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Significance of Basic Fibroblast Growth Factor and Fibroblast Growth Factor Receptor Protein Expression in the Formation of Fibrotic Focus in Invasive Ductal Carcinoma of the Breast

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A fibrotic focus (FF) is a scar-like area within invasive ductal carcinoma (IDC) of the breast, and has been shown to be a marker of high aggressiveness of IDC. In order to investigate the mechanism of FF formation in IDC, expression of basic fibroblast growth factor (bFGF) and fibroblast growth factor receptor (FGFR) was studied. One hundred and forty-nine IDCs were divided into solid tumors and scirrhous tumors. Immunohistochemistry was used to determine the expression of bFGF and FGFR proteins in both tumor cells and fibroblasts forming FF. Scirrhous tumors with FF showed a significantly higher frequency of bFGF protein expression than those without ($P=0.017$), whereas, in solid tumors, the presence of FF was not significantly associated with the frequency of bFGF protein expression ($P=0.143$). In addition, scirrhous tumors showed a significantly higher frequency of FGFR protein expression than solid tumors ($P=0.001$). Among IDCs having FF and expressing bFGF protein, a significantly larger number of fibroblasts expressing FGFR protein within FF was observed in scirrhous tumors than in solid tumors ($P=0.016$). The results of this study suggest that in scirrhous tumors the interaction between tumor cells and stromal fibroblasts plays an important role in the formation of FF, and that there is a paracrine mechanism between bFGF protein from tumor cells and FGFR protein in fibroblasts.

Key words: Invasive ductal carcinoma — Fibrotic focus — Immunohistochemistry — Basic fibroblast growth factor — Fibroblast growth factor receptor

We have reported that invasive ductal carcinomas (IDCs) with fibrotic focus (FF) of the breast have a significantly higher frequency of lymph node metastasis, a significantly higher histologic grade, a significantly higher frequency of c-erbB-2 protein overexpression, and a significantly higher labeling index of proliferating cell nuclear antigen than IDCs without FF.[1)] These facts indicate that IDCs with FF are more likely to be aggressive than IDCs without FF. Therefore, the presence of FF in IDCs is a potentially important histological parameter for predicting the outcome of IDC.

The FF is composed of fibroblasts mixed with various amounts of collagen fibers, and the arrangement of fibroblasts or collagen fibers forming FF appears different from that of the normal breast tissue stroma or that of the surrounding tumor stroma, which is more orderly. Therefore, some factors that induce the proliferation of fibroblasts may play an important role in the formation of FF. Basic fibroblast growth factor (bFGF) is a potent stimulator of fibroblasts,[2,3)] and in the breast tissue the ductal epithelial cells express bFGF mRNA or protein.[4,5)] bFGF acts through high-affinity tyrosine kinase receptors, the fibroblast growth factor receptors (FGFR), that are encoded by at least 4 distinct genes.[6)] The extracellular region of FGFRs has an immunoglobulin-like domain, and these receptors play major roles in organ. development.[7)] A putative autocrine/paracrine role for the bFGF/FGFR system has been suggested in the mitogenic activation of fibroblasts or myofibroblasts.[8,9)]

In the present study, we attempted to clarify the following questions: 1) how many IDCs express bFGF or FGFR protein; 2) whether fibroblasts forming FF express FGFR protein; and 3) whether there is a significant association between bFGF protein expression of tumor cells and FGFR protein expression in fibroblasts forming FF.

MATERIALS AND METHODS

Cases One hundred and forty-nine cases of IDC of the breast that had been consecutively treated by surgery between July, 1992 and June, 1994 at the National Cancer Center Hospital East were included in this study. All the patients were Japanese females ranging in age from 28 to 87 years (average, 53 years), and all had a solitary lesion. Standard radical mastectomy was performed on 38 patients, modified radical mastectomy on 107, extended radical mastectomy on four, quadrantectomy on two, and glandectomy on five. Axillary lymph

