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The Role of Stroma in Breast Carcinoma Growth *In Vivo*

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ABSTRACT

The malignant progression of tumors is thought to be related to the expression of oncogenes and loss of expression of tumor suppressor gene. These factors are intrinsic to the cancer cells themselves. However, carcinomas are also infiltrated by host cells (fibroblasts, endothelial cells, inflammatory cells) and surrounded by an extracellular matrix which is extensively remodeled. The extracellular matrix components and infiltrating host cells provide a microenvi-ronment that conditions both tumor progression and the metastatic process. Transplantation of human tumors into athymic nude mice has become an important experimental approach to study the biology of human cancers. The different models developed so far are beginning to elucidate the role of matrix molecules, growth factors and enzymes as well as fibroblasts in tumor progression. These animal models are likely to provide a useful tool to evaluate new antitumor treatments.

Keywords: Breast carcinoma; extracellular matrix; laminin; fibroblasts; matrix metalloproteinases; *in vivo* tumorigenicity.

INTRODUCTION

Cancer cells are not simply isolated islands of cells residing in a specific organ, they are surrounded by extracellular matrix and by stromal cells both of which influence tumor progression. The stroma around tumors is different from the normal breast stroma and is believed to be critically involved in malignant growth.

The extracellular matrix is a complex meshwork of glycoproteins and proteoglycans that determines tissue architecture and conditions many biological activities. Components of the extracellular matrix provide a large variety of specific signals which directly influence cell growth, migration, morphology, proliferation, differentiation, and biosynthetic activities (1,2). In addition, the extracellular matrix plays essential roles in cell survival, and loss of contact with matrix components results in apoptosis (3). It has become clear that the extracellular matrix is made up of specific structural modules that encode information interpreted by cells through interactions with specific plasma membrane receptors, mostly of the integrin family (1). Although the precise mechanisms of matrix effects remain to be elucidated, there is a considerable evidence that the extracellular matrix influences cell properties and behavior by modification of cytoskeletal organization and activation of second messenger and protein kinase pathways (1). In addition, the extracellular matrix is a site of sequestration of various factors that are likely to affect cell activity such as growth factors, mobility factors, natural proteases and their inhibitors.

The extracellular matrix is involved in both normal and pathological processes. Recent studies have revealed the importance of the basement membrane in the morphogenesis and differentiation of mammary epithelial cells [for review(2)]. For example, laminin, one of the major component of basement membranes acts synergically with lactogenic hormones to activate transcription of a tissue-specific gene, β-casein in normal mouse mammary cells in culture (4, 5) These experiments demonstrate the important role of the microenvironment in the control and maintenance of tissue-specific function.

Factors that affect the extracellular matrix might lead to abnormal cellular function and even to cancer progression. Perturbations in the production, deposition and degradation of matrix components have been observed in mammary carcinomas (2). For example, transgenic mice over-expressing stromelysin-1, an extracellular matrix degrading enzyme, were shown to undergo premature involution of the mammary gland in pregnancy and later, to develop mammary tumors (6, 7). These observations suggest that perturbation of the tissue microenvironment by proteases may be sufficient to induce tumor formation.

Breast carcinomas are often characterized by a stromal reaction that consists of modifications in the composition of both the cellular elements (infiltration of fibroblastic cells, endothelial cells, inflammatory cells) and the extracellular matrix. This reactive stroma actually constitutes a major part of the neoplasm. For a long time, only the neoplastic cells were the focus of interest in cancer research and the stroma was rather considered a reactive component without major significance. However, it has become clear that the stromal cells and their products (matrix components, growth factors, proteases, etc.) condition the phenotype of cancer cells. Tumors thus represent a complex ecosystem where multiple host cells-extracellular matrix and tumor cells-extracellular matrix, as well as tumor cells-host cells interactions lead to reciprocal influences resulting in tumor promotion, invasion and metastasis (8).

In this review, we focus on the role of certain matrix proteins, mainly laminin, and stromal cells in tumor progression. We first consider the stromal reaction observed in most breast neoplasms. We describe *in vivo* models in nude mice developed to test the tumorigenicity of human adenocarcinoma cells. In the second part of this paper, we focus on the importance of the tissue microenvironment, added extracellular matrix components, fibroblasts and their products in tumor growth *in vivo.* Finally, the relevance of such models in evaluation of new anticancer therapy that targets stromal cells rather than cancer cells is discussed.

STROMAL REACTION IN BREAST CARCINOMA

Invasive or infiltrating ductal carcinomas, which represent the most common type of breast cancer, are characterized by a pronounced degree of desmoplasia and are often referred to as "scirrhous carcinoma". Desmoplasia is a common host response to epithelial tumors and is classically described as fibroblast proliferation in conjunction with extracellular matrix remodeling. This reactive stroma exhibits many of the changes observed during wound healing, albeit in an uncontrolled fashion (9). Considering these stromal changes in the neoplastic breast, it is reasonable to suggest that this tissue component plays an important role in the pathogenesis of the disease. Stroma generation is essential to growth of solid tumors, most obviously through its supply of blood vessels required for tumor progression (10, 11). Therefore, the stroma is not a passive barrier to invasive tumors that has to be penetrated, but it is an active player in cancer progression.

The stromal reaction is characterized by both extracellular remodeling and by modification of cellular composition.

A. Extracellular Matrix Remodeling

Stromal connective tissue is composed of interstitial collagens (mainly collagen types I and III), fibro-nectin and various proteoglycans. The "desmoplastic reaction" is characterized by both quantitative and qualitative modifications in the composition of the connective tissue matrix (9, 12, 13). An excessive accumulation of extracellular matrix components including different types of collagen (types I, III, V), fibronectin, elastin and proteoglycans is often observed. In addition, nonbasement membrane type IV collagen was shown to be increased in elastotic breast tumor tissues (14). Interestingly, trimers of α_1 type I collagen chain type (I-trimer) and EB-B+ fibronectin resulting from alternative splicing of pre mRNA, otherwise only found in preadult breast tissue, were re-expressed in infiltrating ductal carcinomas (15). Furthermore, breast tumor cells and stromal cells have been shown to re-express the laminin β_2 chain which is widely distributed in embryonic basement membrane, but missing from mature tissue (16). Bone sialo-protein (BSP),[1] a bone-matrix protein involved in hydroxyapatite crystal formation is ectopically expressed in human breast cancers and results in the formation of microcalcification which can be detected in early lesions (17).

