Histol Histopathol (2002) 17: 599-621
DOI: 10.14670/HH-17.599

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Review

Tumor-associated fibroblasts (Part I): active stromal participants in tumor development and progression?

L.A. Kunz-Schughart and R. Knuechel
Institute of Pathology, University of Regensburg, Regensburg, Germany

Summary. Phenotypic and functional characteristics of tumor associated fibroblasts (TAF) in contrast to normal fibroblasts are reviewed in this first synopsis (part I). Terms as tumor stroma, desmo-plasia, TAF, myofibroblast, and fetal-type fibroblast are defined, and experimental systems to study heterologous cell interactions are presented.

While we only start to gather information on the genotype of TAF, a broad range of data deals with the expression profile of these cells, covering e.g. ECM and ECM-modulating molecules, growth factors and cytokines. Summarizing the recent state of knowledge indicates that TAF provide sources for tumor diagnosis and therapy, that have to be further defined in an organ-specific approach in terms of the functional impact on the tumor cell and its environment (see part II).

Key words: Desmoplasia, Fibroblast, Myofibroblast, Oncofetal extracellular matrix, Matrix metallo-proteinases, Integrins, Cell adhesion molecules, Growth factors, Cytokines, Chemokines, Multicellular spheroid

Introduction: History, nomenclature, and model systems

Tumor stroma

According to the general acceptance of epithelial cell degeneration and transformation as the leading process in carcinogenesis, classical *in-vitro* cancer research focused on the isolated epithelial tumor cell population. However, malignant cells are not isolated islands of genetically-transformed cells with an uncontrolled cell cycle and cell death regulation; they are surrounded by a heterologous peritumoral stroma consisting of diverse host cell types and a modified extracellular matrix (ECM). Thus, the relationship between tumor cells and stroma has been of great interest to pathologists since the introduction of microscopic tissue imaging in the 19th century. Already in 1902 and 1924, Max Borst, a German pathologist involved in early tumor research, postulated some fundamental aspects of tumor-stroma relations. Borst's theory of an interaction between tissue-disintegrating tumor cells and stroma to be involved in tumor invasion was verified decades later *in vitro* and *in vivo* via modern molecular and cell biological techniques (for review: Dhom, 1994).

The complex cellular tumor (micro)environment comprises immunocompetent and inflammatory cells, endothelial cells, and fibroblasts (Fig. 1). All of these cell types may critically influence the multi-step process of carcinogenesis and the malignant phenotype. Tumor defence mechanisms of immune cells such as lymphokine-activated killer cells (LAK) or macrophages and the impact of tumor cells on immune cell activation and maturation have been investigated in the past, initiated by the work of Isaiah J. Fidler, Ronald B. Herberman, and Robert J. North in the early 1970s. They were soon joined by a group of insightful scientists such as Alberto Mantovani, Chou-Chik Ting, and Klaus D. Elgert and the term tumor-associated macrophage (TAM) to describe an 'abnormal' differentiation status of macrophages associated with enhanced tumor growth and propagation rather than with tumor regression was introduced (for review: Mantovani et al., 1992; Mantovani, 1994; Elgert et al., 1998). Roughly at the same time, Judah Folkman published his initial hypotheses on the 'angiogenic switch' during tumor cell growth and his idea of an anti-angiogenic therapeutic concept. Since then, the interest in endothelial cells as the main stromal cell component involved in tumor angiogenesis and invasion consistently increased (reviewed recently by: Carmeliet and Jain, 2000; Cherrington et al., 2000; Kerbel, 2000). Multiple studies have not only gained deeper insight into the mechanisms of endothelial-tumor cell interactions affecting tumor vascularization but a wide panel of anti-angiogenic agents has been identified or designed, some of which have already entered clinical trial (e.g. Folkman, 1997;

Offprint requests to: Dr. Leoni A. Kunz-Schughart, Institute of Pathology, University of Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany. e-mail: leoni.kunz-schughart@klinik.uni-regensburg.de

Brem, 1999; Eatock et al., 2000).

Desmoplasia

In contrast to immune and endothelial cells, fibroblasts were long presumed to be passive structural elements in tumors and were mainly studied as a substrate of tumor cell invasion up to the late 80s with the first implications summarized by van den Hooff (1986, 1988). The phenomenon of tumor-associated desmoplasia characterized by enhanced fibroblast proliferation/accumulation and a modified, collagenized extracellular matrix (ECM), however, is well known to surgical pathologists and often used as a secondary, indirect diagnostic tool. Manifestation and degree of the 'desmoplastic reaction' are highly variable not only for different tumor entities but also within one tumor type. Desmoplasia is most frequently described in squamous cell carcinomas independent of their location and in bilio-pancreatic carcinomas while it is rather rare in lymphomas, melanomas, and brain tumors, in hepatocellular and urothelial tumors as well as in tumors of the pituitary gland. In contrast, fibrotic, desmoplastic reactions are also well-known in invasive adenocarcinomas of breast and ovaries, gastrointestinal tract, and in the lung excluding small cell lung cancers (Fig. 2). According to the morphological image and consistency of a subset of these tumors with a proportion of > 50 % desmoplastic stroma contributing to the tumor mass and a scar-like, central fibrosis, the term 'scirrhous' (Greek: hard) tumor became generally accepted. Due to the histological features of the radial sclerotic, scar-like area alternative names, such as 'cancers with sclerotic foci' or scar cancers were used for some time since scar formation was thought to be a predisposing factor in the development of these cancers but appeared to be a host reaction. The prognostic significance of the desmoplastic response and/or formation of central sclerotic scar and fibrotic focus, respectively, in these tumors is still controversially discussed in the literature (e.g. Halvorsen and Seim, 1989; Janot et al., 1996; Yashiro et al., 1996; Breuninger et al., 1997; Kajiyama et al., 1999; Adachi et al., 2000; Hasebe et al., 2000a,b; Suzuki et al., 2000).

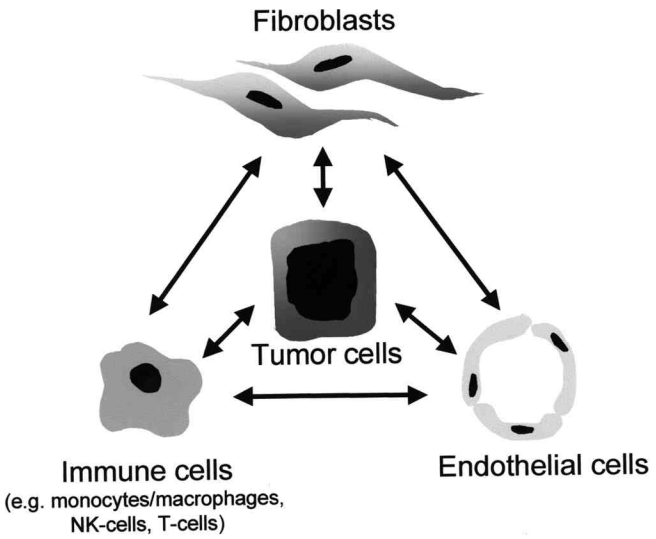

Fig. 1. Heterologous cellular interactions in solid tumors.

