Eur J Cancer Clin Oncol, Vol. 24, No. 9, pp. 1425–1431, 1988.
Printed in Great Britain

0277-5379/88$3.00 + 0.00

Presence and Characterization of Insulin-like Growth Factor 1 Receptors in Human Benign Breast Disease*

J.P. PEYRAT, J. BONNETERRE, J.C. LAURENT, M.M. LOUCHEZ, S. AMRANI, B. LEROY-MARTIN, M.O. VILAIN, A. DELOBELLE and A. DEMAILLE

Centre Oscar Lambret, BP 307, 59020 *Lille Cédex, France*

Abstract—*Insulin-like growth factor* 1 *binding sites were characterized in human benign breast disease. We demonstrated the presence of one high affinity binding site. Chemical cross-linking of* [^{125}I]*IGF*1 *to benign breast disease membranes in reducing condition and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed one band with an apparent* M_r *of* 130,000. *The specificity of the binding was studied: IGF* 2 *was a good competitor whereas insulin competed for binding with a potency lower than* 1/100 *that of IGF*1. *This IGF*1 *binding corresponded to the previously described type I IGF receptor (IGF*1*-R).*

*IGF*1*-R was assayed in* 35 *cases of benign breast disease and two samples of normal breast tissue. Forty-three per cent of the lesions were IGF*1*-R positive. The mean geometric level of specific binding was* 1.98% *in the whole population, it was significantly lower in adenofibromas* (1.55%) *than in epithelial hyperplasia* (2.5%); *it was* 2% *in dystrophic disease. IGF*1*-R was undetectable in normal tissue. Considering our previous results showing that almost all the breast cancers contained IGF*1*-R, these data suggest that the increase in IGF*1*-R could be a marker of malignant tumor development.*

INTRODUCTION

INSULIN-LIKE growth factor 1 (IGF-1), also termed somatomedin C, is a polypeptide whose well-known physiological role is to promote skeletal development via the endocrine pathway [1, 2]. IGF1 is synthesized in many different tissues [3] where it has recently been detected using IGF1-cDNA probes [4]. These results and the demonstration that IGF1 stimulates the growth of many cell types [5] suggest that this factor is important in the control of the cell cycle.

In human breast cancer it has been demonstrated that IGF1 stimulated the growth in all the human breast cancer cell lines tested [6–8]. Immunoreactive IGF1 is secreted by all human breast cancer cells examined to date [9, 10]. Several IGF1 mRNA have also been detected by northern blot analysis using a DNA probe to authentic IGF1 [8]. Utilizing phenol red-free medium an E2 induction of secreted IGF1 was noted [11]. These observations suggest that IGF1 could also act via the autocrine pathway.

The first step of IGF1 action is its binding to membrane receptors. The receptor for IGF1 is remarkably similar to that of insulin, comprising a heterotetrameric structure with two alpha and two beta subunits joined by disulfide bridges [12]. IGF1 receptors of the expected size have been detected by cross-linking studies on cultured human breast cancer cell lines [6]. Research on intact cell IGF1 receptors has been hampered by the presence of a secreted, non-receptor binding protein which interferes in ligand binding assays [13–15]. Recently we have characterized specific IGF1 binding sites in breast cancer cell membranes; we have also demonstrated the presence of IGF1-R in the majority of human breast cancer biopsies, these receptors being correlated with estradiol and progesterone receptors [16]; these results suggest a potential role of IGF1 *in vivo*.

To our knowledge there is no report on the presence of IGF1-R in human benign mammary tumors. In the present study the IGF1 specific binding to membrane preparations of benign breast disease was characterized and then determined in 35 individual cases.

Accepted 28 April 1988.
*This work was supported by the 'Association pour la Recherche sur le Cancer' and by the 'Fédération Nationale des Centres de Lutte contre le Cancer'.