Lysy1 oxidase is involved in collagen and elastin cross-linking. While it is undetectable in normal breast, its expression was observed in newly formed stroma in benign lesions and *in situ* ductal breast carcinoma. It has been postulated to be part of an early host defense mechanism. In contrast, lysyl oxidase was not found in the stroma of invasive tumors (18). The low level of lysyl oxidase-cross-linking may be responsible for a loss of matrix organization and a higher sensitivity to metalloproteases, both could favor tumor invasion. Altogether these observations underline both quantitative and qualitative modifications of the tumor stroma.

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[1] Abbreviations: matrix metalloproteinases (MMP); tissue inhibitor of matrix metalloprotease s (TIMP); Stromelysin-3 (ST-3); bone sialoprotein (BSP).

Quantitative changes in matrix components may be related to an imbalance between their synthesis and degradation. Tumor cells may directly alter the adjacent matrix by producing excessive matrix proteins or proteolytic enzymes. Alternatively, the desmoplastic response may depend on specific interactions between tumor cells and host fibroblastic cells. For example, breast adenocarcinoma cells in culture were shown to produce diffusible factors able to stimulate the synthesis of proteoglycans, different types of collagen and fibronectin by human fibroblasts (19, 20). Indeed, while human breast tumor MCF7 cells were unable to synthesize collagen in culture, they induce a three-fourfold enhancement of collagen production by human fibroblasts (19).

B. Modification of Cellular Composition

The breast neoplastic stroma contains a heterogenous cell population composed of fibroblasts, myofibroblasts, endothelial cells and inflammatory cells. Stromal cells are known to produce a variety of cytokines, growth factors, and proteases which may influence neoplastic cell properties.

The appearance of myofibroblasts expressing smooth muscle a-actin is a prominent feature of the stromal reaction observed in wound healing and carcinomas. In ductal mammary carcinomas, for example, they constitute more than 70% of stromal cells. Ronnov-Jessen *et al.* (21) suggested that the myofibroblasts in breast carcinoma form primarily by differentiation from fibroblasts, but they also may be derived from vascular smooth muscle cells, and occasionally from pericytes. Cytokines such as TGFβ produced by cancer cells or released from the extracellular matrix are likely involved in these differentiation processes (21). In breast cancer, the myofibroblasts have been shown to produce enzymes involved in proteolysis of matrix components such as urokinase, plasminogen activator and stromelysin-3 (22). They are also able to retract collagen bundles and in this way, lead to the "fibrotic and X-ray dense aspect" of the intratumoral stroma. The clinical significance of the complex changes in tumor stroma remains controversial. Pathological observations suggested that this "stromal reaction" resembles wound healing and represents tumor encapsulation and a host defense (9). Others suggest that, by providing a scaffold for newly formed blood vessels and by complex interactions with tumor cells, the stroma facilitates tumor progression and invasion (10).

ROLE OF TISSUE MICROENVIRONMENT IN TUMOR GROWTH *IN VIVO*

Animal models of breast cancer have been used to study different aspects of breast cancer biology and are diverse including chemically or virally induced tumors, human tumor xenografts and trangenic mouse models [for review, see (23, 24)]. We focus here on the transplantation of human tissues in an adequate *in vivo* microenvironment. Experimental models close to the *in situ* environment of human cancers are required in order to study cancer progression, tumor invasion and to evaluate new anti-tumor treatments. The ideal model should allow the growth of tumors presenting histological features of the original cancers and should also mimic the interactions occurring between tumor cells and host factors. Xenografts of established human breast cancer cell lines allow studies of their hormone dependence (MCF7 and T47D cells), hormone-independence (most breast cell lines), drug resistance (MCF7-^{ADR} cells), metastasis (MDA-MB231 and MDA-MB435 cells), and angiogenesis (MCF7 transfected with VEGF or FGF-4) (23, 25, 26). Some cells for example, provide a useful model to study the pathogenesis of malignant ascites (MDA-435 /LCC6 cells) and of proliferative disease (MCF10 A neo T cells) (23, 27).

Transplantation of human tumors *in vivo* requires the use of immunodeficient animals, most commonly athymic or nude mice which do not reject heterotrans-plants of human tumor (23). Human tumors that are able to grow in nude mice generally retain their morphological and biochemical features (28). However, fewer than 10% of breast cancers are transplantable to these animals (28, 29). The nature of the microenvi-ronment appears to be one factor involved in the ability of human tumors to grow in nude mice. The use of the mammary fat pad (orthotopic site) as a site for transplanting breast cancer cells has been shown to improve the tumor incidence or tumor "take" (the percentage of tumor bearing animals), and tumor growth compared to a subcutaneous site [for review, see (28)]. This trophic effect of fat pad is specific for breast cancer cells since no difference has been seen in the growth or take of injected cancer cells derived from colon, kidney, or melanoma when the mammary fat pad was compared with subcutaneous sites. Metastatic behaviour is also known to be enhanced when breast tumor cells are implanted orthotopically (28, 30). Nude mice with mammary fat pad tumors of MDA MB-435 cells developed more frequently metastasis (80- 100% mice) than mice with subcutaneous tumors (20-40% mice) (29). These observations emphasize the important role of the tissue microenvironment for tumor growth and full expression of the metastatic phenotype.

Injection into the mammary fat pad requires general anesthesia and surgical exposure of the fat pad to ensure that cells are injected into the tissue and not into the subcutaneous space [for review, see (31)]. However, subcutaneous inoculation is a quicker and easier procedure and is therefore more often used. An alternative approach to improve the tumorigenicity of human cancers implanted subcutaneously into nude mice is to mix cancer cells with Matrigel, a "reconstituted basement membrane" or with normal human fibroblasts.

ROLE OF BASEMENT MEMBRANE IN TUMORIGENICITY *IN VIVO*

Much of our understanding of the role of the extracellular matrix in tumor growth has come through the use of Matrigel. Matrigel is a solubilized basement membrane matrix extracted from the Engelbreth-Holm-Swarm tumor. Its major components are lami-nin-1, type IV collagen (α1 and α2 chains), heparan sulfate proteoglycans, and entactin.

Laminin is a 850 kDA heterotrimeric cross-shaped molecular complex (Fig. 1). The laminin molecule consists of a large α chain and two different smaller chains, the β and γ chains connected by disulfide bridges. The prototypic laminin is the laminin-1 isolated in 1979 from the Engelbreth-Holm-Swarm tumor (made up of α_1, β_1, and γ_1 chains) and present in Matrigel. It is expressed ubiquitously in epithelium and endothelium. Different laminin isoforms arise from an exchange of single chains (3 α chains, 3 β chains and 2 γ chains) [for review, (15)].

