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The overall aim of our project is to identify paracrine and autocrine mechanisms that regulate stromal-tumor cell interactions in breast cancer. We have shown that platelet-derived growth factor (PDGF) β-receptors, which are high affinity receptors for PDGF-BB, are upregulated in vascular endothelial cells and smooth muscle actin-expressing cells (most likely myofibroblasts) at the peri-epithelial stroma in DCIS of human breast tumors. Our results suggest that breast carcinoma cells, which secrete PDGF-BB, stimulate vascularization by endothelial cells and recruitment of myofibroblasts in the peri-epithelial stromal regions of early stage breast cancer. In contrast to PDGF β-receptors, HGF receptor/Met is expressed on epithelial and some carcinoma cells. Our results that HGF mRNA is produced by some nonmalignant epithelium and by carcinoma cells in the breast, particularly at the migrating tumor front, suggest the intriguing possibility that some invasive carcinoma cells express an HGF autocrine loop. Regions of invasive carcinoma also showed reduced expression of E-cadherin and absence of tight junctions. These results suggest that the type of response to HGF elicited in nonmalignant and malignant epithelium may be different. Using a novel sensitive method developed in our laboratory for purification of HGF from tissues and cell lines, we have identified one mouse carcinoma cell line that co-expresses HGF and HGF receptor/Met. However none of 8 breast epithelial cell lines examined expressed detectable HGF. Information from these studies will possibly be useful in diagnosis of individuals with high risk breast cancer, and in designing and testing of putative antagonists of PDGF- and HGF-dependent steps in breast cancer progression.

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Bruce E. Elliott Oct 15, 1996

PI - Signature Date

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INTRODUCTION:

Growth factors which act in a paracrine or autocrine manner are important regulators of stromal-tumor interactions in breast cancer. Stromal fibroblasts and adipocytes secrete growth factors, such as keratinocyte-derived growth factor (1,2) and hepatocyte growth factor (HGF) (3), which can stimulate in a paracrine manner growth and invasiveness of mammary carcinoma cells. In addition, stimulation of stromal cells by carcinoma cells has recently been shown to induce expression of stromal proteinases (4,5), extracellular matrix proteins (e.g., tenascin (6) and thrombospondin (7)) and certain growth factors (e.g. HGF (8)). However the tumor-derived stimulatory components involved are not well understood.

PDGF BB, which is upregulated in the majority of breast cancer tissues (9-11) and in breast carcinoma cell lines (12,13), is an important candidate molecule in the paracrine stimulation of the stroma by carcinoma cells. PDGF BB has been shown to stimulate a variety of functions including growth (14), chemotaxis (15), and fibronectin matrix assembly (16) in connective tissue cells. PDGF is composed of two chains, A and B, held together by disulphide bonds and present in three dimeric forms AA, AB and BB (17,18). The dimers bind to two separate receptor types: α and ß (19,20). The α-receptors bind to all three isoforms of PDGF, while the ß-receptors have a very high affinity for PDGF BB, bind PDGF AB to some extent, but do not bind PDGF AA at all. The ß-receptor has a ligand-activated protein kinase (21); the receptor is autophosphorylated in response to the appropriate ligand. Each PDGF chain is encoded by a distinct gene. The gene encoding PDGF B has been identified as the normal mammalian analogue of the v-sis oncogene (17,22,23). Fibroblast cells transfected with the PDGF B gene showed a higher transforming activity than cells transfected with the PDGF A gene (24). The PDGF β-receptor may therefore represent an important regulatory pathway in breast cancer. PDGF β-receptor mRNA and protein have been found on most stromal cell types including fibroblasts (25), smooth muscle cells (15), endothelial cells (26), and pericytes (27). Recently, some breast carcinoma cell lines (28), as well as breast cancer tissues (11,29,30) have also been shown to express PDGF β-receptors. In breast cancer tissues, PDGF β-receptors were expressed primarily in the stroma of normal and malignant breast tissue, not on non-malignant epithelial cells and only on a few malignant epithelial cells (11). However, it is not known what stromal cell types in breast cancer express PDGF β-receptors.

In contrast to the PDGF family of growth factors, **HGF** is secreted by stromal cells and acts on epithelial cells (31). HGF is a heterodimer consisting of two noncovalently-linked 65 kDa and 35 kDa chains (32). Like FGF and PDGF families, HGF has heparin-binding affinity and can be sequestered in the extracellular matrix. HGF is a potent mitogen for certain cells (e.g., hepatocytes, kidney epithelial cells, and some breast carcinoma cells (33)), and an inducer of differentiation (e.g. kidney tubule formation (34), breast lumen formation (35)) and of cell motility (36) angiogenesis (37) and invasion (38). The action of HGF is mediated via the c-met tyrosine kinase receptor which mediates a signal exchange from the mesenchyme to the epithelia during development (39). Recent studies have shown that c-met, though expressed in normal breast ductal epithelia is expressed less in certain breast carcinoma cells (40). Based on the morphogenic potency of HGF, it has been proposed that this gene and the corresponding c-met are tumor suppressor genes. Both of these

genes are localized on the long arm of chromosome 7. Monosomy 7 or 7q are often found in certain tumors, and loss of heterozygosity on 7q in breast tumors correlates with significantly shorter metastasis-free survival and overall survival (41,42). However, it has been shown that HGF stimulates cell proliferation and invasion in certain carcinoma cell lines including a murine mammary carcinoma studied in our laboratory (App. III). Therefore a switch of HGF activity to stimulation of cell proliferation and invasion could result in malignant transformation and increased metastasis in certain tumor cells. Thus depending on the HGF-dependent function expressed, the HGF pathway could have tumor suppressive (morphogenic) or tumor promoting (mitogenic) activity. It is not known what regulates HGF or c-met expression, nor what functional response is elicited by triggering of this receptor pathway in breast carcinoma cells.

Our **hypothesis** is that upregulation of PDGF β-receptors in the peri-epithelial stroma of breast tissues is an indicator of possibly malignant epithelium. PDGF BB secreted from breast carcinoma cells could stimulate proliferation, chemotaxis or angiogenesis in the neighbouring stromal cells. In addition, induction of synthesis and secretion of cytokines such as HGF by stromal cells could stimulate growth and invasion of tumor cells. HGF-induced changes such as loss of tight junctions, degradation of surrounding basement membrane, and increased motility and invasion of carcinoma cells might occur; these changes have been shown to be prognostic indicators of an invasive phenotype. In addition, a shift to autocrine production of HGF by carcinoma cells would augment the tumorigenic and metastatic properties of breast carcinoma cells.

Our initial **objectives** were to:

1) Compare nonmalignant, *in situ*, and invasive carcinoma cells for expression of PDGF β-receptors and correlate with stromal cell type, and to establish association of PDGF β-receptor expression with progression of breast cancer;
2) Identify and localize expression of PDGF-BB and HGF in human breast cancer tissues as well as human carcinoma cell lines;
3) Examine cell proliferation and basement membrane degradation, as indicators of invasion, associated with PDGF β-receptor-bearing stromal cells or neighbouring carcinoma cells and to determine the putative functional relationship with HGF; and
4) Determine whether PDGF-BB stimulates proliferation and cytokine secretion in breast-derived myoepithelial cells.

This report will summarize briefly the work in the first two objectives which is now published and will describe more recent work in progress relating primarily to Objective 3. Unexpected results in Objectives 1 and 2 required that the directions of Objectives 3 and 4 be altered as described below.

MATERIALS AND METHODS: Breast carcinoma cell lines used in this study are described in Table II. Details of materials and methods are described in publications referenced in the Results section. See also Figure legends.

RESULTS:

Objective 1: Compare nonmalignant, *in situ*, and invasive carcinoma cells for expression of PDGF β-receptors and correlate with stromal cell type, and to establish association of PDGF β-receptor expression with progression of breast cancer disease: During the first year of this programme, we demonstrated increased expression of PDGF β-receptors in peri-epithelial stroma of ductal carcinoma *in situ* (DCIS) (29 tumor tissues examined). Image analysis was used to assess the coincidence of staining of PDGF β-receptor with epithelial or stromal cells in 13 of the 29 tumor tissues studied. The data are summarized in Table I. Less than 5% of malignant ductal epithelium or myoepithelium showed PDGF β-receptor staining. Analysis with stromal cell type-specific markers indicated significant localization of PDGF β-receptor primarily with α-smooth muscle actin staining cells (32%) and vascular endothelial cells (41%) in the peri-epithelial stroma. PDGF β-receptor positivity was strongly associated with basement membrane surrounding regions of carcinoma *in situ*, but was less intense in regions of invasive carcinoma where basement membrane was degraded. The absence of PDGF β-receptors on carcinoma cells and their presence in the surrounding stroma suggests a paracrine stimulation of adjacent stromal tissue (esp. endothelial cells and smooth muscle cells) by malignant epithelial cells in human breast tumors. Thus increased PDGF β-receptor expression appears to correlate with early stages of the disease (e.g. DCIS) and may stimulate angiogenesis. This work is now published in Clin.Cancer Res. 2:773-782, 1996 (App. I).

Objective 2) Identify and localize expression of PDGF-BB and HGF in human breast cancer tissues as well as human carcinoma cell lines: During the course of this work, other investigators (e.g. (11)) demonstrated that PDGF-BB is secreted by breast carcinoma cells. These observations together with our recent findings support the role of PDGF-BB in the paracrine stimulation by carcinoma cells of the surrounding stroma. We therefore focused our attention in Objective 2 on identifying the cell types in mammary tumors that express HGF, a cytokine known to be produced by the mammary stroma and to stimulate normal mammary ductal development (43).

In situ analysis of HGF and HGF receptor mRNA in human breast carcinomas: Using nonisotopic *in situ* hybridization (ISH), we showed that HGF and HGF receptor/Met mRNA are expressed in both non-malignant and malignant epithelium of primary human breast tissues. Epithelial expression of HGF mRNA was particularly intense in regions of ductal epithelial hyperplasia. Positive expression of HGF (but not HGF receptor/Met) mRNA was also found in adipocytes, endothelial cells, and to varying degrees in stromal fibroblasts. In 12 of 12 cases of ductal carcinoma *in situ* and infiltrating ductal carcinoma, carcinoma cells showed a heterogeneous pattern of expression for both HGF receptor/Met and HGF mRNA. (See Figure 1 for representative example of staining pattern). In infiltrating ductal carcinomas, intense expression of HGF receptor/Met mRNA was not restricted to ductal structures, but was also seen in non-duct-forming carcinoma cells. The same zones of the tumors (most commonly at the advancing margins) that expressed HGF receptor/Met mRNA strongly, often were also strongly positive for HGF mRNA, suggesting a possible autocrine effect. The expression pattern of HGF receptor/Met protein in 25 cases including the same series of tissues used for *in situ* hybridization analysis was similar to that of HGF receptor/Met mRNA, as determined by an immunoperoxidase technique. The finding that HGF receptor/Met is expressed by both benign and malignant epithelium, and is not restricted to duct-forming structures, suggests that although the potential for ligand binding to HGF receptor/Met

is maintained in malignancy, the response to ligand binding at the level of the receptor, or the cellular response to receptor activation, may change at some point during progression. (Tuck et al. Am. J. Path. 148:225-232, 1995; App. II.)

Development of improved methods for detection of HGF in cell lines and tissues: The observation that HGF mRNA is strongly expressed in carcinoma cells at the migrating tumor front is a novel concept since the majority of studies indicate that HGF expression is strongly suppressed in epithelial cells (44). It was therefore important to determine whether HGF protein is produced and is functional in human mammary carcinoma cells. Preliminary IHCS experiments showed that HGF protein is indeed present in mammary tumors (data not shown), however it was difficult to localize the cell types producing the HGF protein. We therefore developed approaches to examine HGF expression and function in freshly isolated breast tissue or carcinoma cell lines. Since HGF protein might be produced only in trace amounts in tissues or conditioned media (CM) from cell lines, we used two approaches to detect HGF. The first involved conventional immunoprecipitation of concentrated CM with polyclonal sheep anti-HGF IgG (obtained from R. Schwall, Genentech). The second involved a new technique developed in our laboratory to separate and identify HGF, based on a unique binding interaction between HGF and Cu(II). In our original study, conditioned medium (CM) from mouse 3T3-L1 adipocytes which secrete HGF (3), or purified human recombinant HGF was used for analysis (Figure 2). 3T3-L1 adipocyte CM was applied to a Cu(II)-affinity column and rinsed with buffer containing 0.02 M Na_2HPO_4 and 1 M NaCl. HGF was then eluted with 10 mM imidazole. Fractions eluted from the column were analyzed by SDS-PAGE. Analysis by silver staining revealed two major bands of 115 kDa and 85 kDa. Further analysis of these bands by western blotting with polyclonal anti-HGF IgG demonstrated that these bands corresponded to pro-HGF and mature HGF, respectively. Human recombinant HGF, when applied to a Cu(II)-affinity column, showed a stronger affinity to Cu (II) than mouse HGF. Human recombinant HGF was not eluted from the Cu(II) column with either 10 mM or 20 mM imidazole, however it was readily eluted with 0.5 mM EDTA. Both mouse and human recombinant HGF eluted from the Cu(II)-affinity column retained their biological activity as measured by HGF-induced cell proliferation of Mv1Lu cells. Our findings provide the first evidence that HGF is a copper-binding protein and that a Cu(II)-affinity column can be used for efficient one-step purification of biologically active HGF. This work is now published (Rahimi et al. Protein Expression and Purification 7:329-333, 1996), and a patent application for this procedure has been filed through Queen's University.

Examination of HGF and HGF receptor expression in mouse and human mammary carcinoma cell lines: To examine the possibility that mammary carcinomas might secrete their own HGF, we have examined a variety of human and mouse nonmalignant and malignant breast epithelial cell lines (Table II and III). One mouse mammary carcinoma cell line, SP1, showed co-expression of both HGF and HGF receptor/Met. SP1 cells, and metastatic variant SP1-3M cells derived from SP1, expressed tyrosine-phosphorylated HGF receptor/Met, and exhibited HGF-dependent spontaneous growth in serum-free medium and invasion through matrigel-coated membranes. The phosphorylation of HGF receptor/Met and cell invasion were inhibited by anti-HGF IgG. Using Northern blot and western blot analysis, we detected expression of HGF mRNA in SP1 cells and a Mr 85,000 HGF protein in

SP1 conditioned medium (Figure 3). These findings suggest the possibility that establishment of an HGF autocrine loop and sustained activation of HGF receptor/Met in breast carcinoma cells could lead to abnormal growth, tumorigenesis and metastasis (Rahimi *et al.* Cell Growth and Diff. 7:263-270, 1996).

The next step in this study was to determine whether the observed HGF autocrine loop in SP1 mammary carcinoma cells was representative of other human and mouse mammary carcinoma cells. We therefore examined a panel of nonmalignant and malignant epithelial cells lines derived from human and mouse breast tissues for expression of HGF and HGF receptor/Met. Lung carcinoma cells were included as a control in this study, since Park *et al.* previously reported that HGF was secreted by human lung bronchioepithelial and carcinoma cell lines (45). As an additional control we used the HEL 299 fibroblast cell line (from ATCC) which we have shown to produce HGF (data not shown). The results showed that none of the 8 mammary carcinoma or 35 lung carcinoma cell lines examined expressed HGF. (See Tables II and III). Most (7/8) mammary carcinoma cell lines expressed HGF receptor/Met, whereas the majority (2/35) of the lung carcinoma cell lines examined did not. In those cell lines where Met was detected, it showed no spontaneous tyrosine phosphorylation but was functionally active, as indicated by tyrosine phosphorylation of Met and cell proliferation following stimulation with HGF (Tables II and III, Figure 4). These findings indicate that the majority of carcinoma cell lines examined in our laboratory do not express HGF protein. Since HGF expression in carcinoma cells may depend on tissue microenvironment, we are currently examining nonmalignant and malignant epithelial cells isolated directly from human breast for expression of HGF mRNA and protein (see also Conclusions below).

Objective 3: To examine cell proliferation and basement membrane degradation, as indicators of invasion, associated with PDGF β-receptor-bearing stromal cells or neighbouring carcinoma cells and to determine the putative functional relationship with HGF. To further assess the possible role of PDGF β-receptors and HGF in early stages in the development of breast cancer, we examined the localization of these receptors with respect to regions of basement membrane degradation, cell proliferation, and differentiation (as indicated by presence of epithelial tight junctions) in nonmalignant epithelium and *in situ* and invasive carcinoma cells.

PDGF β-receptor expression is closely localized to basement membrane regions of DCIS and is decreased in invasive regions: PDGF β-receptor positive cells were found to be closely localized to basement membrane regions of DCIS. In invasive regions, PDGF β-receptor expression remained associated with cells adjacent to fragmented basement membrane or vascular regions; however, where no basement membrane was apparent, expression of PDGF β-receptor was less intense. This finding suggests a loss of PDGF β-receptor expression in peri-epithelial stromal cells (e.g. myofibroblasts) concomitant with degradation of the basement and/or movement of PDGF β-receptor-positive cells toward basement membrane-associated tumor regions. This observation also supports the result in Objective I that PDGF β-receptor-expressing cells in DCIS include vascular endothelial cells which form blood vessels which are also associated with basement membrane. (Clin. Cancer Res. 2:773-782, 1996).

E-cadherin expression is reduced in regions of *in situ* and invasive carcinomas of the breast compared to nonmalignant epithelium although strong Met expression was detected in all tissues: Our finding that HGF receptor/Met is expressed by both benign and malignant epithelium, and is not restricted to duct-forming structures, suggests that HGF may stimulate different functions in nonmalignant versus malignant epithelium. To address this possibility, we have initiated a study to examine cell proliferation markers (cyclin A, cyclin D1 and cyclin E) and differentiation markers, e.g. expression of E-cadherin (cell-cell adhesion molecules that form tight junctions between ductal epithelial cells) in tissues representing different stages of breast cancer development. We have recently shown a marked decrease in expression of E-cadherins, a known response to HGF (46), in regions of invasive carcinoma compared to adjacent nonmalignant ductal epithelium, although strong HGF receptor/Met expression was detected in both regions (Figure 5). In regions of DCIS, cytoplasmic expression of E-cadherin was apparent, however there was very little membrane staining and very few tight junctions were present. These findings support a differential role of HGF receptor Met in malignant versus nonmalignant breast epithelium. In malignancy, HGF may stimulate reduction in E-cadherin expression and consequent disruption of epithelial tight junctions in carcinoma cells, followed by increased cell scatter and invasive activity. In nonmalignant epithelium, Met is either not activated or may be activated in a qualitatively different way leading to maintenance of differentiation. Further experiments to examine the activation state of Met in nonmalignant and malignant epithelial cells and the functional response to HGF is in progress.

Objective 4: To determine whether PDGF-BB stimulates proliferation and cytokine secretion in breast-derived myoepithelial cells: Since myoepithelial cells do not express increased PDGF β-receptors in our human breast tumor samples examined, we have not pursued this objective.

CONCLUSIONS:

The overall aim of our project is to identify paracrine and autocrine mechanisms that regulate stromal-tumor cell interactions in breast cancer. We have focused on two growth factors, PDGF-BB and HGF, since both factors show increased expression in breast cancer. In addition, increased expression of HGF in breast cancer patients has been associated with recurrence and poor survival. However, the cell types expressing PDGF-BB and HGF, and their corresponding receptors in nonmalignant and malignant breast tissues have not been previously described. Results from this project have shown that PDGF β-receptors, high affinity receptors for PDGF-BB, are upregulated at the peri-epithelial stroma in DCIS. The major cell types expressing PDGF β-receptors are vascular endothelial cells and smooth muscle actin-expressing cells (most likely myofibroblasts). PDGF β-receptor-positive cells were found closely localized to basement membrane regions of DCIS and were less evident in regions of basement membrane degradation and tumor cell invasion. Our results support the model that breast carcinoma cells which secrete PDGF-BB (11), stimulate vascularization by endothelial cells and recruitment of myofibroblasts in the peri-epithelial stromal regions of DCIS. PDGF-BB may therefore play an important role in early stage breast cancer by stimulating angiogenesis required for tumor growth. No direct correlation of PDGF β-receptor expression was associated with late stage infiltrating ductal carcinomas (IDC). A future question to address is at what

stage during tumor development (e.g. atypical hyperplasia?) upregulation of PDGF β-receptors occurs in the peri-epithelial stroma.

In contrast to PDGF β-receptors, HGF is considered to be produced by stromal cells and to act in a paracrine manner on neighbouring epithelial cells which express HGF receptor/Met (31). Our results that HGF mRNA is produced by some nonmalignant epithelium and carcinoma cells in the breast are therefore novel and unexpected since HGF expression is strongly suppressed in epithelial cells (44). However, if HGF mRNA is expressed in some breast carcinoma cells, it is still necessary to confirm whether HGF protein is expressed in a biologically active form. Since HGF may be present only in trace amounts in breast tissues and carcinoma cell lines, we decided to develop improved methods for detection of mature HGF protein in biological tissues. Based on the fortuitous observation in our laboratory that HGF is a Cu(II)-binding protein, we developed a novel method for purification of HGF from tissues or conditioned media from cell lines. Recovery of HGF protein by the Cu(II)-affinity chromatography procedure was >90% and the recovery of biological activity as determined in a cell proliferation assay was 100%. Using both Cu(II)-affinity chromatography and conventional immunoprecipitation, we have failed to detect HGF protein in any of 8 breast and 35 lung carcinoma cell lines tested. The apparent discrepancy between these observations and our earlier finding that HGF mRNA is detected in mammary carcinoma cells *in situ* may indicate that 1) selection occurs *in vitro* for carcinoma cells that do not express HGF, 2) stromal interactions are required to maintain HGF expression, and 3) mRNA corresponding to other HGF family members (e.g. macrophage stimulating protein (MSP) (47)) may be detected in the human breast tumor tissues using ISH. These possibilities are currently being investigated, using PCR analysis of HGF mRNA in nonmalignant and malignant epithelial cells isolated from human breast as well as carcinoma cell lines.

