Expression of acidic fibroblast growth factor (aFGF) and fibroblast growth factor receptor 4 (FGFR4) in breast fibroadenomas

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Abstract

Background/Aim—Fibroadenomas are benign tumours composed of both glandular and fibrous tissue. The mechanisms regulating the growth of these tumours and the relation between the stromal and epithelial cells are poorly understood. Acidic fibroblast growth factor (aFGF) is a well known fibroblast activator, which acts through four specific cell surface receptors, among which, fibroblast growth factor receptor 4 (FGFR4) is highly specific. The aim of this study was to evaluate the distribution of aFGF and FGFR4 in specific cell types of fibroadenomas to understand their possible role in the growth of these breast lesions.

Methods—Formalin fixed and paraffin wax embedded tissues from 15 fibroadenomas and peritumoral normal breasts were investigated for the expression of aFGF and FGFR4 using immunohistochemistry. The presence of aFGF mRNA was also investigated using in situ hybridisation.

Results—Immunoreactivity for aFGF and FGFR4 was seen in epithelial cells, but it was lacking in myoepithelial cells of both normal tissues and fibroadenomas. Strong FGFR4 immunoreactivity was found in stromal fibroblasts, which were also weakly positive for aFGF. aFGF mRNA was detected in epithelial cells and in some stromal fibroblasts.

Conclusions—These results suggest a paracrine/autocrine modulation of epithelial and stromal cells of fibroadenomas through an aFGF-FGFR4 interaction. This interaction might regulate various cell functions and the growth of fibroadenomas.

(J Clin Pathol 2001;54:37–41)

Keywords: acidic fibroblast growth factor; fibroblast growth factor receptor 4; fibroadenoma; human breast

Mechanisms controlling the normal growth and differentiation of the mammary gland are poorly understood. In addition to hormonal regulation, a complex system of locally synthesised growth factors is involved in the biological control of mammary gland cells.^{1–2} Although there is some evidence for the involvement of fibroblast growth factors (FGFs) in mammary gland development and tumorigenesis, their exact role remains to be clarified.³

The family of FGFs include at least 20 structurally related peptides^{4–16} and, among

various FGFs, acidic fibroblast growth factor (aFGF or FGF1) is one of the best studied and well known. It is involved in a wide spectrum of biological functions including tissue development and repair, angiogenesis, proliferation of both epithelial and mesenchymal cells, and tumorigenesis.^{7–17} aFGF is widely distributed in human tissues,¹⁸ and it has also been detected in normal breast as well as in benign breast lesions, including fibroadenomas.^{18–21} However, the role of aFGF in breast development and differentiation is poorly understood.

The biological activities of aFGF depend on binding to highly specific cell surface receptors. The family of fibroblast growth factor receptors (FGFRs) includes at least four peptides (FGFR1–4). FGFRs consist of an extracellular ligand binding domain, an intracellular tyrosine kinase domain, and a single transmembrane domain.²² Among the four FGFRs, FGFR4 is a highly specific receptor for aFGF. Partanen and colleagues²³ reported that FGFR4 represents an almost exclusive receptor for aFGF, whereas Ron and colleagues²⁴ found that both aFGF and basic fibroblast growth factor (bFGF or FGF2) can bind to FGFR4, although bFGF binds with a lower affinity. FGFR4 gene expression¹⁹ and related amplification²⁵ were recently identified in breast cancers, but FGFR4 expression in normal breast glands and in benign breast lesions has been poorly investigated.²¹

Fibroadenomas are the most frequent benign breast tumours in young women, which manifest as a proliferation of epithelial and stromal cells, forming a mass that usually measures 2–3 cm in greatest dimension. Clonal analyses have shown that both epithelial and stromal cells of fibroadenomas are polyclonal, suggesting that fibroadenomas are hyperplastic rather than neoplastic lesions.^{26–27} Mechanisms regulating the growth of these tumours and the relation between epithelial and stromal cells are unclear. Interactions between epithelial and stromal cells, through a paracrine mechanism involving different growth factors and related receptors, has been reported in fetal, normal, and neoplastic mammary tissue.³ Considering that aFGF and FGFR4 can be involved in the differentiation and growth of benign breast diseases, it would be interesting to investigate whether they have a role in the modulation of the growth of fibroadenomas. In particular, it is of interest to evaluate their role in the relation between the epithelial and stromal components. To solve this problem we first need to determine the specific cell location of these

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Accepted for publication
12 June 2000

Figure 1 Serial sections showing acidic fibroblast growth factor (aFGF) immunoreactivity (A) in epithelial cells of a breast fibroadenoma. (B) A myoepithelial cell, immunoreactive for actin, is aFGF negative (arrow).

peptides and our study was designed to identify the specific cell types of breast fibroadenomas expressing aFGF and FGFR4.