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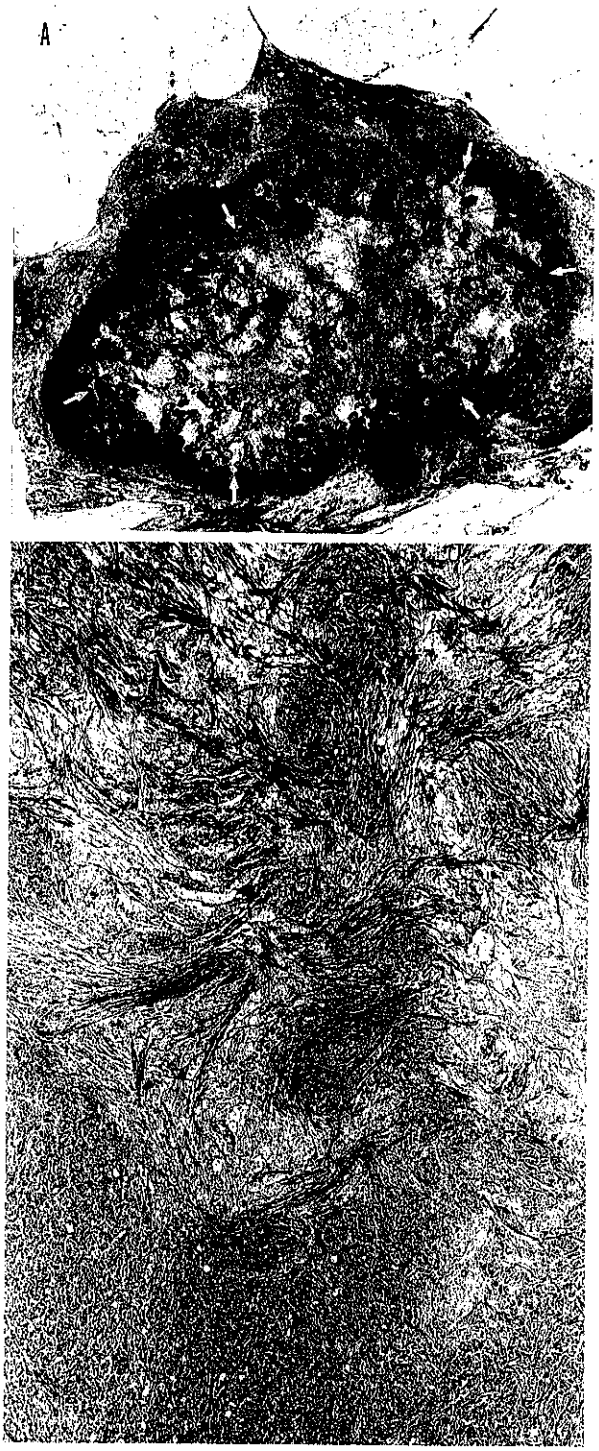

Fig. 1. An example of FF in IDC. A, Panoramic view of IDC with FF. The tumor, measuring 16×15 mm, has a 13×9 mm FF (arrows). B, FF is composed of fibroblasts and collagen fibers showing a storiform-like pattern mixed with tumor cells forming a tubular structure (HE, original magnification ×40).

