J. Steroid Biochem. Molec. Biol Vol. 43, No. 1–3, pp. 87–94, 1992
Printed in Great Britain

0960-0760/92 $5.00 + 0.00

BASIC FIBROBLAST GROWTH FACTOR (bFGF): MITOGENIC ACTIVITY AND BINDING SITES IN HUMAN BREAST CANCER

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Summary—We investigated binding characteristics of basic fibroblast growth factor (bFGF) on membranes prepared from 4 human breast cancer cell lines and 38 primary BC biopsies. Competitive binding experiments were performed and analyzed using the "Ligand" program. Furthermore bFGF mitogenic activity was measured by [^{3}H]thymidine incorporation into DNA from breast cancer cell lines. The presence of high-affinity binding sites was demonstrated in each cell type (MCF-7: $K_d = 0.60$ nM; T-47D: $K_d = 0.55$ nM; BT-20: $K_d = 0.77$ nM; MDA-MB-231: $K_d = 0.34$ nM). The presence of these high-affinity binding sites was confirmed with saturation experiments. A second class of low-affinity binding sites was detected in the 2 hormone-independent cells (BT-20: $K_d = 2.9$ nM; MDA-MB-231: $K_d = 2.7$ nM). bFGF stimulated the proliferation of MCF-7, T-47D, BT-20 but not MDA-MB-231 cell lines. With competition experiments, binding sites were detectable in 36/38 breast cancers; high-affinity binding sites ($K_d < 1$ nM) were present in 19/36 cases and low-affinity binding sites ($K_d > 2$ nM) were present in 29/36 cases (the two classes of binding sites were present in 12 breast cancers). No relation between bFGF binding sites and node involvement, histologic type or grading of the tumor was evidenced. There were negative correlations (Spearman test) between total bFGF binding sites and estradiol receptor ($P = 0.05$) or progesterone receptor ($P = 0.009$). The demonstration of (1) bFGF specific binding sites in breast cancer membranes, and (2) bFGF growth stimulation of some breast cancer cell lines indicates that this factor may be involved directly in the growth of some breast cancers.

INTRODUCTION

Basic fibroblast growth (bFGF) is a potent mitogen and differentiation factor for several mesoderm and neuroectoderm and neuroectoderm derived cells (reviewed Ref. [1]). The action of bFGF at the cellular level is mediated by high-affinity cell membrane receptors with dissociation constants ranging between 10^{-9} and 10^{-11} M. [1] Cross-linking studies have demonstrated that these cell surface receptors are polypeptides with molecular weights ranging from 80 to 165 kDa. The existence of a second cell membrane-associated binding site with dissociation constants of 10^{-8} to 10^{-9} M and related to heparin-like molecules has also been described [1].

bFGF stimulates growth and inhibits casein accumulation in mouse mammary epithelial cells *in vitro* [2] and may be involved in the proliferation of human breast cancer cells [3–5]. Several breast cancer cells have also been reported to express bFGF [6–8]. Moreover bFGF belongs to a growth factor family, some members of which are involved in carcinogenesis [9]. Thus, the two FGF-like oncogenes hst and int2 undergo amplification in 10–20% of breast cancer biopsies [10–12]. bFGF binding sites have been described in normal and tumor rat mammary epithelial cells [13], in the breast cancer cell line MCF-7 [14] and in a first series of breast cancer biopsies [15]. Recently, genes coding for cell surface receptors to FGFs were

Proceedings of the Fourth International Congress on Hormones and Cancer, Amsterdam, The Netherlands, September 1991.

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Abbreviations: bFGF: basic fibroblast growth factor; EGF: epidermal growth factor; IGF: insulin-like growth factor; IGF-R: IGF receptor; ER: estradiol receptor; PgR: progesterone receptor; PDGF: platelet derived growth factor; G-CSF: granulocyte-colony stimulating growth factor; EDTA: ethylenediamine tetraacetic acid; BSA: bovine serum albumin; MEM: minimum essential medium; PAGE: polyacrylamide gel electrophoresis; SDS: sodium dodecyl sulfate.

found amplified in 12% of a population of human breast cancers [16].