MATERIALS AND METHODS

Collection of tumors

Tumor specimens were obtained from 35 patients undergoing surgery for breast tumor. At the time of collection the fat was removed and the samples were divided into two parts: one was immediately frozen for receptor analysis and the other was submitted to pathological examination.

Our series comprised nine adenofibromas, 18 dystrophic diseases (13 fibrose diseases and five cystic diseases) and eight epithelial hyperplasia (four epitheliosis, one adenosis and three atypical hyperplasia).

Moreover two normal breast samples were obtained after plastic surgery in young women.

Tissue processing

The frozen tisues were weighed, pulverized (Spex-Bioblock, France), then homogenized in 25 mM Tris, 10 mM $MgCl_2$, 0.01% azide, 10^{-4} M PMSF (phenylmethylsulfonyl fluoride), pH 7.6 ('Tris' buffer).

The homogenate was centrifuged at 800 ***g*** for 10 min and the supernatant ultracentrifuged at 105,000 ***g*** for 60 min. The pellet ('microsomal' fraction) was resuspended in 'Tris' buffer. The protein concentration was determined by the method of Lowry *et al.* [17] after extraction from the membranes with 1 N NaOH.

Growth factors

The human natural IGF1, IGF2 and the crude mixture of IGF1–IGF2 were a generous gift from Dr. Humbel (Zurich). Human synthetic IGF1 was purchased from Amersham (ARN 4010, Amersham France). A modification of the method of Hunter and Greenwood [18], using 800 ng chloramine T and 1 μg IGF1 with incubation for 50 s at 23°C, was employed to iodinate IGF1. Iodinated IGF1 was purified on a ACA-54 column (LKB, France) and the tubes of radioactivity comprising the protein peak were diluted in assay buffer and could be stored for as long as 2 weeks at 4°C. Specific activities, as calculated by isotope recovery, ranged between 160 and 220 μCi/μg. The quality of iodinated IGF1 was checked after each iodination using a standard laboratory preparation of BT20 breast cancer cell line membrane receptors [16]. When the usual protein membrane concentration was utilized (400 μg), at least 10% of the iodinated IGF1 needed to be specifically bound before the tracer was considered acceptable. The binding capacity of [^{125}I]-IGF1 at 800 μg was always greater than 20% and plateaued for greater concentrations due to membrane interference.

Binding experiments

Unless otherwise specified, for the binding test 400 μg of membrane proteins were incubated 5 h at 4°C with approx. 200,000 cpm of iodinated IGF1. The final incubation volume was 0.5 ml adjusted in 'Tris' buffer containing 0.1% bovine serum albumin (fraction V, ref. A3912; Sigma Chemical Company, St Louis, MO, U.S.A.). The routine receptor assay in tumor biopsies was carried out at the same time. Duplicates were used for total and non-specific determination. The IGF1 specific binding assays were performed twice using IGF1 as unlabeled factor and then controlled once more using insulin for non-specific determination. In each series we included breast cancer cell membranes for which we had determined the IGF1 specific binding level [16] to ensure the assay quality control. A tumor was considered positive when the specific binding was higher than 1% (expressed as the percentage of the total radioactivity added to the tube).

We used 1% as the positive threshold since when the assay was performed on boiled membranes, i.e. on membranes with denatured receptors, the difference beween the binding in the absence or presence of an excess of IGF1 was always less than 1%.