Various growth factors also accumulate in Matrigel including at least epidermal growth factor (EGF), insulin-like growth factor (IGF-1), platelet-derived growth factor (PDGF), transforming growth factor β (TGFβ) and basic fibroblast growth factors (bFGF) (32). Matrigel polymerizes at 37°C to produce a reconstituted, biologically active matrix which aids adhesion and differentiation of cells (33).

When human cancer cells were mixed with Matrigel, tumor developed after subcutaneous transplantation into nude mice. This tumor promoting effect was observed with various tumor cell types including lung, prostatic, mammary, colonic carcinoma cells and melanoma cells (32-37). In the absence of Matrigel, human breast adenocarcinoma MCF7 cells and MCF7/6 cells failed to produce tumor (Table I, Fig. 2). In its presence, tumors appeared rapidly in 100% of the injected animals (Table I, Fig. 2). These findings indicate that for these breast tumor cell lines, interactions with basement membrane components are required for tumor formation. For some other mammary cell lines tested (Table I), Matrigel reduced the latency period for appearance of tumor. In addition, it increased or maintained the percentage of tumor-bearing animals at 100%.

When estrogen-dependent MCF7 cells were inoculated into mice supplemented with estrogen by implantation of an estrogen pellet, tumors developed in 80 - 100% of the animals. The mean tumor volume increased progressively during the study period. In contrast, the tumor incidence was lower in ovariectomized mice (50%) and tumor volume remained static after an initial small increase (Fig. 2). Therefore, although the tumor take for MCF7 cells was increased by addition of Matrigel even in the absence of estrogen, sustained tumor growth required estradiol supplementation, confirming the hormone dependence of these tumors (34).

Since matrigel was shown to enhance the tumorigenic potential of a number of mammary breast cancer cell lines (MCF7, MDA-MB231, T47D) (35, 40), xenografted fresh specimens from primary tumors might also provide interesting data on the microenvi-ronmental factors that promote tumor growth. Cell suspensions or biopsies of human primary breast cancers were transplanted into nude mice (31). When enzymatically dispersed cell suspensions of primary tumors were injected into mice, about 7% of the tumors grew as palpable nodules. In the presence of Matrigel, tumor incidence from xenografted primary breast cancer biopsies reached 50% (26).

The exact mechanism by which Matrigel acts as a tumor promoter is not completely known. First, its effect is not related to its capacity *to form a gel,* since other substances that were able to gel such as type I collagen failed to enhance tumor growth (37, 41). Moreover, when Matrigel was too strongly diluted to form gels, tumor growth was also stimulated in nude mice.

Secondly, since increased vascularization was observed to develop around and within tumors after injection of tumor cells and Matrigel (42), the stimulation of tumor growth by Matrigel has been attributed *to increased angiogenesis.* Matrigel could favor neovascularization, stimulating development of a vascular bed that provides a substratum for tumor cell proliferation. In this regard, the ability of Matrigel to form a gel at body temperature was exploited in order to develop assays for quantifying angiogenesis *in vivo* (41, 43). Injection of Matrigel into BALB/c, nude and SCID mice was sufficient to induce the production of capillary ingrowths which infiltrated the matrix suggesting that Matrigel itself or perhaps even laminin-derived peptides may contribute to

angiogenesis. However, an optimal effect of Matrigel on angiogenesis *in vivo* required recruitment of host cells such as fibroblasts (44). In addition, angiogenesis observed *in vivo* in tumor induced with Matrigel is probably due, at least in the case of HT 1080 cells, to a synergistic effect between a tumor-derived angiogenic factor and Matrigel (43). Thirdly, Matrigel may also stimulate tumor expansion by promoting *the expression or activation of various proteases* such as stromelysin-1 (5), gelatinase A (45) and plasminogen activator (46). The role of these proteases, produced mainly by stromal cells, will be discussed below.

Fig. 1. *Schematic model of laminin-1 molecule. The three chains α_1, β_1, γ_1 are held together by disulfide bonds. Three putative binding sites (YIGSR, RGD and SIKVAV) are indicated. Their promoting (+) or inhibiting (—) effects on cell properties are mentioned.*

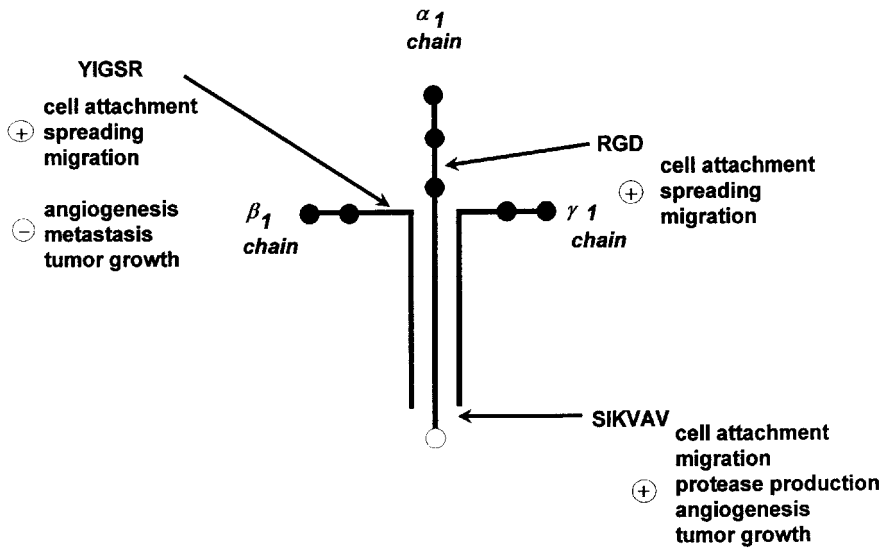
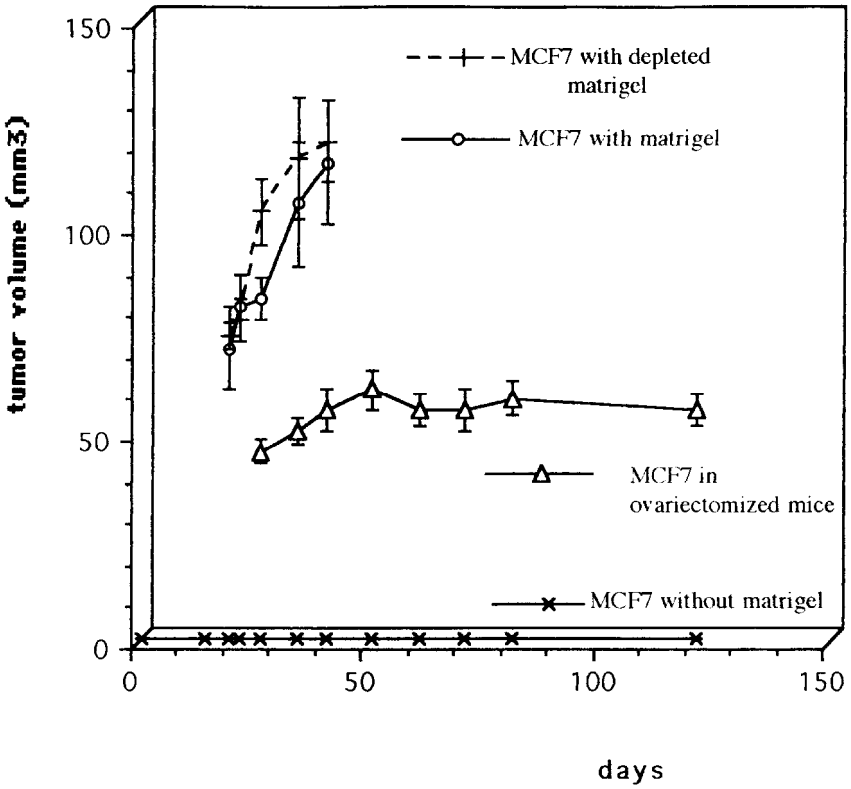