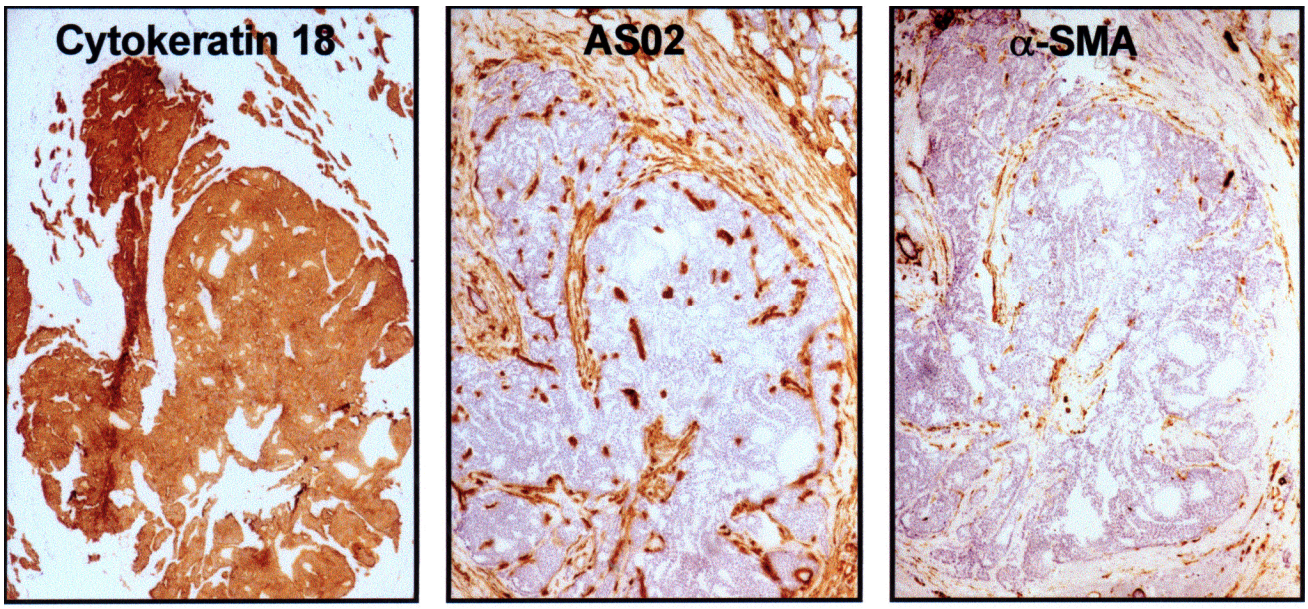

Fig. 2. Poorly-differentiated invasive ductal breast tumor (G3) with desmoplastic reaction. Immunohistochemical staining for cytokeratin 18 (tumor cell marker), AS02 antigen (fibroblast marker that also stains activated endothelial cells), and α-SMA in 5-µm parallel frozen sections. DAB for color development; hematoxilin counterstain. x 100

Although some discrepancy may result from an inconsistent morphological definition of desmoplastic and scirrhous reactions, the tumor-type and organ-specific micromilieu is presumed a critical factor determining the prognostic and therapeutic value of the desmoplastic response.

Tumor-associated fibroblasts

Within the last 15 years, fibroblasts, the quantitatively most abundant stromal cell type in scirrhous tumors and cellular substrate of desmoplasia-inducing tumor cells have been considered active participants and modulators of tumor growth and propagation (for review: Wernert, 1997; Hayward et al., 1998; Elenbaas and Weinberg, 2001; Tlsty and Hein, 2001). Also, there is growing awareness that stromal fibroblasts and their ECM environment may be involved in early tumor development. Olumi et al. (1999) described tumor-derived fibroblasts to direct tumor progression of initiated but not of normal human prostatic epithelium in an animal model system. In the same year, Sternlicht and coworkers showed for the first time that an ECM-modulating molecule (matrix-metalloproteinase-3/stromelysin-1), which is expressed during normal mammary gland development and in post-lactational mammary involution by fibroblasts (Hansen and Bissell, 2000), may act as a 'natural' tumor promotor in breast carcinogenesis (Lochter et al., 1998; Sternlicht et al., 1999, 2000) that also plays a role in epithelial-to-mesenchymal conversion (Lochter et al., 1997). Their data profoundly extend the rather reductional model of multi-step genetic alterations during tumor development and progression by a microenvironmental 'effector' theory as emphasized in Catherine Park's, Mina Bissell's and Mary-Helen Barcellos-Hoff's commendable review article (Park et al., 2000). While they prefer the term 'microenvironmental effector', Kinzler and Vogelstein refer to these carcinogenesis-enabling factors/genes as 'landscapers' (Kinzler and Vogelstein, 1998a) extending their initial 'gatekeeper-caretaker' classification (Kinzler and Vogelstein, 1998b). The different terminology should not, however, deceive that identical processes in tumor development are described.

While there are few data available on the contribution of normal fibroblasts in tumor development, the impact of fibroblasts on tumor growth and dissemination has been investigated more intensively. Some of these functional studies that indicate tumor-associated/-derived fibroblasts to have an 'abnormal' phenotype are highlighted in our second article entitled 'Tumor-associated fibroblasts (part II): functional impact on tumor tissue'. In order to better understand these functional data, the present article aims to stress the phenotypic (and genotypic) differences between normal and tumor-stromal fibroblasts and the impact of tumor cells on the expression profile and behavior of fibroblasts. Thus, fibroblasts contributing to the tumor stroma have been termed peritumoral fibroblasts, reactive stroma, carcinoma-associated (CAF) or tumor-associated fibroblasts (TAF). The attempts to define a specific TAF phenotype with regard to functional status will be outlined. However, the reader should be aware that adult fibroblasts at different anatomical sites are per se an extremely heterogeneous multifunctional cell population in spite of a similar morphology and may give rise to different types of TAF (e.g. Schor et al., 1994; Gregoire and Lieubeau, 1995; Schor, 1995; Spanakis and Brouty-Boyé, 1997).

Tumor-stroma modeling

Cell biologists have put much effort into the establishment of valid model systems to mimic and monitor interactive processes in tumors *in vivo* and *in vitro*. Today, various animal models are applied, e.g. to study tumor angiogenesis and invasion. These models are complemented by multiple more or less complex *in-vitro* systems that all aim to reflect the dynamic interplay in the multifaceted tumor environment. Thus, *in-vitro* coculture systems, e.g. artificial or stromal-cell derived ECM compounds with tumor cells, stromal-cell conditioned media and tumor cells or vice versa, and different stromal and tumor cell types in 2-D and 3-D coculture, have and will considerably contribute to the understanding of the complex network of reciprocal actions in tumors (Fig. 3).

A number of studies have shown that the various 3-D culture systems established to investigate aspects of tumor biology and pathophysiology better reflect the *in-vivo* situation in tissues and tumors with regard to proliferation and differentiation patterns of epithelial cells than monolayer or suspension cultures. Some investigators apply 3-D reconstituted basement membrane culture systems to study the impact of stromal cells on the conversion of normal to malignant tissues (e.g. Rønnov-Jessen et al., 1996). With this model, Bissell's group for example described reversion of a tumorigenic into a rather normal phenotype following functional blockade of the ß1 integrin in the HMT-3522 breast tumor progression series of cells (Weaver et al.,

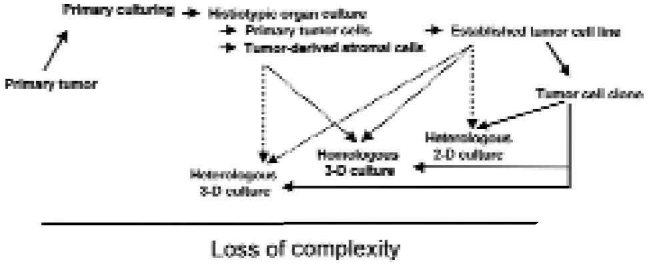

Fig. 3. Cellular complexity of different culturing techniques relative to primary tumor. 3-D cultures are of intermediate complexity and physiologically reflect the *in-vivo* situation to some extent with 3-D cocultures of different cell types resembling cellular heterogeneity.

1997; Wang et al., 1998; Bissell et al., 1999). Another approach is the accumulation of tumor cells and cell mixtures by centrifugation to form 'nodules' which are cultivated on a semisolid agar medium at medium/air interface (Beaupain, 1999). Spheroids represent an alternative 3-D culture model showing histiotypic organization of tumor cells without artificial matrices (Mueller-Klieser, 1997; Kunz-Schughart et al., 1998; Kunz-Schughart, 1999). This unique culture technique has entered tumor research in the early 1970s and is well-known to mimic the three-dimensional micromilieu in solid tumors. Thus, it is considered a valid *in-vitro* tumor model to evaluate novel anti-tumor therapeutic regimes. Consequently, multicellular tumor spheroids have been the topic not only of two books (Spheroids in Cancer Research: Methods and Perspectives, Ed. H. Acker, J. Carlsson, R. Durand, R.M. Sutherland, Springer-Verlag, 1984; Spheroid Culture in Cancer Research, Ed. R. Bjerkvig, CRC Press, 1992) but also of the November 2000 issue of Crit. Rev. Oncol. Hematol. Here, in addition to a fundamental up-to-date review on rather classical aspects in cancer research, some approaches of spheroid cocultures and their contribution to understand heterologous interactions in tumors are discussed, i.e. the relation between tumor and endothelial cells in spheroids or autologous spheroid cultures as a screening tool for tumor invasion. As a major focus of the authors, the potential of spheroid cocultures consisting of different tumor and tumor-associated cell types, in particular stromal fibroblasts, to gain deeper insight into the mechanisms of tumor-stroma cell interactions will be highlighted in the present review article (Fig. 4). It is of particular interest that fibroblasts in 3-D culture are in general cell-cycle arrested. Thus, cocultivation of individually initiated tumor and fibroblast spheroids or of tumor cell suspensions with fibroblast spheroids represent the *in-vivo* state of a tumor cell cluster or of floating/migrating tumor cells affecting quiescent fibroblasts.

