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EVIDENCE OF HIGH AND LOW AFFINITY BINDING SITES FOR BASIC FIBROBLAST GROWTH FACTOR IN MOUSE PLACENTA

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ABSTRACT : The placenta has been shown to contain bFGF, but the presence of specific binding sites for this growth factor in this tissue remained to be established. In order to study the role of bFGF in the placenta growth, we looked for specific binding sites on mouse placental cell membranes at days 12, 14, 16, and 18 of pregnancy. At day 12, Scatchard analyses indicated that two classes of specific interaction sites for bFGF were detected. One class of high affinity binding sites was characterized by an apparent Kd of 10 pM and a binding capacity of 10 fmoles per mg of membrane protein. A second class of low affinity binding sites was detected with an apparent Kd of 60 nM and a binding capacity of 26 pmoles per mg of membrane protein. At days 14, 16 or 18, Scatchard analyses only showed low affinity binding sites with an apparent Kd of 24 nM and a binding capacity of 230 pmoles per mg of membrane protein. The characterization of these binding sites was performed by cross linking experiments that revealed two forms of specific complexes. This result suggested that the high affinity binding sites correspond to putative receptors with relative molecular masses equal to 65,000 and 85, 000. The dramatic decrease of the high affinity receptor number after the 12th day of pregnancy, which is synchronous with the 9-fold increase of the low affinity binding site number, suggests that the biological activity of bFGF could be regulated by a balance between both the numbers of high and low affinity binding sites on placenta cell membranes. Thus, as it was shown for other growth factors, bFGF could only be involved at specific pregnancy stages.

Heparin binding growth factors that are also named fibroblast growth factors (FGFs) consist in polypeptides which display homologous amino acid sequence and an affinity for heparin (1). The growing FGF family includes both acidic and basic FGF (a and bFGF) as well as the int-2 and the hst gene products (2,3), FGF5 (4), FGF6 (5) and KGF (6).

Several particular properties of FGFs have been experimentally shown. 1. Both *in vitro* cell nucleus DNA synthesis and cell division of a large variety of cells from different

Abbreviations : a or b FGF : acidic or basic fibroblast growth factor ; BSA : bovine serum albumin ; DSS : dissuccinimidyl suberate ; PBS : phosphate buffer saline 0.15 M NaCl ; PAGE : polyacrylamide gel electrophoresis; MEM : minimum essential medium ; PMSF : phenyl methyl sulfonic fluoride ; EDTA : ethylenediaminetetraacetic acid.

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tissues belonging to different species were stimulated by FGFs (7). 2. FGFs could be extracted from most of the studied tissues and were described as cell and/or basement membrane associated components, but were not found as in a circulating form (8). 3. They bound to two classes of membrane binding sites, a high affinity binding site class corresponding to receptors that mediated both the mitogenic effect and a few other properties of FGF, and a low affinity binding site class that have an unclear function (9). 4. They were also able to promote *in vivo* angiogenesis and wound healing (7). Hence members of the FGF family appear to play roles in developmental processes, tissue homeostasis and may be involved in enhancing tumor growth and cell invasiveness (8,10). Several other growth factors including TGF, EGF, PDGF and IGF were shown to be expressed during early embryonic growth, at specific times. However, very little is known about the expression of the corresponding receptors (11,12,13,14). Evidence for a role of the members of the FGF family in development was observed in the following cases. Basic FGF could induce blastula ectoderm to give mesoderm in *Xenopus* eggs (15). Int-2 was expressed only up to day 7.5 of the mouse gestation (16). Acidic FGF was found in the embryonic chick brain (17,18). Heparin-binding growth factors can be purified from different developmental stages of the embryonic chick limb bud (19) and newt limb blastema (20). Antisera to bFGF could delay the development of 10 day old rat embryos transplanted into renal capsule (21). Receptors for a and bFGF, were also detected in *Xenopus* eggs (22) and a form of the bFGF receptor with a relative molecular mass of 130 000 was recently purified from 6 day old chick embryos (23). Placental tissues that were known to contain a high level of bFGF (24,25), were highly vascularized and characterized by intense cell proliferation. Therefore, in order to extend our understanding of the FGF role in development, it was of interest to study the bFGF receptor content in placental tissues at different stages of pregnancy.

MATERIALS AND METHODS

Materials

$[^{125}I]$ Na and $[^{3}H]$thymidine were obtained from Oris France, dissuccinimidyl suberate from Pierce chemical Co, high molecular weight standards and polyacrylamide gel electrophoresis (PAGE) reagents from Bio-Rad. Basic FGF was purified from bovine brain as previously described (26).