Table I. *Effect of Matrigel on the In Vivo Tumorigenicity of Human Breast Adenocarcinoma Cells*

Cells	Treatment[a]	Incidence	Latency period days[b]
MCF7	none	0/20	
	matrigel	20/20	16
	"depleted matrigel"	5/5	18
MCF7/6	none	0/10	—
	matrigel	5/5	39
MCF7 gpt	none	10/10	31
	matrigel	10/10	8
MCF7 ras	none	9/10	27
	matrigel	10/10	10
MCF7 (AZ)	none	10/10	54
	matrigel	10/10	10
MCF7 (AZ) TD5	none	10/10	54
	matrigel	10/10	10
MDA-MB231	none	5/10	46
	matrigel	5/5	32

[a] 10^6 cells were injected s.c. in nude mice in the absence (none) or in the presence of matrigel.
[b] The latency period was estimated as the number of days needed to obtain tumors of 200 mm^3.

Fig. 2. *Effect of Matrigel on MCF7 cell tumor growth. MCF7 human breast tumor cells (2.5 x 10^5) were injected subcutaneously in the absence (*) or in the presence of normal Matrigel (continuous line), or "depleted Matrigel" (devoid of main growth factors) (clashed line) into ovariectomized mice (Δ) or estrogen-supplemented nude mice (O,*,+).*



Fourthly, *the release of the numerous active growth factors* accumulated in Matrigel may influence tumor cell properties (32). However, Matrigel devoid of growth factors ("depleted" Matrigel) is as efficient as complete Matrigel in stimulating MCF7 cell growth (Fig. 2) and B16.F10 melanoma growth *in vivo* (37).

Finally *laminin-1,* the major glycoprotein of Matrigel could promote tumor cell activities. Its co-injection with submandibular carcinoma cells can reproduce the effect of Matrigel (42). This may not be true for all tumor cell types since Fridman *et al.* (36) showed that growth of human lung carcinoma cells was not stimulated by laminin. Thus, the tumor promoting effect of laminin appeared to be dependent on the cell types tested. Nevertheless, different laminin-derived peptides have been shown to influence tumor cell growth. As illustrated in Fig. 2, several sites on the cruciform laminin molecule have been identified as binding domains which promote various activities in cultured cells such as adhesion, spreading, migration and differentiation.

For example, the YIGSR site promoted cell adhesion and migration by binding to a 32/67 kDa laminin-binding protein on the cell surface that functions as one putative receptor [for review, see (33)]. The YIGSR peptide inhibited angiogenesis and the tumor promoting effect of Matrigel on prostatic carcinoma cells (41). When co-injected with melanoma cells, or fibrosarcoma HT1080 cells, the YIGSR peptide prevented tumor metastasis and growth in the presence of Matrigel (47, 48).

The RGD site is expected to be biologically active since it is part of the adhesion site that binds fibronec-tin, vitronectin and other proteins. Although its precise role in laminin has not been elucidated, it appears to promote adhesion of endothelial cells to Matrigel (33).

More numerous studies have focused on the SIK-VAV peptide present at the end of the long arm just above the large globule on the α1 chain. Peptides containing this sequence are angiogenic in several *in vitro* models [for review, see (31)]. *In vivo,* it increased the number of metastases of melanoma cells and colon cancer cells to the lung (45,47). This peptide also enhances plasminogen activation and gelatinase A activity (45, 46). It is worth noting that the SIKVAV site is present at a region where the three chains form a coiled-coil structure and where laminin is known to bind to other extracellular matrix components such as heparin and type IV collagen. For this

reason, it has been suggested that the SIK-VAV site is cryptic under some conditions. If this is the case, laminin could possess alternative properties. It is likely that some laminin domains are neither active nor available at all times. In authentic tumors, this peptide could be released by proteolysis of subepithelial basement membrane by serine proteases or matrix metalloproteinases.

Altogether, these observations indicate that in the Matrigel tumor-promoting model, many factors can act in a cooperative way to facilitate tumor growth.

ROLE OF STROMAL CELLS IN *IN VIVO* TUMORIGENESIS

The ability of tumor cells to grow and to metastasize is affected by the presence of adjacent host cells, particularly fibroblasts or myofibroblasts [for review, see (10)]. Co-injection of fibroblasts and parental MCF7 cells in the presence of Matrigel increased tumor growth (Fig. 3) and shortened the latency period (14 days vs. 26 days) (Table II). In these conditions, the tumor incidence always reached 100% and the tumor volume was increased (Table II and Fig. 3). Such a tumor-promoting effect of fibroblasts is also observed with breast cancer MDA-MB231 cells (49). The co-inoculation of fibroblasts with tumoral cells is as efficient in ectopic sites (e.g., subcutaneous injection) as in the orthotopic site (e.g., fat pad) (28).

Fibroblasts can affect mammary cells by secreting several classes of compounds including: (1) matrix proteins, (2) soluble chemokines, cytokines and growth factors, and (3) proteases. Such matrix proteins together with growth factors could promote the proliferation of endothelial cells, tumor cells or both (50, 51). In this regard, co-injection of lethally irradiated fibroblasts with human cancer cells enhances their tumorigenic potential, supporting a role for the matrix itself (52). In addition, fibroblast-conditioned medium injected weekly at the site of inoculation of MCF7 cells and Matrigel partially reproduced the effect of fibroblasts on tumor growth by affecting the rate of tumor growth, but not the latency period (Fig. 3). The failure of the conditioned medium to shorten the latency period may be related to subcutaneous dilution of soluble factors before tumor take. Overall these results suggest the involvement of soluble factors secreted by fibroblasts.