Phenotype of TAF

Myofibroblasts

One consistent feature of fibroblasts in the desmoplastic reaction of tumors is a myofibroblastic phenotype which is based on the synthesis of intracellular smooth muscle markers, in particular α-smooth muscle actin (α-SMA) as indicated in Fig. 2 (Brouty-Boyé et al., 1991; Rønnov-Jessen et al., 1992, 1995; Rønnov-Jessen and Petersen, 1996; Lazard et al., 1993; Chiavegato et al., 1995; Gregoire and Lieubeau, 1995; Gabbiani, 1996; Schürch, 1999). Myofibroblasts were first described by Guido Majno and Giulio Gabbiani in the early 1970s as fibroblastic cells with a muscle cell-like morphology and a strong

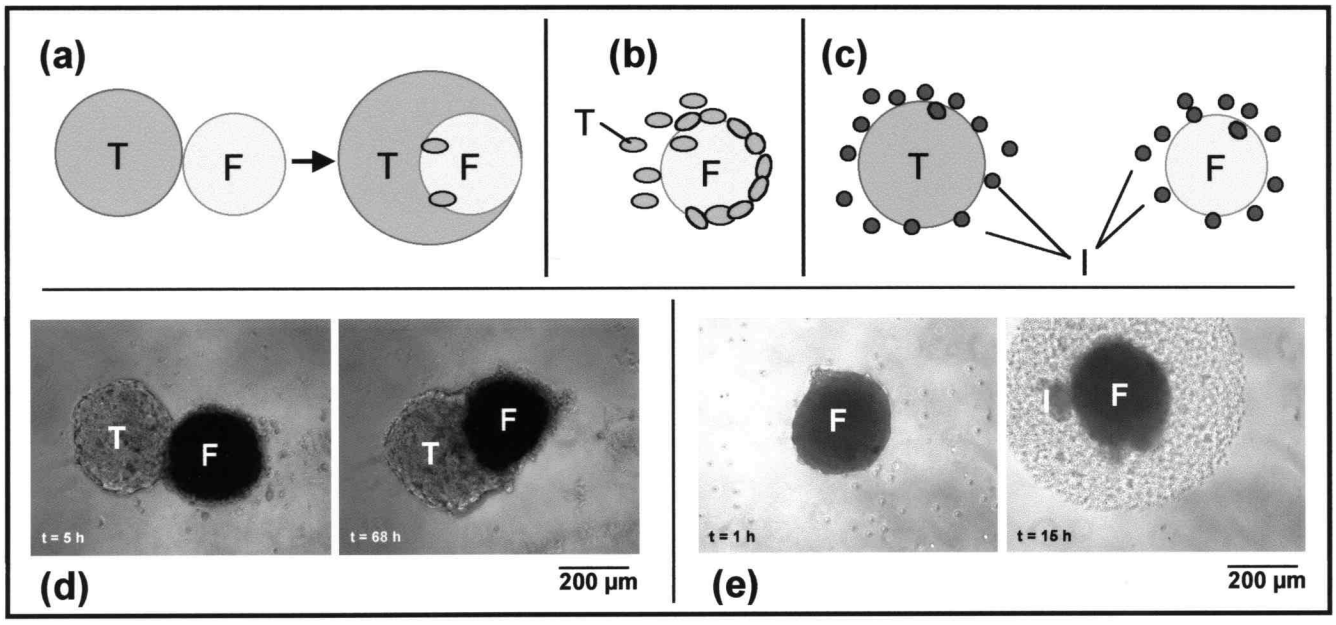

Fig. 4. Spheroid cocultures as *in-vitro* model to investigate heterologous cellular interactions in tumors. The liquid overlay technique in 96-well plates is recommended for cocultivation to monitor and manipulate individual spheroids and spheroid cocultures, respectively throughout growth. **a.** Cocultivation following individual initiation of tumor cell and fibroblast spheroids. **b.** Cocultivation technique to be performed with non-aggregating tumor cells; here, tumor cell suspensions are added to fibroblast spheroids. The same coculturing method is used to investigate immune cell migration and maturation processes in tumor cell spheroids or in spheroids of fibroblasts of different origin (e.g. TAF). **d.** Phase-contrast image of a breast tumor cell/fibroblast coculture after 5 h and 68 h in coculture according to **a**. **e.** Phase contrast image of a fibroblast spheroid 1 h and 15 h after addition of an immune cell (monocytes) suspension. T: tumor cells; F: fibroblasts; I: immune cells.

microfilamentous apparatus resulting in a contractile phenotype. The expression of α-SMA has become the most reliable marker for myofibroblast differentiation (for review: Gabbiani, 1999; Powell et al., 1999a; Schürch, 1999; Serini and Gabbiani, 1999) and is widely used as a single label. Based on immunohistochemical staining of other cytoskeletal filaments several myofibroblast subpopulations have been defined according to the following criteria: V-type myofibroblasts exclusively express vimentin, VD-type myofibroblasts express vimentin and desmin, VAD-type show positive staining for vimentin, α-SMA, and desmin, and VA- and VM-type myofibroblasts secrete vimentin in combination with α-SMA and smooth muscle myosin, respectively (for review: Powell et al., 1999a). A VAM-type has also been proposed and expression of smooth muscle actin heavy chains was for example illustrated in the tumor-associated myofibroblast population (Chiavegato et al., 1995).

Myofibroblasts play an important role and were thus intensively examined in chronic inflammatory diseases, in granulation tissue, as well as during wound healing and scar formation (Desmouliere, 1995; Meister, 1998; Powell et al., 1999a; Serini and Gabbiani, 1999). The presence of myofibroblasts in desmoplastic tumors was first recorded about 20 years ago and was positively correlated with invasive tumor growth behavior (Seemayer et al., 1979, 1980), a characteristic that seems to strengthen Dvorak's theory of a tumor-host interface resembling a non-healing wound (Dvorak, 1986). Breast malignancies were among the first tumor entities studied with regard to tumor-induced fibroblast-myofibroblast differentiation processes and were promptly used as a source for myofibroblasts for *in-vitro* research (Barsky et al., 1984).

Two of the most comprehensive review articles about myofibroblasts have been published by Powell and coworkers (1999a,b) who reference the literature on myofibroblasts in disease and also their distribution and functions in normal tissues and during organo- and morphogenesis. Thus, it is to be emphasized that myofibroblasts are present as a minor fibroblast subpopulation in almost all organs including those that may exhibit tumors with a strong desmoplastic reaction such as pancreas, breast, and gastrointestinal tract. The origin of myofibroblasts, however, is still unclear. Origination from embryonic progenitor stem cells but also differentiation' from resident tissue fibroblasts or smooth muscle cells under various paracrine stimuli have been documented. In desmoplastic tumors, the two latter hypotheses are favored and in particular the differentiation process fibroblast-myofibroblast has been investigated in some detail indicating that growth factors such as TGF-ß (transforming growth factor), PDGF (platelet-derived growth factor), IGF-II (insulin-like growth factor II) but also cytokines such as IL-4 (interleukin-4) are capable of inducing myofibroblastic phenotype.

In spite of the observed coherence of myofibroblastic differentiation and invasive tumor growth, the question whether tumor-associated myofibroblasts result from or cause an invasive phenotype has been examined much later (e.g. Hasegawa et al., 1990; Dimanche-Boitrel et al., 1994). Most recent observations with our 3-D spheroid coculture system of breast tumor cells and fibroblasts imply that myofibroblast induction and invasion are rather independent and not causally related processes. Both invasive and non-invasive tumor cells were identified as potential inducers of α-SMA expression in fibroblasts in the model system (Fig. 5a). Also, fibroblasts were invaded independently of their differentiation status (Kunz-Schughart et al., 2001). While we cannot exclude a supportive effect of myofibroblasts on tumor cell infiltration, our data clearly show fibroblasts of different pathological and non-pathological origin to react differently to tumor cell contact in terms of differentiation in a 3-D environment.