Chemical cross-linking of ^{125}I labeled IGF1

Membrane preparations (800 μg) were incubated with approximately 8×10^5 cpm [^{125}I]IGF1 for 5 h at 4°C in the absence or presence of excess unlabeled IGF1. Membranes were then washed twice by the addition of 3 ml cold 10 mM Na-phosphate buffer pH 7.4 and centrifuged at 3000 ***g*** for 20 min. The pellets were resuspended in 0.5 ml of the same buffer and the non-photoreactive cross-linking agent DSS (disuccinimidyl suberate) (Pierce Chemical Co, Rockford, IL), dissolved in dimethyl sulfoxide, was added to give a final concentration of 500 μM. The tubes were then incubated for 15 min in ice. To terminate the cross-linking reaction, 3 ml cold 10 mM Tris–HCl buffer, pH 7.4, were added and the tubes were centrifuged at 3000 ***g*** for 20 min. The pellets were solubilized with electrophoresis sample buffer (50 mM Tris–HCl, pH 7.4; 2% SDS; 9% sucrose and 0.005% bromophenol blue). The samples were boiled for 5 min in the presence of 100 mM dithiothreitol (DTT). Electrophoresis (SDS-PAGE) was performed on a 5% polyacrylamide gel using the discontinuous buffer system of Laemmli [19]. Gels were stained by 0.2% Coomassie brilliant blue R-250, dried and exposed to Kodak SAR-5 X-ray film for 14 days at −70°C. The molecular weight of each band was determined using calibration kits for molecular weight determination (Pharmacia high molecular weight and low molecular weight calibration kits, Pharmacia,

France). The marker proteins were ferritin (220,000), phosphorylase B (14,000), albumin (67,000), lactate dehydrogenase (36,000).

RESULTS

Saturation of [^{125}I]IGF1 binding in one tumor in the presence of increasing concentrations of the labeled hormone and a constant amount of membrane protein (400 μg) is shown in Fig. 1. We demonstrated a similar high affinity binding site in four other individual tumors.

Results of chemical cross-linking of ^{125}I-labeled IGF1 on the same breast tumor as in Fig. 1 are presented in Fig. 2. SDS, polyacrylamide gel electrophoresis under reducing condition demonstrated one major band with an apparent M_r of 130,000.

Figure 3 illustrates the binding specificity of ^{125}I-labeled IGF1 to membranes from a pool of five benign breast tumors. IGF2 was able to compete for binding; insulin was 200 times less potent than IGF1. The crude preparation of IGF1–IGF2 (2000 ng/ml) had the same potency as 1000 ng/ml IGF1; finally epidermal growth factor (EGF) and other hormones like prolactin, luteinizing hormone, follicle-stimulating hormone and growth hormone had no effect on IGF1 binding (data not shown).

Figure 4 shows that in the whole population 43% (15/35) of the tumors were IGF1 positive; specific binding ranging from 0 to 3.4%.

Figure 5 gives a breakdown of the distribution of human breast tumors as a function of IGF1 specific binding level, taking into account their nature. Four out of nine fibroadenomas, 7/18 dystrophic diseases and 4/8 epithelial hyperplasia were IGF1 specific

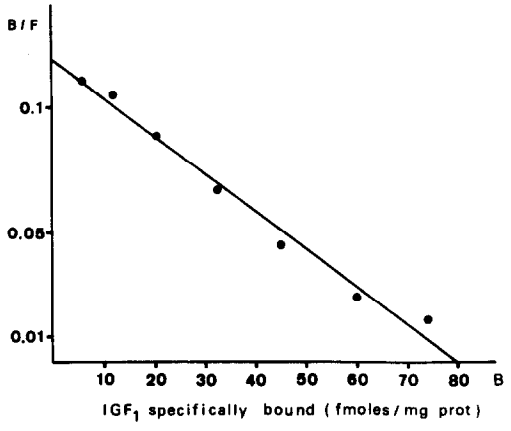

Fig. 1. Scatchard analysis performed by transformation of binding data from the saturation of benign breast disease membrane proteins (400 μg) *with increasing concentrations of* [^{125}I]*IGF1. The slope of this line gives a* K_a *of* 2.38 nM^{-1} *while the intercept on the abscissa gives* 80 fmol/mg *protein.*