Radioiodination of bFGF

bFGF was iodinated using chloramine T method (27) with a few modifications. Briefly, 3 µg of bFGF in 0.1 M phosphate buffer with 0,1% polyethylene glycol were incubated at room temperature for 1 min with 1 mCi $[^{125}I]$ Na and 20 µM chloramine T. The reaction was quenched by adding 100 mM N-acetyl tyrosine. Free iodine was eliminated by heparin-Sepharose chromatography and $[^{125}I]$ bFGF was stored at 4°C before use. The FGF biological activity was controlled by *in vitro* bioassay as previously described (28).

Placental membrane preparations

Placental membranes were prepared according to Ledoux *et al* (29). Mouse placentas from 12,14,16 and 18 days of pregnancy were dissected at 4°C. Immediatly after their dissections, the placentas were homogenized in buffer A (20 mM Hepes pH 7.4, 5 mM EDTA, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 5 kallikrein inhibitor units / ml aprotinin and 0.1 mM PMSF) containing 0.3 M sucrose. The homogenate was centrifuged (1,000 g,

15 min , 4°C) and the supernatant was pelleted (40,000 g, 15 min, 4°C). This pellet was resuspended in buffer A containing 3M $MgCl_2$, and incubated for 30 min at 4°C ; then, the membranes were pelleted once more (40,000 g, 15 min, 4°C), and washed in buffer B (buffer A without EDTA and containing 1mM $MgCl_2$, 1mM $CaCl_2$ and 0.1M NaCl). This procedure was repeated twice. The final pellet, containing the crude membrane preparation,was resuspended in buffer B. The proteic concentration was adjusted to 1 mg/ml and the samples were stored at -70°C before use. The proteic concentration was determined using Bradford's reagents (30) with BSA as a standard.

Binding assays

Binding assays were performed as previously described (31). 30 µg of crude placental membranes was incubated in 500 µl of buffer B containing 0.5% BSA and 0.1 M NaCl with 23 pM of [^{125}I] bFGF for 60 min at 4°C. Simultaneous other incubations were performed in the presence of increasing quantities of unlabeled bFGF ranging from 10^{-11} to 23 nM. After the incubation, the membranes were pelleted by centrifugation and the remaining [^{125}I] bFGF was measured with a gamma counter. Scatchard analyses of the obtained data was performed using the LIGAND fitting program (32).

Kinetic of binding : 23 pM of [^{125}I] bFGF was added to 30 µg of placental membranes and incubated as previously described for 5, 15, 30, 40, 50, 60, 80 minutes. At equilibrium state, 1000-fold excess of unlabeled bFGF were added to reactionnal mixture and centrifuged as described after various times (15, 20, 25 min) of incubation.

Effect of membrane concentration : increasing concentrations of membrane (5 to 100 µg of protein) were incubated with a constant amount (23pM) of [^{125}I] bFGF for 60 min at 4°C and then traited as previously described.

Cross linking experiments

500 µg of crude placental membranes was 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 DSS. After incubation for 15 min at room temperature, the reaction was quenched by 10 mM methylamine. The membranes were repeatedly washed in buffer B, isolated by centrifugation and finally solubilized in Tris-HCl 70 mM, pH 6.8, 10% glycerol, 1% SDS for 15 min at 4°C. The unsolubilized material was pelleted and the supernatant analyzed by SDS PAGE in 5-10% polyacrylamide gel gradient, according to the procedure described by Laemmli (33) and radioautographied at -70°C with Kodak X omat R film. Radioautograms were scanned with a densitophotometer (Helena-France).

RESULTS

Binding assay

Kinetic of binding

The kinetic of [^{125}I] bFGF binding on placental membranes at 4°C is shown in figure 1. Maximal membrane associated radioactivity was obtained after 30 min of incubation. The binding then appeared to have attained a steady state. At this time, a 1000-fold molar excess of unlabeled bFGF added to half of the wells, induce a displacement of ^{125}I bFGF which reached 60 % after 10 min and next to 100 % after 30 min.

Effect of membrane concentration

As shown in figure 2, the specific binding (difference between total binding and non specific binding) increased as a function of the membrane concentration and reached the saturation for 30 µg of placental membrane.