Our observation that E-cadherin tight junctions are decreased in regions of invasive IDC compared to nonmalignant epithelium, although both regions showed strong expression of HGF receptor/Met suggests that HGF receptor might elicit different patterns of response in nonmalignant and malignant epithelial cells. To further test this hypothesis, we will examine whether cell proliferation (as determined by expression of cyclins A, D1 and E which are known to be upregulated in breast cancer (48,49)) correlates with HGF receptor/Met expression in regions of carcinoma cells. Experiments are also in progress to assess the level of HGF receptor/Met activation (as determined by tyrosine phosphorylation) in nonmalignant and malignant breast tissues, and whether different signalling pathways are stimulated by HGF in nonmalignant versus malignant epithelial cells.

Together this study has extended our knowledge of how two growth factors, PDGF-BB and HGF, may act in regulating stroma-tumor cell interactions in breast cancer. Our results suggest that up-regulation and activation of PDGF β-receptors on stromal cells (e.g., endothelial cells, myofibroblasts and smooth muscle cells) adjacent to regions of DCIS in human breast tumors may be an important step in the development of early stage breast cancer. In addition, increased expression of HGF and HGF receptor in breast carcinoma cells, particularly at the migrating tumor front may provide a selective advantage for invasive carcinoma cells. Since upregulation of PDGF-BB and HGF and their corresponding receptors occurs in breast cancer, future studies should be directed at characterizing the types of functional response. Information from these studies will be useful in

designing and testing of possible antagonists of growth factor ligand binding or of downstream regulatory molecules in the PDGF and HGF signalling pathways. These approaches might open novel possibilities for the treatment of breast cancer.

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PATENT APPLIED FOR:

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TABLE 1

Localization of PDGF ß-receptors with various cell markers in biopsies of human breast carcinomas

Tissue[a]	Percentage of cell marker-positive area coincident with PDGF ß-receptor[b]: Keratin (epithelium)	CALLA (myoepithelium)	α-Smooth Muscle Actin	PAL-E (endothelium)
Malignant	2.6 ± 4.4 (9)	4.9 ± 3.8 (7)	31.9 ± 25.2 (10)	41.0 ± 20.2 (13)

Table legend:

a. Frozen sections (6 µm) of malignant human breast biopsies were stained by double immunofluorescence with anti-PDGF ß-receptor IgG and either anti-keratin (epithelial cells), CALLA (myoepithelial cells), anti-α-smooth muscle actin, or PAL-E (endothelial cells) IgGs. See materials and methods for antibody details.

b. Slides were photographed under fluorescence illumination for Texas Red (PDGF ß-receptor) or FITC (cell markers). The total area in pixels of Texas Red and FITC positivity in each section was independently determined using an image analysis system. The percentage of pixels positive for both stains is shown. Percentage values represent the mean ± standard deviation. The number of tumors analyzed for each cell marker is indicated in parentheses.

TABLE II

Expression of HGF and HGF-receptor/MET in human and mouse breast epithelial and carcinoma cell lines

Breast Cell Line	Malignant	HGF Secretion	Scatter Activity[e]	Met Expression	Met Activation[f]		
					–	HGF	Anti-Met Ab
EL-E[a]	+	–	–	+	–	+	ND
HU-E[a]	+	–	–	+	–	+	ND
WO-E[a]	+	–	–	–	–	–	ND
MCF10A1[b]	-	–	–	+	–	+	+
MCF10A1neoT[b]	–	–	–	+	–	+	+
MCF10A1T3B[b]	+/–	–	–	+	–	+	+
TM3[c]	–	–	ND	+	–	+	ND
T-2410L TM6[c]	+	–	ND	+	–	+	ND
SP1[d]	+	+	+	+	+	++	ND
SP1- 3M[d]	+	+	+	+	+	++	ND

Legend:

a) EL-E, HU-E and WO-E are human breast carcinoma cell lines derived from human breast cancer patients (obtained from B. Campling).

b) MCF10A1 cells are subclones of a spontaneously immortalized non-tumorigenic human breast epithelial cell line establised from a long term culture of a breast subcutaneous mastectomy. MCF10A1neoT are a tumorigenic cell line derived following transfection of MCF10A1 with *Ha-Ras*. MCF10A1T3B is a cell line derived from MCF10A1neoT tumor growing in nude mice (obtained from F. Miller).

c) TM3 is a Balb/c mouse-derived mammary epithelial cell line (obatined from D. Medina, Baylor College). T-2410L TM6 is a malignant breast epithelial cell line derived from TM3.

d) SP1 is a murine mammary carcinoma which arose spontaneously in a CBA female mouse. SP1-3M is a highly metastatic variant subclone of SP1 selected by serial passage of a lung metastatic nodule into the mammary fat pad.

e) Scatter activity of conditioned media from cell lines was tested on MDCK epithelial cells.

f) Tyrosine phosphorylation of Met was used as an indicator of Met activation. ND, not done.

TABLE III

Expression of HGF and HGF-receptor/Met in Human Carcinomas

SCLC Cell Line	HGF Secretion	Met Expression
AD-A	–	–
BK-T	–	–
CK-A	–	–
GL-E	–	–
H69	–	–
H69AR	–	–
H128	–	–
H209	–	–
HA-E	–	–
HG-E	–	–
JN-M	–	–
JO-E	–	–
JS-E	–	–
LD-T	–	–

SCLC Cell Line	HGF Secretion	Met Expression
LG-T	–	–
LV-E	–	–
Mar	–	–
MM-I	–	–
MO-A	–	–
OS-A	–	–
RG-I	–	–
SHP-77	–	–
SM-E	–	–
SV-E	–	–
TY-E	–	–
WL-E	–	–
YR-A	–	–

NSCLC Cell Line	HGF Secretion	Met Expression
BH-E	–	–
FR-E	–	–
LC-T	–	–
QU-DB	–	–
SK-LC6	–	–
SK-MES	–	–
SW-900	–	+
WT-E	–	+

Legend:

A series of lung carcinoma cell lines were derived from pleural exudates from lung carcinoma patients. These cell lines were kindly provided by Dr. B. Campling. SCLC; small cell lung carcinoma. NSCLC, non-small cell lung carcinoma. ND, not done.

FIGURE LEGENDS:

Figure 1: Moderately (A-C) and well (D) differentiated ductal carcinomas: Panel A: ISH for HGFR mRNA showing intense cytoplasmic positivity at the advancing margin of the tumor (Original magnification, X630). Panel B: ISH for HGFR mRNA showed much weaker staining in the malignant epithelium in more central regions of the same tumor (Original magnification, X250). Panel C: ISH for HGF mRNA showing strong cytoplasmic positivity of the malignant epithelium at the advancing margin of the same tumor, in the same region which stained intensely for HGFR mRNA (Original magnification, X630) (cf. Fig. 2A). In addition, stromal fibroblasts and endothelial cells showed some cytoplasmic positivity. Panel D: ISH analysis for HGFR mRNA showing IDC surrounding a benign duct. Intensity of cytoplasmic staining is similar in both benign and malignant epithelium, regardless of whether the malignant epithelium forms well-defined ductular structures (Original magnification, X400).

Figure 2: Purification of mouse HGF from 3T3-L1 adipocyte CM: 3T3-L1 adipocyte CM (40 ml), which contains HGF (3), was concentrated to 2 ml using Centricon 10 tubes, and loaded onto a Cu(II)-affinity column. The column was rinsed with equilibration buffer (10 ml). Protein samples were sequentially eluted in 10 ml fractions with equilibration buffer plus 5 mM imidazole (5 mM IM) and 10 mM imidazole (10 mM IM). After concentration of eluates, aliquots corresponding to half of each fraction were separated by 10% SDS-PAGE (non-reducing conditions) and subjected to: silver staining (Panel A), and western blot analysis with anti-HGF IgG (Panel B). Protein molecular weight standards are shown on the right. Tracks from left: 3T3-L1 CM (20 ml equivalent), HGF (0.05 μg), flow through (F.T.), wash, 5 mM IM and 10 mM IM fractions.

Human recombinant HGF was eluted from the Cu(II)-affinity column at 20 mM imidazole.

Figure 3: Detection of HGF protein in SP1 cells: Panel A: *In vitro* translation of HGF RNA transcript: Poly $(A)^+$ RNA was extracted from SP1 cells, translated in a rabbit reticulocyte system and analyzed by western blotting. None, SP1, and Control correspond to: no added RNA, 2 μg RNA from SP1 cells, and unrelated RNA supplied by Amersham as a negative control, respectively. The membrane was probed with polyclonal rabbit anti-HGF IgG and protein bands were detected by ECL.

Panel B: SP1 CM and SP1-3M CM (25 ml each) were concentrated in Centricon-30 tubes, electrophoresed through 10% SDS-PAGE under non-reducing or reducing conditions as indicated, and blotted onto nitrocellulose membrane. The membrane was probed with polyclonal rabbit anti-HGF IgG.

Panel C: Metabolic labeling of SP1 cells: Cells ($5x10^6$) were metabolically labeled with $[^{35}S]$-methionine for 18 h as described in Materials and Methods. CM from SP1 cells (10 ml) was immunoprecipitated with anti-HGF antibody, analyzed by SDS-PAGE and autoradiographed. Protein molecular weight standards are shown on the right.

HGF protein was translated from HGF mRNA purified from SP1 cells, and was secreted into SP1 cell conditioned medium.

Figure 4: Panel A: Detection of HGF receptor/Met in human breast carcinoma cell lines: Cell lysates from three brest carcinoma cell lines EL-E, MCF10AT3B and WO-E were immunoprecipitated using a monoclonal anti-Met IgG. Immunoprecipitates were separated by 10% SDS-PAGE under reducing conditions, and transferred to nitrocellulose membrane. Immunocomplexes were detected by ECL. The membranes were probed with a polyclonal rabbit anti-Met IgG. Molecular weight standards are shown on the right.

A 145 kDa protein was detected, corresponding to HGF receptor/Met, in EL-E and MCF10AT3B but not WO-E.

Panel B: Activation of HGF receptor/Met in human breast epithelial cell lines: Human mammary epithelial cells (MCF10A1) and MCF10AneoT cells (transfected with pSV40neo) were serum-starved for 24 h, and then incubated for 1 h with serum-free growth media (control), HGF (20 ng/ml), or monoclonal anti-Met activating IgG (1 μg/ml). Cell lysates were collected and immunoprecipitated with anti-phosphotyrosine IgG. Immunoprecipitates were subjected to 10% SDS-PAGE and transferred onto nitrocellulose membrane. The membranes were probed with polyclonal rabbit anti-Met antibody and immune complexes were detected by ECL. Molecular size standards are indicated on the right.

Met showed no tyrosine phosphorylation in control cells but became tyrosine phosphorylated following incubation with HGF or anti-Met activating IgG.

Figure 5: E-cadherin expression is downregulated in regions of invasive human breast carcinoma and this downregulation correlates with strong expression of HGF receptor/Met: A series of 12 infiltrating ductal carcinomas of human breast were examined for expression of E-cadherins and HGF receptor/Met using immunoperoxidase staining of paraffin-embedded serial tissue sections. Anti-E-cadherin IgG was from UBI. Anti-human Met IgG was provided by M. Park (McGill U.) Invasive regions of infiltrating ductal carcinoma and adjacent nonmalignant ductal epithelium were examined.

Panel A: Nonmalignant epithelium stained with anti-E-cadherin IgG.
Panel B: Nonmalignant epithelium stained with anti-Met IgG.
Panel C: Region of DCIS stained with anti-E-cadherin IgG.
Panel D: Region of IDC stained with anti-E-cadherin IgG.
Panel E: Region of IDC stained with anti-Met IgG.

Expression of E-cadherin is markedly reduced in regions of invasive breast carcinoma compared to adjacent nonmalignant ductal epithelium, although strong expression of HGF receptor/Met expression was detected in both regions. Regions of DCIS showed cytoplasmic expression of E-cadherin; however there was very little membrane staining. Met expression in DCIS was heterogeneous (data not shown).

FIGURE 1

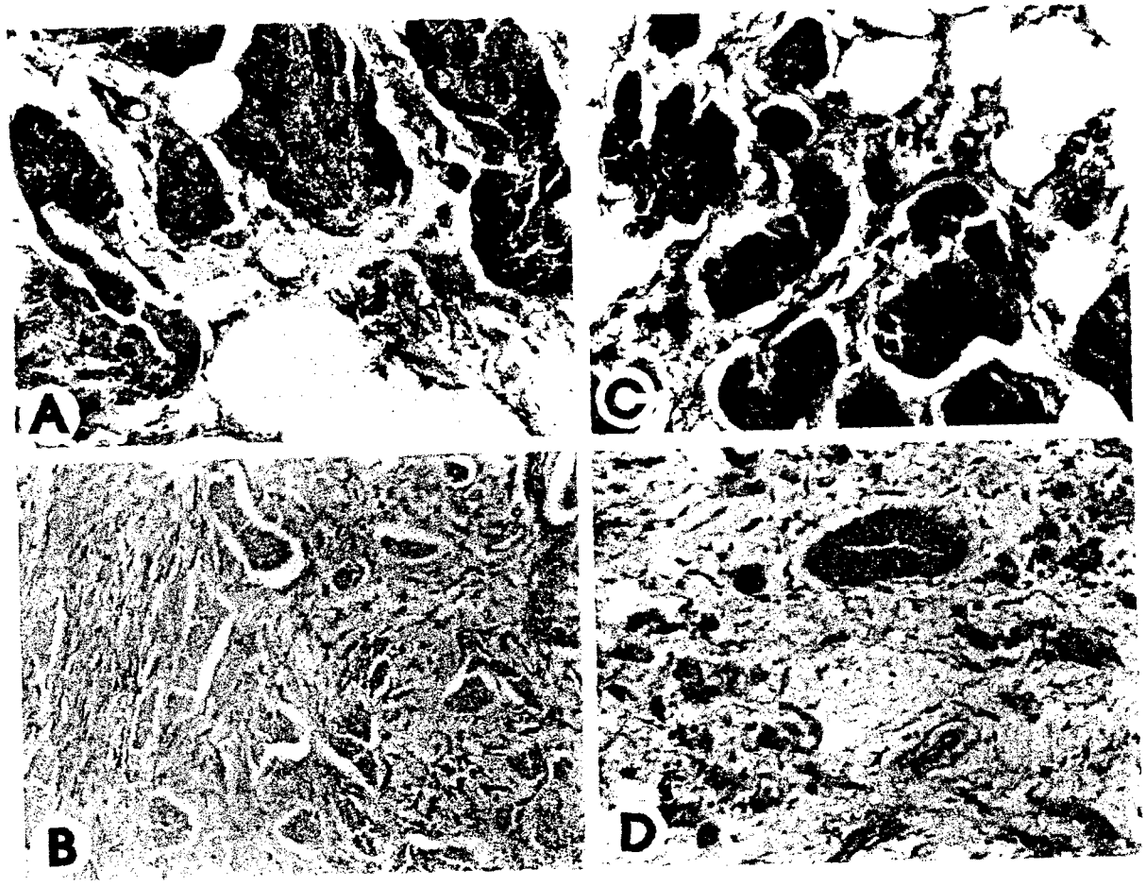
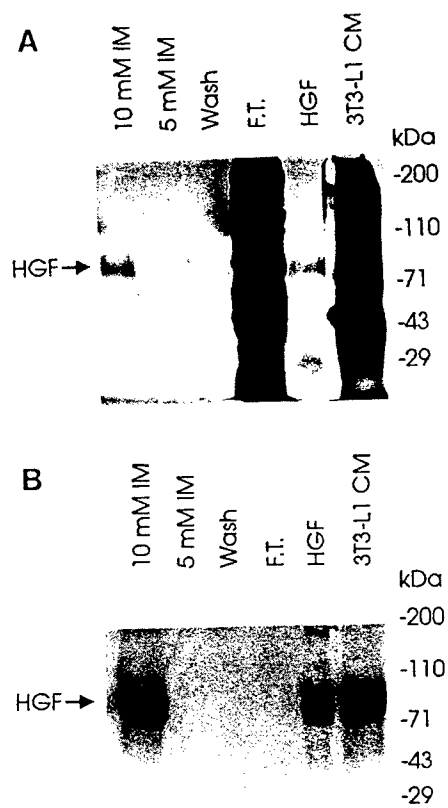


FIGURE 2



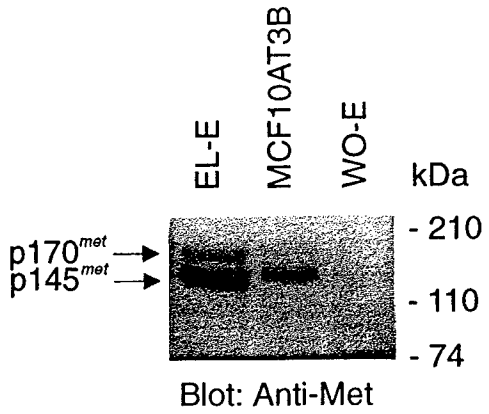
Blot: Anti-HGF

FIGURE 3


A
None
SP1
Control
kDa
- 210
- 110
- 74
- 45
HGF
Blot: anti-HGF
B
SP1
SP1-3M
Non-reduced
Reduced
Non-reduced
kDa
- 214
- 110
- 74
- 45
- 29
HGF
α-HGF
Blot: Anti-HGF
C
kDa
- 210
- 110
- 74
- 45
HGF
Pulse Time (h) 0 18

FIGURE 4

A


EL-E
MCF10AT3B
WO-E
kDa
p170met
p145met
- 210
- 110
- 74
Blot: Anti-Met

B

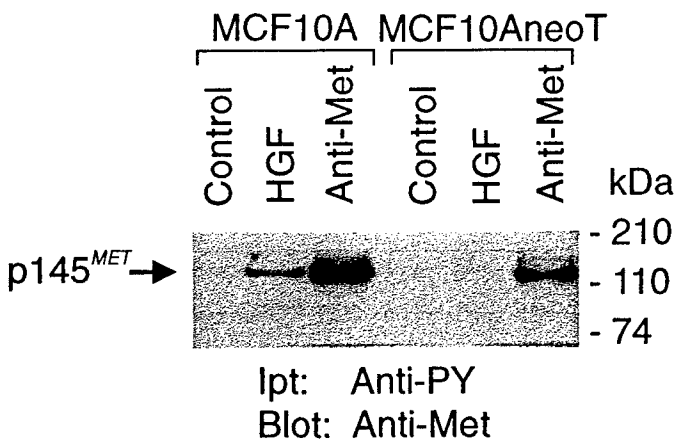
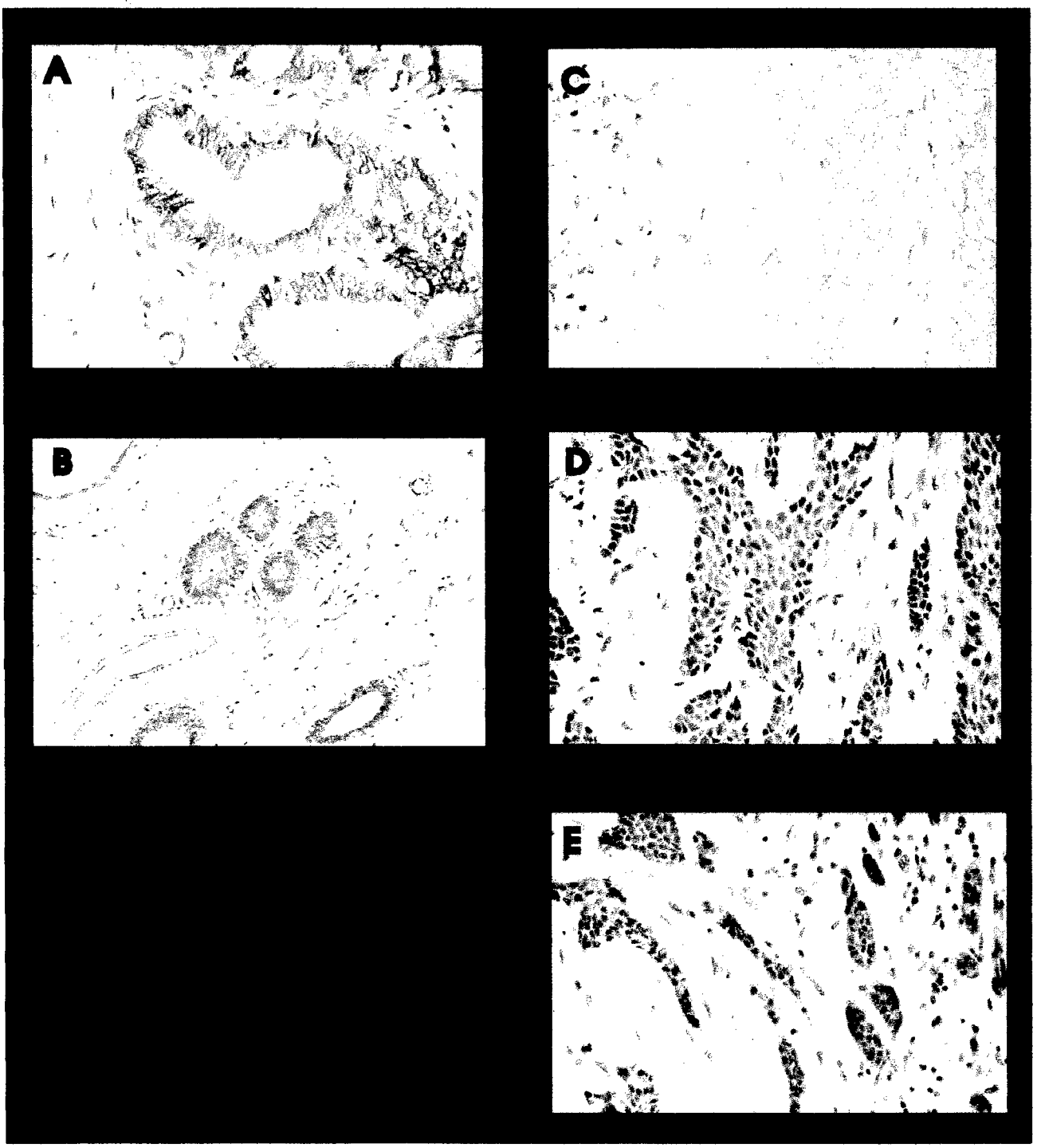

MCF10A
MCF10AneoT
Control
HGF
Anti-Met
Control
HGF
Anti-Met
kDa
p145MET
- 210
- 110
- 74
Ipt: Anti-PY
Blot: Anti-Met

FIGURE 5



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Localization of Platelet-derived Growth Factor β Receptor Expression in the Periepithelial Stroma of Human Breast Carcinoma[1]

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ABSTRACT

Platelet-derived growth factor (PDGF) BB is secreted by most human breast carcinoma cells; however, only recently have PDGF β receptors been demonstrated in malignant breast tissue. In the present study, the tissue localization of PDGF β receptor expression was studied in human breast carcinoma and nonmalignant breast tissues stained using both immunofluorescence and immunoperoxidase techniques. We examined a total of 29 cases of breast carcinomas, which showed both *in situ* and invasive components. PDGF β receptor staining was localized in the periepithelial stroma and was particularly intense in regions immediately adjacent to carcinoma *in situ* components in all tumors examined. A diffuse low level of PDGF β receptor staining was seen throughout the stroma of eight nonmalignant breast tissues as well as of nonmalignant regions of tumor tissues. Image analysis was used to assess the coincidence of staining of PDGF β receptor with epithelial or stromal cells in 13 of the 29 tumor tissues studied. Less than 5% of malignant ductal epithelium or myoepithelium showed PDGF β receptor staining. Analysis with stromal cell type-specific markers indicated significant localization of PDGF β receptor primarily within α smooth muscle actin-staining cells (32%) and vascular endothelial cells (41%) in the periepithelial stroma. PDGF β receptor positivity was strongly associated with periepithelial stromal cells adjacent to the basement membrane surrounding regions of carcinoma *in situ* but was less intense in regions of invasive carcinoma where basement membrane was degraded. The absence of PDGF β receptors on carcinoma cells and their presence in the surrounding stroma suggest a paracrine stimulation of adjacent stromal tissue by malignant epithelial cells in human breast tumors.

