</td>
<td>16</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>69</td>
<td>Post</td>
<td>50</td>
<td>4</td>
<td>Colloid</td>
<td></td>
<td>10</td>
<td>13</td>
<td>9</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>70</td>
<td>Post</td>
<td>60</td>
<td>0</td>
<td>Inf. lob.</td>
<td>I</td>
<td>115</td>
<td>5</td>
<td>59</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>13</td>
<td>73</td>
<td>Post</td>
<td>18</td>
<td>0</td>
<td>Inf. duct.</td>
<td>II</td>
<td>352</td>
<td>192</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>76</td>
<td>Post</td>
<td>70</td>
<td>7</td>
<td>Inf. duct.</td>
<td>III</td>
<td>229</td>
<td>13</td>
<td>35</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>15</td>
<td>77</td>
<td>Post</td>
<td>40</td>
<td>10</td>
<td>Inf. duct.</td>
<td>III</td>
<td>231</td>
<td>13</td>
<td>20</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>78</td>
<td>Post</td>
<td>60</td>
<td>21</td>
<td>Inf. duct.</td>
<td>II</td>
<td>4</td>
<td>2</td>
<td>30</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>17</td>
<td>83</td>
<td>Post</td>
<td>20</td>
<td>0</td>
<td>Inf. duct.</td>
<td>III</td>
<td>584</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>87</td>
<td>Post</td>
<td>37</td>
<td>3</td>
<td>Inf. duct.</td>
<td>III</td>
<td>213</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>19</td>
<td>91</td>
<td>Post</td>
<td>30</td>
<td>6</td>
<td>Inf. duct.</td>
<td>II</td>
<td>140</td>
<td>42</td>
<td>40</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

*Pre, premenopausal; post, postmenopausal; PT, primary tumor size; inf. duct., infiltrating ductal carcinoma; inf. lob., infiltrating lobular carcinoma; DIS, ductal carcinoma in situ; ID papill., intraductal papillary carcinoma; colloid, colloid carcinoma.

The percentage of cells stained by anti-LH/hCG receptor antibody was graded as follows: 0, <14%; 1, 15—24%; 2, 25—64%; 3, >65%.

The staining intensity was divided into four categories: 0, negative; 1, low; 2, medium; 3, high.

ICC, immunocytochemical. The final score is the product of the percentage grade multiplied by the intensity grade.

### Table 2. Immunocytochemical study of LH/hCG receptors in normal breast and in benign breast lesions

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Menopausal status</th>
<th>Histology</th>
<th>Association with breast carcinoma</th>
<th>Stained cells (%)</th>
<th>Staining intensity</th>
<th>ICC score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23</td>
<td>Pre</td>
<td>Normal breast</td>
<td>No</td>
<td>77</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>Pre</td>
<td>Normal breast</td>
<td>No</td>
<td>79</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>Pre</td>
<td>Normal breast</td>
<td>No</td>
<td>72</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>36</td>
<td>Pre</td>
<td>Mild hyperplasia without atypia</td>
<td>No</td>
<td>68</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>37</td>
<td>Pre</td>
<td>Mild hyperplasia without atypia</td>
<td>No</td>
<td>73</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>37</td>
<td>Pre</td>
<td>Mild hyperplasia without atypia</td>
<td>Yes</td>
<td>58</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>38</td>
<td>Pre</td>
<td>Mild hyperplasia; apocrine change</td>
<td>Yes</td>
<td>53</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>47</td>
<td>Pre</td>
<td>Blunt duct adenosis</td>
<td>No</td>
<td>92</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>9</td>
<td>48</td>
<td>Pre</td>
<td>Mild hyperplasia; cystic change</td>
<td>Yes</td>
<td>59</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>51</td>
<td>Post</td>
<td>Mild hyperplasia; cystic change</td>
<td>No</td>
<td>64</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>11</td>
<td>65</td>
<td>Post</td>
<td>Adenosis</td>
<td>Yes</td>
<td>35</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>12</td>
<td>65</td>
<td>Post</td>
<td>Moderate hyperplasia; ductal epithelial change</td>
<td>Yes</td>
<td>66</td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
</table>

*See Table 1, footnote a, for definition.