In order to better understand how bFGF controls the regulation of growth and differentiation of human breast cancer we studied the bFGF membrane binding sites in this tissue. Sites were detected in 4 breast cancer cell lines (BT-20, MCF-7, MDA-MB-231 and T-47D) and the effect of bFGF on the proliferation of these cells was determined. We also detected bFGF binding sites in 38 breast cancers. As we had done in our studies on prolactin and insulin-like growth factor 1 receptor (IGF1-R) [17–19], bFGF binding site concentrations were related to tumor characteristics and steroid receptors.

MATERIALS AND METHODS

Breast cancers

Included in this study were 38 patients undergoing surgery for locoregional primary breast cancer in the Centre Oscar Lambret (Lille, France). The median age of the patients was 64 years (range: 32 to 87 years); 28 (74%) were post-menopausal; 28 (74%) had positive axillary nodes. Clinical tumor diameter was between 2 and 5 cm in 14 (37%) and more than 5 cm in 24 (63%) of the cases. Tumor specimens consisted solely of adenocarcinomas. At the time of collection fat was removed and samples were divided in three parts: (1) was submitted for histological studies, the tumor type, differentiation and histoprognostic grading according to Scarff and Bloom [20] were noted; and (2) and (3) were immediately frozen in liquid nitrogen, one for estradiol receptor (ER), progesterone receptor (PgR) and IGF1-R routine analyses [17–19], and one for FGF binding site determinations. Thirty-one breast cancers (82%) were ductular, 5 (12%) were lobular and 2 (6%) were of other types. Histoprognostic grading was done in ductular breast cancers: 2 (6%) were grade 1; 13 (42%) were grade 2; and 16 (52%) were grade 3.

Growth factors

Pure recombinant bFGF was purchased from Amersham (ARN 12100-Amersham, France). Iodination was performed using the chloramine T method [21] with few modifications; briefly 2 μg of bFGF in 0.1 M phosphate buffer with 0.1% of polyethylene glycol were incubated at room temperature for 1 min with 1 mCi of [^{125}I]Na and 20 μM of chloramine T. The reaction was stopped by the addition of 100 mM *N*-acetyl tyrosine. Free iodine was eliminated by heparin-Sepharose chromatography and [^{125}I]bFGF was stored for as long as 2 weeks at 4°C. The specific activity, calculated by the isotope recovery method, was approx. 100 μCi/μg. The biological activity of iodinated bFGF was checked on fibroblasts (ATCC-CCL39) according the method of Plouet *et al.* [22].

The human natural IGF1 and 2 were a generous gift from Dr Humbel (Zurich, Switzerland). Recombinant IGF1 was purchased from Amersham (ARN 4010). Recombinant IGF2 was a generous gift from Lilly (IN, U.S.A.). The other growth factors and hormones were purchased from Sigma Chemical Company (St Louis, MO, U.S.A.).

Culture of breast cancer cells

The four breast cancer cell lines were routinely grown as monolayer culture [23]. The T-47D cells were maintained in RPMI 1640 medium (Seromed, ATGC-Biopro, France) supplemented with 20 mM Hepes, 2 g/l sodium bicarbonate, 2 mM glutamine, 10% fetal calf serum (FCS), 25 μg/ml streptomycin, 25 U/ml penicillin, 50 μg/ml kanamycin and 0.6 μg/ml human hemi-synthetic insulin (Actrapid HM Novo 1,5 mg/40 IU/ml). The MCF-7, MDA-MB-231 and BT-20 cells were maintained in MEM (Earle's salts) supplemented in the same manner as RPMI except that the insulin concentration was 5 μg/ml. Cells were maintained in a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C.

DNA synthesis

The cancer cells lines were seeded at 20,000 cells/cm^2 in 16 mm wells in appropriate medium (0.5 ml/well) containing 10% of FCS. After 60 h they were starved for 24 h in serum-free medium containing fibronectin (2 μg/ml) and transferrin (30 μg/ml). Finally, bFGF containing medium was added during the last 24 hours. [^{3}H]thymidine was added during the last 4 h of incubation; induction of DNA synthesis was estimated by liquid scintillation spectrophotometry of acid-precipitated material.