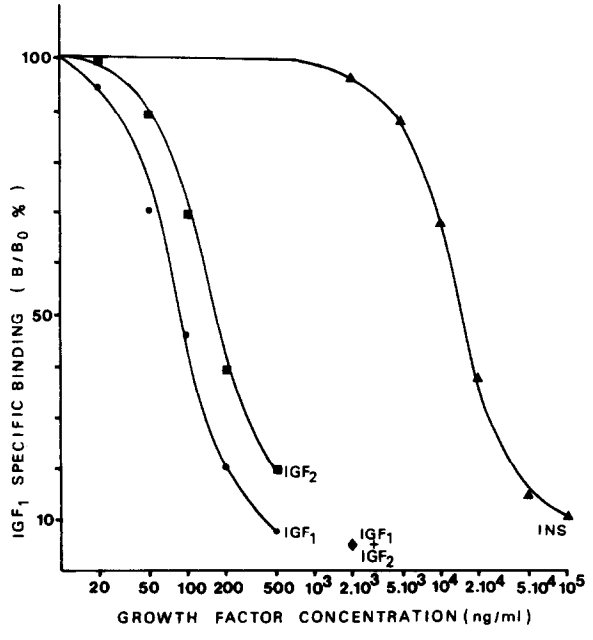

Fig. 3. Growth factor specificity of labeled IGF1 to benign breast disease membranes. Competition for binding of [^{125}I]*IGF1* (100% = *binding in the absence of unlabeled growth factor) in the presence of increasing amounts of unlabeled growth factors.*

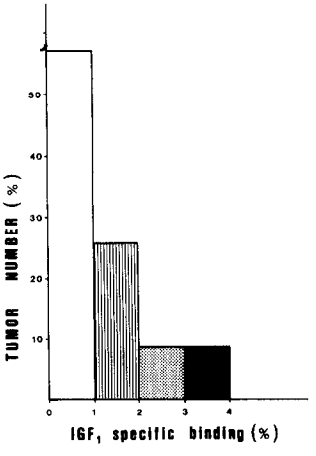

Fig. 4. Distribution of 35 *human benign breast tumors as a function of their IGF1 specific binding level.* 40 μg *of membrane proteins were incubated for* 5 h *at* 4°C *with* 200,000 cpm [^{125}I] *IGF1. IGF1 specific binding is expressed as a percentage of total counts. There is no specific binding in* 20 *tumors; specific binding for* 15 *positive tumors ranges from* 1 *to* 3.4% (*mean goemetric level:* 1.98%).

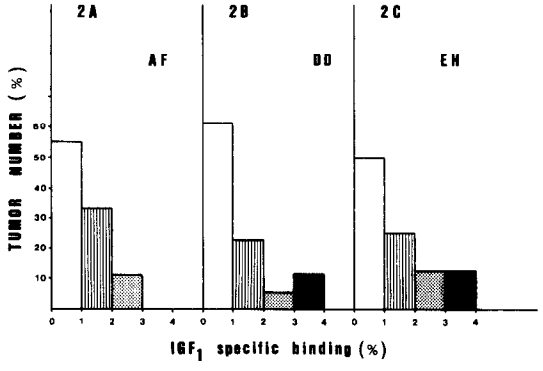

Fig. 5. Distribution of human benign breast tumors as a function of their IGF1 specific binding level (in percentage of total counts per 400 μg *of membrane protein). 2A: Adenofibromas, 2B: dystrophic disease, 2C: epithelial hyperplasia.*

binding positive; there was no difference in the positivity rates among these three groups.

Figure 6 shows the mean geometric level of IGF1 specific binding in the three groups; the concentrations were in increasing order: fibroadenomas, dystrophic diseases, epithelial hyperplasia. The IGF1 specific binding was significantly higher in epithelial hyperplasia than in fibroadenomas or fibroadenomas plus dystrophic diseases together. In the two normal breast tissues IGF1 specific binding was undetectable.