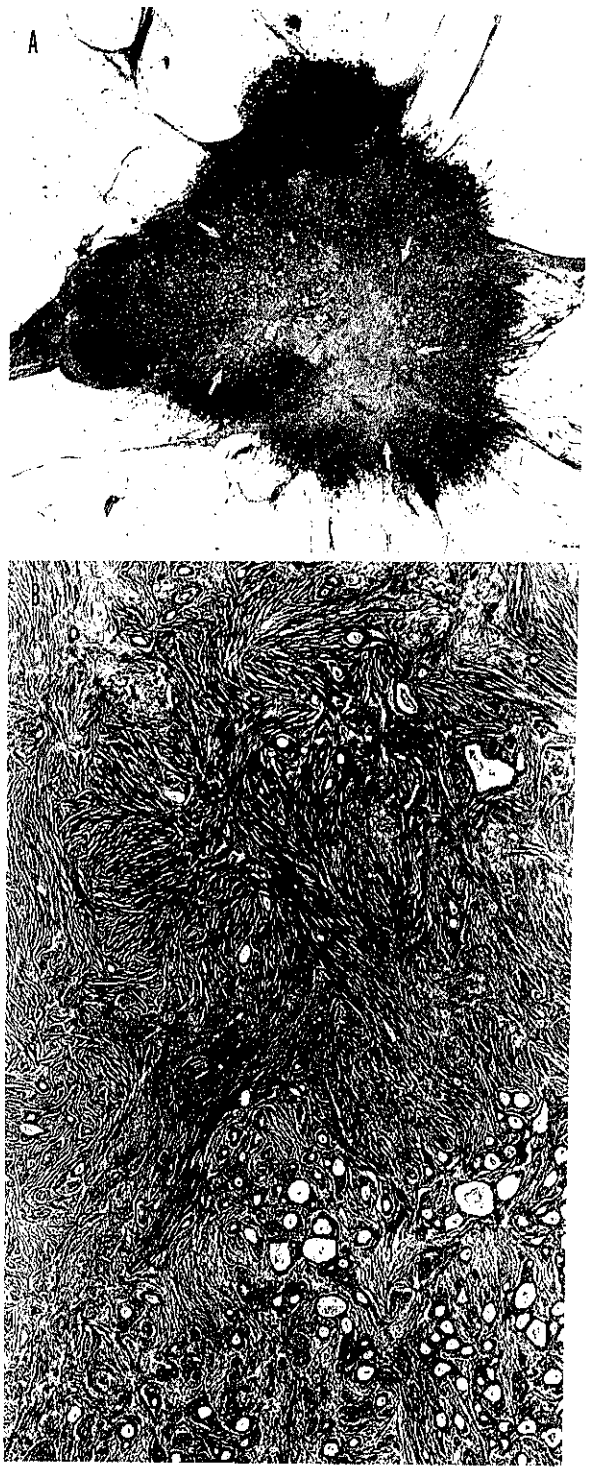

Fig. 2. An example of FF in IDC. A, Panoramic view of invasive ductal carcinoma with fibrotic focus. The 21×19 mm tumor has a large fibrotic focus measuring 17×18 mm (arrows). B, There are tumor cell islands within the FF (HE, original magnification ×40).

node dissection was done in 151 patients, among whom 72 (48%) showed lymph node metastasis. None of the patients received radiotherapy or chemotherapy before surgery.

For the pathological examination, surgically resected tissue specimens were fixed in 10% formalin overnight at room temperature and the entire tumor was cut into slices at intervals of about 0.5 to 0.7 cm. The sections were processed routinely and embedded in paraffin.

Histological examination Serial sections of each tumor were cut from the paraffin blocks. One section was stained with hematoxylin and eosin and examined pathologically to confirm the diagnosis. The remaining sections were used for immunohistochemistry. All the tumors were classified according to the guidelines of the World Health Organization.[10] The tumors were divided into two groups according to their growth pattern: 1) solid type in which the tumor cells grow in solid nests, 2) scirrhous type in which the tumor cells grow in a scirrhous fashion.

Histological examination of FF FF, which consisted of an increased number of fibroblasts and/or collagen fibers, was located within the tumor, and occupied various percentages of the tumor area (Figs. 1 and 2). When the FF was 3 mm or smaller, tumor cells were only infrequently seen within it. However, tumor cells growing in a scirrhous fashion or in solid nests were seen within larger FF. Fibroblasts or collagen fibers in FF were arranged in irregular or storiform-like patterns with increased fibroblast cellularity and/or collagenization. The arrangement of fibroblasts or collagen fibers forming FF appeared different from that of the normal breast tissue stroma or that of the surrounding tumor stroma, which was more orderly. Elastic tissue may be abundant in FF. If several FFs of various sizes and degrees of fibrosis were present within one tumor, the largest one was examined. We defined FF in metastatic lymph node tumor using the same parameters as in the primary tumor (Fig. 3).

Fig. 3. Lymph node metastasis of IDC with FF measuring 1×1 mm (HE, original magnification ×40).

Immunohistochemistry Immunohistochemical staining for bFGF and FGFR proteins was performed by the ABC method.[11] The primary antibodies employed were an affinity-purified mouse monoclonal antibody specific for bovine bFGF protein (Upstate Biotechnology Inc., Lake Placid, NY), used at 1:200 dilution, and a mouse monoclonal antibody against human FGFR (flg) protein (Upstate Biotechnology Inc.), used at 1:50 dilution. Reagents for the ABC method were obtained from Dako (Glostrup, Denmark). Microwave treatment was performed before the immunohistochemical staining for FGFR protein.[12] After the immunostaining, the sections were counterstained with hematoxylin. Sections of IDC positive for bFGF and FGFR proteins were used each time as a positive control. As a negative control, the primary antibody was replaced with normal mouse immunoglobulin. Dark-brown to brown cytoplasmic staining was judged to be positive for bFGF and FGFR protein. When only a few tumor cells showed positive staining for bFGF protein or FGFR protein, it was very difficult to judge its significance. Therefore, brown to dark brown cytoplasmic staining for bFGF or FGFR protein in more than 10% of the tumor cells throughout the tumor was judged to be positive. In order to confirm the specificity and sensitivity of the immunostaining in paraffin-embedded tissues, the immunohistochemical staining pattern for these proteins in frozen sections of the tumor and in non-tumorous tissue was initially examined in 20 IDCs.