INTRODUCTION

Growth factors which act in a paracrine or autocrine manner are important regulators of stromal-tumor interactions in breast cancer. Stromal fibroblasts and adipocytes secrete growth factors, such as keratinocyte-derived growth factor (1, 2) and HGF[5](3), which can stimulate in a paracrine manner growth and invasiveness of mammary carcinoma cells. In addition, stimulation of stromal cells by carcinoma cells has recently been shown to induce expression of stromal proteinases (4, 5), extracellular matrix proteins [*e.g.*, tenascin (6) and thrombospondin (7)], and certain growth factors [*e.g.*, HGF (8)]. However, the tumor-derived stimulatory components involved are not well understood.

PDGF BB, which is up-regulated in the majority of breast cancer tissues (9–11) and in breast carcinoma cell lines (12–14), is an important candidate molecule in the paracrine stimulation of the stroma by carcinoma cells. PDGF BB has been shown to stimulate a variety of functions including growth (15), chemotaxis (16), and fibronectin matrix assembly (17) in connective tissue cells. PDGF is composed of two chains, A and B, held together by disulfide bonds and present in three dimeric forms AA, AB, and BB (18, 19). The dimers bind to two separate receptor types: α and β (18, 19). The α receptors bind to all three isoforms of PDGF, while the β receptors have a very high affinity for PDGF BB, bind PDGF AB to some extent, but do not bind PDGF AA at all. The β receptor has a ligand-activated protein kinase (20); the receptor is autophosphorylated in response to the appropriate ligand. Each PDGF chain is encoded by a distinct gene. The gene encoding PDGF B has been identified as the normal mammalian analogue of the v-*sis* oncogene (19, 21, 22). Fibroblast cells transfected with the *PDGF B* gene showed a higher transforming activity than cells transfected with the *PDGF A* gene (23). The PDGF β receptor may therefore represent an important regulatory pathway in breast cancer.

PDGF β receptor mRNA and protein have been found on most stromal cell types including fibroblasts (24), smooth muscle cells (16), endothelial cells (25), and pericytes (26). Recently, some breast carcinoma cell lines (27), as well as breast

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[5] The abbreviations used are: HGF, hepatocyte growth factor; DCIS, ductal carcinoma *in situ*; ISH, *in situ* hybridization; PDGF, platelet-derived growth factor; α-SMA, α-smooth muscle actin; CALLA, common acute lymphoblastic leukemia antigen.

Table 1 Relative PDGF β-receptor expression in the periepithelial stroma and the remote stroma of nonmalignant and malignant breast tissue

Tissue[a]	n[b]	Periepithelial syroma[c]	Remote stroma[c]
Nonmalignant	14	0.73 ± 0.08	0.71 ± 0.07
Malignant	26	1.00[d] ± 0.01	0.70 ± 0.08

[a] Tissue sections were stained with monoclonal mouse anti-PDGF β receptor antibody and biotinylated goat antimouse IgG detected with rhodamine-conjugated avidin, as described in Fig. 1. Tissues were viewed under epifluorescence illumination and photographed with T-MAX Kodak film.

[b] Number of tissue sections per group analyzed. A total of five nonmalignant lesions and six tumors with regions of DCIS was analyzed.

[c] Image analysis was carried out to determine the ratio of mean intensity of total PDGF β receptor staining to that of the periepithelial stroma or of the remote stroma (*i.e.*, distant from epithelium). A total of 25 comparisons per tissue section were carried out. Results are expressed as mean ± SD.

[d] Significantly greater intensity of PDGF β receptor staining in the periepithelial stroma than in the remote stroma in malignant breast tissue ($P < 0.05$ as determined by ANOVA).

cancer tissues (11, 28, 29), have also been shown to express PDGF β receptors. In breast cancer tissues, PDGF β receptors were expressed primarily in the stroma of normal and malignant breast tissue, not on nonmalignant epithelial cells and only on a few malignant epithelial cells (11). However, it is not known what stromal cell types in breast cancer express PDGF β receptors.

In the present study, we examined the presence of PDGF β receptors in human malignant and nonmalignant breast tissues, and used double immunofluorescence and ISH techniques to localize their expression in specific cell types. Double immunofluorescence was studied using image analysis to quantify the degree of overlap of PDGF β receptor-stained areas with areas stained with antibodies specific for cell types. The results showed greater expression of PDGF β receptor in the periepithelial stroma of regions of DCIS than in stroma remote from epithelial cells. We therefore used specific markers for epithelium (cytokeratin), myofibroblasts and myoepithelial cells (α-SMA; Ref.30), and endothelial cells (PAL-E; Ref. 31) to determine what cell types were involved. This study provides novel information about tissue localization of PDGF β receptor in breast cancer tissue and about possible cell targets for stimulation by PDGF BB.

MATERIALS AND METHODS

Tissues. Tissue from surgical breast biopsies from patients with suspected breast cancer were snap frozen in liquid nitrogen within 20 min after acquisition and stored at −70°C. A total of 27 ductal carcinomas with both *in situ* (DCIS) and invasive (IDC) components, 1 infiltrating lobular carcinoma, and 1 pure DCIS were analyzed. Of the 27 ductal carcinomas examined, 13 showed positive lymph node involvement, and the 1 infiltrating lobular carcinoma was lymph node positive. Nonmalignant breast tissues were obtained from the grossly normal regions surrounding the tumors and from eight nonmalignant breast lesions. Cryostat sections (6 μm) were fixed with cold (4°C) chloroform:acetone (50:50) for 5 min and stored at −70°C for future staining.

Immunofluorescence and Immunoperoxidase Staining. For simultaneous detection of PDGF β receptor and cell-type markers, we used double immunofluorescence staining as described by Sundberg *et al.* (32). This technique allows the simultaneous use of two different primary antibodies. It involves a Texas red avidin detection of a biotinylated secondary antibody in the first step and a FITC-conjugated secondary antibody in the second step. The concentrations of each secondary antibody were preselected such that access of the second FITC-conjugated secondary antibody to the first mouse monoclonal is inhibited by a relative excess of the first biotinylated secondary antibody. The specificity of the staining was confirmed by exchange of primary antibodies with normal mouse IgG and switching of secondary biotinylated and FITC-conjugated antibodies. Staining reactivity of the individual antibody markers, when used alone, did not differ from the reactivities obtained with the same antibodies used in double immunofluorescence. Additionally, in regions of tissue showing double staining, the texture of staining with each antibody was clearly different: PDGF β receptor showed a punctate staining pattern, whereas cell markers showed a continuous, more intense staining pattern.

Staining was carried out as follows. To block endogenous biotin in mammary tissue sections, an avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA) was used. Nonspecific binding sites were blocked with normal horse serum (1:5 dilution). For detection of PDGF β receptors, tissue sections were incubated with mouse monoclonal anti-PDGF β receptor antibody PDGFR-B2 (Ref. 33; kindly provided by Dr. K. Rubin, Uppsala, Sweden) at 1 μg/ml for 1 h, followed by biotinylated horse antimouse IgG (1:200 dilution; Vector Laboratories) and Texas red-conjugated avidin (1:100) or horse radish peroxidase-conjugated avidin (Vector Laboratories). For immunoperoxidase staining, the immunoreaction was detected using a Vector Laboratories staining kit.

For staining cell markers or collagen type IV, nonspecific sites for the second antibody were blocked with normal goat or normal sheep sera (1:5 dilution). The second antibodies used were: (*a*) mouse monoclonal antibody (CAM 5.2) against human cytokeratins 8 and 18 (Becton Dickinson) used at 25 μg/ml to stain epithelium; (*b*) monoclonal anti-α-SMA antibody 1A4 (Sigma Chemical Co.) used at a 1:400 dilution to stain smooth muscle-related components such as myoepithelium and myofibroblasts (30); (*c*) monoclonal antibody PAL-E (Monosan) used at a 1:12 dilution to stain nonarterial capillary endothelial cells (31); (*d*) polyclonal rabbit anti-CALLA antiserum A12 (Ref. 34; kindly provided by S. Carrel, Bern, Switzerland) used at a 1:25 dilution to stain myoepithelial cells; and (*e*) goat anti-collagen type IV IgG used at 25 μg/ml (Southern Biotechnologies Associated). All second antibodies were incubated with tissues for 30 min; binding of second antibodies was detected by affinity-purified FITC-labeled goat antimouse or antirabbit IgG and sheep antigoat IgG used at 1:100 dilution. All washes were done with PBS. Slides were mounted with Fluoromount-G (Southern Biotechnologies Associated) and were examined under a Leica fluorescence microscope equipped with an Hb 100 light source. Each field was viewed with an I3 filter for FITC illumination and an N2.1 filter for Texas red illumination. H&E staining of

Table 2 Localization of PDGF β receptors with various cell markers in human breast carcinomas[a]

% of cell marker-positive pixels coincident with PDGF β receptor[b]			
Cytokeratins 8 and 18 (epithelium)	CALLA (myoepithelium)	α-SMA (smooth muscle-related)	PAL-E (endothelium)
2.6 ± 4.4 (9)	4.9 ± 3.8 (7)	31.9 ± 25.2 (10)	41.0 ± 20.2 (13)

[a] Frozen sections (6 μm) of malignant human breast biopsies were stained using double immunofluorescence with anti-PDGF β receptor antibody and antibody against either cytokeratins 8 and 18 (epithelial cells), CALLA (myoepithelial cells), α-SMA (smooth muscle-related cells), or PAL-E (nonarterial endothelial cells). See "Materials and Methods" for antibody details.

[b] Slides were photographed under epifluorescence illumination for Texas red (PDGF β receptor) or FITC (cell markers). The total area in pixels of Texas red and FITC positivity in each section was independently determined using an image analysis system. The percentage of double-positive pixels divided by the total FITC-positive pixels is shown. Values represent the mean ± SD. Number in parentheses, number of tumors analyzed for each cell marker.

the frozen sections was performed to assess tissue morphology. The endothelial nature of the PAL-E-positive cells was confirmed by staining of adjacent sections with polyclonal rabbit antisera against anti-factor VIII-related antigen at a 1:800 dilution (DAKO Corp.). The results showed a high coincidence of staining of the two markers in endothelial cells of the microvasculature (Fig. 4) similar to previous reports (31, 32).

Image Analysis. Images of identical microscopic fields were photographed under illumination for Texas red or FITC with TMAX 400 film. Black and white negatives of the FITC and Texas red fluorescence were analyzed using an M2 Image Analysis software program (Image Analysis Systems, Brock University, Ontario, Canada). To compare staining of PDGF β receptor in different regions, the ratio of the mean fluorescence intensity of the total positive target to that of the periepithelial stroma or of the remote stroma (*i.e.*, distant from epithelium) was calculated (Table 1). To determine the proportion of tissue area stained with both PDGF β receptor and a specific cell marker, the total number of pixels positive for FITC and Texas red fluorescence in an identical field was calculated. The overlap between FITC- and Texas red-positive pixels was then calculated when the two fields were superimposed. The proportion of pixels corresponding to double staining was expressed as the percentage of double-positive (FITC + Texas red) pixels divided by the total FITC-positive pixels (Table 2).

ISH. ISH was performed by a modification of the procedure of Yang and Park (35). Tissues were fixed in 4% paraformaldehyde, processed in alcohol, and embedded in paraffin. Sections (6 μm) were dewaxed in toluene and rehydrated. Permeabilization was performed by treating with 0.2 M HCl, 0.2% Triton X-100 in PBS, and 40 μg/ml proteinase K. Slides were then washed in 0.1× PBS, refixed in 4% paraformaldehyde, washed again in 0.1× PBS, and acetylated using 0.25% acetic anhydride in 0.1 M triethanolamine HCl. Slides were then dehydrated, delipidated in 100% chloroform followed by absolute ethanol and 95% ethanol, and then air dried.

Digoxigenin-labeled RNA riboprobes were synthesized from a plasmid containing a 1-kb *Pst*I fragment of cDNA corresponding to the external domain of the PDGF β receptor (36). The probe was diluted in prehybridization mix at 15 ng/μl, and hybridization mix (200 μl) was applied to each section. Slides were incubated at 42°C overnight, followed by washing in 1× SSC, rinsing in RNase buffer [0.5 M NaCl, 10 mM PIPES (pH 7.2), and 0.1% Tween 20), and digesting unbound single-stranded RNA by incubation in 20 μg/ml RNase A (Sigma). Slides were then washed in buffer 1 (100 mM Tris-HCl and 150 mM NaCl, pH 7.5) and blocked with 3% normal sheep serum in buffer 1. To detect specific hybrids, slides were incubated with antidigoxigenin antibody conjugated to alkaline phosphatase (1:1000; Boehringer Mannhein), then washed twice with buffer 1, and twice in buffer 2 [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 50 mM $MgCl_2$). Hybrids bound to antidigoxigenin antibody were then visualized by a color reaction containing nitro blue tetrazolium salt, 5-bromo-4-chloro-3-indolyl-phosphate, and levamisole (0.24 mg/ml) in buffer 2 (kit from Vector Laboratories). Slides were then dehydrated, washed in xylene, mounted with permount, and viewed and photographed under bright field illumination with a Leica microscope. RNA specificity of probe binding was tested by treating control slides with 20 μg/ml RNase A in RNase buffer at 37°C prior to hybridization. Specificity of binding was also verified by the pattern of expression in tissues known to express PDGF β receptor [*e.g.*, skin, placenta (48)], and by comparison with tissue compartment-specific expression of other positive control probes [*e.g.*, HGF receptor (Met) expression (47)].

RESULTS

PDGF β Receptor-expressing Cells Are Localized in the Periepithelial Stroma in Regions of Mammary DCIS. Our initial approach was to compare the tissue localization of PDGF β receptor protein in 29 breast tumor tissues with that in nonmalignant breast tissues. Immunofluorescence staining revealed intense PDGF β receptor protein expression localized in the periepithelial stroma, particularly in regions of DCIS (Fig. 1*F*). A similar pattern of staining was observed when tissues were stained using an immunoperoxidase technique (data not shown). ISH analysis showed that cells in the periepithelial stroma and adjacent capillary endothelium of carcinoma *in situ* express PDGF β receptor mRNA (Fig. 2*A*, *C*, and *D*). An RNAse-treated adjacent section showed no specific staining (Fig. 2*B*). In contrast to malignant breast, regions of nonmalignant breast tissue showed a less intense, more diffuse staining pattern of PDGF β receptors in the stromal region (Fig. 1*C*).

To quantify the relative amount of PDGF β receptor protein staining in the periepithelial stroma and in the stroma remote from epithelial cells, we carried out image analysis of malignant (DCIS) and nonmalignant tissue sections stained with

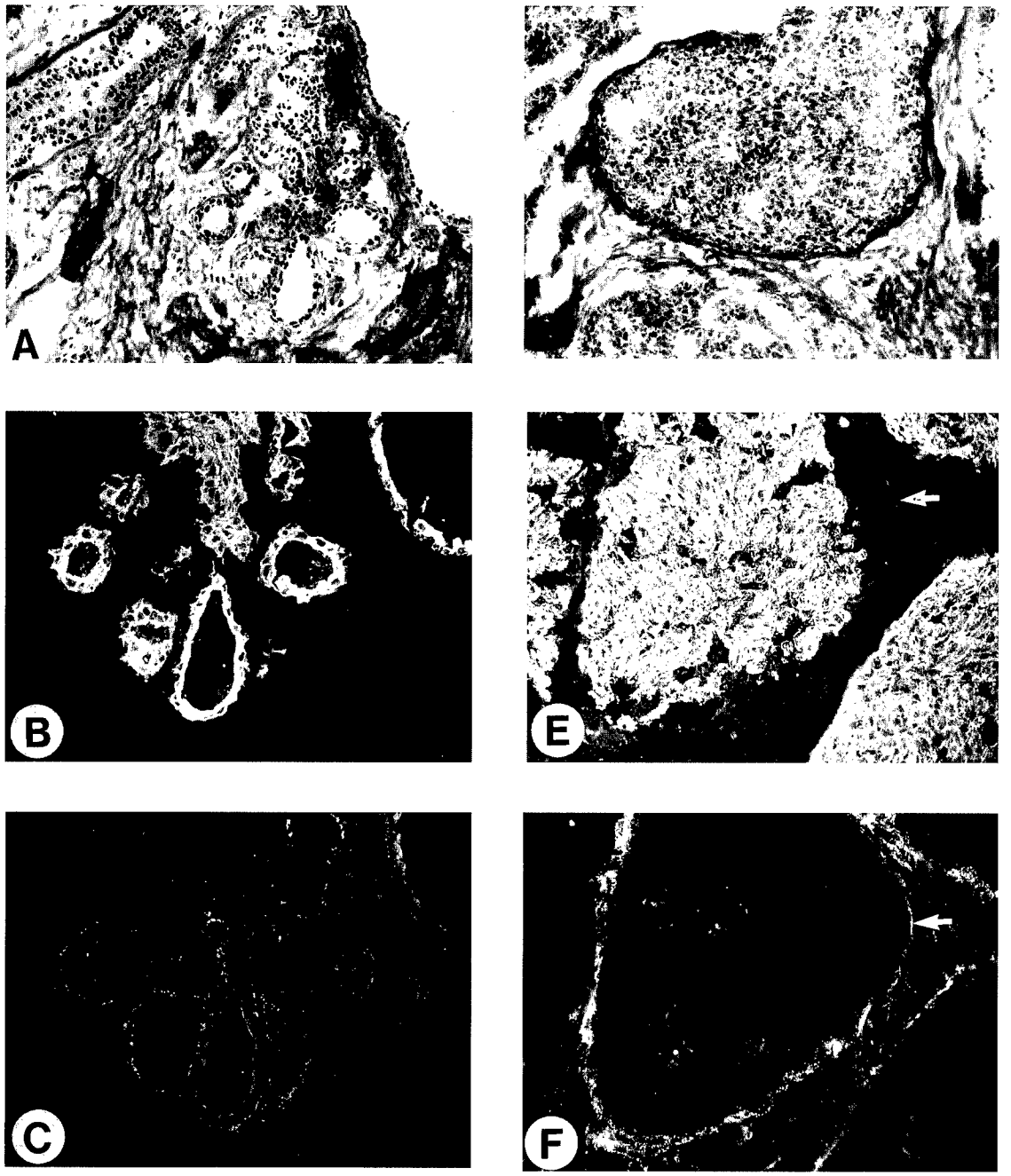

Fig. 1 Localization of cytokeratins 8 and 18 and PDGF β receptor expression in nonmalignant tissue (*A-C*) and DCIS (*D-F*). Sections (6 μm) from malignant and nonmalignant breast tissue were stained using H&E to identify tissue morphology (*A* and *D*). Adjacent sections were also stained using double immunofluorescence as follows: with monoclonal anticytokeratin 8 and 18 antibody detected by FITC-conjugated antimouse IgG to identify epithelial cells (*B* and *E*) and with monoclonal anti-PDGF β receptor antibody detected by biotinylated antimouse IgG and Texas red-avidin (*C* and *F*), as described in "Materials and Methods." The same fields were viewed under FITC illumination (*B* and *E*) and Texas red illumination (*C* and *F*). *Arrows*, regions of PDGF β receptor staining in *F* and lack of keratin staining in the corresponding site in *E*. *A*, ×250 and *B-F*, ×400.

anti-PDGF β receptor antibody (Table 1). The results showed that most PDGF β receptor staining of malignant tissue was localized in the periepithelial stroma. The mean fluorescence intensity of PDGF β receptor staining of the remote stroma was significantly less than that of the periepithelial stroma. In nonmalignant regions, both the periepithelial and the remote stroma showed a mean fluorescence intensity which was significantly less than that of the mean intensity of the PDGF β receptor-

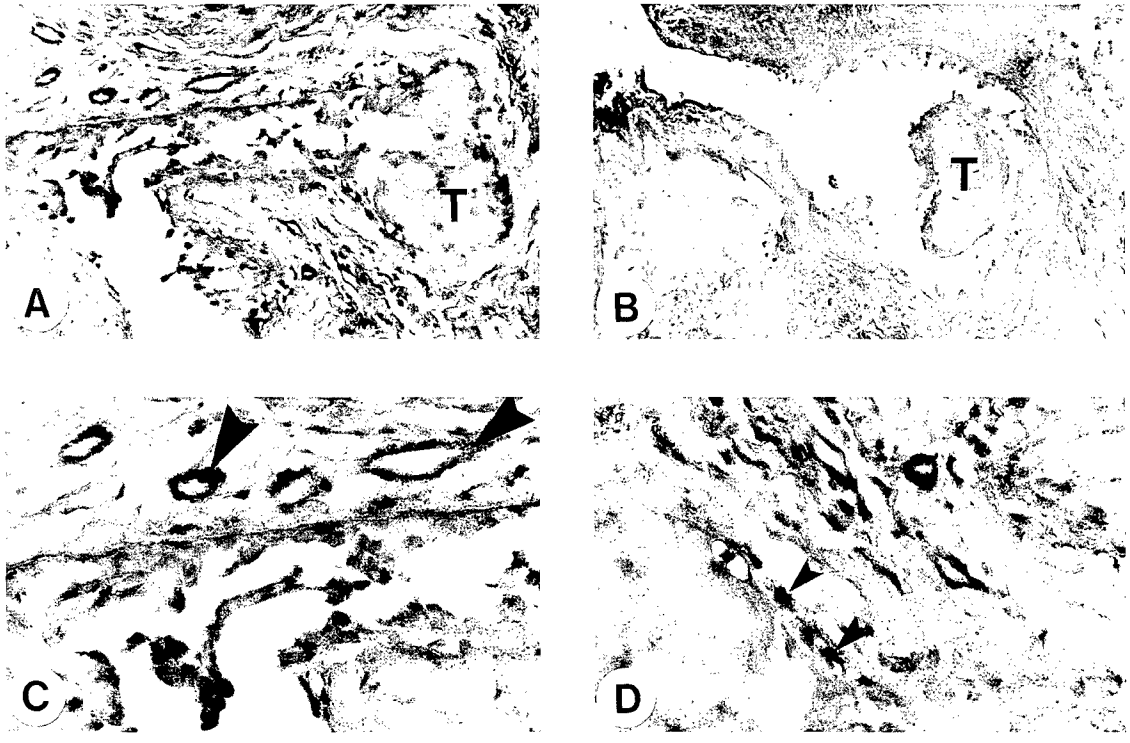

Fig. 2 ISH analysis of PDGF β receptor mRNA expression in DCIS. Breast tissue with DCIS was analyzed for PDGF β receptor mRNA using ISH, as described in "Materials and Methods." *A*, ISH analysis of PDGF β receptor mRNA (×250); *B*, ISH analysis with the same PDGF β receptor probe of an adjacent section treated with RNAse (×250); *C* and *D*, high magnification (×1000) fields of *A*. The results showed strong expression of PDGF β receptor mRNA in capillary endothelia (*large arrowheads*) and in the periepithelial stroma (*small arrowheads*). *T*, tumor tissue.

positive target area. Thus, PDGF β receptor expression was localized to the periepithelial stroma in regions of DCIS and was more diffuse throughout the stroma in nonmalignant breast tissue.