---

**Fig. 1.** Use of anti-LH/hCG receptor monoclonal antibodies for immunolabeling of breast tumors. A, invasive ductal carcinoma (patient 16) immunostained with antibody LHR29. The intraductal malignant cells are labeled. ×100. B, no labeling is visible on a serial section of the same tumor incubated with an unrelated control antibody (IDA 10) of the same subclass and at the same concentration. ×100. C, immunolabeling of a serial section of the same ductal carcinoma with antibody LHR1055, which recognizes a different epitope of the LH/hCG receptor. ×100. D, invasive lobular carcinoma (patient 12) immunostained with antibody LHR29; the malignant intralobular cells adjacent to the basement membrane are stained. ×200. E, no immunostaining is found in the same region of the tumor using the same antibody previously incubated with recombinant LH/hCG receptor ectodomain. ×200.
Fig. 2. Immunocytochemical study of LH/hCG receptors in human breast cancer. A. Intraductal carcinoma (patient 6). Only the basal cells and the cells facing the luminal cavity (L) are intensely stained. ×450. B. Highly cellular invasive ductal carcinoma (patient 17). Only strands of cells adjacent to the fibrovascular septa are labeled. ×1000. Bar, 100 μm. C. Invasive lobular carcinoma (patient 3). All cells are labeled with variable staining intensity. ×200. Bar, 50 μm. D. Papillary carcinoma (patient 7). The expression of LH/hCG receptors is more intense on the peripheral cells. ×400. Bar, 10 μm. E. Intraductal carcinoma (patient 6). The staining is localized to the plasma membrane. ×1420. Bar, 10 μm. F. Colloid carcinoma (patient 11). The immunolabeling is intense and mainly intracytoplasmic, and sometimes has a band-like perinuclear disposition. ×2000. Bar, 10 μm. LHR29 antibody was used.

Infected with an expression vector encoding LH/hCG receptor, whereas no reaction was seen with mock-transfected control cells. Antibodies LHR29 and LHR1055 (both of the IgG1 subclass) were used in this study to identify LH/hCG receptor-bearing cells.

Antibodies against cytokeratin 19 (CK 19 clone BA 17; Dako, Carpinteria, CA), epithelial membrane antigen (clone E 29; Dako), and α-smooth muscle actin (clone 1 A4; Sigma Chemical Co., St. Louis, MO) also were used to differentiate normal and neoplastic epithelial cells from myoepithelial cells in the sections studied. IDA10 antibody (35) was used as a non-receptor related antibody control.

Tissues. Breast cancer specimens were obtained from 19 patients undergoing mastectomy or lumpectomy (Table 1). Samples of benign breast lesions were obtained from four patients with benign breast disease and from five patients undergoing mastectomy for breast cancer (Table 2). Three samples of normal breast tissue were obtained during reductive surgery. The blocks of tissue were snap-frozen in liquid nitrogen and preserved at −196°C until use.

Porcine mammary tissue was obtained from four Large White sows of reproductive age (Institut National de la Recherche Agricole, Nouzilly, France). The animals were treated with a hormonal cycle regulator (Regumate 228, Roussel Uclaf, Romanville, France). Two sows were in the late preovulatory phase (day 1 of the estrus cycle) and two were in the early luteal phase (day 5 of the estrus cycle). The length of the estrus cycle in the sow is about 21 days. The tissue was excised immediately after sacrifice, trimmed of excess fat, cut into blocks of 5 mm width, and snap-frozen in liquid nitrogen after embedding with tissue-Tek OTC embedding medium (Miles Inc., Elkart, IL). The blocks were preserved at −196°C until further use.
chemical procedures, the slides were left unwrapped to thaw for 10 min. Afterward, the beads were incubated 1 h with horseradish peroxidase-conjugated monoclonal anti-ER (D547) or anti-PR (KD68) antibodies. The beads were washed, and then incubated for 30 min with hydrogen peroxide and DAB solution. Absorbance was measured at 492 nm and steroid receptor concentration was derived from a standard curve. Proteins were assessed by Pierce protein assay (Pierce, Rockford, IL). Results were expressed in fmol/mg proteins. Cutoff was set at 15 fmol/mg protein for both receptors.