Fetal-like fibroblasts

The term 'fetal-like' fibroblast to describe the TAF phenotype is based on the *in-vitro* behavior and expression profile of tumor-derived as opposed to normal fibroblasts and implies an aberrant phenotype exclusively if present in adult tissue. This term has e.g. been applied by Schor and associates who observed an abnormally enhanced, fetal-like migratory behavior of breast tumor-derived as opposed to normal adult fibroblasts in a three-dimensional *in-vitro* collagen gel assay in the mid 1980s. These fibroblasts were shown to express and release an autocrine (and paracrine) factor termed migration-stimulating factor (MSF). The putative factor was purified and three biologically active fragments of 120, 60, and 33 kD were identified, with the smallest peptide showing considerable homology to the gelatin-binding domain of fibronectin (FN) (for review: Schor et al., 1993, 1996; Schor, 1995). Later it was verified that a similar fibroblast subpopulation is present during developmental processes and wound healing and also contributes to the intralobular stroma in the adult breast. As a result, an epigenetic clonal modulation model was postulated considering these fibroblasts not to be intrinsically aberrant, but rather an expanded subpopulation of cells also present in the healthy adult (Schor, 1995).

Myofibroblasts and 'fetal-like' fibroblasts share several similar phenotypic characteristics like ECM and cytokine expression (Brouty-Boye et al., 1991; Berndt et al., 1994, 1995; Ueki et al., 2000). However, it has not been verified so far if fetal-like and myofibroblastic subpopulations in normal tissues and tumors, respectively, are identical or derive from each other. Interestingly, myofibroblastic differentiation in tumors was documented to be locally restricted to tumor-adjacent stroma but has not been illustrated in distant areas, whereas fetal-like migratory fibroblasts were found in the skin of about 50% of patients with breast,

colon, lung, and prostate carcinomas, melanomas, or soft tissue sarcomas (Schor, 1995), a phenomenon of 'extended field effect' that still awaits systematic and mechanistic examination.

TAF phenotype and function

The establishment of tumor-associated fetal-like and myofibroblastic phenotypes is associated with a wide range of phenotypic and functional alterations in TAF as opposed to normal adult mesenchyme, some of which have already been outlined in 1995 in a book edited by AE Goldberg and KJ Rosen Epithelial-Mesenchymal Interactions in Cancer (Birkhäuser Verlag, Basel, Switzerland). The alterations include a distinct expression profile not only of intracellular marker molecules such as smooth muscle filaments but also of cell surface proteins, e.g. cell adhesion molecules and integrins, as well as of soluble autocrine and paracrine factors like growth factors and cytokines. In addition, fibroblasts are the main source of the ECM in normal and tumor tissue and are also capable of producing several ECM-modulating factors. Thus, they profoundly contribute to the creation of a tumor microenvironment that inappropriately promotes expansion and dissemination of a (pre)neoplastic epithelial cell population by determining a tumor-specific, fetal/oncofetal ECM profile.

Genotype of TAF

TAF are in general considered phenotypically and

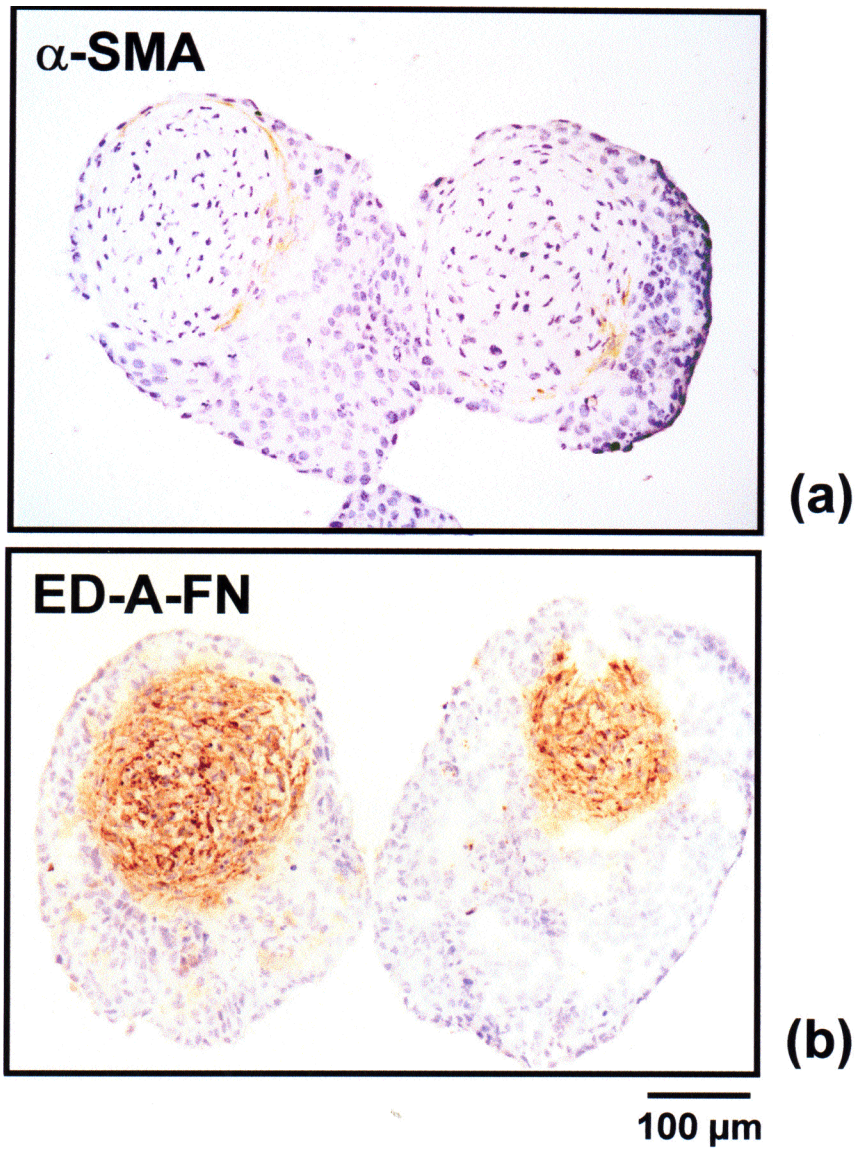

Fig. 5. Immunohistochemical detection of α-SMA **(a)** and oncofetal ED-A fibronectin **(b)** in 5-µm frozen sections of breast tumor/fibroblast spheroid cocultures. Non-invasive T47D breast tumor spheroids were grown in coculture with TAF spheroids for 7-8 days. DAB for color development; hematoxilin counterstain. Myofibroblast differentiation (α-SMA immunoreactivity) is only found at the tumor-fibroblast border indicating that a direct cell-cell contact plays a role in the differentiation process (Kunz-Schughart et al., 2001). This behavior reflects the in vivo situation (see also Fig. 2). Also, TAF, but not normal fibroblasts, produce an ED-A-FN-rich ECM scaffold in 3-D culture and in cocultures. Dim staining is infrequently found in tumor cell areas (unpublished data).

functionally altered but genetically normal host cells in solid tumors. Thus, myofibroblasts in the desmoplastic reaction of epithelial tumors should be discriminated from those in myofibroblastic tumors, which describes a spectrum of benign and malign soft tumor types with myofibroblastic features such as myofibromas, myofibroblastomas, angiomyofibroblastomas, or myofibrosarcomas (e.g. Fletcher, 1998; Mentzel and Katenkamp, 1998). However, there is increasing evidence that carcinoma-adjacent mesenchyme may 'already' be genetically altered (Wernert et al., 1998; Kurose et al., 2001; Moinfar et al., 2000). The genetic markers identified most recently in the stroma of breast and colon carcinomas via LOH (loss of heterozygosity) and mutation analyses following microdissection, e.g. regarding p53 suppressor gene, and determination of microsatellite instability seem to involve both concurrent and independent alterations in epithelial and stromal compartments.

Analogous alterations imply clonal development of epithelial and fibroblastic cells in tumors and may result from an epithelio-mesenchymal transition, a phenomenon that has been known for some time (Hay, 1995) and is hypothesized to play a role in (colon) carcinoma cell invasion and tumor progression (Brabletz et al., 2001). In addition to diverse paracrine effectors and intracellular signal transduction molecules such as TGF-ß and their signal transduction pathway (e.g. Piek et al., 1999), alterations in the cadherin/catenin complex have been identified to play a role in the epithelial-mesenchymal transition (e.g. Birchmeier et al., 1996; Kim et al., 2000; Brabletz et al., 2001). As an alternative explanation, we need to consider that pluripotent (epithelial) stem cells but not the differentiated epithelium in specific organs are the primary target for first hit genetic mutations giving rise to both epithelial and mesenchymal compartments. The hypothesis of stem cells being involved in carcinogenesis is supported by a study of Vortmeyer et al. (1997) who showed a concordance of genetic alterations in poorly differentiated colorectal neuroendocrine carcinomas and associated adenocarcinomas. They concluded that these tumors appear to be derived from the same cell of origin, supposingly a pluripotent epithelial stem cell or an adenocarcinoma precursor cell.