Western blot analysis bFGF protein from frozen tissue samples of IDCs was extracted in 10 m*M* HEPES (pH 7.4) containing 0.4 *M* NaCl, 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, MO) and 1 μg/ml leupeptin (Sigma), and partially purified by using heparin-acrylic beads (CL-6B, Pharmacia

Biotech, Sweden).[13] The proteins binding to the beads were solubilized at 98°C for 3 min in sodium dodecyl sulfate (SDS)-sample buffer containing 100 m*M* Tris HCl (pH 6.8), 4% SDS, 10% β-mercaptoethanol, 0.2% bromophenol blue (BPB), and 20% glycerol. FGFR protein from frozen tissue samples of IDCs was extracted in 20 m*M* PIPES (pH 7.4) containing 250 m*M* sucrose, 1 m*M* EDTA, 1 m*M* EGTA, and 0.5 m*M* PMSF. The tissue lysates were solubilized with 4×SDS-sample buffer (final concentration of SDS-sample buffer, 1×). One hundred and twenty μg aliquots of each of the lysates for bFGF and FGFR were separated by 15% and 8% SDS-polyacrylamide gel electrophoresis (PAGE), respectively, and were transferred to an Immobilon membrane (Millipore, Tokyo). bFGF or FGFR protein was detected by using monoclonal anti-bFGF at 1 : 500 or monoclonal anti-FGFR at 1 : 100, then visualized by reaction of avidin-biotin complex with 3,3′-diaminobenzidine (Dojindo, Kumamoto) as a chromophore. The protein concentrations were determined by using a Bio-Rad assay kit (Bio-Rad Laboratories, Richmond, CA).

RESULTS

Immunohistochemical localization of bFGF and FGFR expression in normal breast tissue The expression of bFGF protein and FGFR protein was observed in the following sites; (1) the mammary ductal or secretory epithelium, (2) the endothelial cells, (3) the smooth muscle cells of vessel walls, and (4) the epidermis. These patterns of expression were confirmed by immunohistochemistry using frozen specimens.

Effect of FF on the expression of bFGF or FGFR in different growth types of invasive ductal carcinomas Scirrhous tumors with FF showed a significantly higher frequency of bFGF protein expression than those without FF ($P=0.017$) (Table I) (Fig. 4B). In contrast, in solid tumors the presence of FF in IDCs was not associated with bFGF protein expression (Table I) ($P=0.143$). No significant difference in the frequency of

Table I. Association of the Growth Types of IDC and FF with bFGF Expression

	No. of patients (%)			
	Total	bFGF expression		*P-value*
		+	−	
All cases	149	104 (70)	45 (30)	
Growth type				
Solid	86	57 (66)	29 (34)	
FF present	42	25 (59)	17 (41)	
FF absent	44	32 (73)	12 (27)	0.143
Scirrhous	63	47 (73)	16 (27)	
FF present	36	31 (86)	5 (14)	
FF absent	27	16 (59)	11 (41)	0.017

IDC, Invasive ductal carcinoma; FF, fibrotic focus; bFGF, basic fibroblast growth factor; +, positive; −, negative.