RT-PCR of LH/hCG Receptor mRNA. Total RNA was extracted with the RNA Flash T (Bioprobe) from human tissues collected at surgery, from T47D, ZR75, and MCF-7 cell lines and from an L-cell line transfected with the anti-LHR primary antibodies. The cDNAs were synthesized using the first-strand cDNA kit from Clontech (4 ng of total RNA in a 20-μl standard reaction). RNA of LR recombinant L-cells was diluted 100-fold with RNA extracted from mock-transfected L-cells. PCR was carried out (volume, 100 μl) with 5 μl of the cDNA mixture and the following synthetic oligonucleotides (upstream/downstream primers): nucleotides 170–190 (exon 2), 836–858 (exon 4), 11089–1111 (exon 11), and 500–524 (exon 5)/1099–1031 (exon 11); nucleotides were numbered from the initiation ATG). After 35 cycles of amplification, DNA fragments (one-thirtieth of the PCR reaction) were electrophoresed in 1% agarose and analyzed by Southern blot using an internal 32P-labeled oligonucleotide probe (100 cpm/ml; the probes corresponded to nucleotides 283–310, respectively). Control experiments were performed to assess β-actin mRNA content in human tissue samples (not shown).

RESULTS

Specificity of LH/hCG Receptor Immunostaining in Human Breast Tissue. We used for these studies both neoplastic and normal breast tissue. Representative experiments are shown in Fig. 1. Immunolabeling of an invasive ductal carcinoma (Fig. IA) and an invasive lobular carcinoma (Fig. 1D) was observed using LR hCG recombinant antibody. If we used a control unrelated monoclonal antibody of the same IgG1 subclass, there was no staining (Fig. 1B). A similar result was obtained if the primary antibody was omitted (not shown) or if it was previously incubated with recombinant LH/hCG receptor ectodomain (Fig. 1E).

Furthermore, we compared the staining obtained with antibody LR hCG receptor with that observed with antibody LR hCG receptor. These two antibodies bind additively to the antigen, showing that they recognize different epitopes. As shown in Fig. 1, A and C, the labeling observed on consecutive sections was similar for the two antibodies.

Immunocytochemical Study of LH/hCG Receptor in Breast Cancers. Intraductal carcinomas were frequently strongly immunolabeled either on the majority of the cells or on both the basal layers and the layers close to the lumen. In the latter case, the intermediate layers of cells appeared devoid of LH/hCG receptor (Fig. 2A). In ductal invasive cancers, the number of stained cells varied widely between carcinomas of the same histological type. A stronger staining intensity was often observed in the cells situated at the

Fig. 3. Immunocytochemical study of LH/hCG receptor in human breast cancer cell lines. A, permanently transfected L-cells uniformly expressing the LH/hCG receptor. ×200. B, T47D breast cancer cells. The receptor is found in only a fraction of the cells, and the staining is strong. ×200. C, MCF7 breast cancer cells. The expression of the receptor is more abundant. D, ZR75 breast cancer cells. Only a small minority of the cells are stained. ×200. LHR29 antibody was used.

Breast Cancer Cell Lines. MCF7 (ATCC, Rockville, MD; HTB 22), T47D (HTB 33), and ZR75–1 (CRL 1500) were used. The cells were cultured in DMEM with 10% FCS, 2 mm glutamine, 2 mm sodium pyruvate and 400 μl of insulin (Umsuline Rapide, Lilly, France) in an incubator at 37°C with 5% carbon dioxide. Before immunohistochemistry, the cells were plated in Labtek chamber slide culture chambers (Nunc Inc., Naperville, IL) at a concentration of 105 cells per chamber. Just before confluence (18–24 h growth) the cells were removed from the incubator and washed twice with PBS. The upper structure of the chamber slides was removed, and the slides were fixed with cold (−20°C) acetone for 5 min and then rehydrated in PBS prior to immunostaining.

Immunohistochemistry. Six-μm-thick serial frozen sections were cut with an OTM56 Bright cryomicrotome, air dried for 30 min at room temperature, fixed in cold (−20°C) acetone for 5 min, and dried for 20 min. The sections were individually wrapped in cellulose and tinfoil and stored at −20°C until further use; the storage time never exceeded 3 months. Before immunohistochemical procedures, the slides were left unwrapped to thaw for 10 min at room temperature and then rehydrated in two successive baths of 10 mm PBS, pH 7.2. The sections were then pretreated for 20 min with 3% hydrogen peroxide and o-phenylenediamine. Antibody incubation was performed overnight in a humid chamber at room temperature, and then with streptavidin-biotin complexes. Aminoethylcarbazole (Sigma Chemical Co.) was used as a chromogen. The sections were lightly counterstained with Meyer's hematoxylin and mounted with an aqueous medium (Glycergel, Dako). Replacement of the specific primary monoclonal antibody with preimmune mouse immunoglobulins (Sigma Chemical Co.) or IIDA01 antibody (35) at the same concentration resulted in absence of epibetal staining, as did the replacement of the primary antibodies with other unrelated monoclonal antibodies of the same subclass, used at the same concentrations (anti-common leukocyte antigen, Dako LC, clones PD 26 and 2B11).