Cell membrane preparation for bFGF binding site assays

The method used was that described by Courty *et al.* [24, 25]. Breast cancer tissues were

weighed and then pulverized (Spex-Bioblock, France). Confluent cells were mechanically harvested in phosphate buffer saline (PBS) and then centrifuged for 10 min at 3500 *g*; the pellet was pulverized. Powder was homogenized in buffer A (20 mM Hepes pH 7.4, 5 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 5 kallikrein inhibitor U/ml aprotinin and 0.1 mM phenylmethylsulfonyl fluoride) all protease inhibitors were from Sigma. The homogenate was centrifuged (14,300 *g*, 60 min, 4°C) and the resulting pellet resuspended in 2 vols of buffer A containing 0.3 M sucrose using a Teflon–glass homogenizer. The suspension was centrifuged at 3000 *g* for 15 min, and the resulting supernatant pelleted (40,000 *g*, 30 min, 4°C). The pellet was taken up by 10 vol of buffer A in the presence of 3 M $MgCl_2$, incubated for 30 min at 4°C, and washed in buffer B (buffer A without EDTA and containing 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.1 M NaCl) by centrifugation at 40,000 *g* for 30 min. This procedure was repeated twice. The final pellet, containing the crude membrane preparation was resuspended in buffer B. The protein content was assayed according to the method of Lowry [26] using bovine serum albumin (BSA) as standard. The suspension was stored at −70°C until the binding assay was performed.

Binding assays

Unless otherwise specified, binding assays were performed as described previously [24, 25]. 50 μg of breast cancer cell membranes were incubated with 200 pg of [^{125}I]bFGF for 60 min at 4°C in a final volume of 500 μl buffer B containing 0.5% BSA (fraction V, Ref. A 3912, Sigma). Simultaneous incubations were done in parallel, in the presence of increasing quantities of unlabeled bFGF. The bFGF total concentrations were 0.05, 0.1, 0.15, 0.25, 0.5, 1, 2.5, 5, 10, 20, 50 and 100 nM. After incubation, the membranes were pelleted by centrifugation and the remaining [^{125}I]bFGF measured. Scatchard analyses of the data obtained were performed using the "Ligand" fitting program [27]. The choice of the presence of one or two classes of binding sites was based on the best fitting of the two models.

Binding assays were also performed using increasing concentrations of [^{125}I]bFGF (10, 20, 50, 100 and 200 pM). The non-specific binding was determined in the presence of a 100-fold excess of cold bFGF; the low-affinity binding sites ($K_d > 1$ nM) cannot be characterized using this method [13].

Cross-linking experiments

Five hundred micrograms of crude breast cancer cell membranes were incubated in buffer B with 1.2 nM of [^{125}I]bFGF with or without a 100-fold molar excess of unlabeled bFGF, for 60 min at 4°C. Cross-linking reaction was initiated by addition of 0.1 mM disuccinimidylsuberate. After incubation for 15 min at room temperature, the reaction was quenched by 10 mM methylamine. The membranes were washed by repeated centrifugations in buffer B, and solubilized by incubation in Tris 70 mM, pH 6.8, 10% glycerol, 1% SDS (15 min at room temperature). The unsolubilized material was pelleted and the supernatant analyzed by SDS–PAGE on a 10% polyacrylamide gel, according to the procedure described by Laemmli [28] and autoradiographied at −70°C with Kodak X omat R film.

RESULTS

DNA synthesis stimulation

bFGF stimulated the [^{3}H]thymidine incorporation in the three cell lines T-47D ($ED_{50} = 0.50$ ng/ml), BT-20 ($ED_{50} = 0.50$ ng/ml) and MCF-7 ($ED_{50} = 0.25$ ng/ml) (Fig. 1). In each cell line, the maximal stimulation was obtained for 2.5 ng/ml of bFGF; then, with higher bFGF concentrations (until 50 ng/ml) we observed a plateau (data not shown). Finally, in each of these cell lines, we noted that the maximal stimulation was lower (500%) than that obtained with IGF1 (1000%).

No stimulation was observed in the MDA-MB-231 cell line; the [^{3}H]thymidine incorporation in the control was much higher (300,000 cpm) than in the three other cell lines (T-47D: 60,000 cpm, MCF-7: 70,000 cpm; BT-20 20,000 cpm). In MDA-MB-231 insulin stimulation was low (30%).

bFGF specific binding characteristics in breast cancer membranes

Kinetics. Maximal membrane-associated radioactivity was obtained after 30 min of incubation. Then, the binding appeared to have attained a steady state. After 1 h, a 1000-fold molar excess of unlabeled bFGF added to half of the wells, induced a displacement of [^{125}I]bFGF which reached 75% after 15 min and then decreased slowly.