PDGF β Receptor Is Expressed by α-SMA-positive Cells and Endothelial Cells in the Periepithelial Stroma of Breast Cancer Tissues. To identify the stromal cell type(s) expressing PDGF β receptors, we carried out a double staining procedure with anti-PDGF β receptor antibody and antibodies against cytokeratins 8 and 18 (epithelial cells; Fig. 1), α-SMA (myoepithelium, myofibroblasts, or pericytes; Fig. 3), or PAL-E (nonarterial capillary endothelial cells; Fig. 4). Controls with no primary antibody showed very low background staining (Fig. 3, *C* and *E*; data not shown). Image analysis revealed <5% overlap of the cytokeratin-positive cells with PDGF β receptor-staining regions (Table 2). In contrast, an average of 32% of the α-SMA-staining areas in all tumors examined showed PDGF β receptor positivity, particularly surrounding regions of DCIS. In addition, strong colocalization (averaging 41%) of PAL-E-staining areas with PDGF β receptor-staining areas was also observed in both regions of DCIS and invasive carcinoma. The pattern of PAL-E-positive staining was very similar to factor VIII-stained vascular endothelium (Fig. 4, *B* and *C*), and a significant presence of microvessels was evident in the adjacent stroma (Figs. 2 and 4, *B* and *C*). No significant coincidence of PDGF β receptor staining with CALLA-expressing myoepithelial cells was detected (Table 2). We conclude from these studies that PDGF β receptor is present in smooth muscle-related cells (myofibroblasts or pericytes) and nonarterial capillary endothelial cells in the periepithelial stroma of malignant breast tissue.

PDGF β Receptor-expressing Cells Are Associated with Basement Membrane. To further assess the anatomical location of PDGF β receptor-staining cells within the gland, we determined their position with respect to basement membrane. Double immunofluorescence staining for PDGF β receptor and collagen type IV, a prominent basement membrane protein, was therefore carried out. The results showed close association of collagen type IV (Fig. 5*B*) and PDGF β receptor staining (Fig. 5*C*) in the periepithelial regions of all DCIS tumors examined. These results indicate that PDGF β receptor-positive cells are associated with basement membrane structures. Basement membrane of invasive regions became fragmented and disorganized (Fig. 5, *E* and *F*). In these invasive regions, PDGF β receptor expression was detected primarily in cells associated with remaining basement membrane fragments, but was decreased or absent in regions where basement membrane was degraded (Fig. 5*F*).

DISCUSSION

PDGF BB is secreted by many breast cancer cell lines and has recently been shown to be up-regulated in breast cancer (9–11). However, only recently have PDGF β receptors been demonstrated in malignant tissue (11). In the present report, we showed that PDGF β receptor protein is essentially absent in both nonmalignant and malignant breast epithelial cells. How-

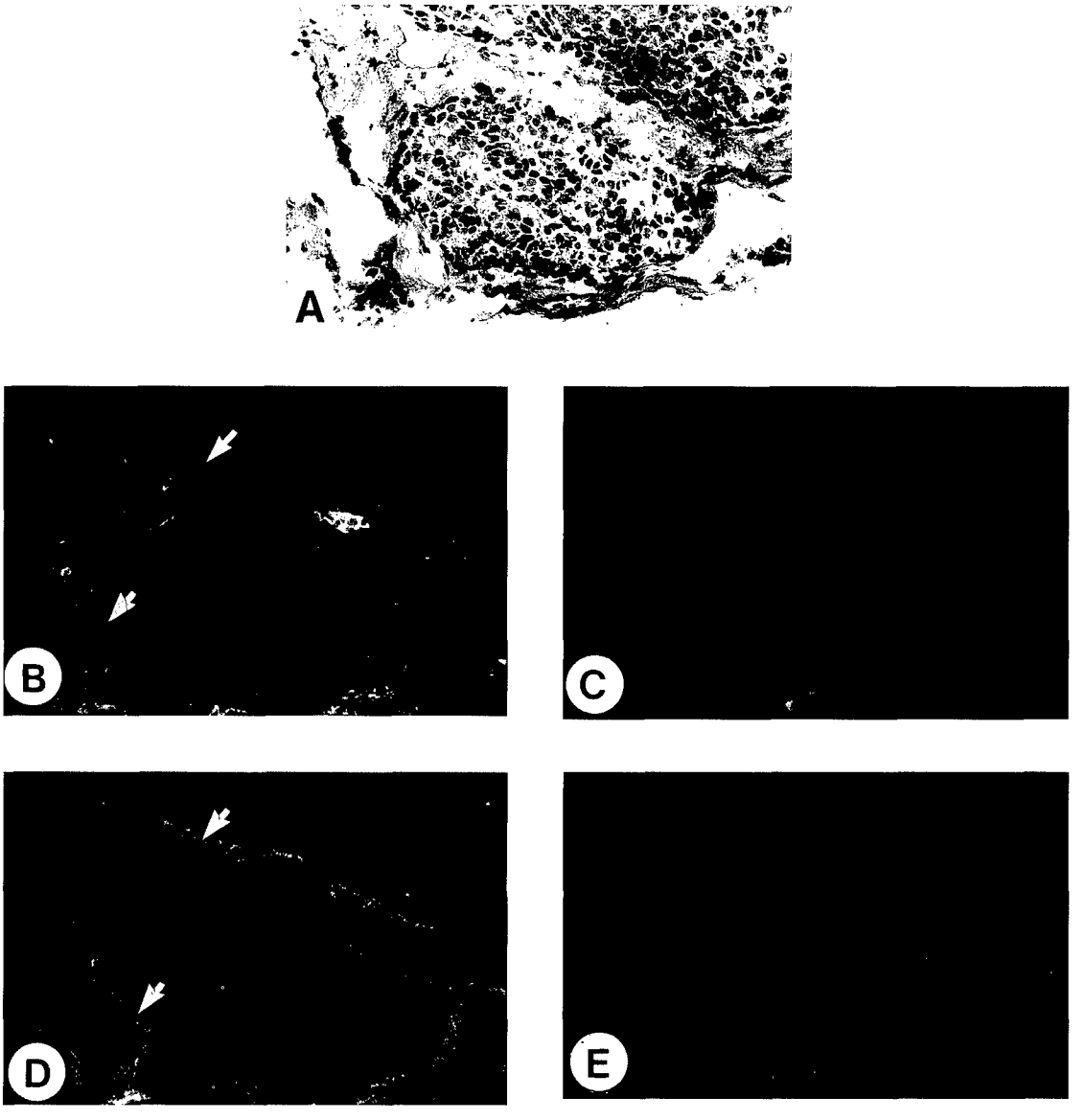

Fig. 3 Localization of α-SMA and PDGF β receptor expression in DCIS. A breast tumor tissue section showing DCIS was stained using H&E (*A*) to identify tissue morphology. An adjacent section was also stained using double immunofluorescence with monoclonal anti-α-SMA antibody (*B*), which identifies myofibroblasts, myoepithelial cells, and pericytes, and with monoclonal anti-PDGF β receptor antibody (*D*), as described in Fig. 1. *Arrows*, regions of double staining. Controls with no primary antibody for FITC-conjugated antimouse IgG and biotinylated antimouse IgG and Texas red-avidin are shown in *C* and *E*, respectively. *A*, ×250 and *B-E*, ×400.

ever, PDGF β receptor expression was intense and localized in the periepithelial stroma in regions of DCIS, whereas regions of nonmalignant stroma displayed a more diffuse, weaker expression of PDGF β receptors. These findings coincide with the recent report by Coltrera *et al.* (11) that the PDGF β receptor is expressed in fibroblastic stromal cells in nonmalignant breast tissue and DCIS. We have further demonstrated that the intense PDGF β receptor staining in malignant breast tissue was colocalized with α-SMA-staining cells and endothelial cells near intact basement membrane surrounding malignant ducts and capillaries, respectively, but not with myoepithelial cells. In regions of invasive carcinoma, PDGF β receptor expression was less intense where basement membrane was degraded. These results imply a paracrine stimulation by malignant epithelium of specific cell types in adjacent stroma.

PDGF β receptor-expressing α-SMA-positive cells could be myoepithelial cells, myofibroblasts, or vascular smooth muscle cells (30). Double staining for PDGF β receptor and the myoepithelial cell marker CALLA (34) showed that myoepithelial cells, although adjacent to PDGF β receptor-positive areas, did not themselves express PDGF β receptor. Recently, Ronnov-Jessen *et al.* (37) have shown that malignant breast epithe-

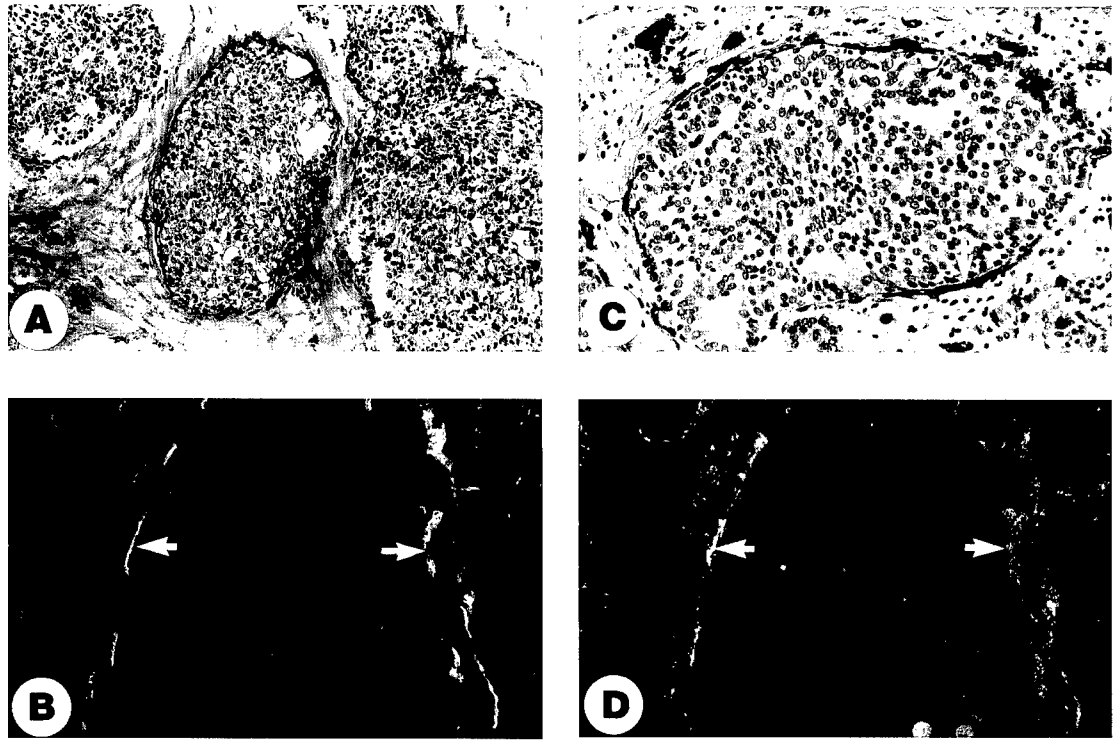

Fig. 4 Localization of PAL-E (endothelial) marker and PDGF β receptor expression in DCIS. A breast tumor tissue section showing DCIS was stained using H&E (*A*) to identify tissue morphology. An adjacent section was also stained using double immunofluorescence with monoclonal anti-PAL-E antibody (*B*), which identifies nonarterial capillary endothelial cells, and with monoclonal anti-PDGF β receptor antibody (*D*), as described in Fig. 1. A similar section was stained with polyclonal anti-factor VIII-related antigen (*C*) using an immunoperoxidase technique. *Arrows*, regions of double staining. *A*, ×250 and *B-D*, ×400.

lium can stimulate differentiation of α-SMA-expressing myofibroblasts from fibroblasts, concomitant with migration of myofibroblasts toward the carcinoma cells in a serum-free three-dimensional "tumor environment" culture system with purified cell types. Thus, myofibroblasts may be attracted to the vicinity of the basement membrane by malignant epithelial cells which produce PDGF BB. PDGF BB could be involved either directly by stimulating chemotaxis (15, 38) or indirectly by stimulating production of other growth factors [*e.g.*, transforming growth factor β (17), basic fibroblast growth factor (39), and insulin-like growth factor I (40, 41)]. Thus, stimulation of fibroblast proliferation and chemotaxis by PDGF BB may be at least partially responsible for the pronounced desmoplastic response in breast cancer.

Strong colocalization of PDGF β receptors with vascular endothelial cells (PAL-E positive) was also observed. Most of the PDGF β receptor-expressing endothelial cells are associated with capillary-like structures which are frequently located near or adjacent to regions of DCIS and in invasive carcinoma, as confirmed independently by factor VIII immunohistochemical staining (Fig. 4*C*). Our ISH analysis of PDGF β receptor mRNA expression in vascular endothelial cells adjacent to carcinoma *in situ* corroborated this finding. These findings are consistent with the observation that PDGF BB, which binds to PDGF β receptors, can stimulate angiogenesis *in vitro* and *in vivo* (42, 43). Although the mechanism by which PDGF BB stimulates angiogenesis *in vivo* is not known, recent reports suggest that thrombospondin, which is secreted in response to PDGF (44), may be involved (45). In support of this hypothesis, Clezardin *et al.* (7) have shown that thrombospondin and its receptors CD36 and CD51 are up-regulated in hyperplastic and neoplastic human breast tissue. We have also demonstrated increased expression of thrombospondin in the periepithelial regions of DCIS and invasive carcinoma (data not shown). PDGF BB may therefore exert its angiogenic effect by stimulating thrombospondin production.

PDGF β receptor-positive cells were found to be closely localized to basement membrane regions of DCIS. In invasive regions, PDGF β receptor expression remained associated with cells adjacent to fragmented basement membrane or vascular regions; however, where no basement membrane was apparent, expression of the PDGF β receptor was less intense. This finding suggests a loss of PDGF β receptor expression in periepithelial stromal cells (*e.g.*, myofibroblasts) concomitant with degradation of the basement membrane and/or movement of PDGF β receptor-positive cells toward basement membrane-associated tumor regions.

We also observed occasional expression of PDGF β receptor mRNA in malignant epithelium (data not shown), similar to recent results reported by Coltrera *et al.* (11). However, PDGF β receptor protein was not detected on malignant or nonmalignant epithelium in our study. This observation may reflect the fact that our staining procedure was carried out on unfixed

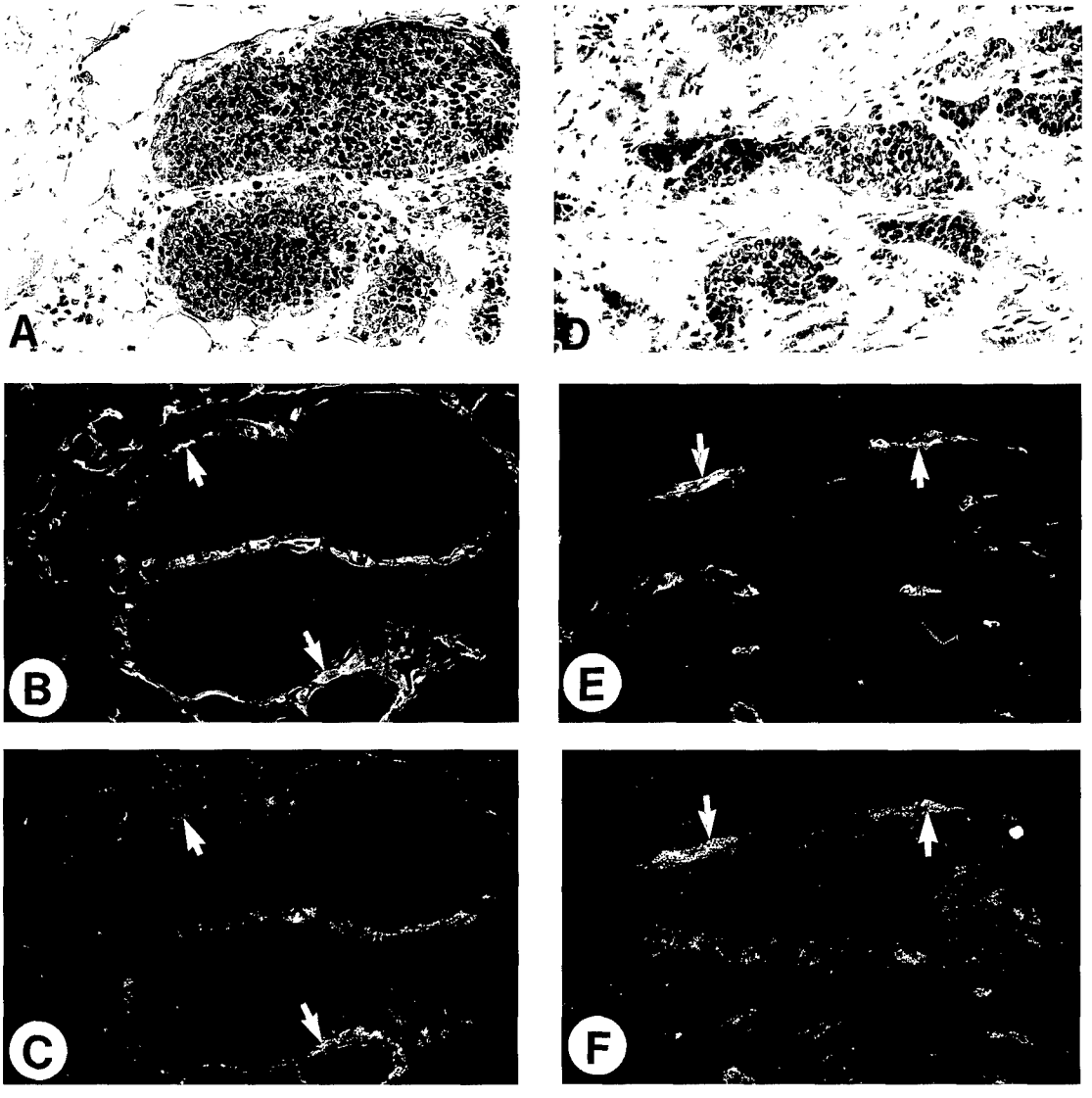

Fig. 5 Localization of collagen type IV and PDGF β receptor expression in DCIS (*A-C*) and in invasive carcinoma (*D-F*). Breast tumor tissue sections showing DCIS and invasive carcinoma were stained using H&E (*A* and *D*) to identify tissue morphology. Adjacent sections were also stained using double immunofluorescence as follows: with anticollagen type IV IgG (*B* and *E*), which identifies basement membrane, and with monoclonal anti-PDGF β receptor antibody (*C* and *F*), as described in Fig. 1. *A-C*, region of DCIS, and *D-F*, region of invasive carcinoma. *Arrows*, regions of double staining. ×400.

frozen sections and would therefore detect primarily membranous receptors.

Recently, basal cell carcinoma (46) and colorectal carcinoma (32) have been independently shown to express PDGF AB/BB, and the corresponding receptors can be detected on adjacent stromal components. Ponten *et al.* (46) observed expression of PDGF α and β receptors in the specific stromal components surrounding basal cell carcinoma. The A and B chains of PDGF were found mainly in the basal cell carcinoma cells, hair matrix, and sweat gland epithelium. Likewise, Sundberg *et al.* (32) have demonstrated that microvascular pericytes express PDGF β receptors in human healing wounds and in colorectal adenocarcinomas. PDGF β receptors and PDGF B chain-expressing cells were found to be in close proximity, suggesting a role for PDGF in the physiology of the microvasculature in these lesions. PDGF has also been shown to be directly responsible for induction of vascular connective tissue stroma by xenotransplanted human melanoma cells which produce PDGF BB (43). Our results provide strong support for a similar mechanism for stimulation of the stroma by adjacent carcinoma cells in breast cancer.