Competitive binding assays

Incubation of membrane preparations of placentas from 12, 14, 16 and 18 days of pregnancy in buffer with various [^{125}I] bFGF isotopic dilutions, indicated that each

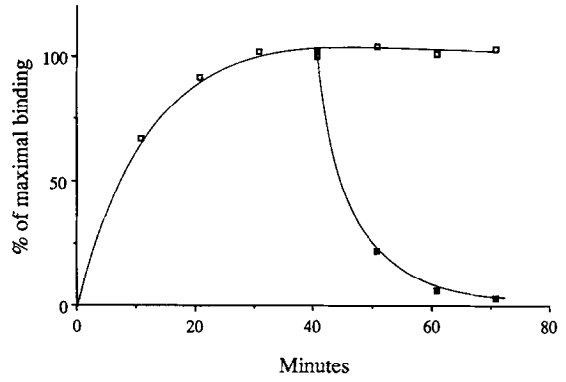

Fig.1 . Association and dissociation kinetics of [^{125}I] bFGF specific binding to placental membrane preparation.

30 µg of membrane preparation (14 days of pregnancy) was incubated in the presence of 23 pM of [^{125}I]bFGF at 4°C for the time intervals indicated, for dissociation experiments, membranes were initially incubated with [^{125}I] bFGF for 40 min at 4°C, followed by a second incubation in presence of 1000-fold excess of unlabeled bFGF for various times indicated. Each point represents triplicate determinations of a single experiment.

preparation exhibited specific [^{125}I] bFGF binding. Scatchard analyses performed using LIGAND fitting program showed the presence of two classes of specific interaction sites on placental membrane, at day 12 of pregnancy (Fig.3 A). The first class of sites for bFGF presented an apparent Kd value of 10 pM and a binding capacity of 10 fmoles per mg of membrane protein (Table 1). The second class of sites had an apparent Kd of 60 nM and a binding capacity of 26 pmoles per mg of membrane protein (Table 1). At days 14, 16, 18 of pregnancy (Fig.3 B) only one binding site class was detected with an

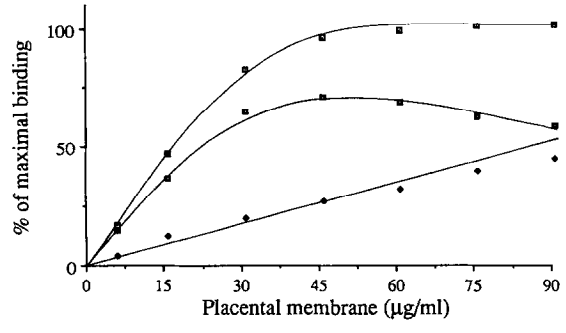

Fig.2 . Effect of increasing placental membrane concentration.

Increasing concentrations of membranes (5 to 90 µg of protein) were incubated for 1 hour at 4°C with a constant amount (23pM) of [^{125}I]bFGF with (♦) or without (■) 1000 fold excess of unlabeled bFGF. Bound radioactivity was determined as described in "Materials and Methods".Specific binding (■) (the difference between total binding (□) and non specific binding (■)) was the average of duplicate determination.

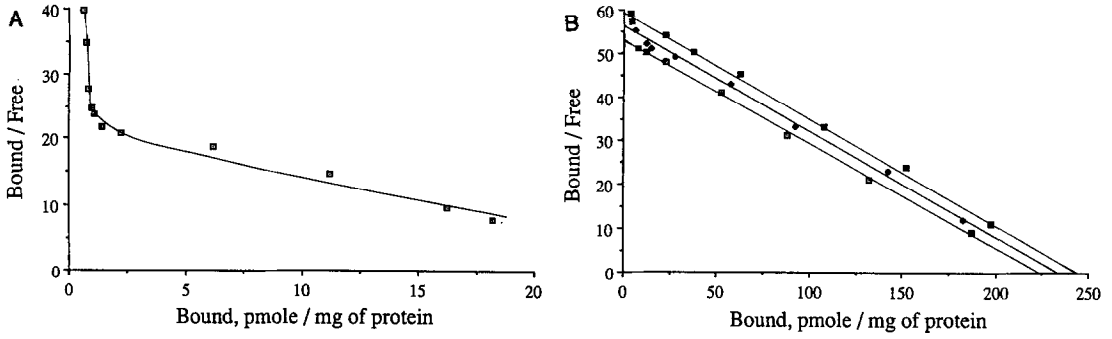

Fig.3 . Scatchard analysis of competitive binding study. 30µg of mouse placental membranes of days 12 (A) ; 14▫,16◆, 18■, (B) ; of pregnancy was incubated with an isotopic dilution of [^{125}I] bFGF. Bound radioactivity was measured as reported in "Materials and Methods" and data obtained were analyzed using a LIGAND fitting program. For each isotopic dilution measurements were done in triplicate. An average of 10 dilutions were needed for each membrane preparation. Each curve presented in the figure are composites of three independent experiments. Three independent membrane preparations were performed for each stage of pregnancy. Each study at day 12 was done from a pool of 100 placentas while at day 18 only 10 placentas were needed.

apparent Kd mean value of 25 nM and a binding capacity of 230 pmoles per mg of membrane protein (Table1). Student t test analyses of these data indicated that there were no significant differences for both Kd and binding capacities measured at days 14, 16 and 18.