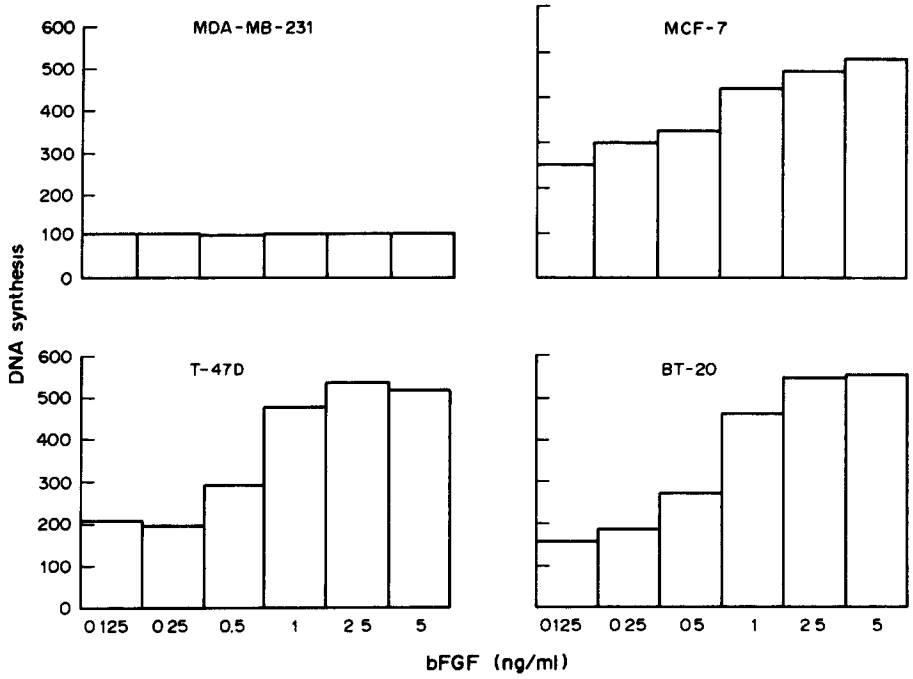

Fig. 1. Stimulation of DNA synthesis by bFGF in MCF-7, T-47D, BT-20 and MDA-MB-231 breast cancer cell lines. Cells were cultured as detailed in Materials and Methods; [^{3}H]thymidine was added during the last 4 h and the acid-precipitated material was counted. Results are the mean of 6 determinations expressed as percentage of the control.

Effect of membrane concentration. The specific binding of labeled bFGF increased with the membrane concentration, reaching saturation for 100 μg per tube; it then decreased.

Specificity. [^{125}I]bFGF binding was competed with increasing concentrations of cold bFGF or aFGF. The growth factors: EGF, insulin, IGF1, IGF2, PDGF, G-CSF and the hormones: human and ovine prolactin, human growth hormone and human chorionic gonadotropin, had no effect on bFGF binding.

Cross-linking experiments. Chemical cross-linking of ^{125}I-labeled bFGF and then SDS–PAGE demonstrated a major band of relative M_r 105,000 for membranes prepared from the four cell lines and some samples of biopsies. Cross-linking carried out in the presence of a 100-fold excess of unlabeled bFGF prevented labeling.

Binding site assays

Breast cancer cell lines. The data of the competitive binding curves were transformed by Scatchard analysis (Fig. 2). A class of high-affinity binding sites was demonstrated in each cell type (MCF-7: $K_d = 0.60$ nM, N = 0.84 pmol/mg protein; $n = 175{,}000$ sites/cell; T-47D: $K_d = 0.55$ nM, N = 0.90 pmol/mg protein; $n = 180{,}000$ site/cell; BT-20: $K_d = 0.77$ nM,