Moinfar and associates (2000) and Kurose et al. (2001) also documented some independent genetic alterations. Accordingly, Kurose et al. proposed an extended genetic model of multistep carcinogenesis of breast cancers involving epithelium and stroma with the assumption that genetic alterations occur in the epithelial compartment prior to LOH in the stromal compartment. Also interesting: the observations of van den Berg et al. in a neoplasm with mixed carcinomatous and sarcomatous growth pattern, the pancreatic mucinous cystic tumor with sarcomatous stroma. They documented identical allelic losses in epithelial and sarcomatous areas but in 1/3 cases investigated gene mutation was found in the second allel in the epithelial compartment only (van den Berg et al., 2000). From their data they hypothesized monoclonal origin with subsequent divergence of neoplastic epithelial and sarcomatous portions. This may support the theory of an initial pluripotent stem cell transformation during carcinogenesis.

Some questions, however, remain unsolved: (1) why do pathologists not diagnose alliance of carcinomas and sarcomas more frequently? (2) Why have scientists using cultured tumor-derived fibroblasts never decribed a 'tumorigenic' behavior of these fibroblasts but only a supportive effect on epithelial tumor development and dissemination in animal models? (3) Why do tumor-derived fibroblasts show cell senescence and contact inhibition in culture? The answer might be that the preliminary stromal component may still be non-tumorigenic in spite of potential genetic alterations. While the causal relation between epithelial tumor cells and fibroblastic stroma remains ambiguous and the data referenced need further evaluation, observations with tumor stromal fibroblasts imply an important if not a key role in the neoplastic transformation process. This key role of phenotypically and functionally altered fibroblast might be independent from genetic or epigenetic reasons.

Expression profile of TAF

Fibroblasts are a major source of ECM and ECM-modulating factors. The tumor-associated ECM is an abnormal but still complex meshwork of collagens, fibrillar glycoproteins, and proteoglycans that determine an aberrant tumor architecture. Perturbations in the production, deposition and degradation of matrix components have been observed in many human tumors including breast carcinomas (Lochter et al., 1997). Therefore, this paragraph will primarily focus on the TAF-dependent ECM composition. In addition, TAF expression of cell matrix receptors and cell adhesion molecules as well as of growth factors and cytokines will be discussed.

Oncofetal ECM components

The neoexpressed embryonic-type ECM in desmoplastic carcinomas frequently contains tenascin-C (TN-C) and several oncofetal fibronectin (FN) variants. Both members of the FN and TN families are high-molecular-weight, multifunctional ECM glycoproteins and cell-substrate adhesion molecules with large disulfide-linked subunits composed of multiple structural modules.

Out of the five human TN family members identified to date, only the prototype TN-C (alternative names: cytotactin, hexabrachion, myotendinous antigen, neuronectin, glioma mesenchymal extracellular matrix protein, or J1220/200) seems to play a relevant role in epithelial tumors. TN-C has been reported to be reexpressed in pathological conditions such as

inflammation, healing wounds and tissue repair, and also in the stroma and neovasculature of many undifferentiated tumors (e.g. Erickson and Bourdon, 1989; Lightner et al., 1990; Natali et al., 1991; Chiquet-Ehrismann, 1993; Rettig et al., 1994). It was detected in mammary tumors (e.g. Mackie et al., 1987; Ferguson et al., 1990; Ishihara et al., 1995), squamous cell carcinomas of the skin (Lightner et al.,1990), lung carcinomas (Oyama et al., 1991; Zeromski et al., 1995), gastric cancers (Ilunga and Iriyama, 1995), and epithelial tumors of the female reproductive system and prostate (e.g. Vollmer, 1994; Xue et al., 1998; Pilch et al., 1999). In breast tumors, TN-C was originally considered a stromal marker of malignancy since it was either not detected in normal adult mammary gland (Chiquet-Ehrismann et al., 1986; Mackie et al., 1987) or its distribution was restricted to areas around ducts and ductules but not around acini (Shoji et al., 1992, 1993). Later investigations, though, revealed that TN is frequently 'over'expressed in breast hyperplasia and dysplasia as well as in benign breast neoplasms (Ferguson et al., 1990; Howeedy et al., 1990; Moch et al., 1993). It was also detected in diffuse chronic gastritis, glandular atrophy and intestinal metaplasia and thus seems to correlate with infection and inflammation rather than with tumor port and prognosis (Ilunga and Iriyama, 1995; Schenk et al., 1995).

In addition to fibroblasts, normal and malignant breast epithelial cells have been described as a source of TN-C (Yoshida et al., 1995; Jahkola et al., 1998a,b). In fact, carcinoma, in contrast to normal mammary epithelial cells, may express higher levels of TN-C and incorporate little into an underlying matrix (Lightner et al., 1994). The hypothesis of alternative carcinoma cell-specific TN-C splice variants is supported by the observation of a distinct TN-C variant in squamous lung tumors containing an additional FN type III repeat as opposed to the normal counterparts (Oyama et al., 1991). Retrospective studies with tumor biopsies indicate that tumor cell but not stromal expression of TN-C, in particular at the invasion border of small infiltrating breast carcinomas may predict both recurrence after conservative breast surgery and distant metastases (Ishihara et al., 1995; Yoshida et al., 1995; Jahkola et al., 1998a,b). A correlation between tumor cell TN-C and erb-B2 overexpression, another prognostic and therapeutically relevant marker, has also been documented (Ishihara et al., 1995).

It could be concluded that stromal cells are rather irrelevant players in this TN-associated tumor scenario. However, the observation that carcinoma cell TN-C expression was observed in the margin of cancer nests at the site adjacent to the stroma led to the hypothesis that the underlying mesenchyme may induce changes in epithelial TN-C expression. A number of independent *in-vitro* and *in-vivo* studies verified that epithelial-mesenchymal interactions stimulate expression of stromal TN-C (Chiquet-Ehrismann et al., 1989; Ekblom and Aufderheide, 1989; Talts et al., 1999) but also induce expression of TN-C in defined tumor cells such as MCF-7 (breast), A431 (epidermoid), HEp-2 (larynx) presumably via a paracrine TGF-ß-dependent mechanism; WiDr (colon) and A549 (lung) carcinoma cells were not inducible (Hiraiwa et al., 1993; Sakai et al., 1994; Yoshida et al., 1995).

In contrast to TN-C, expression of oncofetal FN isoforms is largely restricted to embryonic tissues and reappears in granulation tissues, fibroses, and tumor stroma (Nicolo et al., 1990). Oncofetal FNs have been shown to correlate with poor differentiation status, grading and staging, and/or poor prognosis in oral squamous cell carcinomas (Mandel et al., 1992, 1994; Kosmehl et al., 1999; Lyons and Cui, 2000). A similar correlation was documented in epithelial ovarian cancers (Menzin et al., 1998), renal cell carcinomas (Lohi et al., 1998), in colorectal carcinomas, where it was also positive in the majority (> 90%) of liver metastases studied (Inufusa et al., 1995), and also in breast carcinomas (Loridon-Rosa et al., 1990; Kaczmarek et al., 1994). Here, invasive lobular breast tumors showed less intense and less frequent staining than invasive ductal carcinomas. In addition, oncofetal FN was found in post-radiation fibrosis of breast tumor patients in contrast to types I, II, V collagens, FN, and vimentin that were ubiquitously expressed (Brouty-Boye et al., 1991). Codistribution of the different oncofetal FN variants has been documented and expression was observed in tumor stroma, connective tissue and in tumor vessels. In advanced epithelial ovarian cancer, oncofetal FN was recorded in the stroma surrounding epithelial tumor nests and a more delicate fibrillar staining was described within tumor nests (Menzin et al., 1998). These features were reflected in our breast tumor fibroblast coculture model (Kunz-Schughart et al., 1998, 2001; and unpublished data). Here, fibroblast spheroids showed an intense staining for ED-A FN in spheroid mono- and coculture (Fig. 5b). Breast tumor spheroids of T47D and BT474 cells were negative in monoculture but a dim staining was reproducibly seen in some tumor cell areas in cocultures. Notably, other tumor spheroids, e.g. MCF-7 and ZR-75-1 were immunonegative in coculture with fibroblasts. These data are supported by the observation that not only fibroblasts (Brouty-Boye and Magnien, 1994) but also tumor and potentially endothelial cells may be a source of oncofetal FNs as shown for ED-B FN in vitro and in a tumor xenograft model (Midulla et al., 2000).