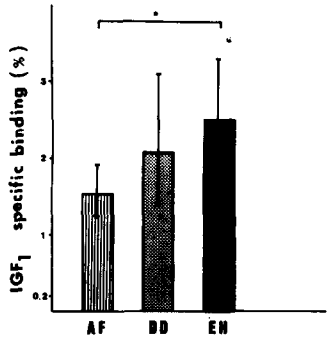

*Fig. 6. Mean geometric level of IGF1 specific binding in adenofibromas (AF), dystrophic disease (DD) and epithelial hyperplasia (EH). *Statistically significant difference (AF < EH, P = 0.05; AF + DD < EH, P = 0.05).*

DISCUSSION

The binding and chemical cross-linking experiments reported here indicate that in benign breast disease IGF1 binds to a type I IGF-R. This receptor has a K_d, a binding specificity and a binding unit molecular weight similar to that of type I receptors on other cell lines and tissues [6, 12].

The present results show for the first time that the IGF1-R positivity rates and the levels are lower in benign breast disease than in cancer [16]. Moreover, we have not detected any binding in normal tissue. IGF1 or insulin can be used as unlabeled hormone in the assay; this is in agreement with results discussed above, demonstrating that IGF1 specific binding corresponds to IGF1-R, and not to IGF2 receptor, insulin receptor or tissue- or serum-derived non-receptor IGF1 binders [14, 15].

Both positivity rates and levels were very different in benign breast tumors from those obtained in human breast cancer, as in the latter population (1) 93% of the tumors were IGF1 specific binding positive, (2) the specific binding ranged from 1 to 16.4% and (3) the geometric mean level of IGF1-R was 3.9% [16].

Different authors have studied hormone receptors in human benign breast tumors: there is no clear difference in positive rates or concentrations between benign and cancer tumors. De Souza *et al.* [20], studying eight benign tumors by the immunoperoxidase method, were unable to find any positive prolactin reaction; the same kind of result was found by Holdaway and Friesen [21] but conversely Di Carlo *et al.* [22], studying a larger population, reported that 34.4% of the tumors contained prolactin receptors. These results were close to the 43% (n = 432) of prolactin receptor positivity in human breast cancer [23, 24]. The presence of estradiol or progesterone receptors has generally been shown in benign breast diseases [25–27]. Conversely, Giani *et al.* [28] found ER only in 13% of fibrocystic disease and 19% of fibroadenomas (the figure was respectively for RPg 31% and 71%). The steroid receptor level depends on the epithelial cellularity of the tumor [29] and is, in most cases, lower in fibrocystic disease than in fibroadenoma [25, 27,28]. The mean level and positivity rate of RE and RPg in benign diseases were not clearly different from the mean level in breast cancer [25–27].

Although the number of biopsies is too small for a definite conclusion, it is interesting to note that the mean level of IGF1-R was higher in those cases carrying the highest risk of cancer development (hyperplasia) [30].

Our results suggest that IGF1 may be less important in stimulating the growth of benign breast tumors than malignant ones. The higher levels of IGF1-R in those benign tumors which carry a high risk of cancer leads to the hypothesis that IGF1-R increase might be a step in the malignant transformation.

REFERENCES

1. Furnaletto RW, Underwood LE, Van Wyk JJ, D'Ercole AJ. Estimation of somatomedin C levels in normals and patients with pituitary disease by radiommunoassay. *J Clin Invest* 1977, **60**, 648–657.
2. Zapf J, Walter H, Froesch ER. Radioimmunological determination of insulin-like growth factors I and II in normal subjects and in patients with growth disorders and extrapancreatic tumor hypoglycemia. *J Clin Invest* 1981, **68**, 1321–1328.
3. D'Ercole AJ, Stiles AD, Underwood LE. Tissue concentrations of somatomedin C: further evidence for multiple sites of synthesis and paracrine or autocrine mechanism of action. *Proc Natl Acad Sci USA* 1984, **81**, 935–939.
4. Murphy AJ, Bell GI, Friesen HG. Tissue distribution of insulin-like growth factor I and II messenger ribonucleic acid in the adult rat. *Endocrinology* 1987, **120**, 1279–1282.
5. Moses AC, Pilistine SJ. Insulin-like growth factors. In: *Control of Animal Cell Proliferation.* New York, Academic Press, 1985, Vol. I, 91–120.
6. Furnaletto RW, Di Carlo JN. Somatomedin C receptors and growth effects in human breast cells maintained in long-term tissue culture. *Cancer Res* 1984, **44**, 2122–2128.