Table 1. Apparent dissociation constant and binding capacity of interaction sites for bFGF on placental membrane preparations. These values were calculated from Scatchard analysis reported in Fig.1. Mean values were calculated from three to five independant experiments analyzed by Scatchard representation . Apparent Kd for high affinity binding sites at day 14 to 18 were below 1 pM and could not be measured.

	HIGH AFFINITY BINDING SITES		LOW AFFINITY BINDING SITES	
DAY OF PREGNANCY	Kd (pM)	BINDING CAPACITY (fmol/mg)	Kd (nM)	BINDING CAPACITY (pmol/mg)
12	10 ±4	10 ±4	60 ±10	26± 9
14	ND	ND	24 ± 8	225±8
16	ND	ND	24 ± 6	227± 10
18	ND	ND	26 ± 6	240±11

ND : not detected.

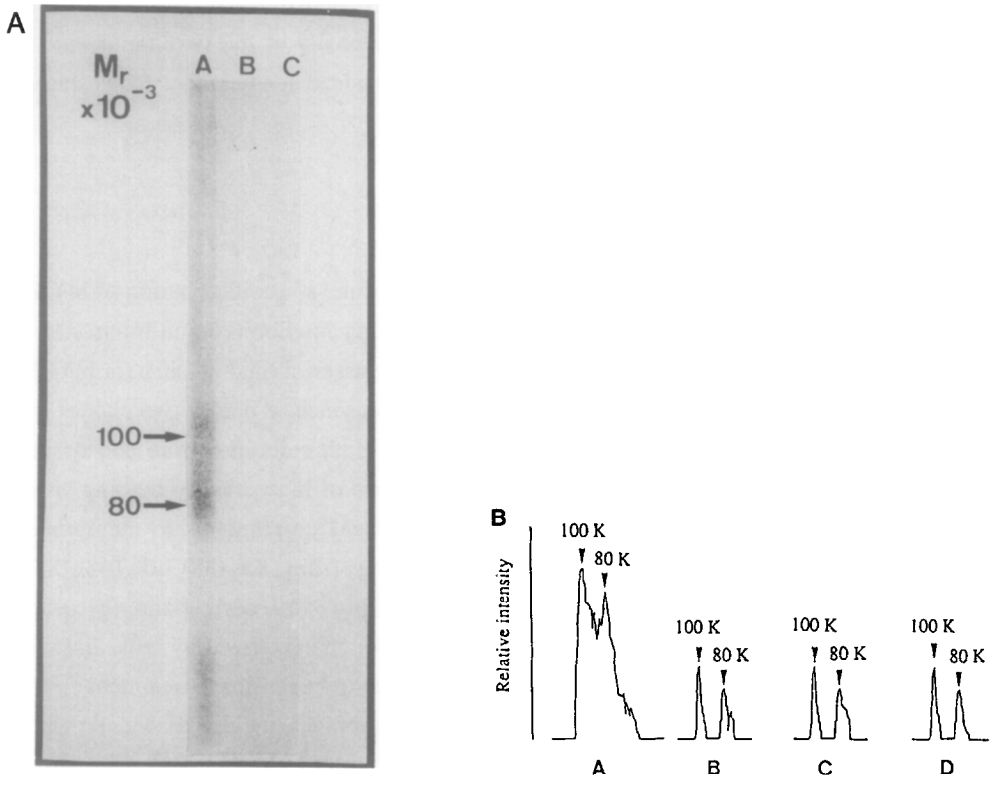

Fig.4 . Cross linking experiments. For each pregnancy day studied, 500 ng of placental membrane preparations were incubated with 10 ng of [^{125}I]bFGF for 60 min at 4°C. The cross linking reaction was performed with 0.1 mM DSS and samples were analyzed by PAGE-SDS and radioautographied. In figure 4.A, placental membranes prepared from 12 days of pregnancy are presented. Lane A (1 ng [^{125}I]bFGF), Lane B same experiment is performed in presence of a 100-fold excess of unlabelled bFGF (laneB) or in absence of DSS (lane C). The molecular weight markers used are myosin (200,000), galactosidase (116,000), phosphorylase b (92,000) and bovine serum albumin (66,000). Cross linking radioautograms obtained for day 12 (A), 14 (B), 16 (C) and 18 (D) of pregnancy were analyzed by densitrometry as shown in figure 4.b.