Materials and methods

Fifteen specimens of fibroadenomas and peritumoral normal breast tissues, collected at surgery, were fixed in buffered formalin (4%

wt/vol formaldehyde and 0.005 M acetate buffer) for six hours and embedded in paraffin wax.

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Immunohistochemical staining was performed using the following primary antibodies: anti-aFGF (UBI, Lake Placid, New York, USA),

Figure 2 Double label immunostaining showing that actin positive (red) myoepithelial cells are negative for antibodies directed against (A) acidic fibroblast growth factor (aFGF) and (B) fibroblast growth factor receptor 4 (FGFR4), which stain epithelial cells brown.

used at a 1/100 dilution; anti-FGFR4 (Santa Cruz Biotechnology, Santa Cruz, California, USA), used at a 1/100 dilution; and anti-actin (Biogenex, San Ramon, California, USA), used undiluted. Briefly, 3 µm thick sections were mounted on to poly-L-lysine coated slides and then dewaxed and hydrated through graded alcohols to water. Endogenous peroxidase activity was inhibited by treating sections with 3% hydrogen peroxide for 10 minutes. Primary antibody incubations were carried out at 4°C for 18–20 hours, followed by the avidin–biotin peroxidase complex (ABC) procedure according to Hsu *et al.*²⁸ Immunoreactions were developed using 0.03% 3,3' diaminobenzidine tetrahydrochloride and, successively, sections were counterstained with Harris's haematoxylin. Colocalisation studies were performed using double labelled immunostains, according to Mason and Sammons.²⁹ Sections stained for aFGF and FGFR4 were pretreated with 0.01 M citrate buffer, pH 6 (10 minutes), in a microwave oven at 650 W. Specificity controls consisted of absorption of antibodies and antisera with their related antigens, the omission of the first layer, and the use of control tissues with or without the pertinent antigen, as described previously.³⁰

IN SITU HYBRIDISATION

The preparation of tissue sections was performed using the method described by Pringle *et al.*³¹ Dewaxed slides were incubated in 100% and 95% ethanol for five minutes each and then washed in 2× saline sodium citrate (SSC) at room temperature. Sections were treated with 0.2 M HCl for 20 minutes and with 5 µg/ml of proteinase K for five minutes at 37°C. Each slide was treated with 0.25% acetic anhydride in triethanolamine for 10 minutes, followed by incubation with prehybridisation buffer (40% formamide, 1× Denhart's, 10% dextran sulphate, 0.5 µg/ml sonicated salmon sperm DNA, 1 mg/ml yeast tRNA, 10 mM dithiothreitol (DTT), and 4× SSC) for one hour at 42°C. A 28 base oligonucleotide probe (5'-TGAGCTGCA GCTGAATGTGCTGGTCGCT-3') complementary to bases 166–193 of the coding region of human aFGF messenger RNA³² (Biognostik, Göttingen, Germany) was labelled with biotin using an oligonucleotide tailing kit purchased from Boehringer Mannheim (Mannheim, Germany). The efficiency of the tailing reaction was routinely checked by direct detection using a Boehringer Mannheim detection kit. For hybridisation, slides were incubated in a moist chamber at 42°C overnight with labelled probes diluted in the prehybridisation buffer (0.4 ng/µl; 20 µl/slide) and covered with a coverslip. After 18 hours, coverslips were gently removed in 2× SSC and slides were washed for 30 minutes in 2× SSC and for 30 minutes in 1× SSC at 37°C. Biotinylated probes were detected using the Gen-Point kit (Dako, Copenhagen, Denmark). Controls for specificity were performed using a sense aFGF probe and slides hybridised without probes.

Figure 3 In situ hybridisation demonstrating that acidic fibroblast growth factor (aFGF) mRNA is present within epithelial and stromal cells. Some myoepithelial cells are negative for aFGF mRNA.

Results

The average age of patients, none of them in menopausal state, was 28 years (range, 16–41). The mean diameter of fibroadenomas was 3.6 cm (range, 1.5–12 cm).

aFGF immunoreactivity was mainly restricted to epithelial cells and was cytoplasmic (fig 1A). In addition, stromal cells were also weakly positive. Myoepithelial cells, identified between epithelial cells and basal lamina by their clear cytoplasm with haematoxylin–eosin stain, were negative for aFGF. This result was also confirmed by colocalisation studies, using anti-actin and anti-aFGF antibodies (figs 1B, 2A). In situ hybridisation revealed the presence of aFGF mRNA in epithelial and stromal cells, whereas myoepithelial cells appeared negative (fig 3).