Laminins (LNs) are a family of trimeric glycoproteins involved in basement membrane architecture. The LN chain constitution of the basal lamina depends on tissue type but generally switches from embryonic to adult, differentiated tissues reflected by a reduction in LN-1 (α1ß1γ1) and the LN γ2 subunit. Out of the twelve known laminin heterotrimers each combined of one of five α, three ß, and three γ subunits (for review: Colognato and Yurchenco, 2000), only LN-1 and LN-5 (α3ß3γ2) and their respective integrin receptors have been implicated in tumor progression and

invasion including colorectal, breast, oral squamous, and lung adenocarcinomas (Berndt et al., 1997; Lohi et al., 2000). The corresponding fetal-type LN subunits are universally expressed by different cell types or derive from epithelial cells, i.e. subunits α3, and γ3. As a result, LN distribution in tumor tissue is critically determined by tumor cells rather than fibroblasts and will not be discussed herein.

Other ECM components

In addition to the qualitative fetal-like modifications in the composition of the connective tissue ECM in desmoplastic tumors described above, several quantitative alterations have frequently been documented, i.e. an excessive accumulation of ECM molecules such as FN, collagen types I, III, and V, elastin, and diverse proteoglycans. For example, promotion of collagen production by human fibroblasts as a result of interaction with tumor cells *in vivo* and *in vitro* is well known since many years ago (e.g. Naito et al., 1984; Ohtani et al., 1992; Noel et al., 1993).

Hyaluronic acids (HA) are linear glycosaminoglycans of different molecular weight and a major class of biosynthetic products of fibroblasts. Migration-stimulating factor (MSF), one of the factors overexpressed in fetal-like TAF was shown to stimulate the synthesis of a high molecular weight HA species. This observation was in accordance with previous studies showing that coculture of tumor cells and fibroblasts resulted in an increased HA production in fibroblasts (Knudson et al., 1984; Knudson and Knudson, 1990). Notably, cell migration and HA synthesis were differentially regulated by exogenous cytokines/growth factors in adult as opposed to fetal fibroblasts (Ellis et al., 1992, 1997). With regard to the HA scenario in tumors (Fig. 6) it should also be mentioned that tumor cells may produce elevated levels of HA-degrading enzymes (hyaluronidases). In some tumor types such as prostate and bladder cancers hyaluronidase and/or HA levels and profile, respectively, correlate with tumor grade and a HA-HAase urine test has been proposed recently as a sensitive diagnostic tool for urothelial tumors (Lokeshwar et al., 1996, 1997, 2000). Among others, activity of both immobilized HA and HA-derived fragments is modulated by the HA binding protein hyaluronectin which impaires the binding to endothelial cells inhibiting the stimulatory effect of HA *in vitro* (Trochon et al., 1997). Interestingly, hyaluronectin originates from tumor-associated stromal fibroblasts and presumably from monocytes (Delpech et al., 1997). Hyaluronectin is not related to the cell surface receptor of HA CD44, which is an ubiquitously expressed transmembrane glycoprotein involved in cell-cell and cell-matrix interactions. In scirrhous gastric cancer cells TAF-derived TGF-ß upregulated the expression of the CD44 H splice variant associated with an enhanced metastatic spread (Koyama et al., 2000a,b). The complex nature of CD44, i.e. multiple splice variants, posttranslational regulation, binding of various ligands including ECM components expressed by TAF such as collagens, LN, and FN, and the clear evidence of alternative splicing and overexpression in different neoplastic epithelial cells have been detailed in several recent review articles (e.g. Naot et al., 1997; Rudzki and Jothy, 1997; Sneath and Mangham, 1998; Herrlich et al., 2000).

The expression pattern of various heparan sulfate proteoglycans such as diverse syndecans, glypican, perlecan, and versican that are differentially expressed during developmental processes, organogenesis and wound healing was sporadically discussed with regard to

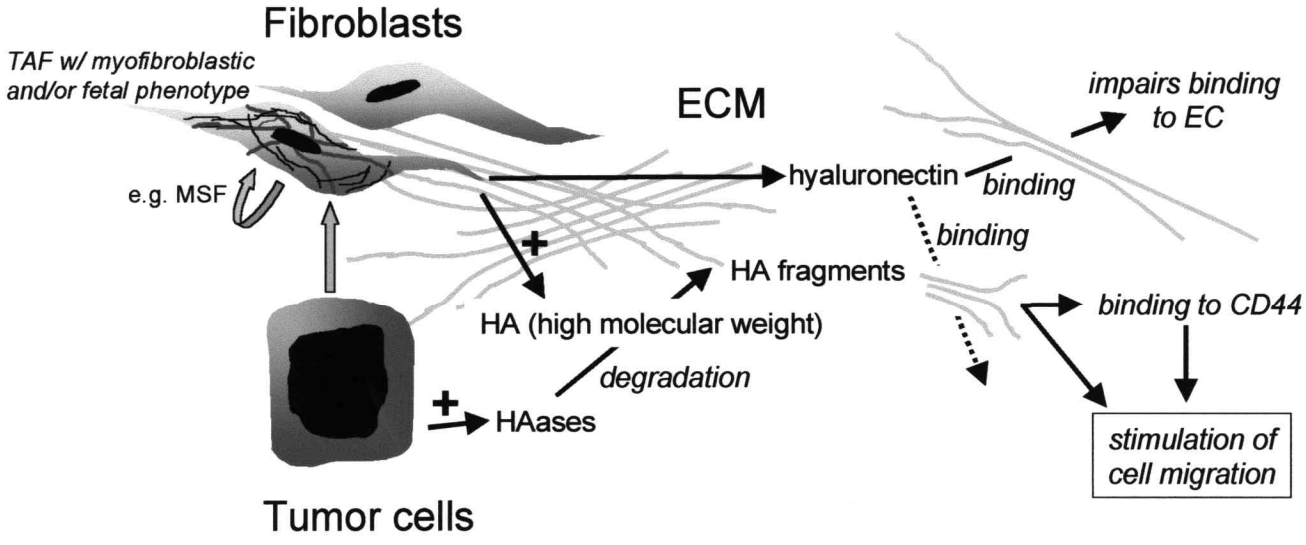

Fig. 6. Schematic illustration of the possible involvement of TAF in the hyaluronidase scenario in solid tumors leading to an affecting migratory activity not only of tumor cells but also of normal host cells such as endothelial and immune cells.

tumor prognosis. Deficiency or significant reduction in syndecan-1, an integral cell surface proteoglycan which contains an extracellular matrix-binding domain and a cytoskeleton-associated domain, has for example been demonstrated in particular in poorly differentiated head and neck squamous cell carcinomas (Inki and Jalkanen, 1996) and colorectal adenocarcinomas (Day et al., 1999). However, as shown for LN-5 subunits, these ECM molecules are primarily expressed by epithelial cells (Roskams et al., 1998). Other fibroblast-originated proteoglycans, i.e. the chondroitin sulfate proteoglycans decorin and versican, have rarely been studied in tumors. The few data, however, imply that expression of versican, a molecule with reported anticellular adhesive properties, but not of decorin, may become an independent predictive marker for prostate cancer progression (Ricciardelli et al., 1998).

ECM-modulating molecules

Turnover and temporal variations of the ECM are controlled by several enzymes (ECM proteases) and provide a large variety of signals that affect biosynthetic activities and directly influence cell proliferation and survival, differentiation and morphology as well as migration of various cell types. It has been known for some time that these enzymes can be secreted by neoplastic cells (Nicolson, 1991), but it has also become clear in recent years that a major fraction is also produced by stromal fibroblasts as a host response to tumor (e.g. Noel et al., 1994; Borchers et al., 1997).

ECM proteases expressed by TAF include several soluble and membrane-bound matrix metalloproteinases (MMPs). Transcripts of the interstitial or type I collagenase (MMP-1), the two type IV collagenases or gelatinase-A (Mr = 72 kD; MMP-2) and gelatinase-B (Mr =92 kD; MMP-9), as well as stromelysin 1 and 3 (MMP-3 and MMP-11, respectively) have all been demonstrated in tumor-adjacent fibroblasts of different human tumors such as oral and squamous cell carcinomas, skin, lung, breast, colon, and ovarian cancers (Basset et al., 1993; Poulsom et al., 1992; Pyke et al., 1992, 1993; Wernert et al., 1994; Gallegos et al., 1995; Okada et al., 1995; Heppner et al., 1996; Bolon et al., 1997; Rudolph-Owen and Matrisian, 1998; Maatta et al., 2000).