Cross linking experiments

[^{125}I] bFGF cross linked to placental membrane proteins formed complexes that were analyzed by SDS-PAGE and radioautographied. Figure 3 is a radioautogram of the gel obtained with day 12 membranes. Although there is a large spreading of the bands, two major peaks corresponding to the relative molecular masses of 80,000 and 100,000 can be detected. The specificity of this reaction is controlled by adding a 100-fold excess of unlabeled bFGF (Fig.3, lane B) or without the cross linking agent (Fig.3, lane C). Similar experiments were done at days 14, 16 and 18 of pregnancy. The same two peaks at 80,000 and 100,000 of molecular mass were detected, with however a smaller

intensity (data not shown). The figure 4 represents the densitometric analysis of the radioautograms obtained at day 12 (Fig.4, A). The intensity of the two major bands obtained at day 12 is 9-fold more intense than the one obtained at any other studied stages (Fig.4B, C, D)

DISCUSSION

This study is concerned with establishing, for the first time, a specific binding of bFGF on placental membranes. After optimization of the binding conditions (equilibrium state, saturation state) we demonstrated the presence of two classes of binding sites for bFGF, by competitive binding and Scatchard analyses. The presence of one or two classes of binding sites to bFGF had already been reported for both cells in culture and tissue membrane preparations (29,31, 34,35,36,37). The class of high affinity binding sites with an apparent Kd value ranging from 10 to 300 pM corresponds to membrane glycoproteins of relative molecular mass ranging from 85,000 to 165,000 (29,31,34,35,36,37). The class of low affinity binding sites corresponding to an apparent Kd ranging from 3 to 30 nM has not yet been characterized by cross linking experiments. However, these binding sites are sensitive to heparitinase treatments (38) and were found not only at the surface of cellular membranes but also associated with extracellular matrix. These low affinity binding sites may represent a storage and a regulatory mechanism controlling bFGF bioavailability (38).
On placental membrane, these two classes of bFGF binding sites are present and their number appears to change during pregnancy. High affinity binding sites have a Kd of 10 pM and a capacity of 10 fmoles per mg of membrane protein. They are detected only on the 12th day. Low affinity binding sites have a Kd range of 20-60 nM with a membrane capacity of 26 pmoles per mg of protein at day 12 and 225-240 pmoles per mg of protein at days 14,16,18.

For each studied stage, cross linking experiments reveal two forms of putative receptors with apparent molecular masses (Mr) of 65000 and 85000. These Mr appear lowest than those of FGF receptors studied in other systems (29,31,34,35, 36,37). These differences suggest that receptors might undergo a degradation under our experimental conditions. However, using the same conditions, we have reported a bFGF receptor Mr of 150,000 in bovine brain (31) and 130,000 and 160,000 in guinea pig brain (29). In addition, a form of bFGF receptor with a Mr of 85,000 has previously been reported from fetal hippocampal neurons (35) while a receptor with a Mr of 65,000 was also copurified with a 130,000 Mr receptor by affinity chromatography on immobilized bFGF of adult brain membrane protein preparations (39). By densitometric analyses of cross linking radioautograms, we have shown that the intensity of ^{125}I-labeled bands (Mr of 65000 and 85000) becomes nine times weaker between day 12 and day 14 of pregnancy. These data are in agreement with the non detection of bFGF high affinity binding sites by Scatchard analysis at day 14 and later.

The main conclusion from this paper is that the decrease of the number of high affinity binding sites for bFGF (9 times) between the day 12 and the day 14 is synchronous of an increase of the number of low affinity binding sites (10 times). These data suggest that low affinity binding sites could completely trap the available bFGF and therefore may act as negative regulators of the biological activity. The mitotic activities in the whole placenta decrease significantly by 50% between the days 12 and 14 of pregancy and remain unchanged later (data not shown). This fact may be correlated to the decrease of the number of bFGF high affinity receptors - which are known to mediate the FGF mitogenic activity and the parallel increase of the number of low affinity binding sites. Nevertheless, several other growth factors have been shown to be present in the placenta and to be involved in embryogenesis (11,12,13, 14), then, it is difficult to further establish a more direct correlation between the decrease of cellular proliferation and the corresponding decrease of number of bFGF high affinity receptors. This problem is currently under investigation.

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