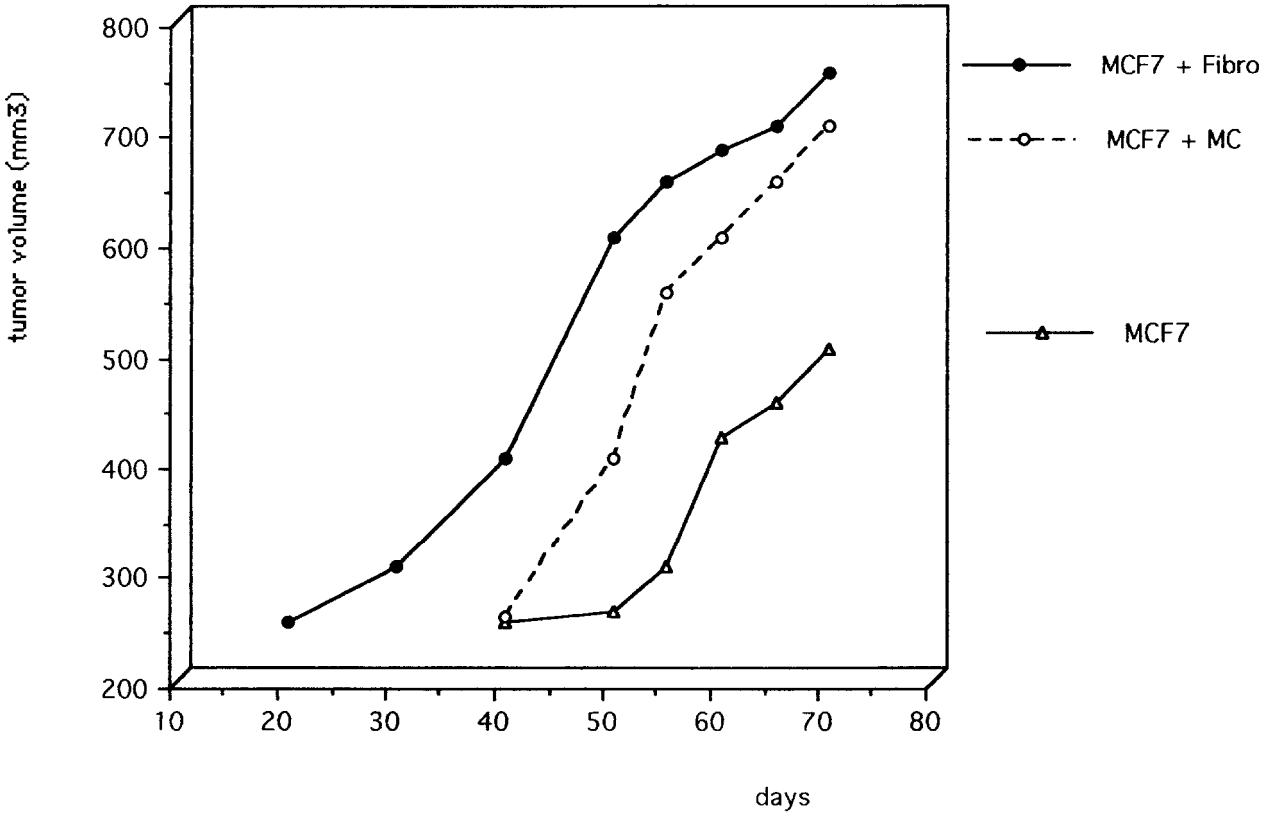
THE USE OF *IN VIVO* MODELS TO EVALUATE THE ROLE OF STROMAL PRODUCTS IN CANCER PROGRESSION

Evidence that stromal proteinases play a role in tumor progression has generated a great deal of interest and opened new therapeutic perspectives. Conventional cancer treatments are based on the use of cytotoxic agents targeting cancer cells themselves. Targeting stromal cells rather than tumor cells may facilitate drug delivery since tumor stroma is easily accessible from the blood stream. In addition, stromal cells may be less susceptible to the development of drug resistance.

Among the soluble factors produced by fibroblasts, stromal matrix metalloproteinases (MMPs) represent putative contributors to the stromal involvement in tumor progression. The MMPs are a family of zinc-dependent endopeptidases with a broad spectrum of proteolytic activities towards extracellular matrix components [for review, see (53); and (7) in this issue).

Although MMPs were first thought to be produced by tumor cells themselves, the mRNA's for stro-melysin-3, gelatinase A, interstitial collagenase and MT1-MMP has been associated with stromal cells (54, 55). Furthermore, gelatinase A activation and MT1-MMP expression correlated with an epithelial to mesenchymal transition by breast cancer cell lines (56). Studies with natural MMP inhibitors, the Tissue Inhibitors of MMPs (TIMPs), and synthetic MMP inhibitors have demonstrated that MMP activity is required for tumor progression and metastasis in several model systems [for review, see (57, 58)]. In this context, reduction of tumor growth and local invasion was observed when TIMP2 was overexpressed in tumor cells, either by transfection of TIMP2 cDNA (59) or retroviral-mediated gene transfer (60). Batimastat, an inhibitor of MMPs, inhibited human breast cancer growth and metastasis in nude mice models (58, 61). The overexpression of TIMP-4 in human MDA-MB 435 breast carcinoma cells reduced *in vivo* tumor growth, angiogenesis and (62).

Fig. 3. *Effect of fibroblasts on MCF7 cells tumor growth. MCF7 cells (2.5 x 10^5) were injected subcutaneously without fibroblasts (Δ), with fibroblasts (●, continuous line), or with medium conditioned by fibroblasts (O, dashed line).*



That proteases specifically produced by stromal cells play a role in cancer progression is supported by the observation that stromelysin-3 (ST-3) promoted the tumor formation by MCF7 cells transfected with cDNAs encoding either human or murine stromelysin-3 (55). In addition, high ST3 expression levels are predictive of a poor clinical outcome in breast cancer (63). MCF7 cells infected with a retroviral vector containing TIMP-2 have been developed. No differences in tumor incidence or latency period were observed when MCF7 cells transfected with vector alone (MCF7 *puro)* or TIMP2 producing MCF7 cells were injected with Matrigel (Table II). This result is consistent with the lack of MMP production by MCF7 cells. However, the co-injection of these TIMP2-producing MCF7 cells with fibroblasts and Matrigel abolished the tumor promoting effect of fibroblasts (Table II). The main effect was to enhance the latency period suggesting that stromal MMPs mainly affect early events of tumor progression.