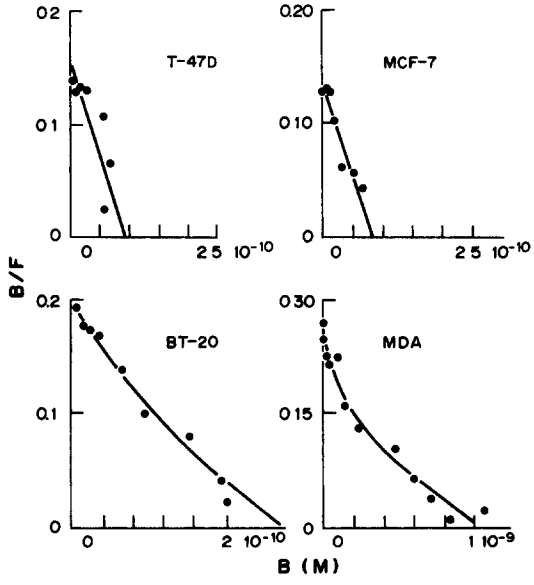

Fig. 2. Scatchard analysis of competitive binding study. 50 μg of MCF-7, T-47D, BT-20 and MDA-MB-231 (MDA) membrane preparation were incubated with an isotopic dilution of [^{125}I]bFGF. Bound radioactivity was measured as reported in 'Materials and Methods' and data obtained were analyzed using the "Ligand" fitting program. For each isotopic dilution, measurements were done in triplicate. Twelve dilutions were needed for each membrane preparation. Each curve presented in the figure represents composites of 3 independent experiments.

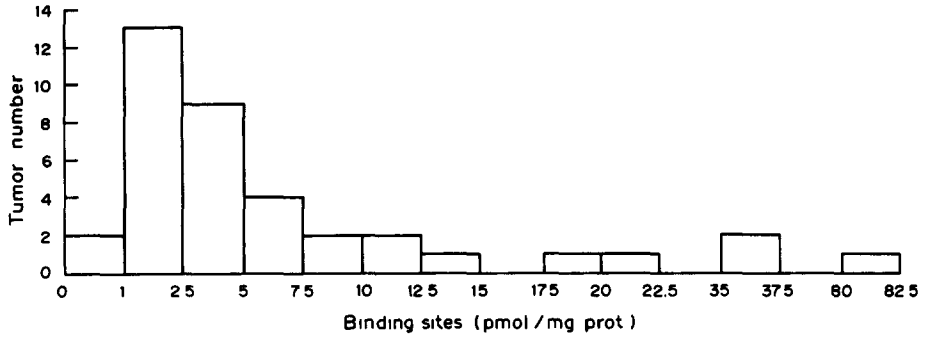

Fig. 3. Distribution of 38 human breast cancers as a function of their total (HA: high-affinity + LA: low-affinity) bFGF binding site concentrations.

N = 1.05 pmol/mg protein; n = 185,000 sites/cell; MDA-MB-231: K_d = 0.34 nM; N = 0.37 pmol/mg protein; n = 85,000 sites/cell). The presence on these high-affinity binding sites was confirmed with saturation experiments (MCF-7: K_d = 0.24 nM, N = 0.42 pmol/mg protein; n = 84,400 sites/cell; T-47D: K_d = 0.43 nM, N = 0.14 pmol/mg protein; n = 28,000 sites/cell; BT-20: K_d = 0.07 nM, N = 0.99 pmol/mg protein; n = 19,800 sites/cell; MDA-MB-231: K_d = 0.15 nM; N = 0.205 pmol/mg protein; n = 41,000 sites/cell). A second class of low-affinity binding sites was detected in the 2 hormone independent cells lines, as the fit with a two-class binding-site model was better (BT-20: K_d = 2.86 nM, N = 1.70 pmol/mg protein; n = 303,000 sites/cell; MDA-MB-231: K_d = 2.73 nM; N = 9.09 pmol/mg protein; $n = 2 \times 10^6$ sites/cell).

Breast cancer biopsies. With competition experiments, binding sites were detectable in 36/38 breast cancers. High-affinity binding sites (K_d < 1 nM) were present in 19/36 cases: the medium K_d was 0.21 nM (range: 0.01–0.99 nM), the median concentration of binding sites was 281 fmol/mg protein (range: 13–2972 fmol/mg protein). Low-affinity binding sites (K_d > 2 nM) were present in 29/36 cases: the median K_d was 3.42 nM (range: 1.09–23.5 nM), the median concentration of binding sites was 4295 fmol/mg protein (range: 632–90254 fmol/mg protein). The two classes of binding sites were present in 12 breast cancers: there was no correlation (Spearman test) between low-affinity binding site concentrations and high-affinity binding site concentrations (P = 0.22). The distribution of total (high- + low-affinity) bFGF binding sites is represented in Fig. 3. Total binding site concentrations were correlated to low-affinity (P < 0.0001) and not to high-affinity (P = 0.36) binding site concentrations.