Both, TAM and TAF may significantly contribute to MMP-2 synthesis and many studies have demonstrated enhanced MMP-2 expression and activity to correlate with the invasive properties of tumor cells *in vitro* and a malignant phenotype, poor prognosis, and metastasis *in vivo* (for details: Curran and Murray, 1999, 2000; Ellerbroek and Stack, 1999; Seiki, 1999; McCawley and Matrisian, 2000). In head and neck squamous cell carcinomas (HNSCC) the activation ratio of MMP-2/proMMP-2 significantly correlated with cervical lymph node metastasis. *In-vitro* coculture experiments revealed MT1-MMP expression in both tumor and stromal cells with a stronger expression in HEp-2 cells resulting in an activation of stromal proMMP-2 following tumor-cell contact (Tokumaru et al., 2000). This is in accordance with previous observations in different human invasive carcinomas showing expression of MT1-MMP, a key activator of MMP-2, in both tumor and fibroblastic stromal cells with a more prominent staining in adenocarcinomas with severe fibrotic response (desmoplasia) (Imamura et al., 1998; Chenard et al., 1999; Seiki, 1999).

It was shown earlier that collagen-1 induces MMP-2 activation in human breast cancers in a highly specific manner and is inhibited by metalloproteinase inhibitors (Thompson et al., 1994). In a more recent study using an ovarian tumor/fibroblast coculture model it was verified that carcinoma cells stimulated proMMP-2 release from fibroblasts but not vice versa. In this system, collagen-1 and inhibition of integrin ß1 subunit induced activation of proMMP-2 in tumor-derived but not normal fibroblasts (Boyd and Balkwill, 1999). In fact, culture on collagen I gel seems to activate proMMP-2 via upregulation of MT1-MMP (Preaux et al., 1999). A recent *in-vitro* study with human dermal fibroblasts again suggested a role of the α2ß1 integrin receptor in the collagen-induced activation process of proMMP-2 via MT1-MMP (Zigrino et al., 2000).

Coculturing of an MMP-2-negative colon cancer cell line with fibroblasts resulted in an increased expression of active MMP-2 protein in fibroblasts. In parallel, tumor xenografts in nude mice, developed following injection of the MMP-2 non-expressing tumor cell line, were also positive for MMP-2. Tumor cell-conditioned medium was also inductive suggesting a soluble factor to be involved in this interaction (Ornstein et al., 1999). Activation of fibroblast-derived MMP-2 was also documented in a coculture model of colon cancer cells (CaCo-2, LoVo) and the colon fibroblast line CCD18-Co. In both contact and non-contact coculture the quantity of MMP-2 in conditioned media was unaltered but activity was enhanced by tumor cells or tumor-cell membrane extracts containing intact MT1-MMP (Ko et al., 2000). A similar effect, i.e. enhanced MMP-2 activity accompanying increase in tumor MT1-MMP expression was shown in a coculture model of human squamous carcinoma cells and dermal fibroblasts (Sato et al., 1999). Here, the cell-cell contact rather than a soluble factor was suggested to enhance MT1-MMP expression in tumor cells. In the ovarian tumor cell/fibroblast coculture system of Boyd and Baldwill (1999) carcinoma cells stimulated not only expression of proMMP-2 but also release of tissue inhibitor of metalloproteinase TIMP-2 from fibroblasts (Boyd and Balkwill, 1999). Also, in lung adenocarcinomas stromal fibrosis and desmoplasia with central scar formation correlated with the destruction of the basal membrane and the expression of MMP-2 and TIMP-2 (Kitamura et al., 1999).

A few other studies shall be highlighted to point out other MMPs to be potentially overexpressed in tumor-associated stroma. In fact, most of the novel family

members identified within the last year such as MMP-25 (membrane type MMP: MT6-MMP), MMP-26 (matrilysin-2), and MMP-28 have shown to be overexpressed in diverse established carcinoma cells and/or in tumor tissues, while expression in TAF has not explicitly been studied (Park et al., 2000; Uria and Lopez-Otin, 2000; Velasco et al., 2000; Marchenko and Strongin, 2001). The relation of MMP-1, MMP-3, MMP-9, and/or MMP-11 with poor prognosis and survival in different tumor entities and even in identical tumor types documented in the literature is rather inconsistent (for review: Curran and Murray, 1999, 2000; Koblinski et al., 2000). This may result from the fact that expression profile (mRNA expression), protein level, and activity status of MMPs in tumor tissue do not necessarily correlate. MMP-9, however, is an interesting candidate in the tumor-associated ECM modulation, as it is activated via different overlapping proteolytic cascades: enzymatic cleavage through MMP-2 or MMP-3 which is potentially converted into an active form via the urokinase-type plasminogen activator (uPA)-plasminogen-plasmin system (Schmitt et al., 1992; Murphy and Gavrilovic, 1999; Koblinski et al., 2000). Pro-uPA as well as the plasminogen-activator inhibitors PAI-1 and PAI-2 are primarily expressed by stromal fibroblasts (Pyke et al., 1991; Koretz et al., 1993; Wernert et al., 1994; Bianchi et al., 1995; Borgfeldt et al., 2001) while the receptor, a membrane-bound glycosylphosphatidylinositol-anchored cell-surface molecule at which pro-uPA is proteolytically activated, may also be expressed on tumor cells and stromal macrophages (Pyke et al., 1991; Bianchi et al., 1995; Saito et al., 2000).

In addition to the serine protease uPA, cathepsin B is involved in the upstream regulation of proMMPs both directly (e.g. activation of MMP-1 and MMP-2) and indirectly via activation of pro-uPA. Cathepsin B was one of the first proteases found to be localized in TAF and in the ECM at the invasive front *in vivo* in a rabbit tumor model and to be affected by the tumor-stroma interaction (Graf et al., 1981; Baici et al., 1984, 1988). This was verified much later in human tumors including breast and colon carcinomas (Campo et al., 1994; Castiglioni et al., 1994). Cathepsin B also appears to be active in the downstream regulation of MMPs as it leads to fragmentation of TIMP-1 and TIMP-2 resulting in a loss of MMP-inhibitory and anti-angiogenic activities (Kostoulas et al., 1999).

Squamous cell carcinoma cells have been used to examine the molecular mechanisms of tumor-induced MMP-1 expression by stromal fibroblasts showing a paracrine-induced activation at the transcriptional level crucially regulated via the p38 mitogen-activated protein kinase and c-Jun NH2-terminal kinase (JNK) pathway (Westermarck et al., 2000). An epithelial-derived inducer of MMPs (EMMPRIN) has been identified that is prominently expressed by various malignant cell populations (Lim et al., 1998; Caudroy et al., 1999) and stimulates MMP-1, MMP-2, and MMP-3 in fibroblasts (Kataoka et al., 1993; Guo et al., 1997). In bronchopulmonary and breast lesions a positive correlation of EMMPRIN presence and MMP-2 expression in stromal fibroblasts was documented (Caudroy et al., 1999). In fact, many different factors including hormones, growth factors, and cytokines which are secreted by tumor and/or stromal cells have been shown to regulate metalloproteinases at the transcriptional level. e.g. at the AP-1 binding site. Interestingly, the c-ets1 transcription factor, which is induced by tumor-conditioned medium and known to play a relevant role in MMP activation, seems to be expressed in TAF but not in fibroblasts of non-invasive lesions (Wernert et al., 1994; Wernert, 1997; Bolon et al., 1995, 1996; Gilles et al., 1996). However, transcriptional regulation of the MMP genes in different cell types is complex and still not well understood (Curran and Murray, 1999).

One additional, quite specific factor involved in the proteolytic cleavage of the ECM that is expressed by TAF should be mentioned: fibroblast activation protein (FAP). FAP is a serine integral membrane proteinase with gelatinase activity that is expressed on the surface of reactive stromal fibroblasts in 90% of lung, breast, and colon tumors, during wound healing and in granulation tissue (Scanlan et al., 1994; Park et al., 1999). Interestingly, the expression of FAP, in contrast to other ECM proteases, was shown to correlate with a longer overall and disease-free survival in patients with invasive ductal breast tumors (Ariga et al., 2001). The cell surface expression of FAP on TAF has led to the first design of a therapeutic tumor-stroma targeting using an FAP-specific antibody (Mersmann et al., 2001). Studies to better understand FAP function are ongoing.

In summary, the observations unambiguously indicate that the expression and activation of ECM-modulating molecules in human cancers result from complex reciprocal interactions between malignant cells and their non-malignant stroma.