One contribution of stromal proteases could be the release of biologically active factors from the extracellular matrix. Indeed, as mentioned above, various growth factors (IGFs, FGFs and TGF-βs) are associated with matrix proteins or with heparan sulfate groups on proteoglycan [for review, see(62)]. Their release by MMPs from matrix and/or activation of their latent forms may result in their binding to and stimulation of tumor cells. MMPs are indeed able to cleave growth factor controlling molecules, such as insulin-like growth factor-binding protein 3 (IGFBP-3) or the ecto domain of the fibroblast growth factor receptor 1 (FGFR1) (65, 66). These activated growth factors could act indirectly on cancer cells by promoting migration of stromal cells, proliferation, and bio-synthetic activities. In addition, some of these factors have angiogenic activities and contribute to the angiogenic effect of Matrigel.

CONCLUSIONS

Altogether these data emphasize the importance of stromal cells and their products such as proteases during cancer progression. Targeting stromal cells for therapeutic purpose rather than cancer cells themselves could be important since, if the functions of the stroma can be abrogated, tumors should be deprived of important and essential support needed for angiogenesis, growth and metastasis. In this context, stromal MMPs are attractive targets since by their mode of action, the toxicity profile of MMP inhibitors would differ markedly from those of

cytotoxic drugs. Several synthetic MMP inhibitors have demonstrated antitumor activity in various experimental models developed in nude mice [for review, see (56)] and phase III clinical trials have been already initiated. The next step will be to identify more specific and/or more potent inhibitors for each MMP expressed in human breast carcinomas.

Table 2. *Effect of TIMP2 on the Tumor-Promoting Effect of Fibroblasts on MCF7 Cells In Vivo Tumorigenicity*

	Latency period (days)[a]		Tumor volume at the end of the assay (mm^3)[2b]	
	without fibroblasts	with fibroblasts	without fibroblasts	with fibroblasts
7 parental	24	12	114 ± 10	210 ± 15
7 puro (n =?)[c]	26	14	96 ± 14	179 ± 15
7.TIMP2 clone 23[d] (n = 5)[c]	28	28	114 ± 10	96 ± 6
7.TIMP2 clone 24[d] (n = ?)[c]	26	26	109 ± 16	118 ± 18

[a] Latency period = number of days between injection and development of tumor larger than 80 mm^3 in 100% of injection sites.
[b] Mean volume of tumors at the end of the assay.
[c] N indicates the number of injected mice.
[d] Two clones (23 and 24) of TIMP2 producing MCF7 cells *(MCF7. TIMP2),* their corresponding control cells *(MCF7 puro)* or parental. MCF7 cells were injected s.c. with or without fibroblasts, in the presence of matrigel.

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References

1. S. E. LaFlamme and K. L. Auer (1996). Integrin signaling. *Sem. Cancer Biol.* 7:111-118.

2. V. M. Weaver, A. H. Fisher, O. W. Peterson, and M. J. Bissel (1997). The importance of the microenvironment in breast cancer progression: recapitulation of mammary tumorigenesis using a unique human mammary epithelial cell model and a three-dimensional culture assay. *Biochem. Cell Biol.* 74:833-851.

3. S. M. Frisch, and H. Francis (1994). Disruption of epithelial cell-matrix interactions induces apoptosis. *J. Cell Biol.* 124:619-626.

4. C. H. Streuli, C. Schmidhauser, N. Bailey, P. Yurchenco, A. P. N. Skubitz, C. Roskelley, and M. J. Bissell (1995). Laminin mediates tissue-specific gene expression in mammary epithelia. *J. Cell Biol.* 129:591-603.

5. C. Streuli and G. Edwards (1998). Control of normal mammary epithelial phenotype by integrins. *J. Mam. Gland Biol. Neoplasia* 3:xx-xx.

6. A. Lochter, A. Srebrow, C. J. Sympson, N. Terracio, Z. Werb, and M. J. Bissell (1997). Misregulation of stromelysin-1 expression in mouse mammary tumor cells accompanies acquisition of stromelysin-1-dependent invasive properties. *J. Biol. Chem.* 272:5007-5015.

7. L. A. Rudolph-Owen and L. M. Matrisian (1998). Matrix metal-loproteinases in remodeling of the normal and neoplastic mammary gland. *J. Mam. Gland Biol. Neoplasia* 3:177-190.

8. M. Mareel, M. E. Bracke, F. Van Roy, and P. de Baetselier (1997). Molecular mechanisms of cancer invasion. In *Encyclopedia of Cancer,* (Vol. II), Academic Press, Inc., pp. 1072-1083.

9. H.F. Dvorak (1986) Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N. Engl. J. Med.* 315:1650-1659.

10. J. Folkman (1995). Angiogenesis in cancer, vascular, rheumatoid, and other disease. *Nature Med.* 1:27-31.

11. M. C. Rio, O. Lefebvre, M. Santavicca, A. Noël, M. P. Chenard, P. Anglard, J. A. Byrne, A. Okada, C.H. Régnier, R. Masson, J. P. Bellocq, and P. Basset (1996). Stromelysin-3 in the biology of the normal and neoplastic mammary gland. *J. Mam. Gland Biol. Neoplasia* 1:231-240.

12. M. Grégoire and B. Lieubeau (1995). The role of fibroblasts in tumor behavior. *Cancer Metastasis Rev.* 14:339-350.

13. N. Wernert (1997). The multiple roles of tumor stroma. *Vir-chows Arch.* 430:433-443.

14. D. Verhoeven, N. Bourgeois, A. Noël, J. M. Foidart, and N. Buyssens (1990). The presence of a type IV collagen skeleton associated with periductal elastosis in breast cancer. *J. Histo-chem. Cytochem.* 38:245-255.

15. H. Kosmehl, A. Berndt, and D. Katenkamp (1996). Molecular variants of fibronectin and laminin: Structure, physiological occurrence and histopathological aspects. *Virchows Arch.* 429:311-322.

16. K. Henning, H. Kosmehl, A. Berndt, P. Rousselle, and D. Gabier Katenkamp (1995). Differential expression of laminin chains is benign and malignant lesions of the breast. *Pathol. Res. Pract.* 191:175.

17. A. Bellahcène, S. Menard, R. Bufalino, L. Moreau, and V. Castronovo (1996). Expression of bone sialoprotein in primary human breast cancer is associated with poor survival. *Int. J. Cancer* 69: 350-353.

18. S. Peyrol, M. Raccurt, F. Gerard, C. Gleyzal, J. A. Grimaud, and P. Sommer (1997). Lysyl oxidase gene expression in the stromal reaction to *in situ* and invasive ductal breast carcinoma. *Am. J. Pathol.* 150:497-507.