Our findings are consistent with a paracrine mechanism in which PDGF β receptor is up-regulated in stromal cells immediately adjacent to tumor cells which have been shown previously to produce PDGF BB (11). This up-regulation of PDGF β receptors was found to be localized with vascular endothelial

cells and α-SMA-positive myofibroblasts associated with basement membrane structures in the periepithelial stromal region of breast carcinomas. These results suggest that PDGF BB acts during early stages of breast cancer, *e.g.*, at the level of DCIS, on adjacent PDGF β receptor-expressing myofibroblasts and endothelial cells. Activation of the PDGF β receptor pathway in these cells could result in the desmoplastic and angiogenic response of adjacent stroma in breast cancer. Future studies will examine the possible functional role of PDGF β receptor activation in epithelial-stromal interactions in breast cancer.

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Appendix II

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Coexpression of Hepatocyte Growth Factor and Receptor (Met) in Human Breast Carcinoma

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Expression of hepatocyte growth factor (HGF) and HGF receptor (HGFR, product of the* met *proto-oncogene) mRNA were examined by nonisotopic* in situ *hybridization in a spectrum of benign and malignant human breast tissues. mRNA for both HGFR and HGF was detected in benign ductal epithelium. Epithelial expression of HGF mRNA was particularly intense in regions of ductal epithelial hyperplasia. Positive expression of HGF (but not HGFR) mRNA was also found in adipocytes, endothelial cells, and to varying degrees in stromal fibroblasts. In 12 of 12 cases of ductal carcinoma* in situ *and infiltrating ductal carcinoma, carcinoma cells showed a heterogeneous pattern of expression for both HGFR and HGF mRNA. In infiltrating ductal carcinomas, intense expression of HGFR mRNA was not restricted to ductular structures but was also seen in non-duct-forming carcinoma cells. The same zones of the tumors (most commonly at the advancing margins) that expressed strongly HGFR mRNA often were also strongly positive for HGF mRNA, suggesting a possible autocrine effect. The expression pattern of HGFR protein in 25 cases including the same series of tissues used for* in situ *hybridization analysis was similar to that of HGFR mRNA, as determined by an immunoperoxidase technique. The finding that HGFR is expressed by both benign and malignant epithelium, and is not restricted to duct-forming structures, suggests that, although the potential for HGF/HGFR binding is maintained in malignancy, the response to ligand binding at the level of the receptor or the cellular response to receptor activation may change at some point during progression. (Am J Pathol 1996, 148:225–232)

Hepatocyte growth factor receptor (HGFR), also known as the product of the *met* proto-oncogene, is expressed by a variety of epithelial (and a few non-epithelial) cell types, including mammary epithelium.[1–4] The ligand, hepatocyte growth factor (HGF) was originally described as a mitogen for hepatocytes[5] and later found to be identical to scatter factor, a modulator of intercellular organization.[6] HGF has been found to be produced and secreted by cells of the mesenchymal/stromal compartment,[7–12] although recent evidence has shown epithelial expression of HGF in certain instances.[13–16] HGF has been shown to control a variety of cell functions including cell growth,[10,17,18] movement,[19–22] invasiveness,[21,22] and cell-cell adhesion,[23,24] as well as morphogenesis of epithelial (eg, ductular or tubular) structures.[2,25–27] Many of these functions have been shown to require activation of the Met receptor.[28,29]

There is presently controversy over the possible role of HGFR in human breast cancer. Both the HGF[6] and HGFR[30] genes have been mapped to human chromosome 7q. Loss of heterozygosity at 7q has been found to be a frequent event in human breast cancer (40% of informative cases), and loss of heterozygosity at 7q in human breast tumors has been found to correlate with shorter metastasis-free and overall survival times.[31] In addition, Tsarfaty et al[2] have reported that HGFR protein is expressed in benign ductal epithelium and is expressed at lower levels in the immediately adjacent malignant ductal epithelium. Although collectively these results are consistent with a possible tumor suppressor role for

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HGF and/or HGFR in human breast carcinoma, this hypothesis has yet to be proven. Other studies have shown HGF to act as a motogen or morphogen in most breast carcinoma cell lines tested,[2,22] and there are a few reports of HGF acting as a mitogen for mammary epithelial cells.[10,18] Wang et al[16] reported that HGF mRNA is expressed in both benign and malignant mammary epithelium and that the most abundant expression in benign epithelium was in regions of proliferative activity. Although they suggested a possible autocrine role for HGF in inducing proliferation of benign and malignant mammary ductal epithelium, they had not examined expression of HGFR mRNA or protein in these same tissues to support this contention. Finally, the clinical import of HGF in human breast carcinoma was highlighted recently in the work of Yamashita et al,[32] who reported that a high level of expression of HGF protein is an even more significant factor in predicting poorer relapse-free and overall survival than is lymph node status. However, this study did not examine the stromal or epithelial source of the HGF in breast cancer tissues.

We thus regarded it to be important to establish more clearly the cellular expression pattern of both HGF and HGFR in benign and malignant human breast tissues. We examined expression of both genes at the mRNA level in serial sections of the same tissues, using *in situ* hybridization (ISH).

Materials and Methods

Tissues

Mastectomy and segmentectomy/lumpectomy specimens were obtained fresh immediately after excision. Sections of both benign and malignant breast taken for ISH were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 4 hours, followed by alcohol cycle processing and embedding in paraffin. Sections of normal skin were taken for use as controls for HGFR expression from associated (usually nipple region) skin. Placental tissues as controls for HGF expression were obtained fresh from normal term deliveries and also fixed immediately in 4% paraformaldehyde for 4 hours. For ISH, tumor specimens included 2 cases of ductal carcinoma *in situ* alone and 2 cases of well differentiated, 5 cases of moderately differentiated, and 3 cases of poorly differentiated infiltrating ductal carcinoma (IDC). For immunohistochemistry, an additional 13 specimens, including 1 well differentiated, 7 moderately differentiated, and 5 poorly differentiated IDCs were analyzed. Access to tissues satisfied the requirements of the Kingston General Hospital ethics committee.

Plasmids

Both the HGF and HGFR (*met*) plasmids used for generation of riboprobes consisted of a cDNA fragment of the gene of interest cloned into a Bluescript KSII$^+$ vector between the T3 and T7 promoters, each with the 5′ end of the cDNA downstream to the T7 promoter. The HGF cassette consisted of the 540-bp *Bam*HI-*Xho*I fragment of the human HGF cDNA.[33] The HGFR cassette consisted of the 800-bp *Eco*RI-*Eco*RV fragment of the human *met* cDNA.[34] The platelet-derived growth factor α-receptor plasmid, used for generation of control riboprobe, consisted of the 1.5-kb *Eco*RI-*Pst*I fragment (extracellular domain) of the cDNA cloned into pGEM-Blue between the T7 and SP6 promoters, such that the 5′ end lay downstream of the T7 promoter. The platelet-derived growth factor α-receptor plasmid was obtained as a kind gift from Dr. Lena Welsh and Dr. Keiko Funa.[35]

Riboprobes

Riboprobes were generated by *in vitro* transcription from linearized templates with the appropriate phage RNA polymerase (Promega Corp., Madison, WI) in the presence of digoxigenin-UTP (Boehringer Mannheim, Montreal, Canada). Antisense riboprobes for HGF and HGFR were generated by transcription from the T3 promoter, whereas platelet-derived growth factor α-receptor antisense (control) riboprobes were generated by transcription from the SP6 promoter. Riboprobes generated from vector-only templates were also used as negative controls.

In Situ *Hybridization*

ISH was performed by a modification of the procedure of Yang and Park.[36] Six-micron paraffin sections were cut onto baked slides coated with triethoxysilane (Sigma Chemical Co., St. Louis, MO), dewaxed in toluene, and rehydrated. Permeabilization was performed by treating at room temperature sequentially with 0.2 mmol/L HCl, 0.2% Triton X-100 in PBS, and 40 μg/ml proteinase K for 10 minutes each. Slides were then washed in 0.1X PBS, refixed for 30 minutes at room temperature in 4% paraformaldehyde, washed again in 0.1X PBS, and acetylated with 0.25% acetic anhydride in 0.1 mmol/L triethanolamine HCl. Slides were then dehydrated, delipidated in 100% chloroform for 15 minutes followed by

absolute ethanol for 5 minutes and 95% ethanol for 15 minutes, and then air dried.

Probes were diluted in prehybridization mix at 15 ng/μl, and 200 μl of hybridization mix was applied to each section. Slides were incubated in a humid chamber at 42°C overnight, washed in 1X standard saline citrate (SSC) at 55°C for 30 minutes, rinsed in RNAse buffer (0.5 mol/L NaCl, 10 mmol/L PIPES (pH 7.2), 0.1% Tween 20) at room temperature for 10 minutes, and incubated in 20 μg/ml RNAse A (Sigma) for 30 minutes at 37°C to remove unbound single-stranded RNA. Slides were washed in buffer 1 (100 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.5) at room temperature for 10 minutes and blocked with 3% normal sheep serum in buffer 1 at room temperature for 30 minutes. To detect specific hybrids, slides were incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim; 1 to 1000 dilution in buffer 1 with 3% normal sheep serum) for 30 minutes, and then washed twice (10 minutes each) with buffer 1 and twice (5 minutes each) in buffer 2 (100 mmol/L Tris-HCl (pH 9.5), 100 mmol/L NaCl, 50 mmol/L $MgCl_2$). Hybrids bound to anti-digoxigenin antibody were then visualized by a color reaction containing nitroblue tetrazolium salt, 5-bromo-4-chloro-3-indolylphosphate, and levamisole (0.24 mg/ml) in buffer 2. An alkaline phosphatase substrate kit IV (Vector Laboratories, Burlingame, CA) was used. Color was allowed to develop for 12 hours in the dark. Slides were then dehydrated, washed in xylene, mounted with permount, and viewed and photographed by a light microscope. Positive areas showed brown-purple cytoplasmic staining (Figures 1 and 2).

Testing for RNA specificity of probe binding was performed by (1) treating sets of control slides with 20 μg/ml RNAse A in RNAse buffer for 30 minutes at 37°C before hybridization and (2) hybridizing with vector-only riboprobes generated from the parent vector without the HGF or HGFR cassette, transcribed in the same direction as the antisense probes. Controls for cell specificity of binding included testing of the pattern of binding of antisense HGFR and HGF probes to known positive tissue targets (eg, placenta (ie, trophoblast) for HGFR mRNA and skin (ie, epidermis and adnexal epithelium) for HGF mRNA).

Immunohistochemistry

Paraffin-embedded sections of formalin-fixed breast tissues from 25 cases of ductal carcinoma *in situ* and IDC including the same set of surgical pathological specimens used for ISH analysis were assessed for HGFR expression by an immunoperoxidase technique. Samples were immunostained with a polyclonal anti-Met antibody raised against a COOH-terminal peptide (1:200),[37] by a modification of the avidin-biotin peroxidase complex method previously described.[38] The immunoreaction was visualized with diaminobenzidine and H_2O_2. The antibody used has been shown previously to detect human Met (HGFR) specifically by both immunoprecipitation and Western blotting.[15,37]

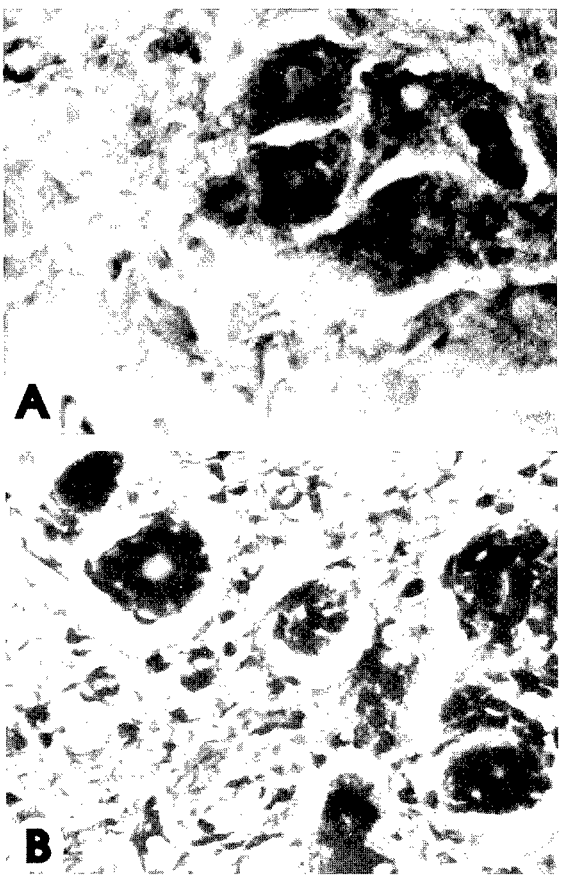

Figure 1. *Nonmalignant breast tissue.* A: *ISH for HGFR mRNA showing a strong cytoplasmic staining in ductal epithelial cells but negligible cytoplasmic staining of stromal cells (original magnification, ×630).* B: *ISH for HGF mRNA showing variable cytoplasmic staining of benign epithelium, with focal areas of strong positivity. Some cytoplasmic staining is also apparent in stromal fibroblasts and endothelial cells (original magnification, ×630).*

Results

Both HGFR and HGF mRNA were expressed cytoplasmically in regions of nonmalignant ductal epithelium in all tissues examined (Figure 1). Expression of HGF mRNA in benign ductal epithelium was variable but appeared most intense in regions showing architectural evidence of ductal epithelial hyperplasia. Expression of HGF (but not HGFR) mRNA was also

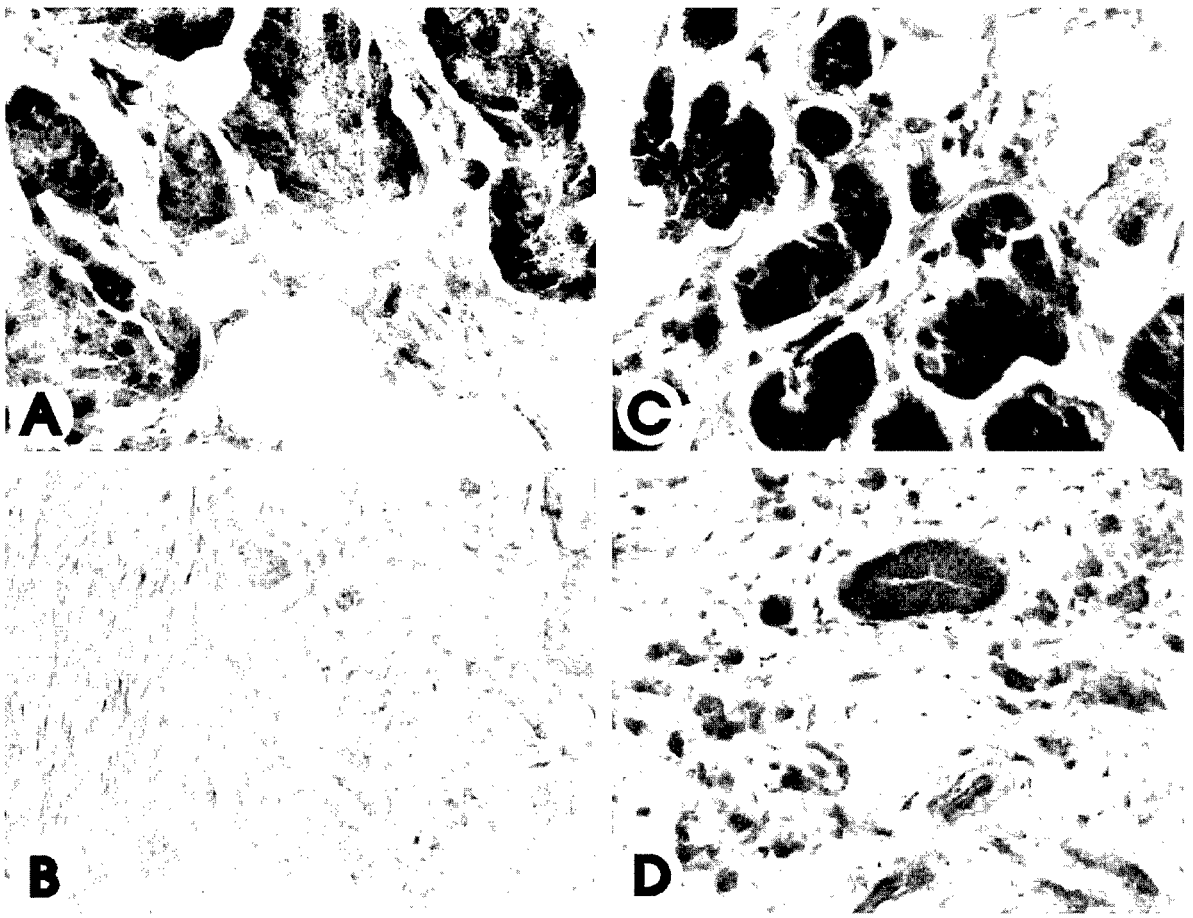

Figure 2. *Moderately* (A to C) *and well* (D) *differentiated ductal carcinomas.* A: *ISH for HGFR mRNA showing intense cytoplasmic positivity at the advancing margin of the tumor* (*original magnification,* ×*630*). B: *ISH for HGFR mRNA showed much weaker staining in the malignant epithelium in more central regions of the same tumor* (*original magnification,* ×*250*). C: *ISH for HGF mRNA showing strong cytoplasmic positivity of the malignant epithelium at the advancing margin of the same tumor, in the same region that stained intensely for HGFR mRNA* (*original magnification,* ×*630; cf* A). *In addition, stromal fibroblasts and endothelial cells showed some cytoplasmic positivity.* D: *ISH analysis for HGFR mRNA showing IDC surrounding a benign duct. Intensity of cytoplasmic staining is similar in both benign and malignant epithelium, regardless of whether the malignant epithelium forms well defined ductular structures* (*original magnification,* ×*400*).

found to varying degrees in adipocytes, endothelial cells, and stromal fibroblasts (Figure 1B). Ductal carcinoma *in situ* and IDC cells in all tissues examined showed a heterogeneous pattern of expression of both HGFR and HGF mRNA (Figure 2). In 10 of 10 IDCs examined, intense expression of HGFR mRNA was not restricted to ductular structures but was seen also in non-duct-forming carcinoma cells (Figure 2D). There was no apparent difference in patterns of HGF mRNA and HGFR mRNA in IDCs of different grades. The same zones of a tumor that were strongly positive for HGFR mRNA were commonly also strongly positive for HGF mRNA expression, suggesting a possible autocrine loop effect (eg, Figure 2, A and C). Where heterogeneity of expression for HGF and HGFR mRNA was apparent within a tumor, strongest positivity for both was usually seen at or near the advancing margins of the tumor (Figure 2, A and C), whereas expression in more central regions of the tumor was less intense (Figure 2B). This was true for 9 of 10 tumors, which included sampling of the advancing margin. This effect was seen most commonly in the absence of morphological evidence of ischemia/necrosis in more central regions of the tumor, such that higher level expression of mRNA for both HGF and HGFR at the advancing margins is unlikely a result of the ischemia effect alone.

Pretreatment of slides with RNAse A eliminated all positive signals. Likewise, riboprobes generated from the parent vector lacking the HGF or HGFR insert showed no positivity (not shown). ISH of skin sections for HGFR mRNA showed the expected pattern of strong positivity in immature layers of the epidermis and in the adnexal epithelium,[5,39] and expression of HGF mRNA in placental tissue showed the expected pattern of strong positivity in the trophoblast[40] (not shown). These controls verify that

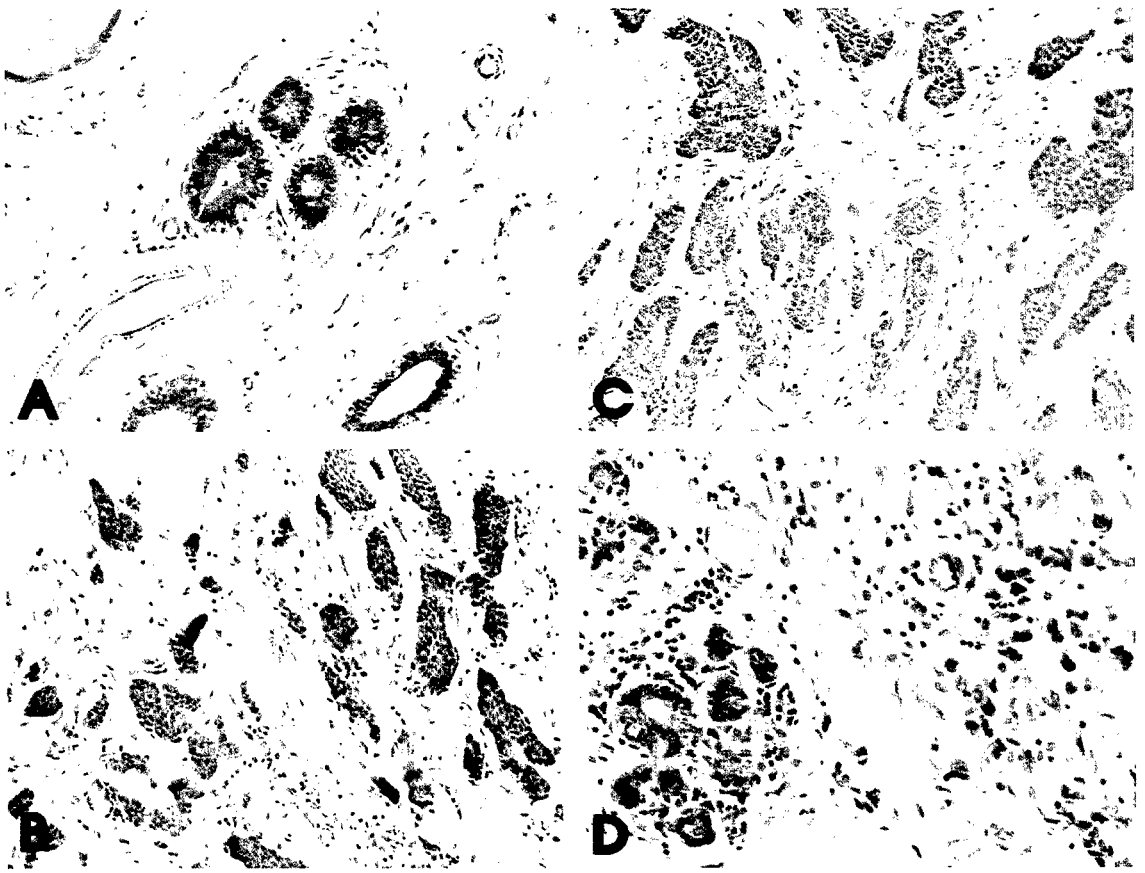

Figure 3. *Immunoperoxidase localization of HGFR protein expression.* A: *Benign ducts showing strong cytoplasmic/membrane positivity (brown; original magnification, ×250).* B: *Moderately differentiated IDC showing strong cytoplasmic/membrane positivity at the advancing margin (original magnification, ×250).* C: *Same tumor as in* B, *showing gradient of staining intensity: strongest closer to the advancing margin* (top) *and weakest in more central regions (bottom) of the tumor (original magnification, ×250).* D: *Poorly differentiated IDC (right) adjacent to nonmalignant ducts (left) showing comparable staining intensity in both malignant (non-duct-forming) and nonmalignant (ductal) epithelium (original magnification, ×400).*

HGF and HGFR probes were in fact binding to the corresponding mRNA. Analysis of breast tissue with a control antisense platelet-derived growth factor α-receptor riboprobe showed the predicted pattern of weak positive expression in stromal fibroblasts, endothelial cells, and inflammatory cells, with no expression in benign or malignant ductal epithelial cells (not shown). Similarly, analysis of skin sections for platelet-derived growth factor α-receptor expression showed positivity in dermal fibroblasts and endothelial cells, with no expression in the epidermis, as reported previously by Pontén et al[41] (not shown). Collectively, these controls confirmed the specificity of the ISH procedure.