For the immunocytochemical scoring, at least 300 epithelial cells were counted at high magnification in three different areas of each biopsy. These areas were assigned to four grades according to the percentage of cells stained (Table 1). The staining intensity also was assessed and subdivided into the following four grades: negative (grade 0), low (grade 1), medium (grade 2), and high (grade 3). The final score was the product of the percentage grade multiplied by the intensity grade (36).

ERs and PRs were assayed on cytosolic extracts of the surgical specimens, using Abbott enzyme immunoassay kits (ER-EIA and PR-EIA, Abbott Laboratories, Abbott Park, IL). Briefly, the tumor material was homogenized and centrifuged at 150,000 × g for 1 h. ERs and PRs were assayed in the supernatant (cytosol). Beads coated with monoclonal anti-ER (H222) or progesterone (Z0B39) antibodies were incubated overnight with aliquots of tumor cytosol or standard solutions of ER and PR. After washing, the beads were incubated 1 h with horseradish peroxidase-conjugated monoclonal anti-ER (D547) or anti-PR (KD68) antibodies. The beads were washed, and then incubated for 30 min with hydroxyperoxide and DAB solution. Absorbance was measured at 492 nm and steroid receptor concentration was derived from a standard curve. Proteins were assessed by Pierce protein assay (Pierce, Rockford, IL). Results were expressed in fmol/mg proteins. Cutoff was set at 15 fmol/mg protein for both receptors.

RT-PCR of LH/hCG Receptor mRNA. Total RNA was extracted with the RNA Flash T (Bioprobe) from human tissues collected at surgery, from T47D, ZR75, and MCF-7 cell lines and from an L-cell line transfected with the anti-LHR primary antibodies. The cDNAs were synthesized using the first-strand cDNA kit from Clontech (4 μg of total RNA in a 20-μl standard reaction). RNA of LR recombinant L-cells was diluted 100-fold with RNA extracted from mock-transfected L-cells. PCR was carried out (volume, 100 μl) with 5 μl of the cDNA mixture and the following synthetic oligonucleotides (upstream/downstream primers): nucleotides 170–190 (exon 2), 836–858 (exon 4), 11089–1111 (exon 11), and 500–524 (exon 5)/1099–1031 (exon 11); nucleotides were numbered from the initiation ATG). After 35 cycles of amplification, DNA fragments (one-thirtieth of the PCR reaction) were electrophoresed in 1% agarose and analyzed by Southern blot using an internal 32P-labeled oligonucleotide probe (100 cpm/ml; the probes corresponded to nucleotides 283–310, 1099–1031, and 836–858, respectively). Control experiments were performed to assess β-actin mRNA content in human tissue samples (not shown).

RESULTS

Specificity of LH/hCG Receptor Immunostaining in Human Breast Tissue. We used for these studies both neoplastic and normal breast tissue. Representative experiments are shown in Fig. 1. Immunolabeling of an invasive ductal carcinoma (Fig. 1A) and of an invasive lobular carcinoma (Fig. 1D) was observed using LRhCG monoclonal antibody. If we used a control unrelated monoclonal antibody of the same IgG1 subclass, there was no staining (Fig. 1B). A similar result was obtained if the primary antibody was omitted (not shown) or if it was previously incubated with recombinant LH/hCG receptor ectodomain (Fig. 1E).

Furthermore, we compared the staining obtained with antibody LRhCG receptor with that observed with antibody LRhCG receptor. These two antibodies bind additively to the antigen, showing that they recognize different epitopes. As shown in Fig. 1, A and C, the labeling observed on consecutive sections was similar for the two antibodies.

Immunocytochemical Study of LH/hCG Receptor in Breast Cancers. Intraductal carcinomas were frequently strongly immunolabeled either on the majority of the cells or on both the basal layers and the layers close to the lumen. In the latter case, the intermediate layers of cells appeared devoid of LH/hCG receptor (Fig. 2A).