19. A. Noël, C. Munaut, A. Boulvain, C. M. Calberg-Bacq, Ch. A. Lambert, B. Nusgens, Ch. M. Lapière, and J. M. Foidart (1992). Modulation of collagen and fibronectin synthesis in fibroblasts by normal and malignant cells. *J. Cell. Biochem.* 48:150-161.

20. M. J. Merrilees and G. J. Finlay (1985). Human tumor cells in culture stimulate glycosaminoglycan synthesis by human skin fibroblasts. *Lab. Invest.* 53:30-36.

21. L. Ronnov-Jenssen, O. W. Petersen, V. E. Koteliansky, and M. J. Bissell (1995). The origin of the myofibroblasts in breast cancer: Recapitulation of tumor environment in culture unravels diversity and implicates converted fibroblasts and recruited smooth muscle cells. *J. Clin. Invest.* 95:859-873.

22. A. B. Unden, B. Sandstedt, K. Bruce, M. A. Hedblad, and M. Ståhle-Bäckdahl (1996). Stromelysin-3 mRNA associated with myofibroblasts is overexpressed in aggressive basal cell carcinoma and in dermafibrosarcoma but not in dermatofibrosar-coma. *J. Invest. Dermatol.* 107:147-153.

23. R. Clarke (1996a). Human breast cancer cell line xenografts as models of breast cancer—the immunobiologies of recipient mice and the characteristics of several tumorigenic cell lines. *Br. Cancer Res. Treat.* 39:69-86.

24. R. Clarke (1996b). Animal models of breast cancer: Their diversity and role in biomedical research. *Br. Cancer Res. Treat.* 39:1-6.

25. A. L. Harris, H. Zhang, A. Moghaddam, S. Fox, P. Scott, A. Pattison, K. Gatter, I. Stratford, and R. Bicknell (1996). Breast cancer angiogenesis— new approaches to therapy via antiangio-genesis, hypoxic activated drugs, and vascular targeting. *Br. Cancer Res. Treat.* 38:97-108.

26. S. W. McLeskey, L. Zhang, S. Kharbanda, J. Kurebayashi, M. E. Lippman, R. B. Dickson, and F. G. Kern (1996). Fibroblast growth factor overexpressing breast carcinoma cells as models of angiogenesis and metastasis. *Br. Cancer Res. Treat.* 39:103-117.

27. S. R. Wolman, G. H. Heppner, and E. Wolman (1997). New directions in breast cancer research. *FASEB J.* 11:535-543.

28. J. E. Price (1996). Metastasis from human breast cancer cell lines. *Br. Cancer Res. Treat.* 39:93-102.

29. R. R. Mehta, J. M. Graves, G. D. Hart, A. Shilkaitis, and T. K. Das Gupta (1993). Growth and metastasis of human breast carcinomas with Matrigel in athymic mice. *Br. Cancer Res. Treat.* 25: 65-71.

30. N. Brünner, E. W. Thompson, M. Spang-Thomsen, J. Rygaard, K. Danø, and J. A. Zwiebel (1992). LacZ transduced human breast cancer xenografts as an *in vivo* model for the study of invasion and metastasis. *Eur. J. Cancer* 28A: 1989-1995.

31. C. Manzotti, R. A. Audisio, and G. Pratesi (1993). Importance of orthotopic implantation for human tumors as model systems: relevance to metastasis and invasion. *Clin. Exp. Metastasis* 11:5-14.

32. M. Taub, Y. Wang, T. M. Szcesny, and H. K. Kleinman (1990). Epidermal growth factor or transforming growth factor a is required for kidney tubologenesis in Matrigel cultures in serum-free medium. *Proc. Natl. Acad. Sci. U.S.A.* 87:4002-4006.

33. S. Baatout (1997). Endothelial differentiation using Matrigel (Review). *Anticancer Res.* 17:451-456.

34. A. Noël, V. Borcy, M. Bracke, C. Gilles, J. Bernard, P. Birem-baut, M. Mareel, and J. M. Foidart (1995). Heterotransplantation of primary and established human tumour cells in nude mice. *Anticancer Res.* 15:1-8.

35. A. Noël, N. Simon, J. Raus, and J. M.Foidart (1992). Basement membrane components (Matrigel) promote human breast adenocarcinoma MCF7 cells tumorigenicity and provide an *in vivo* model to assess cell responsiveness to estrogen. *Biochem. Pharmacol.* 43:1263-1267.

36. R. Fridman, G. Giaccone, T. Kanemoto, G. R. Martin, A. F. Gazdar, and J. L. Mulshine (1990). Reconstituted basement membrane (Matrigel) and laminin can enhance the tumorigenicity and the drug resistance of small cell lung cancer cell lines. *Proc. Natl. Acad. Sci. U.S.A.* 87:6698-6702.

37. R. Fridman, M. C. Kibbey, L. S. Royce, M. Zain, T. M. Sweeney, D. L. Jicha, J. R. Yannelli, G. R. Martin, and H. K. Kleinman (1991). Enhanced tumor growth of both primary and established human and murine tumor cells in athymic mice after coinjection with Matrigel. *J. Natl. Cancer Inst.* 83:769-775.

38. P. Topley, D. C. Jenkins, E. A. Jessup, and J. N. Stables (1993). Effect of reconstituted basement membrane components on the growth of a panel human tumour cell lines in nude mice. *Brit. J. Cancer* 67:953-958.

39. T. Lopez-Conejo, N. Olmo, J. Turnay, J. Navarro, and A. Lizarbe (1996). Characterization of tumorigenic sub-lines from a poorly tumorigenic human colon-adenocarcin oma cell line. *Int. J. Cancer* 67:668-675.

40. P. Mullen, A. Ritchie, S. P. Langdon, and W. R. Miller (1996). Effect of matrigel on the tumorigenicity of human breast and ovarian carcinoma cell lines. *Int. J. Cancer* 67:816-820.

41. A. Passaniti, J. T. isaacs, J. A. Haney, S. W. Adler, T. J. Cujdik, P. V. Long, and H. K. Kleinman (1992). Stimulation of human prostatic carcinoma tumor growth in athymic mice and control of migration in culture by extracellular matrix. *Int. J. Cancer* 51:318-324.

42. D. S. Grant, M. C. Kibbey, J. L. Kinsella, M. C. Cid, and H. K. Kleinman (1994). The role of basement membrane in angiogenesis and tumor growth. *Path. Res. Pract.* 190: 854-863.

43. Y. Ito, Y. Iwamoto, K. Tanaka, K. Okuyama, and Y. Sugioka (1996). A quantitative assay using basement membrane extracts to study tumor angiogenesis *in vivo. Int. J. Cancer* 67:148-152.