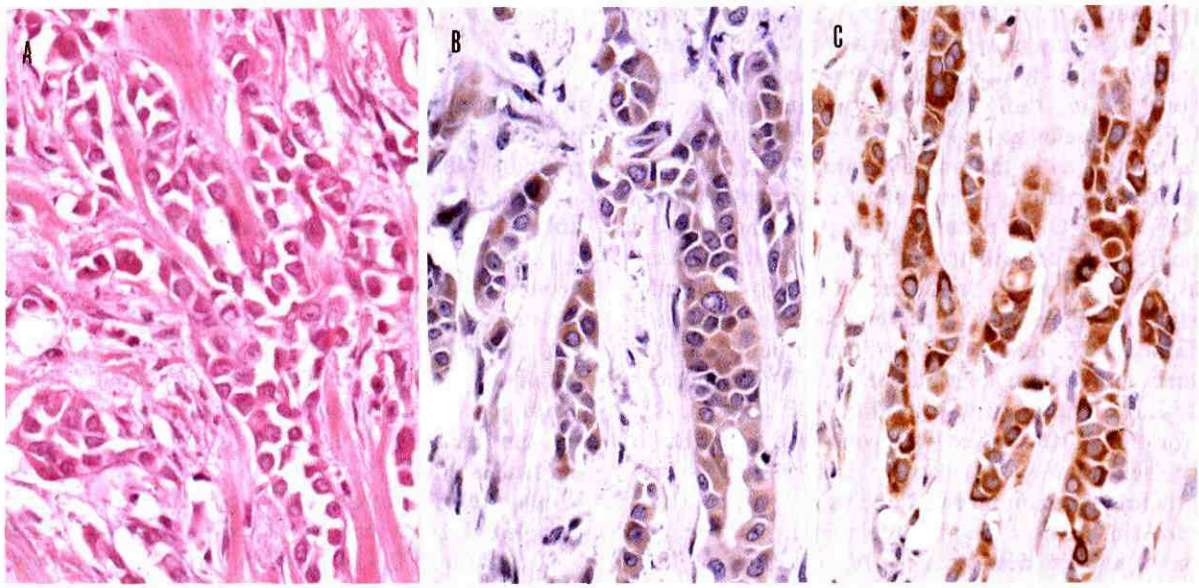

Fig. 4. A, Scirrhous tumor with bFGF and FGFR expression. (HE, original magnification ×200). B and C, Ductal carcinoma cells show intense cytoplasmic staining for bFGF and FGFR protein (immunostaining for bFGF(B) and FGFR(C), original magnification ×200).

Table II. Association of the Growth Types of IDC with FGFR Expression

	No. of patients (%)			
	Total	FGFR expression		*P*-value
		+	−	
All cases	149	104 (70)	45 (30)	
Growth type				
Solid	86	51 (59)	35 (41)	
Scirrhous	63	53 (84)	10 (16)	0.001

IDC, Invasive ductal carcinoma; FGFR, fibroblast growth factor receptor; +, positive; −, negative.

Table III. Association of bFGF Expression of Tumor Cells with FGFR Expression of Fibroblasts Forming FF in IDC of Different Growth Types

	No. of patients (%)			
	Total	Tumor cells		*P*-value
		bFGF (−)	bFGF (+)	
IDC with FF	78	23 (29)	55 (71)	
Solid				
Fibroblasts/FF	42	17 (40)	25 (60)	
FGFR (+)	13	6 (46)	7 (54)	
Scirrhous				
Fibroblasts/FF	36	6 (17)	30 (83)	
FGFR (+)	23	2 (9)	21 (91)	0.016

FF, Fibrotic focus; bFGF, basic fibroblast growth factor; FGFR, fibroblast growth factor receptor; +, positive; −, negative.

bFGF expression between solid and scirrhous tumors was observed ($P=0.274$) (Table I).

IDCs growing in a scirrhous fashion showed a significantly higher frequency of FGFR protein expression than those growing in solid nets ($P=0.001$) (Table II) (Fig. 4C). The presence of FF within IDC was not associated with a significant difference in the frequency of FGFR expression in solid or scirrhous tumors (data not shown).

Correlation between bFGF expression in tumor cells and FGFR expression in fibroblasts forming an FF in invasive ductal carcinomas Among IDCs with FF that express bFGF protein, a significantly larger number of fibroblasts expressing FGFR protein within FF was observed in the scirrhous tumors than in solid tumors ($P=0.016$) (Table III) (Fig. 5).

Frequency of bFGF and FGFR expression in primary and metastatic tumors in the lymph node Of 72 IDCs with lymph node metastasis, 30 (42%) had FF in lymph node metastases, and 24 (80%) of them had FF in the primary lesion. Only 23 (55%) of 42 IDCs without FF in lymph node metastases had FF in the primary. IDCs with FF in lymph node metastases had a significantly higher frequency of FF in the primary lesion than did IDCs without FF in metastases ($P<0.03$).

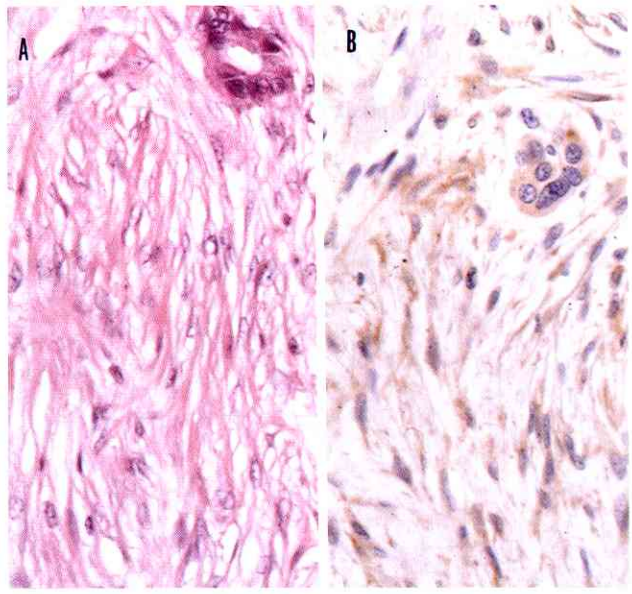

Fig. 5. FGFR expression by fibroblasts in FF. A, Fibroblasts forming a fibrotic focus grow in a storiform-like pattern mixed with ductal carcinoma cells (HE, original magnification ×200). B, Fibroblasts show intense cytoplasmic staining for FGFR protein (immunostaining, original magnification ×200).