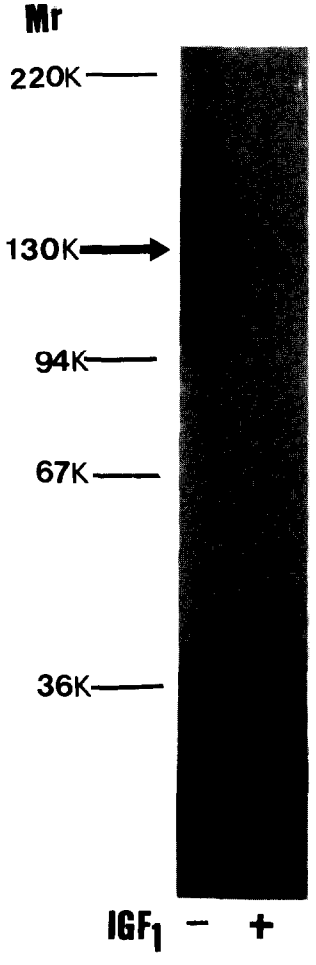

Fig. 2. *Autoradiogram showing the size of* [^{125}I]*IGF*1 *binding unit complexes from benign breast disease membranes as analyzed by SDS-PAGE under reducing conditions. Membrane proteins* (800 μg) *were incubated with* [^{125}I]*IGF*1 *in the absence* (−) *and in the presence of unlabeled IGF*1 (+, 4 μg/ml). *Cross-linking was performed with dissuccinimidyl suberate* (500 μM). *The radioligand-binding site complexes were solubilized with SDS containing dithiothreitol and analyzed by SDS-PAGE and autoradiography. The position of* M_r = 130,000 (130 K) *complexes are indicated by an arrow.*