Immunoperoxidase analysis of HGFR protein expression showed a pattern of staining closely reflecting that seen for expression of the corresponding mRNA (Figure 3, A–D). In 25 of 25 cases examined, strong expression of HGFR protein was seen in both benign and malignant epithelium and was not restricted to ductal or acinar structures. Most intense positive staining for HGFR protein was apparent at or near the advancing margins of invasive carcinomas (Figure 3B).

Discussion

The ISH data reported here show that HGF mRNA is expressed by benign and malignant mammary ductal epithelium, as well as variable expression by stromal cells (fibroblasts, adipocytes, and endothelial cells), in a manner similar to that described by Wang et al.[16] Also in agreement with Wang et al,[16] we found intense expression of HGF mRNA in benign ductal epithelium in regions of ductal epithelial hyperplasia as well as in malignant epithelium of different histological grades.

Expression of HGFR mRNA was detected in both benign and malignant ductal epithelium, consistent with the demonstration by Tsarfaty et al[2] of HGFR protein expression in similar tissues. However, in

contrast to what has been described previously,[2] we have found that expression of HGFR mRNA and protein levels are not decreased uniformly in malignant epithelium, and expression of HGFR is not limited to cells lining ductules or tubules. In fact, we have found that both *in situ* and invasive ductal carcinomas of all grades show a heterogeneous pattern of HGFR expression. Individual malignant cells or groups of cells of poorly differentiated IDCs were commonly found to stain strongly for both HGFR mRNA and protein. Level of expression of HGFR in itself is thus not necessarily associated with degree of ductal differentiation.

As HGFR appears to be expressed by both benign and malignant epithelium and is not restricted to duct-forming structures (suggesting that the potential for HGF/HGFR binding is maintained in malignancy), it may be that functional differences exist between benign and malignant breast tissue relating to HGF/HGFR. Such differences could include the activity of the ligand or receptor itself and post-receptor signal transducer/effector systems. For example, cellular responsiveness to HGF has been shown to be influenced by factors (eg, urokinase) regulating HGF activation[42] or other extracellular mediators (transforming growth factor-β or extracellular matrix components (eg, heparan sulfate)).[43,44] Alternatively, HGFR activation in benign epithelium may, as suggested by Tsarfaty et al[2] result in activation of a morphogenic program leading to duct formation. In malignancy, defective regulation of, or alteration of, components of this program (for example, loss of proper cell-cell adhesion) may result in an incomplete or dysfunctional program. In fact, scatter activity of HGF on invasive carcinomas has been found to be influenced by the integrity of the cadherin system.[45–47] Decreased expression of E-cadherin in more poorly differentiated carcinomas may render them more susceptible to the scatter activity of HGF. Thus, whereas HGF may induce ductal morphogenesis in the presence of an intact cadherin system in areas of well differentiated carcinoma, disruption of cadherin expression in more poorly differentiated areas may instead result in disaggregation, scatter, and more invasive behavior. Such a difference in pattern of response to HGF in different areas within a given tumor or by a given tumor at different times (during progression) could also explain what appears at first glance to be contradictory evidence in the literature for tumor-suppressing *versus* tumor-promoting effects of HGF ligand and receptor.

Zonal coexpression of HGF ligand and receptor mRNA was also found in both benign and malignant epithelium and would suggest the possibility of an HGF autocrine loop. This finding is consistent with recent evidence that HGFR expression may be upregulated by HGF.[48] A similar autocrine loop effect for HGF and HGFR in malignant (and some nonmalignant, proliferative) epithelial cells has been described previously in other systems.[13–15] The higher levels of coexpression of ligand and receptor observed in ductal epithelial hyperplasia suggests that HGF may act in an autocrine role in non-neoplastic ducts as well, especially those undergoing benign proliferation. In the malignant situation, a normal reactive mechanism may be altered as suggested above, such that autocrine stimulation itself is no longer properly regulated or the response to autocrine stimulation is altered, with the end result of invasion and metastasis (malignant conversion) rather than morphogenesis and ductular differentiation (tumor suppression). Also in keeping with this hypothesis is the finding that, in the majority of the tumors examined, highest levels of expression of both HGF and HGFR mRNA were found at the advancing margins of the tumor, where active invasion is taking place. This pattern of expression has been reported for other peptides, eg, transforming growth factor-β,[49] platelet-derived growth factor β-receptor,[50] cathepsin B,[51] and plasminogen activator,[52] which may be involved in invasiveness at the tumor front. Interestingly, urokinase-type plasminogen activator is also an activator of HGF.[42]

Additional work (both *in vivo* and *in vitro*) is necessary to elucidate the role of HGF in the control of these aspects of epithelial cell behavior and to study possible alterations of the response to HGF in malignancy. The recent evidence in the literature for a strong potential prognostic role for the level of HGF expression[32] adds an extra level of significance and urgency to unraveling the role of HGF in human breast carcinoma.

Acknowledgments

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Identification of a Hepatocyte Growth Factor Autocrine Loop in a Murine Mammary Carcinoma[1]

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Abstract

Constitutive activation of growth factor receptors through autocrine/paracrine mechanisms occurs frequently in human cancers and is thought to play an important role in carcinogenesis. We have demonstrated previously that hepatocyte growth factor (HGF) is a potent mitogenic factor for murine mammary carcinoma (SP1) cells *in vitro*. We report here an autocrine HGF loop in SP1 cells. HGF receptor/Met is expressed in SP1 cells and is constitutively tyrosine phosphorylated. The phosphorylation of HGF receptor/Met is inhibited when cells are exposed to suramin or anti-HGF IgG. This finding suggests that constitutive tyrosine phosphorylation of HGF receptor/Met is sustained by an extracellular factor, most likely HGF. Using Northern blot and Western blot analysis, we detected expression of a 6-kb HGF mRNA in SP1 cells and a M_r 85,000 HGF protein in SP1-conditioned medium, respectively. *In vitro* translation of mRNA from SP1 cells and metabolic labeling confirmed expression and synthesis of HGF by SP1 cells. SP1 cells also invade through Matrigel-coated transwell membranes in an *in vitro* invasion assay, and invasion of these cells was inhibited by neutralizing anti-HGF IgG. In addition, SP1-conditioned medium induced scatter activity of Madin-Darby canine kidney epithelial cells, and this activity was inhibited by neutralizing anti-HGF IgG. We have also shown that several signaling molecules including phosphatidylinositol 3-kinase, Src, focal adhesion kinase, and phospholipase C-γ in SP1 cells are constitutively tyrosine phosphorylated, suggesting that coexpression of HGF and HGF receptor/Met may in part contribute to sustained tyrosine phosphorylation of these cytoplasmic proteins in SP1 cells. Our observations in the SP1 model suggest that HGF contributes to growth and invasive phenotypes of mammary carcinomas via both paracrine and autocrine mechanisms.

Introduction

Aberrant expression of growth factors and their receptors has been shown to be associated with many types of human cancers (1–4), and in some instances, deregulation in growth factor-mediated signaling pathways may contribute to the malignant transformation of human cancers. It is known that such aberrant expression may be due to structural alteration of the corresponding growth factor receptors (2, 5) or to the creation of an autocrine loop (6–9). Recently, attention has focused on the role of HGF[3] receptor/Met in human cancers. HGF is a heterodimeric cytokine with a molecular weight of M_r 85,000 (10) and exhibits pleiotropic biological functions as a mitogen, motogen, morphogen, and angiogenic factor (11, 12). HGF has been detected primarily in fibroblasts and other mesenchymal/stromal cells and acts on epithelial cells in a paracrine mechanism (13–16). However, it has been shown that HGF (17) or a variant of HGF (18, 19), produced by nondifferentiated human keratinocytes (17), human normal bronchial epithelial cells, and lung carcinoma cells (18, 19), may act as an autocrine factor in these cells.

In contrast to its ligand, the HGF receptor/Met, a product of the *met* proto-oncogene (20, 21), is expressed most prominently in epithelial cells (22, 23). The HGF receptor/Met protein is a heterodimer of M_r 190,000 composed of a M_r 145,000 β and a M_r 50,000 α subunit. The β-subunit extends through the membrane and contains the tyrosine kinase catalytic domain (24, 25). There is also evidence that the *met* proto-oncogene is overexpressed (22, 23, 26–28), and amplified (23) in a significant number of human carcinomas. In some cases, constitutive activation of HGF receptor/Met was also observed (29). Recently, our laboratory has shown that HGF and HGF receptor/Met mRNA are coexpressed by human breast carcinoma cells *in situ*, particularly in regions of invasive carcinoma (30), suggesting a possible autocrine HGF loop in human breast cancer. However, the existence of an HGF autocrine loop in breast carcinomas and its putative role in growth and metastasis of breast cancer are not known.

To better understand the possible paracrine and autocrine role of HGF in the growth of mammary carcinomas, we have previously developed a model to examine the influence of

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[2] To whom requests for reprints should be addressed. Phone: (613) 545-2825; Fax: (613) 545-6830.

[3] The abbreviations used are: HGF, hepatocyte growth factor; PI, phosphatidylinositol; CM, conditioned medium; MDCK, Madin-Darby canine kidney; FBS, fetal bovine serum; FAK, focal adhesion kinase; PLC-γ, phospholipase C-γ; PY, phosphotyrosine; EGF, epidermal growth factor.

stromal cells, particularly adipocytes, on the growth of a murine mammary carcinoma, SP1 (31). We have subsequently shown that a fully differentiated 3T3-L1 adipocyte cell line secretes HGF, which stimulates growth of SP1 carcinoma cells by a paracrine mechanism (32). In the present study, we demonstrated that HGF and HGF receptor/Met are coexpressed in SP1 cells. Therefore, we examined the activation of HGF receptor/Met and the functional activity of secreted HGF in SP1 cells. Our observations in the SP1 model suggest that HGF may contribute to continuous growth and invasiveness of mammary carcinomas via paracrine and autocrine mechanisms.

Results

Constitutive Tyrosine Phosphorylation of HGF Receptor/Met in SP1 Cells. As a first step to assess the presence of an operative HGF autocrine loop in breast carcinomas, we determined the tyrosine phosphorylation status of HGF receptor/Met expressed in the mammary carcinoma, SP1. Using immunoprecipitation and Western blotting of proteins from SP1 cell lysates with anti-Met IgG directed to the carboxyl terminus of HGF/Met receptor, we showed that SP1 cells express a p170^{met} (Met precursor) and p145^{met} (β chain of mature HGF receptor/Met) (Fig. 1*A*). SP1 cells also express a M_r 110,000 protein that was immunoprecipitated with anti-Met IgG. The M_r 110,000 protein corresponds to an enzymatic cleavage product of p145^{met}, which was formed during the cell lysis and immunoprecipitation procedure (data not shown). Immunoprecipitation of all three HGF receptor/Met proteins was inhibited by co-incubation of cell lysates with a peptide corresponding to the carboxyl terminus of HGF receptor/Met (32). Reprobing of the same membrane with anti-PY IgG showed that p145^{met} and p170^{met} are tyrosine phosphorylated in SP1 cells incubated without exogenous HGF, and the level of tyrosine phosphorylation of these proteins increased following incubation of SP1 cells with exogenous HGF (Fig. 1*B*). In addition, a metastatic variant of SP1, SP1–3M, showed constitutive tyrosine phosphorylation of p145^{met} (Fig. 1*C*). These results suggest that constitutive tyrosine-phosphorylation of HGF/Met receptor in these cells may be sustained by an extracellular factor, most likely HGF protein.

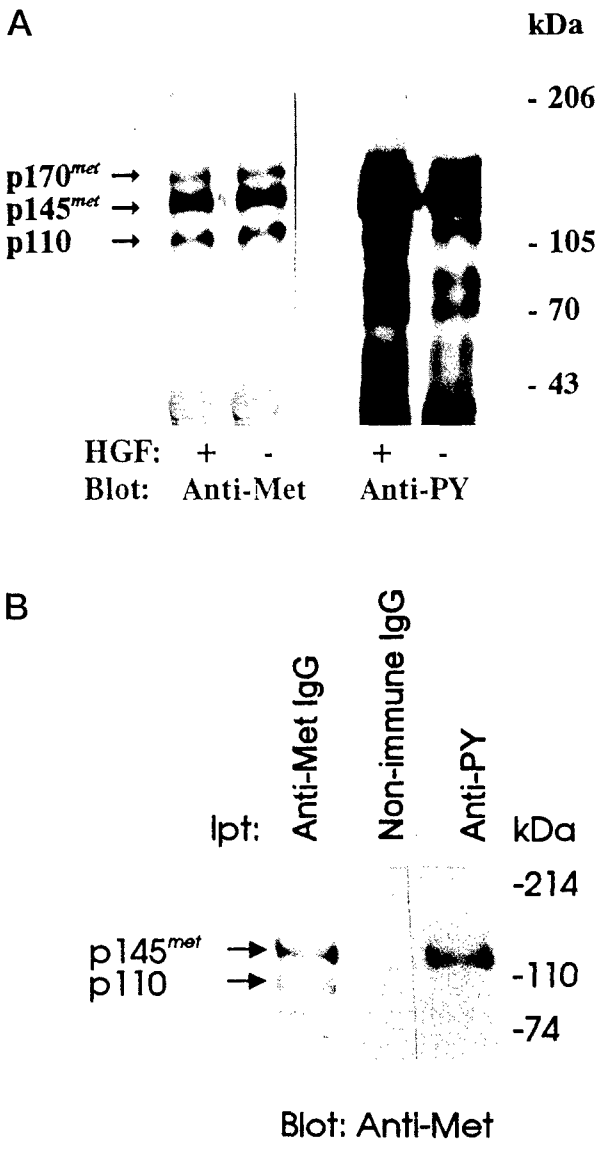


Fig. 1. Tyrosine phosphorylation of HGF receptor/Met in SP1 cells, untreated or treated with exogenous HGF. Nonmetastatic SP1 cells were serum starved for 24 h, were incubated without (−) or with (+) HGF for 20 min, and were lysed as described in "Materials and Methods." *A*, proteins from SP1 cell lysates were immunoprecipitated with anti-Met IgG, separated on 8% SDS-PAGE under reducing conditions, and subjected to Western blot analysis. The nitrocellulose membrane was immunoblotted with anti-Met IgG. Immune complexes were detected by ECL. *Right*, protein molecular weight standards. *B*, the same nitrocellulose membrane was stripped of antibodies and reprobed with anti-PY antibody. *C*, metastatic SP1–3M cells were serum starved, lysed as in *A*, and immunoprecipitated with anti-Met IgG, nonimmune IgG, or anti-PY, separated by SDS-PAGE under reducing conditions, and blotted with anti-Met IgG.

Tyrosine Phosphorylation and Activation of Signal Transducers in SP1 Cells. Receptor tyrosine kinases, such as HGF receptor/Met, upon activation by ligand undergo autophosphorylation due to activation of their intrinsic tyrosine kinase activities. In addition, activated HGF receptor/Met associates with, and phosphorylates, a number of cytoplasmic proteins that contain SH2 domains, including phospholipase C-γ, Src, Shc, and the p85 subunit of PI 3-kinase (33). Since HGF receptor/Met is constitutively tyrosine phosphorylated in SP1 cells, we therefore investigated whether some of these signal transducers are also tyrosine phosphorylated in SP1 cells (Fig. 2). Proteins from lysates of serum-starved SP1 cells were immunoprecipitated with anti-PLC-γ, anti-PI 3-kinase, anti-FAK, or anti-Src antibodies; immune complexes were subjected to SDS-PAGE under reducing conditions (except for Src; see Fig. 2 legend) and immunoblotted with anti-PY antibody. The results showed that all four signal transducer molecules are tyrosine phosphorylated in serum-starved SP1 cells without exogenous growth stimulator. However, the cytokine-activated signaling proteins JAK1, JAK2, JAK3, STAT1 (34), or the EGF receptor substrate, Eps15 (35), although present, were not detected in a tyrosine-phosphorylated form in SP1 cells (data not shown). These results further support the presence of an

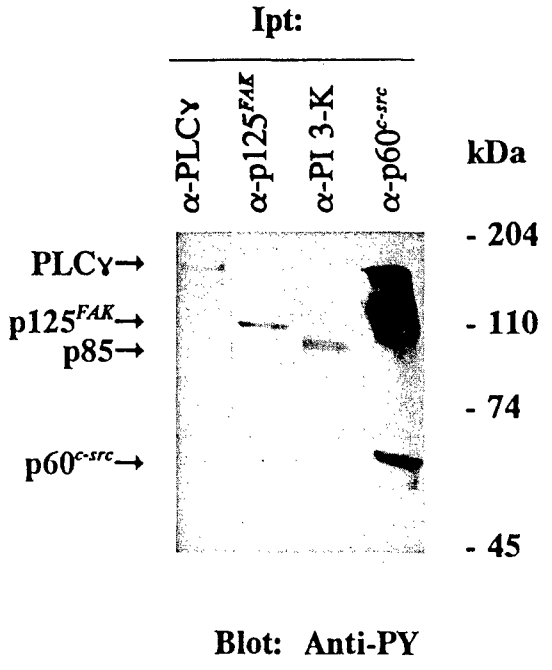
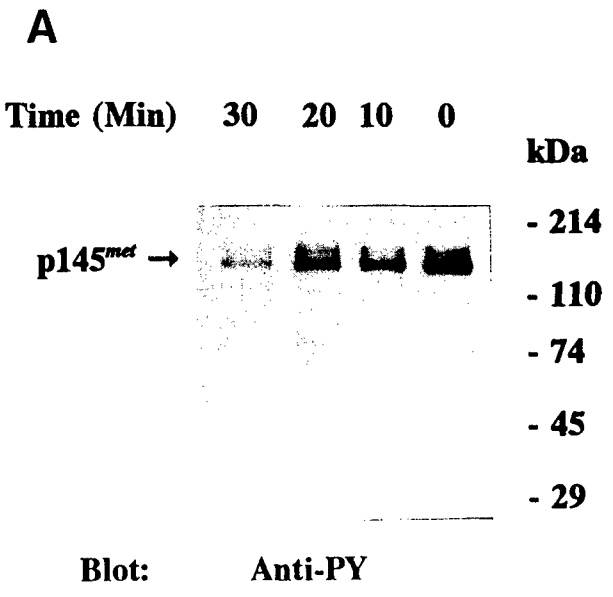


Fig. 2. Tyrosine phosphorylation state of Src, PI 3-kinase, PLC-γ, and FAK in SP1 cells. SP1 cells were serum starved for 24 h. Proteins from SP1 cell lysates were immunoprecipitated with polyclonal rabbit anti-PLC-γ, anti-FAK, anti-PI 3-kinase, or anti-Src IgG and separated on 8% SDS-PAGE under reducing conditions (except for Src, which requires nonreducing conditions since Src and IgG heavy chains have similar molecular weights). Proteins were transferred to a nitrocellulose membrane and immunoblotted with anti-PY antibody. Immune complexes were detected by ECL. *Right,* protein molecular weight standards. Distinct proteins, p150, p125, p85, and p60, were detected in each track, corresponding to the known molecular weight of PLC-γ, FAK, p85 subunit of PI 3-kinase, and Src, respectively.