No relation between total bFGF binding sites and node involvement, histologic type or grading of the tumor was evidenced. In this population 15 (39%) breast cancers were ER-negative (< 10 fmol/mg protein) and 17 (45%) were PgR-negative (< 10 fmol/mg protein). There were negative correlations (Spearman test) between total bFGF binding sites and ER (P = 0.05; n = 36) or PgR (P = 0.009; n = 36). There was no correlation between total bFGF binding sites and IGF1-R (P = 0.22; n = 25). In the population we found the correlations we had previously observed [17–19]: ER–PgR (P = 0.0001; n = 38), ER–IGF1-R (P = 0.05; n = 25) and PgR–IGF1-R (P = 0.05; n = 25).

DISCUSSION

We found bFGF growth stimulation (DNA synthesis) in the three breast cancer cell lines MCF-7, T-47D and BT-20; the stimulation was observed as low as 0.125 ng/ml and was maximal (600%) for 2.5 ng/ml. Our results are in agreement with those of Takahashi *et al.* [3] who found that bFGF (1 ng/ml) stimulates breast cancer cells in primary culture. In MCF-7, Karey and Sirbasku [4] found a maximal stimulation and an ED_{50} twice as low as ours; we confirmed their finding that maximal stimulation was higher with IGF1 or insulin than with bFGF. Studies by Daly *et al.* [5] also showed that bFGF stimulates the proliferation (cell count) of T-47D but they observed increasing stimulation from 1 to 100 ng/ml suggesting a low bFGF sensitivity in their line.

In contrast, bFGF did not stimulate DNA synthesis in MDA-MB-231. However, this cell line had a higher proliferation rate than the three other lines, as suggested by thymidine

incorporation in the absence of bFGF stimulation. The bFGF autonomy of these hormone-independent cells is similar to that reported by Daly *et al.* [5] in long-term steroid-deprived (steroid-independent) T-47D cells where an increased basal growth rate was observed and sensitivity to bFGF abolished.

This study demonstrates bFGF specific binding sites in membranes prepared from the 4 human breast cancer cell lines and 36/38 human breast cancers. The potential interference of endogenous membrane associated bFGF was avoided by the pretreatment of extracted breast cancer membranes with $MgCl_2$ 3 M [24].

Scatchard analyses of the isotopic dilution of labeled bFGF revealed 2 subgroups of breast cancer cell lines: for T-47D and MCF-7 binding data are fitted by a straight line, suggesting the presence of only one class of binding sites, for BT-20 and MDA-MB-231 data are best fitted by a two-class binding site model. In each of the breast cancer cell, one class of binding site with an apparent dissociation constant (K_d) of about 0.5 nM was found. The existence of these high-affinity binding sites was confirmed with saturation experiments. In these experiments, however, K_d and binding site concentrations were lower than those obtained with competition studies. Such differences between results obtained with competition and with saturation experiments have already been found in breast cancer studies on EGF receptors [29]. We hypothesized, and this will be discussed later, that competition experiments did not allow a good discrimination between the two classes of binding sites; unfortunately the low-affinity binding sites were not determined with saturation analysis as high concentrations of bFGF were not available. The class of high-affinity binding sites has already been described on various cell types in tissue culture [1]. In each breast cancer cell line membrane-preparation, cross-linking experiments suggested that this class of high-affinity binding sites corresponded to a protein of M_r 85,000. This is lower than what is generally reported for FGF receptors but is in agreement with those obtained for fetal hippocampal neurons [30], bovine and guinea pig adult brain [24, 31] and mouse placenta [25]. For this low molecular weight, we cannot exclude the action of a protease not responsive to the added inhibitors. However, Ruta *et al.* [32] have detected polypeptides of 90 and 110 kDa that are possibly primary translational products of FGF receptor; very recently, Eisemann *et al.* [33] have demonstrated different isoforms of the bFGF cloned receptor, the shorter isoform being more abundant in mammary cancer cells, among other tissues.