7. Myal Y, Shiu RPC, Bhaumick B, Bala M. Receptor binding and growth-promoting activity of insulin-like growth factors in human breast cancer cells (T47-D) in culture. *Cancer Res* 1984, **44**, 5486–5490.
8. Huff KK, Kaufman D, Gabbay KH, Spencer EM, Lippman ME, Dickson RB. Secretion of insulin-like growth factor I related protein by human breast cancer cells. *Cancer Res* 1986, **46**, 4613–4619.
9. Baxter RC, Maitland JE, Raisur RL, Reddel R, Sutherland RL. High molecular weight somatomedin C (IGF1) from T47-D human mammary carcinoma cells: immunoreactivity and bioactivity. In: Spencer EM, ed. *Insulin-like Growth Factors/Somatomedins*. Berlin, Walter de Gruyter, 1983, 615–618.
10. Dickson RB, Huff KK, Spencer EM, Lippman ME. Induction of epidermal growth factor related polypeptides by 17 beta-estradiol in MCF-7 human breast cancer cells. *Endocrinology* 1985, **118**, 138–142.
11. Huff KK, Knabbe C, Kaufman D, Gabbay KH. Hormonal regulation of insulin like growth factor I (IGF1) secretion from MCF-7 human breast cancer cells. Proceedings of the 68th Annual Meeting of the Endocrine Society, Anaheim, CA, 1986 (Abst. 205).
12. Rosenfield RG, Hintz RL. Somatomedin receptors: structure, function and regulation. In: *The Receptors*. New York, Academic Press, 1986, Vol. III, 281–329.
13. Dickson RB, Lippman ME. Estrogenic regulation of growth and polypeptide growth factor secretion in human breast carcinoma. *Endocrine Rev* 1987, **8**, 29–43.
14. Clemmons DR, Elgin RG, Han VKM, Casella SJ, D'Ercole AJ, Van Wyk JJ. Cultured fibroblast monolayers secrete a protein that alters the cellular binding of somatomedin-C/ insulin-like growth factor. *J Clin Invest* 1986, **77**, 1548–1556.
15. Clemmons DR, Han VKM, Elgin RG, D'Ercole J. Alterations in the synthesis of a fibroblast surface associated 35 K protein modulates the binding of somatomedin-C/ insulin like growth factor 1. *Mol Endocrinol* 1987, **1**, 339–347.
16. Peyrat JP, Bonneterre J, Vennin P, Dusanter-Fourt I, Djiane J, Demaille A. Insulin-like growth factor I receptors (IGF1-R) in human breast cancers: relation with estradiol and progesterone receptors (ER, PgR). In: *Progress in Cancer Research and Therapy*. New York, Raven Press (in press).
17. Lowry OH, Rosebrough NJ, Farr A, Randall RJ. Protein measurement with the folin phenol red reagent. *J Biol Chem* 1951, **193**, 265–275.
18. Peyrat JP, Djiane J, Kelly PA, Vandewalle B, Bonneterre J, Demaille A. Characterization of prolactin receptors in human breast cancer. *Breast Cancer Res Treat* 1984, **4**, 275–281.
19. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, **227**, 680–685.
20. De Souza I, Hobbs JR, Morgan L, Salih H. Localization of prolactin in human breast tumors. *J Endocrinol* 1977, **73**, 17P.
21. Holdaway IM, Friesen HG. Hormone binding by human mammary carcinoma. *Cancer Res* 1977, **37**, 1946–1952.
22. Di Carlo R, Muccioli G, Di Carlo F. Presence and characterization of prolactin receptors in human benign breast tumours. *Eur J Cancer Clin Oncol* 1984, **20**, 635–638.
23. Bonneterre J, Peyrat JP, Beuscart R, Demaille A. Correlation between prolactin receptor (PRL-R) estradiol (ER) and progesterone receptors (PgR) in human breast cancer. *Eur J Cancer Clin Oncol* 1986, **22**, 1331–1336.
24. Bonneterre J, Peyrat JP, Beuscart R, Lefebvre J, Demaille A. Prognostic significance of prolactin receptors (PRL-R) in human breast cancer. *Cancer Res* 1987, **47**, 4724–4728.
25. Allegra JC, Lippman ME, Green L *et al.* Estrogen receptor values in patients with benign breast disease. *Cancer* 1979, **44**, 228–231.
26. Kouyoumdjian JC, Feuilhade F, Pinaudeau Y, Rymer JC. Estradiol and progesterone receptors in normal human mammary gland and in patients with benign disease. *Bull Cancer* 1986, **73**, 120–123.
27. Barthelemi H, Bressot N, Cavalie-Barthez G *et al.* Récepteurs des oestrogènes, de la progestérone et des glucocorticoïdes. Comparaison entre mastopathies fibrokystiques et fibroadénomes. *Int J Breast Mammary Pathol Senologia* 1983, **2**, 211–217.
28. Giani C, D'Amore E, Delarue JC *et al.* Estrogen and progesterone receptors in benign breast tumors and lesions: relationship with histological and cytological features. *Int J Cancer* 1986, **37**, 7–10.
29. Martin PM, Kutten F, Serment H, Mauvais-Jarvis P. Progesterone receptors in breast fibroadenomas. *J Steroid Biochem* 1979, **11**, 1295–1298.
30. Dupont WD, Page DL. Risk factors for breast cancer in women with proliferative breast disease. *N Engl J Med* 1985, **312**, 146–151.