Because the tumor cells in lymph nodes disappeared, seven and eight cases could not be stained for bFGF and FGFR expression, respectively. Lymph node metastases of IDCs expressed bFGF in 19 of 22 cases (86%) when the primary was negative for bFGF, and in 41 of 43 cases (95%) when the primary was positive (Fig. 6A). Similarly, many of the lymph node metastases (49/64, 77%) showed FGFR expression regardless of the presence of FF, or expression of FGFR in the primary lesion (Fig. 6B).

FGFR expression in fibroblasts within an FF and within fibrotic stroma in lymph node metastases Twenty (48%) of 42 lymph node metastases without FF had fibrotic stroma (FS). Fibroblasts forming FF expressed FGFR protein more frequently than those forming FS (Fig. 7), and the difference was statistically significant ($P=0.018$) (Table IV).

Western blot analysis Western blotting detected bands of 18 kDa bFGF proteins from the tumor tissues which were immunohistochemically positive for bFGF protein. The intensity of the bands correlated with the immunohistochemical results. No bFGF protein was detected in

immunohistochemically negative tumors (Fig. 8A). Masses of 68, 71, and 79 kDa were observed on blots for FGFR protein from the tumor tissues which were immunohistochemically positive for FGFR protein (Fig. 8B). The tumors weakly positive for FGFR protein immunohistochemically showed weakly stained 68 and 71 kDa bands on blots(Fig. 8B), whereas those negative for FGFR protein immunohistochemically did not show bands of FGFR protein on western blots (Fig. 8B).

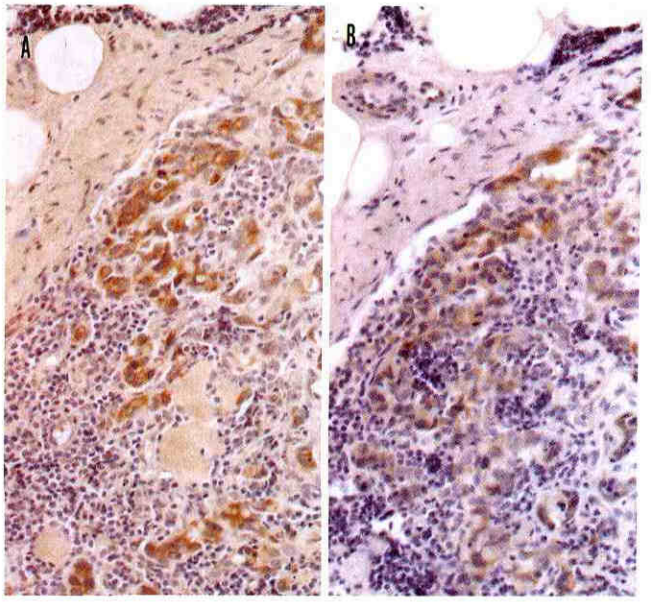

Fig. 6. bFGF expression by IDC. Ductal carcinoma cells in the lymph node show intense cytoplasmic staining for bFGF (A) and FGFR protein (B). (immunostaining, original magnification ×200).

DISCUSSION

The present study clearly demonstrated that bFGF protein expression in the primary lesion did not depend on the type of tumor, but was associated with the presence of FF in the scirrhous tumors. On the other hand, a

Table IV. Frequency of FGFR Expression in Fibroblasts Forming FF and Those Forming Fibrotic Stroma in Metastatic Lymph Node Lesions in Invasive Ductal Carcinomas with and without FF

	No. of patients (%)			
	Total	FGFR expression		*P*-value
		−	+	
IDCs in LN				
IDCs with FF				
Fibroblast within FF	30	20 (67)	10 (33)	
IDCs with FS*				
Fibroblast within FS	20	19 (95)	1 (5)	0.018

FF, Fibrotic focus; FS, fibrotic stroma; FGFR, fibroblast growth factor receptor; LN, lymph node; +, positive; −, negative.
* IDCs that have fibrotic stroma, but no fibrotic focus.