In BT-20 and MDA-MB-231, a second class of binding sites was found and the K_d (3 nM) was in agreement with the values obtained in other cultured cell systems [34–36]. This class of low-affinity binding sites was not detected in MCF-7 and T-47D, in contrast with Briozzo *et al.* [14] who claimed low-affinity binding sites in MCF-7 with an unusually low K_d (17 nM). Low-affinity binding sites are considered as cell associated heparin-like molecules involved in bFGF cell effects [1, 9]. Yayon *et al.* [37] and Rapraeger *et al.* [38] suggest that low-affinity receptor is a molecule required for bFGF binding to the high-affinity sites and, consequently, required for the regulation of biological activity of this growth factor: this corroborates the hypothesis that, in MCF-7 and T-47D, low-affinity binding sites are present but not detected with competition analysis. It is important to note that specific differences between MDA-MB-231 and MCF-7 with respect to glycosaminoglycan composition have been demonstrated. MCF-7 produces significantly lower amounts of heparan sulfate than MDA-MB-231 and retains much less of this glycosaminoglycan on the cell surface [39]. This could explain the high amount of low-affinity binding sites in MDA-MB-231 and probably the absence of detection by Scatchard analyses of these binding sites in MCF-7 (and T-47D). Interestingly high concentrations of low-affinity binding sites were precisely detected in the breast cancer cells devoid of hormone sensitivity: BT-20 and MDA-MB-231 [23]. For these two breast cancer cell lines, low-affinity/high capacity binding sites have also been described concerning another growth factor (EGF); conversely, for MCF-7 and T-47D, high-affinity/low capacity EGF binding sites have been found [40]. The exact significance of the cell differences in high and low-affinity binding site concentrations, in relation to their hormone-dependence, is not known.

Scatchard analyses of the isotopic dilution of labeled bFGF allowed the detection of bFGF binding sites in 36/38 cases and revealed 3 subgroups of positive bFGF binding-site breast cancers. High-affinity binding ($K_d < 1$ nM) were present alone in 7/36 cases and low-affinity binding sites ($K_d > 1$ nM) were present in 17/36 cases, the two classes of binding sites were

present in 12 breast cancers. The significance of these classes of binding sites has been discussed in detail above. The finding of only one class of binding site does not rule out the possible existence of two classes of binding sites either with small, undetectable, K_d differences or with one being in a large excess. Further studies would be useful to determine the exact tissular localization of bFGF binding sites. Considering that bFGF is a multipotential factor [1, 9] it is likely that binding sites are present on normal cellular components of the tumor tissue like fibroblasts. bFGF is also a factor involved in angiogenesis [1, 9], a very active process in cancer, and bFGF binding sites are probably also localized on endothelial cells. The different localization of bFGF binding sites could add to the heterogeneity of binding site classes in tumors.

Almost all the human breast cancers we studied contained bFGF binding sites. This finding suggests that most tumors could respond to bFGF. No relation between bFGF binding sites and node involvement, histologic type or grading of the tumor was evidenced. The reason was perhaps the low number of studied cases. There were negative correlations between total FGF binding sites and ER and PgR. To our knowledge such results are reported for the first time. They are in agreement with our findings in breast cancer cell lines. The mechanism by which bFGF binding sites are regulated in breast cancer are not known. The opposite expression of bFGF binding sites and steroid receptors could suggest a common regulation for these receptors. When we studied IGF1-R [18, 19] in breast cancers we found positive correlations with ER and suggested the reason was that the IGF1 production was low and the IGF1-R down-regulation reduced in ER–IGF1-R positive tumors. Following the same reasoning the present results would suggest an increase in bFGF production and bFGF binding site down-regulation in ER positive tumors.

In conclusion, the demonstration of bFGF specific binding sites in breast cancer membranes indicates that this factor could be involved in the growth and the metastasis potential [41] of most breast cancers. The presence of bFGF binding sites, and the bFGF growth stimulation in some breast cancer cell lines suggest that bFGF is able to act directly on the cancer cells.

Acknowledgements—This work was supported by a grant from the Fédération Nationale des Centres de Lutte Contre le Cancer (Ligue Nationale Contre le Cancer funds, Paris, France). We thank John Hall for the English revision of the manuscript.

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