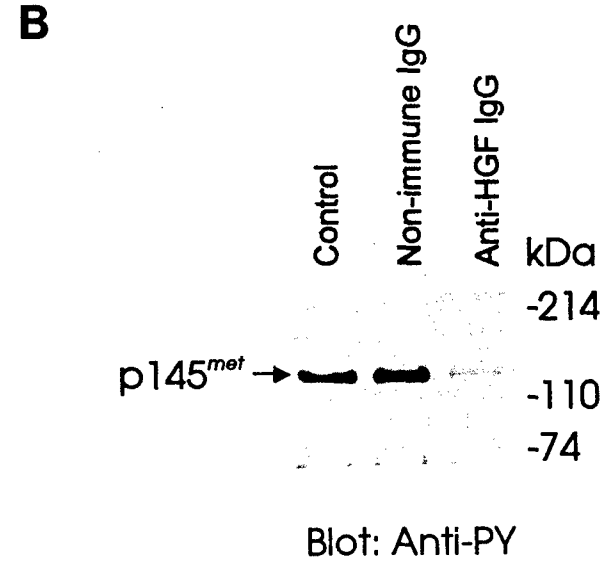


Fig. 3. Effect of suramin or anti-HGF IgG on tyrosine phosphorylation state of HGF receptor/Met in SP1 cells. *A,* SP1 cells were serum starved for 24 h and incubated in 100 μM suramin for the indicated times, lysed, and immunoprecipitated with anti-Met IgG. Immunoprecipitates were separated on 8% SDS-PAGE under reducing conditions and subjected to Western blot analysis. The nitrocellulose membrane was immunoblotted with anti-PY antibody. Immune complexes were detected by ECL. *Right,* protein molecular weight standards. *B,* serum-starved SP1 cells were washed with 0.5 M NaCl and RPMI (pH 3.7) and subsequently incubated with medium alone (*Control*), nonimmune IgG, or anti-HGF IgG (each at 100 μg/ml) for 4 h. Immunoprecipitation and Western blotting was carried out as in *A.*

operational autocrine loop in SP1 cells and suggest that signaling pathways stimulated by growth hormone; prolactin; erythropoeitin; interleukins 1, 2, 4, and 9; interferons; or EGF are not operating in SP1 cells in an autocrine manner.

Inhibition with Suramin and Anti-HGF IgG of Tyrosine Phosphorylation of HGF Receptor/Met in SP1 Cells. Detection of constitutive tyrosine phosphorylation of HGF/Met receptor in SP1 cells prompted us to perform experiments to interfere with such a putative autocrine activation of HGF/Met receptor in these cells. Preliminary results showed that treatment of serum-starved SP1 cells with erbastatin, a tyrosine kinase inhibitor, or low pH (3.7), a condition that has been shown to be effective in dissociating growth factor ligand/receptor complexes (36, 37), caused dephosphorylation at tyrosine residues of HGF receptor/Met in a time-dependent and reversible process (data not shown). We now show that constitutive tyrosine phosphorylation of HGF receptor/Met was significantly decreased in a time-dependent manner by suramin, a known blocker of autocrine loops (Refs. 29 and 38; Fig. 3*A*). In addition, tyrosine phosphorylation of HGF receptor/Met was decreased by treatment of cells with anti-HGF IgG (Fig. 3*B*). It should be pointed out that anti-HGF IgG treatment alone had little effect on tyrosine phosphorylation of HGF receptor/Met at the concentration used (10–30 μg/ml). However, when serum-starved SP1 cells were washed with 0.5 M NaCl and RPMI (pH 3.7) to remove extracellular HGF and subsequently incubated with anti-HGF IgG (100 μg/ml) for 4 h, a sustained dephosphorylation of HGF receptor/Met was observed; in contrast, cells

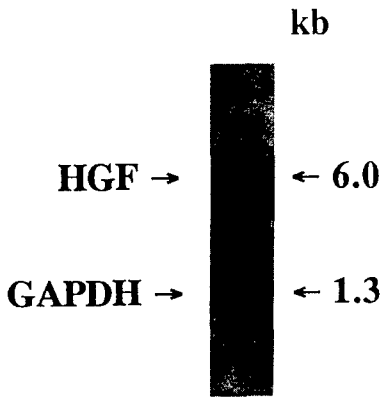


Fig. 4. Detection of HGF mRNA in SP1 cells. Poly(A)$^+$ RNA extracted from SP1 cells was subjected to gel electrophoresis (1 μg/lane), transferred to Zeta probe membrane, and hybridized with an HGF cDNA probe. A 6-kb HGF mRNA was evident. A probe for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used to evaluate relative amounts of mRNA transferred to the membrane.

incubated in parallel cultures with nonimmune IgG showed no change in tyrosine phosphorylation of HGF receptor/Met (Fig. 3*B*). Together, these data suggest that constitutive tyrosine phosphorylation of HGF receptor/Met in SP1 cells may be sustained by an extracellular factor, most likely HGF ligand, and that dephosphorylation of the HGF receptor/Met can be effectively achieved by dissociation of the HGF ligand/HGF receptor complex.

Detection of HGF mRNA in SP1 Cell Lysate and of HGF Protein and Functional Activity in SP1 CM. Detection of constitutively active HGF/Met receptor in SP1 cells prompted us to investigate expression of HGF in SP1 cells. Therefore, we evaluated the expression of HGF in SP1 cells both at mRNA and protein levels. Poly(A)$^+$ mRNA was extracted from SP1 cells and subjected to Northern blot analysis with a mouse HGF cDNA probe. We found that SP1 cells express a 6-kb HGF mRNA transcript (Fig. 4). In addition, total mRNA from SP1 cells was subjected to *in vitro* translation and assayed for the presence of HGF using Western blot analysis. A single band with molecular weight of M_r 80,000 was detected (Fig. 5*A*). Western blot analysis of CM from SP1 and SP1–3M cells with anti-HGF IgG also confirmed the presence of a protein with molecular weight of M_r 85,000 in nonreducing conditions and M_r 69,000 in reducing conditions, corresponding to the mature and α-subunit of HGF protein, respectively (Fig. 5*B*). To analyze further the synthesis of HGF by SP1 cells, we performed metabolic pulse-chase labeling experiments. Pulse-chase analysis and immunoprecipitation with anti-Met IgG showed a protein with a molecular weight of M_r 85,000 (Fig. 5*C*). A longer exposure showed an additional protein with apparent molecular weight of M_r 110,000 (data not shown). The latter band may correspond to the pro-HGF protein.

To assess functional activity of SP1 tumor-derived HGF, we evaluated the involvement of the secreted HGF in cell invasion, growth, and scatter. Invasion of SP1 cells was measured in a transwell and was expressed as the relative number of cells that penetrated the Matrigel-coated membrane during the course of the assay (Table 1). SP1 cells showed significant spontaneous invasion in low-serum (0.1%) medium; this invasion was strongly inhibited by anti-HGF IgG and was enhanced by exogenous HGF. In addition, SP1 cells grew continuously in serum-free or low-serum medium, and this growth was inhibited by suramin (data not shown). It is noteworty that two different preparations of anti-HGF IgG and nonimmune IgG were mitogenic for SP1 cells and, therefore, provided no definitive information about a role of HGF in spontaneous growth of SP1 cells. It is possible that our inability to inhibit growth of SP1 cells with anti-HGF IgG is due to the presence of growth-promoting agents in the sample or that IgG itself is mitogenic for these cells, since normal rabbit IgG, normal turkey IgG, and normal horse IgG all showed variable mitogenic effects on SP1 cells. Whatever the case, these findings suggest that both HGF and HGF receptor/Met are expressed by SP1 cells and that growth and invasive phenotypes of SP1 cells may be facilitated by an autocrine growth factor loop. We have, therefore, independently evaluated the biological activity of HGF secreted by SP1 cells by assaying SP1 CM for stimulation of scatter activity of MDCK cells. SP1 CM induced a strong scatter activity of MDCK cells, compared to RPMI alone (Fig. 6, *A* and *B*). The identification of HGF in SP1 CM as the scatter-inducing factor was confirmed by the inhibition of scatter activity of SP1 CM by neutralizing anti-HGF IgG (Fig. 6*D*), whereas control IgG had no effect (Fig. 6*C*).

Discussion

In this study, we have presented evidence for the presence of an autocrine HGF loop in SP1 mammary carcinoma cells on the basis of three criteria: (*a*) SP1 cells express constitutively-activated HGF receptor/Met, and this activity is significantly inhibited by dissociation of HGF ligand from HGF receptor/Met; (*b*) a 6-kb HGF mRNA and a M_r 85,000 HGF protein, corresponding to the reported sizes of HGF mRNA and mature protein (10), respectively, are produced by SP1 cells; and (*c*) SP1 CM induces strong invasion of SP1 cells and strong scatter activity of MDCK cells, and induction of these functions is inhibited by anti-HGF IgG.

Evidence supports the involvement of different signal transduction pathways in HGF-dependent functions (33). We have demonstrated constitutive tyrosine phosphorylation of PLC-γ, p85 subunit of PI 3-kinase, and FAK in SP1 cells. These results suggest that creation of an HGF autocrine loop in SP1 cells induces specific signaling pathways, some of which may contribute to proliferation and invasion of these cells. However, the cytokine-activated signaling proteins JAK1, JAK2, JAK3, STAT1 (34), or the EGF receptor substrate Eps15 (35), although present, were not tyrosine phosphorylated in SP1 cells (data not shown). Thus, interleukins 1, 2, 4, and 9 and EGF receptor-mediated signaling pathways are not activated in an autocrine manner in SP1 cells under the culture conditions used. Whether cooperative stimulation via other growth factors or extracellular matrix components contributes to autocrine activation of signaling molecules in SP1 cells remains to be investigated.

Autocrine growth factors appear to be important in malignancy and metastasis. Many types of tumors release growth

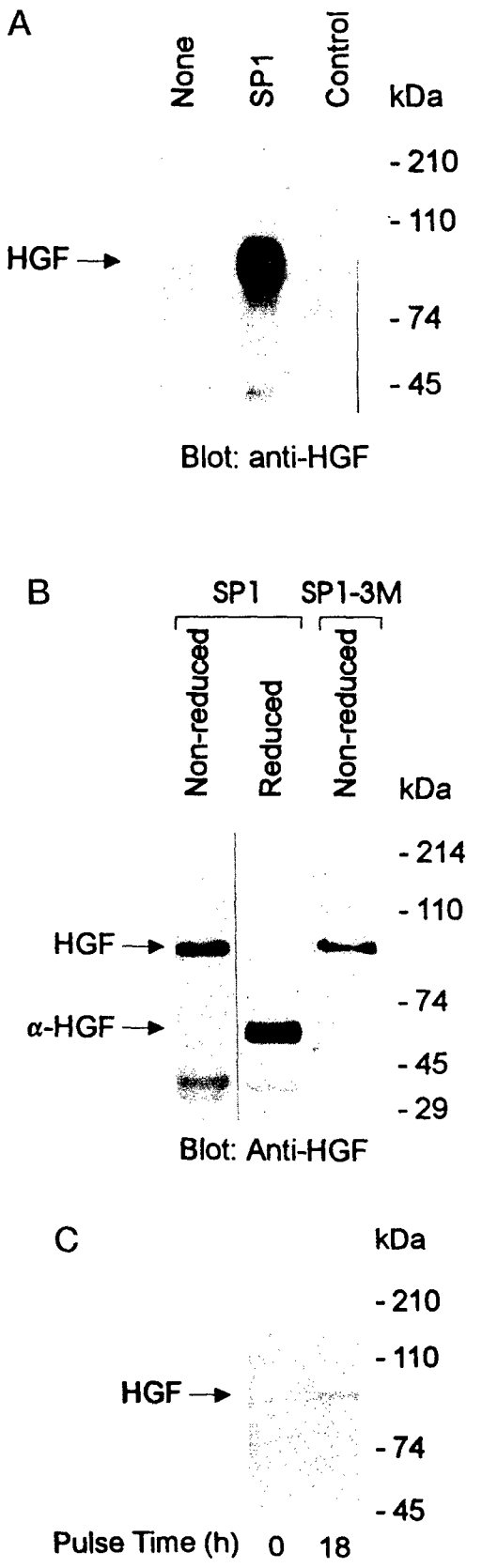


Fig. 5. Detection of HGF protein in SP1 cells. *A, in vitro* translation of HGF RNA transcript. Poly(A)$^+$ RNA was extracted from SP1 cells, translated in a rabbit reticulocyte system, and analyzed by Western blotting. *None*, *SP1*, and *Control*: no added RNA, 2 μg RNA from SP1 cells, and unrelated RNA supplied by Amersham as a negative control, respectively. The membrane was probed with polyclonal rabbit anti-HGF IgG, and protein bands were detected by ECL. *B*, SP1 CM and SP1–3M CM (25 ml each) were concentrated in Centricon 30 tubes, electrophoresed through 10% SDS-PAGE under nonreducing or reducing conditions as indicated, and blotted onto nitrocellulose membrane. The membrane was probed with polyclonal rabbit anti-HGF IgG. *C*, metabolic labeling of SP1 cells. Cells (5×10^6) were metabolically labeled with [^{35}S]methionine for 18 h as described in "Materials and Methods." CM from SP1 cells (10 ml) was immunoprecipitated with anti-HGF antibody, analyzed by SDS-PAGE, and autoradiographed. *Right*, protein molecular weight standards.

Table 1 Autocrine stimulation of cell invasion in SP1 cells is inhibited by anti-HGF IgG

Conditions[a]	Relative no. of cells invaded/well[b]
SP1 CM	24.6 ± 12.9
SP1 CM + anti-HGF IgG	3.0 ± 2.6[c]
rHGF	70.0 ± 10

[a] Invasion assays were performed in Matrigel-coated transwells as described in "Materials and Methods." SP1 CM with or without polyclonal rabbit anti-HGF IgG (15 μg/ml) or rHGF (20 ng/ml) was added to the lower well (800 μl). SP1 cells (10^4) were added in 200 μl RPMI with 0.2% FBS to the upper well. Cells that had initiated invasion were stained and counted on the underside of the membrane after 36 h.
[b] Values represent the mean ± SD of at least three wells/group.
[c] Significant reduction in the number of invading cells compared to the group with SP1 CM alone ($P = 0.0296$). A two-tailed Student *t* test was used. No difference between medium alone, SP1 CM, without or with nonimmune IgG, was observed (data not shown).

factors, including transforming growth factor α, platelet-derived growth factor, bombesin, and transforming growth factor β; these growth factors can act in an autocrine manner (8). Furthermore, an autocrine mechanism of transformation has been demonstrated for a variety of growth factors including v-sis, colony stimulating factor-1, and interleukin 3 (39–41). HGF and HGF receptor/Met might also play a significant role in tumor development and growth. This view was strongly supported by the fact that transfection of a full-size *met* cDNA into NIH 3T3 fibroblasts results in establishment of an HGF autocrine loop and induces transformation and tumor growth of these cells in nude mice (42). Furthermore, the *met* proto-oncogene was found to be overexpressed in a significant number of human cancers of epithelial origin (22, 23, 26–28). Experiments are in progress to determine the possible role of an HGF autocrine loop in growth and metastasis of breast carcinoma cells.

The clinical import of HGF in human breast cancer was highlighted recently in the work of Yamashita *et al.* (43, 44), who reported that a high level of expression of HGF is an even more significant factor in predicting poor relapse-free and overall survival than is lymph node status. However, what cells produce HGF in breast tumors and the potential role of HGF in breast cancer is not known. Tsarfaty *et al.* (45) have reported that HGF receptor/Met protein is expressed in both benign and malignant breast epithelium and that various breast cancer cell lines respond to HGF. Wang *et al.* (46) reported that HGF mRNA is expressed in both benign and malignant mammary epithelium, and that the most abundant expression in benign epithelium was in regions of prolifera-

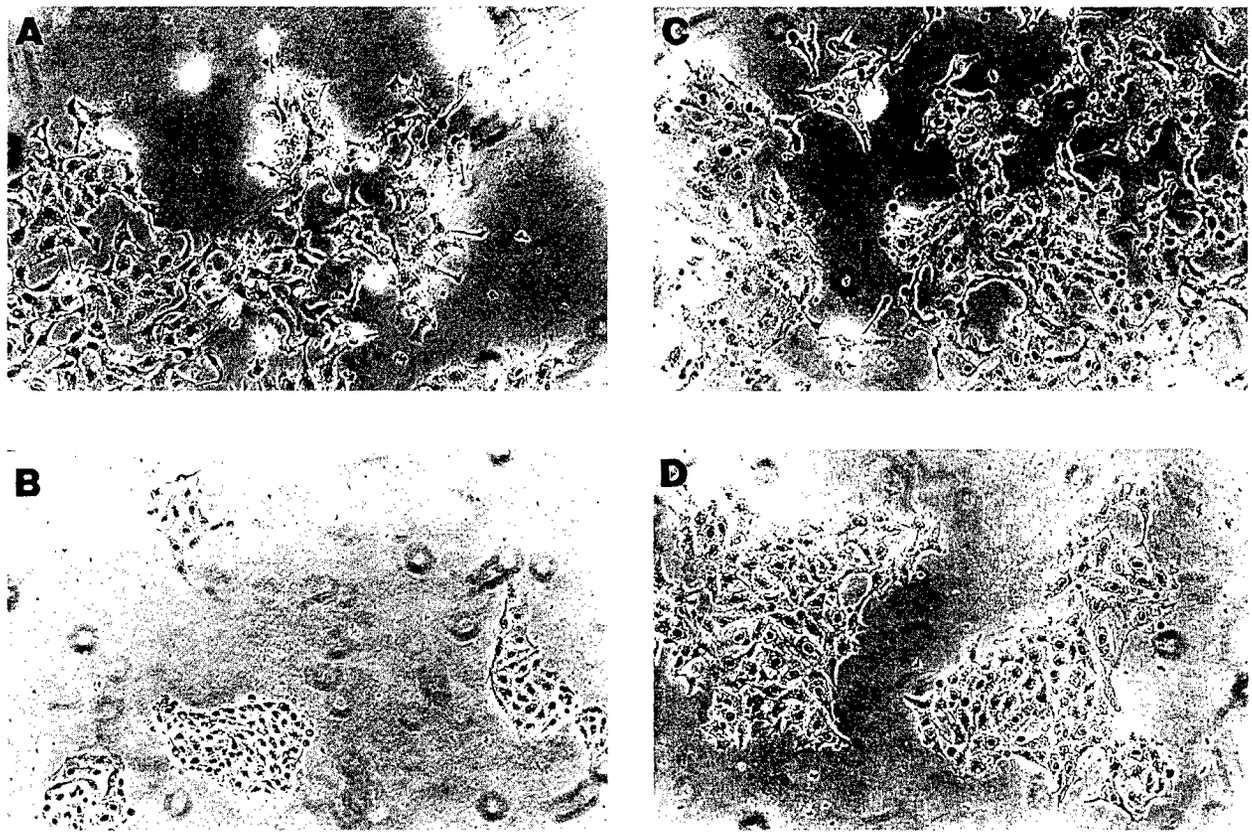


Fig. 6. Effect of SP1 CM on scatter activity of MDCK cells. MDCK cells (5000/well) were plated in 24-well Linbro plates and incubated at 37°C for 24 h in SP1 CM (*A*), RPMI medium (*B*), SP1 CM plus control rabbit IgG (*C*), and SP1 CM plus rabbit anti-HGF IgG (*D*) (both IgGs were at 15 μg/ml). SP1 CM was concentrated 10× using Centricon 30 tubes. Cells were viewed under a Leica inverted microscope and photographed with Kodak Technical Pan film. ×100.

tive activity. Our laboratory (30) has recently shown coexpression of HGF and HGF receptor/Met mRNA in benign and malignant epithelium; expression was particularly intense at the migrating tumor front. Together, these results support a possible autocrine role of HGF in human breast cancer.

We have demonstrated previously that adipocytes, a dominant stromal cell type in glandular tissues, support growth of SP1 cells, and subsequently, we have reported that HGF is the major mitogenic factor for SP1 cells in 3T3-L1 adipocyte CM (32). These findings suggest a paracrine role for HGF in breast cancer (32). Recent data from Wang *et al.* (46) and our laboratory (30) support a possible autocrine role of HGF in human breast carcinomas *in situ*. Our present results provide further support of an HGF autocrine loop in the mammary carcinoma cell line, SP1. Therefore, coexpression of HGF and HGF receptor/Met may provide a selective advantage for the progression of mammary carcinoma cells toward a more aggressive phenotype, making them independent of their surrounding tissues. Our present report indicates that both paracrine and autocrine HGF loops exist in SP1 mammary carcinoma cells and provides a physiologically relevant model for assessing the role of paracrine and autocrine pathways for HGF in breast cancer development.

Materials and Methods

Reagents. Monoclonal mouse anti-PY antibody was purchased from Transduction Laboratories. Polyclonal rabbit anti-Src, anti-PI 3-kinase, and anti-FAK IgG were purchased from UBI. Polyclonal rabbit anti-PLC-γ was kindly provided by C. Ellis (Glaxo Research and Development, Ware Hertfordshire, United Kingdom) (47). Polyclonal rabbit antimurine Met IgG was prepared as described (48), and sheep antihuman HGF IgG (which cross-reacts with the α-subunit of mouse HGF) was obtained from Genentech, Inc. Enhanced chemiluminesence (ECL) reagent was from Amersham. Prestained molecular weight standards were from GIBCO. Suramin was from FBA Pharmaceutical, Inc.

Cell Culture. The SP1 tumor cell line is a spontaneous nonmetastatic murine mammary intraductal adenocarcinoma isolated from an 18-month-old CBA/J female. The characteristics of the SP1 cell line have been described elsewhere (49). SP1–3M cells are a metastatic variant cell line derived from SP1 cells (31). Maintenance medium for SP1 and SP1–3M cells was RPMI (GIBCO) supplemented with 20 mM L-glutamine and 7% FBS (GIBCO).

Scatter Assay. Scatter assays were performed as described previously (18). In brief, SP1 CM (10 ml), concentrated to 1 ml using microconcentrating Centricon 30 tubes (Amicon), was added to MDCK cells, which had been plated overnight in DMEM plus 5% FBS at 3×10^3 cells/well in 24-well Linbro plates. After a 24-h incubation, the degree of scattering was assessed visually. Photographs were taken using a Leica inverted microscope with Kodak Technical Pan film.

Invasion Assay. Invasion assays were performed in transwells (Corning Costar) with a 6.5-mm diameter polycarbonate filter of 8 μm pore size, as described previously (50). Filters were precoated with Matrigel (Collaborative Research; 20 μg/ml in RPMI) and dried overnight. SP1 cells

(1×10^4) in 200 μl 0.2% FBS/RPMI were added to the upper well, and 800 μl various media as indicated were added to the lower well. After incubation for 36 h at 37°C, transwells were removed, and the tumor cells were fixed with 1% gluteraldehyde in PBS for 20 min. Cells were stained with hematoxylin, and the cells and Matrigel on the upper surface were removed with a cotton swab. Filters were dried, and the number of cells in a constant central area of each well was assessed using an inverted light microscope. In this way, cells that had invaded through the membrane could be directly assessed.