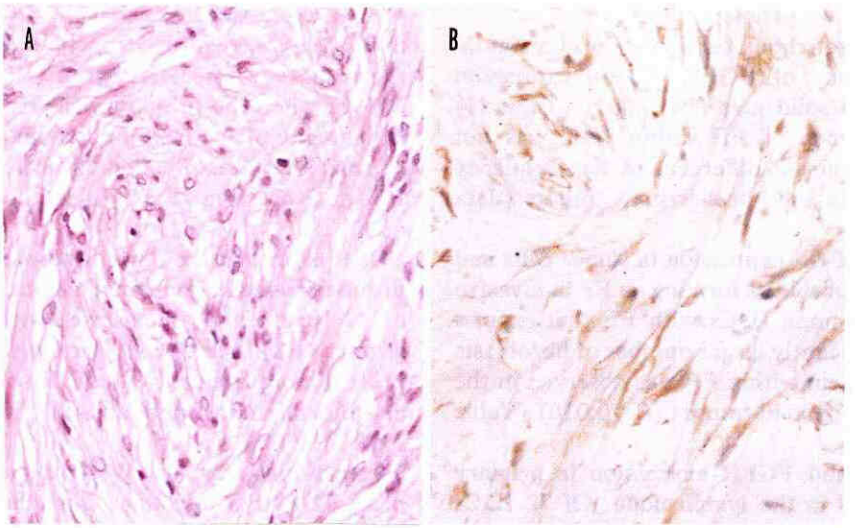

Fig. 7. FF in lymph node metastasis. A, Fibroblasts forming the FF show a storiform-like pattern (HE, original magnification ×200). B, Intense cytoplasmic staining for FGFR protein is present in fibroblasts (immunostaining, original magnification ×200).

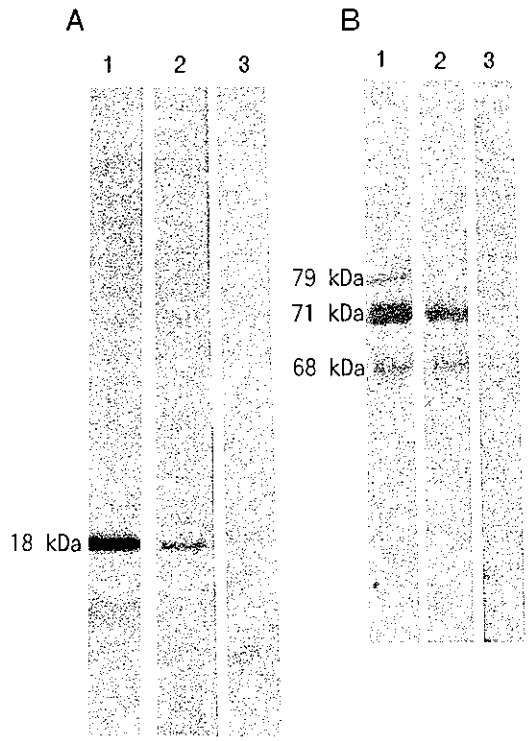

Fig. 8. Western blot analysis for bFGF and FGFR protein. A, Ductal carcinoma cells immunohistochemically positive and weakly positive for bFGF protein show a distinctly and faintly stained 18 kDa bFGF protein, respectively (lanes 1 and 2), whereas those immunohistochemically negative for bFGF protein do not (lane 3). B, Ductal carcinoma cells immunohistochemically positive for FGFR protein show three bands (68, 71, and 79 kDa) of FGFR proteins. Ductal carcinoma cells immunohistochemically weakly positive for FGFR protein show weakly stained bands of 60 and 71 kDa, and lack a 79 kDa band. In ductal carcinoma cells immunohistochemically negative for FGFR protein, no protein band is observed. Bars indicate mobilities of molecular weight markers in kDa. A, Lanes 1, 2, and 3, immunohistochemically positive, weakly positive and negative for bFGF protein, respectively; B, Lanes 1, 2, and 3, immunohistochemically positive, weakly positive and negative for FGFR protein, respectively.