In ductal invasive cancers, the number of stained cells varied widely between carcinomas of the same histological type. A stronger staining intensity was often observed in the cells situated at the
LH/hCG RECEPTORS IN BREAST CANCER

Fig. 4. Immunocytochemical study of LH/hCG receptor in benign breast lesions without atypia. A. adenosis (patient 11). About 30% of the epithelial cells of the hyperplastic lobule are stained (arrow). ×120. Bar, 100 μm. B. epitheliosis (patient 12). All of the benign proliferating epithelial cells lining the duct are stained. ×100. Bar, 100 μm. C. apocrine change (patient 7). The metaplastic epithelial cells are lightly labeled; the staining is more evident on the basal cells. ×400. D. blunt duct adenosis (patient 8). The epithelial cells lining the blunt ducts are uniformly stained. ×100. E. small cyst (patient 10). All of the epithelial cells are immuno-stained. ×800. Bar, 10 μm. F. larger cyst showing a discontinuous epithelial layer (patient 9). Some of the epithelial cells are stained (arrows). ×200. Bar, 50 μm. LHR29 antibody was used.

The great majority of tumoral cells were stained on the plasma membrane (Fig. 2E). In only one case of colloid carcinoma, the staining observed in some cells appeared very intense and mainly intracytoplasmic, sometimes forming a band parallel to the nucleus (Fig. 2F). Such a localization has been described for this family of receptors in cases in which immature precursors of the receptor accumulate (5).

Nineteen different biopsies were examined (7 premenopausal and...
12 postmenopausal). There was no obvious relation between the LH/hCG receptor immunostaining score and the histological type, the size of the tumor, the presence and number of invaded lymph nodes, the content of ERs and PRs, or the histological grading (Table 1).

Immunocytochemical Study of LH/hCG Receptors in Breast Cancer Cell Lines. T47D, MCF7, and ZR75 breast cancer cell lines were studied. As a control, L-cells permanently transfected with an expression vector encoding LH/hCG receptor also were examined. The latter were uniformly labeled (Fig. 3A) because all of the cells expressed the receptor. Only one-third of the T47D cells were intensely labeled by the anti-LH/hCG receptor antibody (Fig. 3B). About two-thirds of MCF7 cells were stained less intensely (Fig. 3C). Less than 1% of the ZR75 cells were immunolabeled; the staining was intense (Fig. 3D). The immunolabeling was identical using antibodies LHR29 and LHR1055, whereas a nonrelated antibody (of the same IgG1 subclass) did not stain these cells (not shown).

Immunocytochemical Studies of LH/hCG Receptors in Benign Breast Lesions. Twelve different biopsies were analyzed. Seven were taken from patients with benign breast disease, whereas 5 consisted of nonmalignant dysplastic breast tissue obtained during surgery for breast cancer. The lesions that were observed included foci of adenosis, hyperplasia of ductal epithelium, foci of apocrine metaplasia of epithelial cells, and small cysts.

In foci of adenosis, about 30% of the cells were labeled by anti-LH/hCG receptor antibodies (Fig. 4A). The hyperplastic ductal epithelium was more intensively stained (Fig. 4B). In most cases, all of the hyperplastic layers were labeled, whereas in some cases, only the luminal and the basal layers were immunostained.

The foci of apocrine metaplasia were only focally and faintly stained (Fig. 4C). All of the epithelial cells of lesions of blunt duct adenosis were labeled (Fig. 4D). In cysts, there was a contrast between the intensely...
labeled cuboidal epithelium lining the smaller cysts (Fig. 4E) and the faintly stained (or negative) cells lining the larger cysts (Fig. 4F).

Immunocytochemical Study of LH/hCG Receptors in Normal Human Breast Tissue. Observation of LH/hCG receptors in breast cancers led us to ask whether their presence was due to the disease or whether they also existed in normal breast tissue. In normal human breast, labeling was observed that increased from the acini to the small ducts (Fig. 5A) and to the large ducts (Fig. 5B). The proportion of labeled cells varied from one lobule to another: some lobules were more intensely stained than others. Only epithelial cells were immunostained; no labeling was found on myoepithelial cells or fibroblasts. In individual cells, there was no polarization of the receptor toward the apical or the basal pole.