FGFR4 immunoreactivity was found in endothelial cells and in both stromal and epithelial cells, but was lacking in myoepithelial cells (fig 4). This was also confirmed by double label immunostaining using anti-FGFR4 and anti-actin antibodies (fig 2B). FGFR4 positivity was cytoplasmic, with a more intense staining at the cell membrane in some cells.

Although immunoreactivity for aFGF and FGFR4 was found in all cases investigated, the staining intensity varied. Some fibroadenomas were strongly immunoreactive, whereas others were less positive, with a few cases showing very weak immunoreactivity. Sometimes, the intensity of staining varied within the same tumour. These different patterns of immunoreactivity did not correlate with any morphological feature of the epithelial or stromal cells, including cellularity, nuclear size, and mitotic count.

Figure 4 Serial sections showing that fibroblast growth factor receptor 4 (FGFR4) (A) is expressed by epithelial and stromal cells, but not by (B) actin positive myoepithelial cells (arrows) of a breast fibroadenoma.

Histologically normal peritumoral breast tissue showed the same pattern of positivity found in fibroadenomas; that is, the epithelial cells of ducts and lobules were positive for aFGF and FGFR4, and the myoepithelial cells negative.

Discussion

Fibroadenomas are benign tumours of the breast typically composed of stromal and epithelial cells. Mechanisms controlling their development and growth are poorly understood. In addition to the well documented role of oestrogen and progesterone receptors expressed by epithelial cells,³³ recent studies suggest a possible role for growth factors and their receptors in the pathogenesis and growth of benign breast diseases, including fibroadenomas, suggesting that multiple receptor signalling pathways could be involved in the growth and differentiation of benign breast lesions. Acidic fibroblast growth factor is a well known stimulator of fibroblast, epithelial, and endothelial cell proliferation.⁴ Its biological actions depend on binding to highly specific cell surface receptors; FGFR4 is highly specific for aFGF and was recently found to be amplified in breast carcinomas.²⁵ In previously published studies, the expression of aFGF and FGFR4 has been investigated mainly in malignant breast tumours with the aim of evaluating the possible role of these molecules in the pathogenesis, growth, and aggressiveness of breast carcinomas.^{19 20 25 34-36} However, the results varied greatly and were sometimes contradictory. In contrast, the expression of aFGF and FGFR4 in normal breast tissue and in benign breast diseases has been poorly investigated. Among benign lesions of the breast, fibroadenomas represent a good biological model for evaluating the expression of aFGF and FGFR4, because the stromal and epithelial components are clearly separated. Our results indicate that both aFGF and FGFR4 are

expressed in epithelial cells, suggesting that these peptides are involved in a paracrine/autocrine regulation of tumour growth or, at least, in the modulation of cell functions. In addition, FGFR4 was also strongly expressed in stromal cells and this finding suggests a paracrine/autocrine control of stromal proliferation through interaction of aFGF, mainly produced by epithelial cells, and FGFR4, expressed by stromal fibroblasts. Interestingly, myoepithelial cells were negative for both aFGF and FGFR4, indicating that this growth factor-receptor system is not involved in the modulation of the functions of these cells. These results are in keeping with those reported previously, which indicate that aFGF is expressed by epithelial cells, but that it is lacking in myoepithelial cells.^{18 20 21 37} However, the study of aFGF expression in breast derived cell lines showed that both non-malignant epithelial and myoepithelial cells express aFGF.²⁰ Perhaps myoepithelial cells do not express aFGF under normal conditions, but are able to do so as a result of changes related to their growth in tissue culture conditions.

It has been reported³⁷⁻³⁹ that myoepithelial cells express bFGF, another member of the fibroblast growth factor family. This protein has biological actions similar to those of aFGF, and can bind to FGFR4, although with a lower affinity than aFGF.²⁴ The close proximity of myoepithelial cells to the basement lamina in the breast could be important for controlling epithelial cell proliferation and extracellular matrix formation during breast growth and development. This hypothetical pathway might be mediated principally by bFGF.

In addition to FGFR4, other FGFRs might be involved in the modulation of cellular function in normal breast tissue and fibroadenomas; in fact, it has been found that epithelial cells of normal breasts and of benign breast lesions also express FGFR1, FGFR2, and

FGFR3.⁴⁰⁻⁴² Taken together, these results suggest that there is a functional interaction among myoepithelial, epithelial, and stromal cells through the secretion of aFGF and bFGF, which can bind to various FGFRs.

In conclusion, the results of our study suggest that aFGF and FGFR4 might be involved in the modulation of normal breast cell functions and, in addition, that they might regulate the growth of both epithelial and stromal cells of fibroadenomas.

This work was supported by a grant of the University of Insubria, Varese, Italy.

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