44. R. D. Bonfil, A. Vinyals, O. D. Bustuoabad, A. Llorens, F. J. Banavides, M. Gonzales-Garrigues, and A. Fabra (1994). Stimulation of angiogenesis as an explanation of Matrigel-enhanced tumorigenicity. *Int. J. Cancer* 58:233-239.

45. T. Kanemoto, R. Reich, L. Royce, D. Greatorex, S. H. Adler, N. Shiraishi, G. R. Martin, Y. Yamada, and H. K. Kleinman (1990). Identification of an amino acid sequence from the laminin-A chain that stimulates metastasis and collagenase-IV production. *Proc. Natl. Acad. Sci. U.S.A.* 87:2279-2283.

46. S. Stack, R. D. Gray, and S. V. Pizzo. (1991). Modulation of plasminogen activation and type IV collagenase activity by a synthetic peptide derived from the laminin A chain. *Biochemistry* 30:2073-2077.

47. R. S. Bresalier, B. Schwartz, Y. S. Kim, Q. Y. Dull, H. K. Kleinman, and P. M. Sullam (1995). The laminin al chain Ile-Lys-Val-Ala-Val (IKVAV)-containing peptide promotes liver colonization by human colon cancer cells. *Cancer Res.* 55:2476-2480.

48. Y. Iwamoto, M. Nomizu, Y. Yamada, Y. Ito, K. Tanaka, and Y. Sugioka (1996). Inhibition of angiogenesis, tumour growth and experimental metastasis of human fibrosarcoma cells HT1080 by a multimetric form of the laminin sequence Tyr-Ile-Gly-Ser-Arg (YIGSR). *Brit. J. Cancer* 73:589-595.

49. A. Noël, H. Emonard, M. Polette, P. Birembaut, and J. M. Foidart (1994). Role of matrix, fibroblasts and type IV collagen-ases in tumor progression and invasion. *Pathol. Res. Pract.* 190:934-941.

50. A. Noël, M. C. De Pauw-Gillet, G. Purnell, B. Nusgens, Ch. M. Lapière, and J. M.Foidart (1993). Enhancement of tumorige-nicity of human breast adenocarcinoma cells in nude mice by Matrigel and fibroblasts. *Brit. J. Cancer* 68:909-915.

51. C. E. P. van Roozendaal, B. van Ooijen, J. G. M. Klijn, C. Claassen, A. M. M. Eggermont, S. C. Henzen-Logmans, and J. A. Foekens (1992). Stromal influences on breast cancer cell growth. *Brit. J. Cancer* 65:77-81.

52. J. L. Camps, S. M. Chang, T. C. Hsu, M. R. Freeman, S. J. Hong, H. E. Zhau, A. C. von Eschenbach, and L. W. K. Chung (1990). Fibroblast-mediated acceleration of human epithelial tumor growth *in vivo. Broc. Natl. Acad. Sci. U.S.A.* 87:75-79.

53. H. Birkedal-Hansen (1995). Proteolytic remodeling of extracellular matrix. *Curr. Opin. Cell Biol.* 7:728-735.

54. K. J. Heppner, L. M. Matrisian, R. A. Jensen, and W. H. Rodgers (1996). Expression of most matrix metalloproteinase family members in breast cancer represents a tumor-induced host response. *Am. J. Pathol.* 149:273-282.

55. A. Noël, O. Lefebvre, E. Maquoi, L. Vanhoorde, M. P. Chenard, M. Mâreel, J. M. Foidart, P. Basset, and M. C. Rio (1996). Stromelysin-3 expression promote tumor take in nude mice *J. Clin. Invest.* 97:1924-1930.

56. H. Pulyaeva, J. Bueno, M. Polette, P. Birembaut, H. Sato, M. Seiki, E. W. Thompson (1997). MT1-MMP correlates with MMP-2 activation potential seen after epithelial to mesenchymal transition in human breast carcinoma cells. *Clin. Exp. Metastasis* 15:111-120.

57. J. R. MacDougall and L. M. Matrisian (1995). Contributions of tumor and stromal matrix metalloproteinases to tumor progression, invasion and metastasis. *Cancer Metastasis Rev.* 14:351-362.

58. D. C. Talbot and P. D. Brown (1996). Experimental and clinical studies on the use of matrix metalloproteinase inhibitors for the treatment of cancer. *Eur. J. Cancer* 14:2528-2533.

59. Y. A. DeClerck, N. Perez, H. Shimada, T. C. Boone, K. E. Langley, and S. M. Taylor (1992). Inhibition of invasion and metastasis in cells transfected with an inhibitor of metalloproteinases. *Cancer Res.* 52:701-708.

60. S. Imren, D. B. Kohn, H. Shimada, L. Blavier, and Y. A. DeClerck (1996). Overexpression of tissue inhibitor of metallo-proteinases-2 by retroviral-mediated gene transfer *in vivo* inhibits tumor growth and invasion. *Cancer Res.* 56:2891-2895.

61. J. A. Low, M. D. Johnson, E. A. Bone, and R. B. Dickson (1996). The matrix metalloproteinase inhibitor Batimastat (BB-94) retards human breast cancer solid tumor growth but not ascites formation innude mice. *Clin. Cancer Res.* 2:1207-1214.

62. M. Wang, Y. E. Liu, J. Greene, S. Sheng, A. Fuchs, E. M. Rosen, Y. E. Shi (1997). Inhibition of tumor growth and metastasis of human breast cancer cells transfected with tissue inhibitor of metalloproteinase 4. *Oncogene* 14:2767-2774.

63. M. P. Chenard, L. O'Siorain, S. Shering, N. Rouyer, Y Lutz, C. Wolf, P. Basset, J. P. Bellocq, and M. J. Duffy (1996). High levels of stromelysin-3 correlate with poor prognosis inpatients with breast carcinoma. *Int. J. Cancer (Pred. Oncol.)* 69:448-451.

64. J. Taipale and J. Keski-Oja (1997). Growth factors in the extracellular matrix. *FASEB J.* 11:51-59.

65. J. L. Fowlkes, J. J. Enghild, K. Suzuki, and H. Nagase (1994). Matrix metalloproteinases degrade insulin-like growth factor-binding protein-3 in dermal fibroblast cultures. *J. Biol. Chem.* 269:25742-25746.

66. E. Levi, R. Fridman, H. Q. Miao, Y. S. Ma, A. Yayon, and I. Vlodavsky (1994) Matrix metalloproteinase 2 releases active soluble ectodomain of fibroblast growth factor receptor 1. *Proc. Natl. Acad. Sci. U.S.A.* 93:7069-7074.