Immunocytochemical Studies of LH/hCG Receptor in Pre- and Postovulatory Sow Breast. If LH/hCG receptors play an important physiological role in breast function, they should be present in various species. Furthermore, the use of an animal model should allow an easier analysis of a possible hormonal regulation of these receptors.

Because high-affinity antibodies for pig LH receptors were available (37, 38), we studied the breast tissue of sows biopsied during either the follicular or the luteal phase. A clear-cut difference was observed: during both phases, only the epithelial cells, not the stromal or myoepithelial cells, were immunostained. During the follicular stage, the ducts (Fig. 6A) were labeled, whereas a heterogeneity of labeling was observed between lobules and in the same lobule between acini.

After ovulation, during the luteal phase (Fig. 6B), a marked increase in labeling was observed: all of the acini in all of the lobules were now immunostained.

These results indicate that the LH receptor in sow breast undergoes a hormonal regulation, suggesting either up-regulation by the LH peak or induction by progesterone.

No labeling was observed whenever antibody LHR38 was replaced with a control unrelated antibody of the same subclass at the same concentration.

LH/hCG Receptor mRNA in Human Breast. We used RT-PCR to study the presence of LH/hCG receptor mRNA in breast tissue. Three pairs of primers were used, spanning regions of the receptor encoded by exons 2–4, 5–11, and 9–11 (the LH/hCG receptor gene contains 11 exons; Ref. 39). Positive controls included mRNA prepared from human ovaries, testes, and an L-cell line permanently expressing human LH/hCG receptor (Fig. 7). Negative controls included human liver and L cells not transfected with human LH/hCG receptor. Hybridization was performed with internal 32P-labeled oligonucleotide probes.

Using this methodology, LH/hCG receptor mRNA was detected in normal breast tissue, in 10 different breast cancers, and in MCF7, ZR75, and T47D breast cancer cell lines (Fig. 7). In all cases, the size of the amplified DNA corresponded to that observed in ovariess and testes.

DISCUSSION

Resolution of the three-dimensional structure of hCG has shown this hormone to be part of the structural superfamily of cystine knot growth factors (17). Indeed, hCG has been shown to exert either stimulatory (11, 12, 16, 22, 40) or inhibitory (24, 27, 41, 42) effects on the growth of various cancer cell lines.

In the breast, it has been proposed that hCG provokes differentiation, which in turn renders the cells less susceptible to neoplastic transformation (13, 14, 30). This mechanism would explain the decreased occurrence of breast cancer in women who had an early pregnancy (25, 26). Such a hypothesis is compatible with the observation of the presence of LH/hCG receptors in normal breast tissue. The receptors seem to undergo a hormonal regulation; their concentration is increased after ovulation.

In breast cancer, the number of cells immunostained with anti-LH/hCG receptor antibodies and the intensity of the staining varied among individual cancers. There was no obvious relation to the histological type. The receptor was also detected at various concentrations in different breast cancer cell lines. Further studies in a greater number of patients will be necessary to determine whether the presence and the concentration of LH/hCG receptors are correlated with the aggressiveness of the tumor and the prognosis of the patients. The preparation of high-affinity antibodies against the human LH/hCG receptor, able to immunostain the receptor even in tissues and cells where its concentration is relatively low, will allow such studies.

The receptor present in breast tumors interacts with two different antibodies recognizing different epitopes. The RT-PCR method allowed detection of the receptor messenger corresponding to exons 2–4, 5–11, 9–11. This suggests that the receptor present in the breast...
is similar to that expressed in the gonads; however, total sequencing would be necessary to definitively solve this question.

It is also unknown if the LH/hCG receptor is activated only by circulating hCG (and possibly LH) or if there exists a local secretion of this hormone and thus paracrine regulations occurring through the LH/hCG receptor.

REFERENCES


3. Xie, J. B., Wang, H., and Segaloff, D. L. Extracellular domain of lutropin/choriogonadotropin receptor is similar to that expressed in the gonads; however, total sequencing would be necessary to definitively solve this question.


7. Xie, J. B., Wang, H., and Segaloff, D. L. Extracellular domain of lutropin/choriogonadotropin receptor is similar to that expressed in the gonads; however, total sequencing would be necessary to definitively solve this question.
Luteinizing Hormone/Human Chorionic Gonadotropin Receptors in Breast Cancer

Geri Meduri, Nathalie Charnaux, Hugues Loosfelt, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/57/5/857

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.