Immunoprecipitation and Immunoblotting. SP1 cells were grown to confluency and serum starved for 24 h. Cells were rinsed with cold PBS buffer three times and lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 1 mM sodium orthovanadate, 50 mM NaF, 2 mM EDTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 μM pepstatin. Lysates were centrifuged for 10 min at 14,000 rpm in an IEC/Micromax centrifuge at 4°C. The supernatants were incubated with 5 μl rabbit anti-Met IgG or the indicated antibodies at 4°C for 1 h. Immunoprecipitates were collected on protein A-Sepharose, washed three times with lysis buffer, separated on 8% SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was blocked with 3% BSA and probed with anti-PY (1:500) or anti-Met (1:200) antibodies. The membrane was washed four times with TBST buffer [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20] for 1 h, incubated with horseradish peroxidase-labeled secondary antirabbit or antimouse IgG antibodies (1:5000) for 1 h, and washed with TBST three times. Immune complexes were detected using ECL.

RNA Extraction and Northern Analysis. The Micro-Fast Track system (InVitrogen) was used to extract poly(A)$^+$ RNA from cultured cells. mRNA was subjected to denaturing electrophoresis on a 1.4% agarose gel and then transferred to Zeta-Probe membranes (Bio-Rad). The hybridization method used was a modification of that of Sambrook *et al.* (51). Blots were prehybridized for 4 h at 42°C in standard hybridization solution (50% formamide, 4× Denhardt's solution, 0.25× SSC, 0.1 mg/ml sheared herring testes DNA, 1% SDS, 5 mM EDTA, and 50 mM NaH_2PO_4). ^{32}P-labeled probes were prepared by a nick translation method (Nick Translation Kit; GIBCO) and hybridized with the membrane for 24 h at 42°C in standard hybridization solution. Membranes were washed for 15 min at room temperature with each of the following: 2× SSC/0.1% SDS, 0.5× SSC/0.1% SDS, and 0.1× SSC/0.1% SDS. The cDNA probe for HGF was obtained from T. Nakamura (Osaka University School of Medicine, Osaka, Japan) (10).

***In Vitro* Translation.** Poly(A)$^+$ was extracted from SP1 cells, and *in vitro* translation was carried out by using a rabbit reticulocyte system (Amersham). The resulting polypeptide products were subjected to Western blot analysis.

Metabolic Labeling and Immunoprecipitation. SP1 cells were metabolically labeled for 18 h with [^{35}S]methionine (Dupont) at 50 μCi/ml in methionine-free RPMI (GIBCO). CM was concentrated and immunoprecipitated with anti-HGF IgG. Immune complexes were analyzed by SDS-PAGE, fluorographed, dried, and exposed to film for autoradiography.

Acknowledgments

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Hepatocyte Growth Factor (HGF) Is a Copper-Binding Protein: A Facile Probe for Purification of HGF by Immobilized Cu(II)-Affinity Chromatography[1]

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Hepatocyte growth factor (HGF) is a multifunctional protein expressed in a variety of cell types and tissues. Here we describe a novel one-step method to separate and identify HGF, based on a unique interaction between HGF and Cu(II). Conditioned medium (CM) from mouse 3T3-L1 adipocytes which contains HGF or purified human recombinant HGF was used for analysis. Mouse 3T3-L1 adipocyte CM was applied to a Cu(II)-affinity column and rinsed with equilibration buffer. HGF was then eluted with 10 mM imidazole. Fractions eluted from the column were analyzed by SDS–PAGE. Analysis by silver staining revealed an 85-kDa protein. Further analysis by Western blotting with polyclonal anti-HGF IgG demonstrated that this protein corresponded to HGF. Human recombinant HGF, when applied to a Cu(II)-affinity column, showed a stronger affinity to Cu(II) than did mouse HGF. Human recombinant HGF was not eluted from the Cu(II) column with either 10 or 20 mM imidazole; however, it was readily eluted with 40 mM imidazole. The percentages of recovery of both human and mouse HGF were greater than 90%. Both mouse HGF and human recombinant HGF eluted from the Cu(II)-affinity column retained their biological activity as measured by HGF-induced cell proliferation of Mv1Lu cells. Our findings provide the first evidence that HGF is a copper-binding protein and that a Cu(II)-affinity column can be used for efficient one-step purification of biologically active HGF.

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Hepatocyte growth factor (HGF) is a heterodimeric glycoprotein composed of two subunits, an α chain (69 kDa) and a β chain (34 kDa) (1), and exhibits pleiotropic functions. HGF stimulates DNA synthesis in various cell types including human and mouse mammary epithelial cells (2,3). HGF is also a morphogen that induces epithelial tubular formation and angiogenesis, a process that forms cords and tubes of endothelial cells *in vitro* (4). In addition to its mitogenic and morphogenic functions, HGF also induces epithelial cell motility (5,6). The receptor for HGF was identified as the product of the *met* proto-oncogene (7,8) and is involved in the transduction of cell regulatory signals in response to HGF (8,9).

Although a great deal of knowledge about *in vitro* functions of HGF has been demonstrated, at present the physiological role of HGF *in vivo* remains largely unknown. This is partly due to the lack of an efficient method for purification of preparative amounts of HGF from specific tissues and cell lines, and of sufficient quantities of HGF for functional studies *in vivo*. To date attempts to purify HGF have been costly, time consuming, and laborious: Platelets from more than 3000 rats were used to purify 60 μg of HGF by a three-step procedure involving cation-exchange chromatography, heparin–Sepharose affinity chromatography, and a C_4 column (10). More than 100 liters of rabbit serum or conditioned medium from MRC-5 fibroblasts was used to purify 50–100 μg of HGF; this purification involved heparin–Sepharose affinity chromatography, anion-exchange chromatography, and reversed-phase HPLC (11,2). Also, attempts to express HGF cDNA in the COS cell line resulted in relatively small yields (12,10).

In this study we demonstrate evidence that HGF is a copper-binding protein. HGF showed high-affinity

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binding to copper when an immobilized Cu(II)-affinity column was used. This unique interaction between HGF and Cu(II) provides a novel method for efficient one-step purification of HGF and HGF-related proteins.

MATERIALS AND METHODS

Materials. The following chemicals were obtained from Fisher Scientific Co. (Ottawa, Canada): imidazole, ~~NaCl~~, EDTA, Na_2HPO_4, and $CuSO_4$. Iminodiacetic acid coupled to epoxy-activated Sepharose 6B was obtained from Pharmacia (Uppsala, Sweden). Rabbit anti-sheep IgG conjugated to horseradish peroxidase was from Bio/Can Scientific (Mississauga, Canada). ECL and Hyperfilm were from Amersham (Oakville, Canada). Silver staining reagents and nitrocellulose membrane were from Bio-Rad (Mississauga, Canada). Human recombinant HGF and affinity-purified sheep anti-human HGF IgG, which reacts with both human and mouse HGF, were kindly provided by Dr. R. Schwall (Genentech, Inc., San Francisco, CA). Conditioned medium (CM) from 3T3-L1 adipocytes was collected following incubation of cells for 24 h in serum-free DMEM as described previously (3). Mv1Lu cells are a mink epithelial cell line obtained from ATCC (Rockville, MA).

Cu(II)-immobilized affinity chromatography. Cu(II)-affinity chromatography was performed with iminodiacetic acid coupled to epoxy-activated Sepharose 6B (Pharmacia) (1 ml packed volume) according to the manufacturer's instructions. The column was loaded with 1 ml $CuSO_4$ (0.1 M) and was equilibrated with an equilibration buffer (10 ml) containing 0.02 M Na_2HPO_4, 1 mM imidazole, and 1 M NaCl (pH 7.2). 3T3-L1 adipocyte CM as a source of mouse HGF or purified recombinant human HGF was mixed with equilibration buffer and directly loaded onto the column; the column was then washed with the equilibration buffer (10 ml). Mouse HGF and recombinant human HGF were eluted at a flow rate of 1 ml/2 min with the equilibration buffer plus 10 and 40 mM imidazole, respectively. Fractions (10 ml) were collected and subsequently subjected to biochemical and functional analysis.

Western blotting. Proteins were separated by electrophoresis on 10% SDS–PAGE and transferred electrophoretically to a nitrocellulose membrane. The nitrocellulose membrane was incubated with sheep anti-HGF IgG (1:1000) and was visualized by successive incubations with rabbit anti-sheep IgG conjugated to horseradish peroxidase (1:5000). The ECL kit (Amersham) was used to detect the protein.

SDS–PAGE and silver staining. The protein samples ~~were analyzed by electrophoresis on 10% polyacrylamide gels~~. The proteins were visualized by a silver stain kit (Bio-Rad) which was used according to the manufacturer's instructions.

Cell proliferation assay. Mv1Lu cells were plated at 2×10^4 cells per well in 24-well plates under the various conditions indicated. DNA synthesis was measured at 2 days by adding 0.2 μCi [^{3}H]thymidine (Amersham) at 24 h. After an additional 24 h, cells were harvested with trypsin/EDTA. Aliquots of cells were placed in 96-well microtiter plates and transferred to filters using a Titertek cell harvester (Flow Laboratories), and [^{3}H]thymidine incorporation was measured in a scintillation counter (Beckman). Results are expressed as the mean cpm/well ± SD of triplicates.

RESULTS AND DISCUSSION

Immobilized metal-affinity chromatography, also known as metal chelate-affinity chromatography, has been used to identify interactions between certain proteins and divalent cations (13–15). Using immobilized Cu(II)-affinity chromatography, we demonstrated that HGF bound with high affinity to a Cu(II)-charged column. Our initial experiments demonstrated that retention of HGF on the column containing iminodiacetic acid coupled to epoxy-activated Sepharose 6B matrices required the presence of immobilized Cu(II): HGF was not eluted under typical elution conditions (0.02 M Na_2HPO_4 alone or plus 1 M NaCl and 1 mM imidazole). Instead, efficient elution of HGF required the presence of higher concentrations of imidazole, a specific competitor for bound copper, or EDTA, a chelator of divalent cations, in the elution buffer (data not shown). These results indicated that HGF bound to Cu(II) with high affinity and/or that multiple sites on the protein combined to form a highly stable interaction with immobilized Cu(II). We therefore decided to take advantage of this unique interaction between HGF and Cu(II) to purify HGF from physiological samples.

Previously we have demonstrated that mouse mammary 3T3-L1 adipocytes secrete biologically active HGF at high levels (3). In this study, 3T3-L1 adipocyte CM was used as a source of mouse HGF. 3T3-L1 adipocyte CM was concentrated and loaded directly onto a Cu(II)-affinity column. The column was rinsed with equilibration buffer (0.02 M Na_2HPO_4, 1 mM imidazole, and 1 M NaCl), and protein samples were eluted from the column with increasing concentrations of imidazole. A base amount of imidazole (1 mM) was added to the equilibration buffer to avoid nonspecific interactions of proteins present in the 3T3-L1 adipocyte CM. Aliquots of the eluates were analyzed by SDS–PAGE under nonreducing conditions followed by silver staining (Fig. 1A). Identical samples were also analyzed by Western blot analysis with anti-HGF IgG (Fig. 1B). The results revealed that HGF bound Cu(II) with high affinity and was not eluted from the column with the

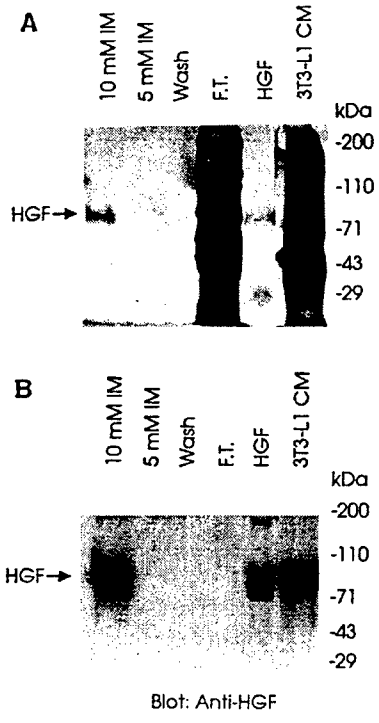

FIG. 1. Purification of mouse HGF from 3T3-L1 adipocyte CM. 3T3-L1 adipocyte CM (40 ml) was concentrated to 2 ml using Centricon 10 tubes and loaded onto a Cu(II)-affinity column. The column was rinsed with equilibration buffer (10 ml). Protein samples were sequentially eluted in 10-ml fractions with equilibration buffer plus 5 mM imidazole (5 mM IM) and 10 mM imidazole (10 mM IM). After concentration of eluates, aliquots corresponding to half of each fraction were separated by 10% SDS–PAGE (nonreducing conditions) and subjected to (A) silver staining and (B) Western blot analysis with anti-HGF IgG. Protein molecular weight standards are shown on the right. Tracks from ~~left~~ right: 3T3-L1 CM (20-ml equivalent), HGF (0.05 μg), flowthrough (F.T.), wash, and 5 and 10 mM IM fractions.

equilibration buffer (0.02 M Na_2HPO_4, 1 M NaCl, and 1 mM imidazole) or the equilibration buffer plus 5 mM imidazole. Efficient elution from the column of mouse HGF protein with an apparent molecular weight of 85 kDa was achieved with 10 mM imidazole (Figs. 1A and 1B). The flowthrough and fractions eluted with 5 mM imidazole did not contain any HGF as analyzed by Western blot analysis (Fig. 1B). However, non-copper-binding proteins readily came off the column in the flowthrough (Fig. 1A). Based on protein concentrations, the purification of HGF was at least 28-fold (Table 1). The same procedures have been successfully used to purify HGF from CM of a murine mammary carcinoma cell line, SP1 (data not shown).

We also evaluated the affinity of recombinant human HGF binding to the Cu(II)-affinity column. Interestingly, analysis of the elutes by silver staining revealed that recombinant human HGF bound Cu(II) with an even higher affinity than mouse HGF, and was not eluted from the column with the flowthrough, wash

TABLE 1

Recovery of HGF Purified from 3T3-L1 Adipocyte Conditioned Medium

Fraction	Volume	Total protein (mg)[a]	Recovery[b]	Purity (fold)[a]
Original material	40 ml	24.6	100%	1
10 mM IM eluate	10 ml	0.8	95%	28

[a] 3T3-L1 adipocyte conditioned medium was separated on a Cu(II)-affinity column as described for Fig. 1A. The amount of protein in each fraction was determined using spectrophotometry at 280 nm and the fold purification of HGF protein eluted at 10 mM imidazole compared to total protein in the original sample was calculated.

[b] The percentage of recovery of HGF was determined by comparing the density of the protein bands corresponding to HGF in the sample eluted with 10 mM imidazole and in the original sample in Fig. 1B.

(equilibration buffer), or equilibration buffer containing either 10 (data not shown) or 20 mM imidazole (Fig. 2A). However, elution of recombinant human HGF from the column was effectively achieved with equilibration buffer containing 40 mM imidazole (Fig. 2A) or 5 mM EDTA (data not shown). Subsequent elutions with equilibration buffer containing 80 mM imidazole or 5 mM EDTA yielded no additional HGF. The percentages of recovery of both mouse HGF and recombinant human HGF from the column were greater than 90% (Figs. 1 and 3). The difference observed in the affinity of human and mouse HGF for Cu(II) may be due to the minor differences in the composition of primary amino acid sequences between human and mouse HGF. The amino acid sequences of HGF appear to be highly conserved across species and the overall amino

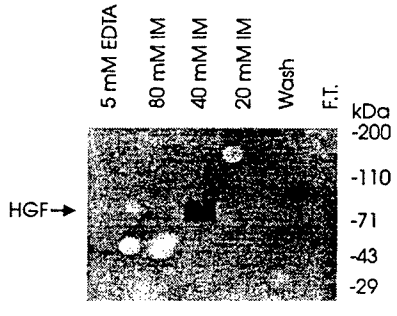

FIG. 2. Detection of human recombinant HGF eluted from a Cu(II)-affinity column. Purified human recombinant HGF (0.1 μg) was applied to a Cu(II)-affinity column. The flowthrough (F.T.) was collected and the column was rinsed with equilibration buffer (Wash). Protein samples were sequentially eluted with equilibration buffer containing 20 mM imidazole (20 mM IM), 40 mM imidazole (40 mM IM), 80 mM imidazole (80 mM IM), or 5 mM EDTA. The fractions were concentrated and aliquots corresponding to half of each fraction were subjected to silver staining as for Fig. 1. Protein molecular weight standards are shown on the right.

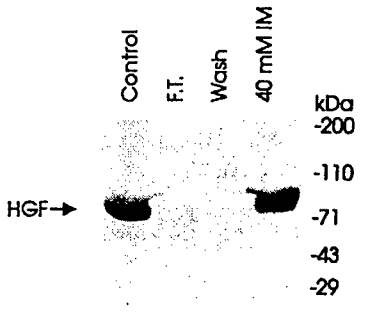

FIG. 3. Evaluation of efficiency of recovery of recombinant human HGF from a Cu(II)-affinity column. Purified human recombinant HGF (0.1 μg) was applied to a Cu(II)-affinity column. The flowthrough (F.T.) was collected and the column was rinsed with equilibration buffer (Wash). Protein samples were subsequently eluted with equilibration buffer containing 40 mM imidazole (40 mM IM). After concentration of eluates, half of each fraction or 0.05 μg of HGF (control) was loaded into each well and separated by 10% SDS–PAGE (nonreducing conditions) and subjected to silver staining. Protein molecular weight standards are shown on the right.

acid identity of mouse and human HGF is about 90% (16). The 10% differences in amino acid sequences or posttranslational glycosylation of HGF may be reasons for such differences. Positively charged amino acids in particular histidine residues are most likely involved in mediating binding of HGF with copper. Identification of amino acids responsible for this interaction is currently being investigated. Overall, these results clearly indicate that both mouse and human HGF bind Cu(II) with high affinity, and/or that multiple sites on the protein combined to form a highly stable interaction with immobilized Cu(II).

Both mouse HGF and recombinant human HGF eluted from the column also showed biological activity in a cell proliferation assay (Figs. 4A and 4B). Aliquots of the eluates were assayed for growth factor activity by measurement of DNA synthesis in MlvLu cells. The mitogenic activity (100%) of 3T3-L1 adipocyte CM [previously shown to be HGF-induced (3)] and of recombinant human HGF was recovered in the fractions eluted with 10 and 40 mM imidazole, respectively, but not in the flowthrough (Figs. 4A and 4B). These results suggest that HGF was eluted specifically from the Cu(II)-affinity column with imidazole and is biologically active.

It has been demonstrated that exposure of some growth factors such as fibroblast growth factor (FGF) 1 and FGF2 to copper results in formation of intermolecular disulfide bonds by copper-induced oxidation of sulfhydryl residues of FGFs and subsequent loss of biological activity (17). Our results demonstrated that both mouse and human HGF eluted from a Cu(II)-affinity column retained biological activity and that no dimerization of HGF was detected in silver staining and Western blot analysis. Overall, these results indicate that interaction between HGF and copper is not the result of copper-induced formation of intermolecular disulfide bonds in the HGF molecule.

Our findings provide the first evidence that HGF is a copper-binding protein and that a Cu(II)-affinity col-

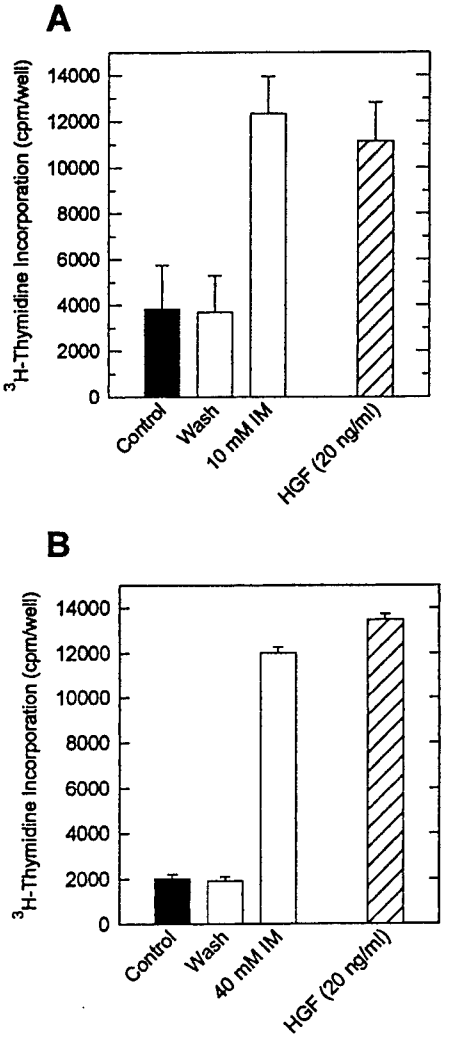

FIG. 4. Evaluation of biological activity of mouse and human recombinant HGF eluted from a Cu(II)-affinity column. Aliquots of the eluates of 3T3-L1 adipocyte CM and of human recombinant HGF from a Cu(II)-affinity column were used for a proliferation assay with Mv1Lu cells. (A) Biological activity of mouse HGF eluted from the column. Cells were plated (2×10^4/well) in serum-free DMEM in the absence of eluates (control) or in the presence of eluates (one-sixth volume per well) washed with equilibration buffer alone (Wash) or equilibration buffer plus 10 mM imidazole (10 mM IM) or in the presence of 20 ng recombinant human HGF. (B) Biological activity of recombinant human HGF eluted from the column. Cells were plated (2×10^4/well) in serum-free DMEM in the absence of eluates (control) or in the presence of eluates (one-sixth volume per well) washed with equilibration buffer alone (Wash) or equilibration buffer plus 40 mM imidazole (40 mM IM) or in the presence of 20 ng recombinant human HGF. DNA synthesis was measured as incorporation of [^{3}H]thymidine (0.2 μCi/well). The results are expressed as the mean cpm/well ± SD of triplicates.

umn can be used for efficient purification of biologically active HGF. This method provides a simple, inexpensive, and novel method to purify HGF in large quantities. Our results also raise the possibility that copper-binding properties of HGF may play a role in HGF functions.

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