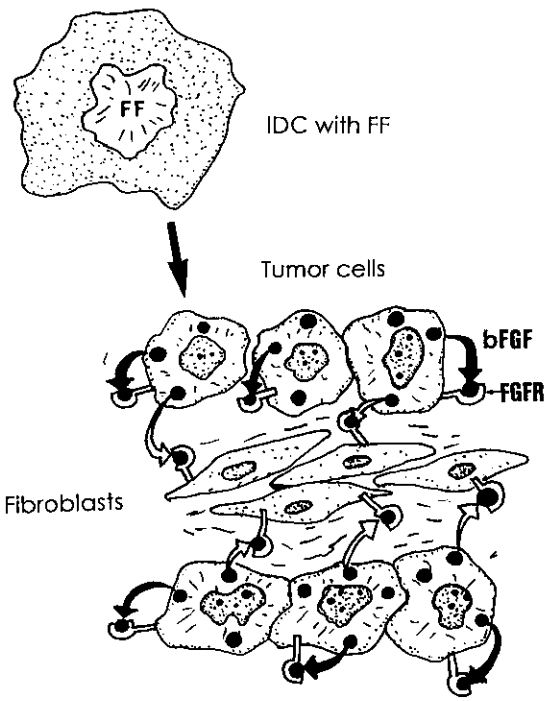

Fig. 9. Schematic illustration of the autocrine or paracrine theory of bFGF and FGFR actions between tumor cells and fibroblasts in the formation of FF in IDC. ↗ autocrine; ↙ paracrine.

statistically significant correlation between the growth pattern of the tumor cells and FGFR expression was observed. bFGF is an autocrine growth factor,[13-16] and acts through high-affinity tyrosine kinase receptors, FGFRs.[6] Autocrine growth stimulation via FGFR may therefore contribute to the growth of scirrhous tumors rather than solid tumors, and IDCs with FF growing in a scirrhous fashion may have a stronger autocrine function than IDCs without FF (Fig. 9). IDCs with FFs show a significantly higher aggressiveness,[1] and significantly higher relative risks of tumor recurrence and death than IDCs without FFs in multivariate analysis.[17] The prognostic significance of bFGF protein expression in breast cancer has been reported.[18] Therefore, the autocrine growth stimulation between bFGF and FGFR appears to increase the degree of biological malignancy of IDCs.

There was a significant correlation between FGFR expression by the fibroblasts forming FF and bFGF expression by the tumor cells of scirrhous tumors. bFGF is a potent stimulator of fibroblasts,[2, 3] and it plays an important role in the physiological fibroproliferative process of granulation tissue formation during wound healing,[19, 20] in the fibroproliferative disorder of alveolar fibrosis after lung injury[21] or in chronic pancreatitis.[22] Therefore, in the scirrhous tumor, a paracrine action of bFGF and FGFR may exist between the tumor cells and the fibroblasts, and the interaction between them appears to be important for the formation of FF (Fig. 9).

We have already reported that coagulation necrosis of the tumor cells within FF is more frequently observed in solid tumors than in scirrhous tumors,[1] which suggests that FFs in the solid tumors are made up of fibroblasts or collagen fibers absorbing or replacing tumor necrosis that may be caused by a lack of blood flow, or hypoxia.

Therefore, the mechanism of formation of FF in IDC may differ between tumors growing in a scirrhous fashion and those growing in a solid nest.

Nodal status is known to be the most important prognostic factor for IDCs.[23,24] In the present study, almost all metastatic tumors in the lymph node stained positive for bFGF or FGFR protein, independently of their expression in the primary lesion. This indicates that metastatic tumors in the lymph nodes appear to acquire an ability to express both proteins, and that an autocrine mechanism between bFGF and FGFR protein may accelerate the spread of metastatic tumors in the lymph node, resulting in less favorable outcomes for patients with lymph node metastases.

This study clearly showed that FF in lymph node metastases was more frequently seen in IDCs with FF than in those without FF, which indicated that IDCs with FF appear to have a greater propensity to form FF in different biological environments than those without FF. Although the formation of FF in lymph node metastases may depend on a similar bFGF/FGFR paracrine mechanism to that in the primary lesion, the number of fibroblasts expressing FGFR in the tumor stroma appears to be more important for the formation of FF in lymph node metastases than the degree of bFGF expression by the tumor cells. This notion was supported by the fact that tumors expressing bFGF in lymph node metastases did not have FF. Therefore, the biological characteristics of fibroblasts forming FF appear to be different from those of fibroblasts forming ordinary stromal fibrosis. Since IDCs with FF in lymph node metastases resulted in a significantly shorter disease-free survival than those without FF in lymph node metastases,[17] the interaction between tumor cells and the stromal fibroblasts may contribute to the high malignant potential of IDCs with lymph node metastasis.

In conclusion, the interaction between tumor cells and stromal fibroblasts plays an important role in the formation of FF in scirrhous tumors. This indicated that there is a paracrine mechanism between bFGF expression by the tumor cells and FGFR expression by the fibroblasts forming FF. In lymph node metastases, almost all tumor cells may express bFGF or FGFR protein, and a paracrine mechanism, similar to that in the primary lesion, between the tumor cells and the stromal fibroblasts may be important for the formation of FF.

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