Paracrine/autocrine peptide growth factors

Fibroblasts are a relevant source of multiple peptide growth factors. However, the expression profile extremely depends on fibroblast location and functional status and it is therefore difficult to define a specific expression pattern for TAF as opposed to normal fibroblasts. Based on the observation of myofibroblastic and fetal-like phenotypes in tumors, some information is provided by investigators studying these phenotypes in various normal and pathological settings. Myofibroblasts of different origin, for example, secrete diverse growth factors, including TGF-ß, PDGF-AA and -BB, ßFGF, IGF-I and II (insulin-like growth factors), KGF (keratinocyte growth factor), HGF (hepatocyte growth factor), NGF (nerve growth factor), SCF (stem cell factor), EGF (epithelial growth factor), and TGF-α (transforming growth factor-alpha) and cytokines CSF-1 (colony-stimulating factor) and GM-CSF (granulocyte-

macrophage colony stimulating factor) (for review: Powell et al., 1999a,b). Myofibroblasts in tumors were also shown to express tissue factor (TF), the cellular initiator of the protease blood coagulation cascade (Vrana et al., 1996). In this study a strong correlation between progression to invasive cancer and expression of the TF antigen in macrophages and myofibroblasts, in particular at the invasion front, was documented. All of the growth factors depicted, directly and/or indirectly affect carcinoma growth. Some of them are not exclusively expressed by TAF but rather accompany myofibroblastic phenotype in general. Also, they do not only show paracrine effects but are also implicated in autocrine loops according to the expression pattern of the respective receptors on the fibroblast cell surface, e.g. TGF-ß R types I and II, PDGF-α and -ß and IGF type I R.

The desmoplastic reaction in human breast carcinomas as a host myofibroblast-mediated collagenous response is, for example, considered to be initiated by tumor-derived PDGF (Shao et al., 2000). Since myofibroblasts produce relevant amounts of PDGF, an autocrine stimulation following tumor cell-fibroblast interaction and myofibroblast induction seems reasonable. In general, PDGF, a homo- or heterodimeric molecule of two structurally-related A- and B-polypeptide chains is a major mitogen for connective tissue that induces changes in cell shape and motility involving reorganization of the actin filament system (for review: Heldin and Westermark, 1999; Powell et al., 1999a; Ostman and Heldin, 2001). Sasaki et al. (2000a,b) described PDGF to induce MMP-1 production in human lung fibroblasts and to enhance MMP-3 and MMP-9 activity when combined with TNF-α and IL-1 treatment. In contrast, active TGF-ß1 (and -ß3) reduced MMP-1 and TIMP-2 secretion in analogous fibroblasts and increased collagen type I, TIMP-1, and PAI-1 expression leading to an enhanced ECM deposition (e.g. Laiho et al., 1987; Overall et al., 1989, 1991; Eickelberg et al., 1999).

TGF-ß is another potent paracrine/autocrine inducer of myofibroblast differentiation *in vitro* and *in vivo* and is implicated in tumor-associated myofibroblastic phenotype (e.g. Rønnov-Jessen and Petersen, 1993; Desmouliere et al., 1993, 1995; Serini et al., 1998; Serini and Gabbiani, 1999; Powell et al., 1999a; Yokozeki et al., 1999; Lanning et al., 2000; Vaughan et al., 2000). Recently, the ED-A fibronectin splice variant has been identified as a mediator molecule in this process (Serini et al., 1998; Serini and Gabbiani, 1999; George et al., 2000; Vaughan et al., 2000), Three TGF-ß isoforms are known. However, in contrast to TGF-ß1 and -3, an impact of TGF-ß2 on tumor-associated myofibroblastic phenotype and fibroblast-myofibroblast differentiation has not been demonstrated. One study showed TGF-ß2-induced transformation of corneal endothelium into myofibroblasts in organ culture (Petroll et al., 1998), and another one described the involvement of TGF-ß-RIII and TGF-ß2 in the epithelial-mesenchymal transformation of cardiac endothelial cells in an *in-vitro* chicken atriventricular cushion explant model (Brown et al., 1999). Our own observations using fibroblast spheroid cultures indicate that fibroblasts do not express relevant amounts of TGF-ß-RIII in a 3-D environment. Binding of TGF-ß2 to TGF-ß-R type II followed by signal transduction through TGF-ß-R type I, however requires binding to and mediation through TGF-ß R type III. From these data we concluded that TGF-ß2 and TGF-ß-RIII may play a role in transdifferentiation or transition processes but not in tumor-induced myofibroblastic phenotype (unpublished data).

TGF-ßs are usually secreted in large latent complexes consisting of an immature TGF-ß homodimer and a covalently-attached latent TGF-ß binding protein (LTBP). Four different LTBPs have been described; three of these may be subject to alternative mRNA splicing. LTBP1, -2, and -4 are secreted by human fibroblasts and may directly associate with the ECM (for recent review: Öklü and Hesketh, 2000). Thrombospondin-1, an ECM glycoprotein that is known to modulate tumor growth, angiogenesis, and metastasis, is involved in the liberation and activation of the TGF-ßs and is present in the desmoplastic tumor stroma of invasive ductal breast carcinomas (Bertin et al., 1997). Another mechanism with potential relevance in the tumor-associated activation of TGF-ß has been described recently by Munger and coworkers (Munger et al., 1999). Their data indicate that latent TGF-ß1 complexes may bind via the TGF-ß1 latency-associated peptide (TGF-ß1 LAP) to αvß6 integrin. Subsequently, the cytoplasmic domain of the ß6 subunit becomes accessible for binding to the actin cytoskeleton and the cytoskeleton-associated integrin induces a conformation change of the latent complex allowing access of mature TGF-ß1 to TGF-ß receptors. Integrin αvß6 is epithelium-restricted and has been shown to be overexpressed following malignant transformation, e.g. in oral squamous and desmoplastic colon carcinomas (Jones et al., 1997; Agrez et al., 1999; Hamidi et al., 2000; Koivisto et al., 2000). αvß6 integrin binds to RGD sites in tenascin, vitronectin, and FN (Weinacker et al., 1994); these ECM compounds are all secreted by fibroblasts.

Stromal-epithelial interactions in human cancers are also mediated by IGFs, e.g. IGF II which is often overexpressed in tumor stroma (Ellis et al., 1994; Shao et al., 2000). IGF-II belongs to an ubiquitously expressed family of pleiotropic growth factors that is enhanced in many tumor types (Werner and Le Roith, 2000; Yu and Rohan, 2000; Zhang and Yee, 2000). Its activity is controlled by at least 7 different IGF-binding proteins (IGFBPs) that serve to transport the IGFs, prolong their half-lives, and modulate their biological action depending on IGFBP species and cell and tumor type, respectively (for review: Hwa et al., 1999; Grimberg and Cohen, 2000; Khandwala et al., 2000). IGFBPs are regulated via IGFBP proteases including MMP-1, MMP-3, and MMP-9 (e.g. Fowlkes et al., 1994,

1995; Manes et al., 1999). These MMPs are potentially secreted by TAF (see above).

The particular role of the growth factors mentioned above in epithelial-stromal interaction during prostate tumorigenesis has been reviewed recently (Wong and Wang, 2000).

Cytokines/chemokines

According to the complex regulation and expression pattern of peptide growth factors in fibroblasts, TAF are poorly characterized and categorized according to their cytokine expression profile. In fact, TAM, but not TAF, have been considered the major source of many inflammatory and pro-inflammatory cytokines in tumors that are also capable of producing autocrine anti-inflammatory, immune-suppressive mediators, depending on their differentiation and activation status. For example, an autocrine loop of IL-10-mediated defective IL-12 production in TAM has been demonstrated most recently, with IL-12 representing an important cytokine in the activation of inflammation and generation of Th1-type responses (Sica et al., 2000). Tumor-derived IL-10 was shown to block macrophage cytotoxicity much earlier (Alleva et al., 1994; Kambayashi et al., 1995; Elgert et al., 1998) but fibroblasts as potential intermediators between tumor cells and monocytes and their highly variable cytokine/chemokine production and release were largely ignored.

Again, the phenotypic tumor-induced alteration towards myofibroblast and the data known for this phenotype provide some information on potential cytokine expression by TAF. According to Powell's review article (1999a), myofibroblasts are capable of producing a number of cytokines such as IL-1, IL-6, TNF-α (tumor necrosis factor-alpha), and IL-10 that may all affect immune-cell function in tumors. Also, a number of chemokines, small molecules defined as chemoattractants for specific leukocyte subpopulations with various other effects that are potentially relevant for tumor growth and propagation, are released by myofibroblasts. These include the C-X-C (or α) chemokines IL-8, GRO-α (growth-regulated oncogene alpha), and ENA-78 (epithelial neutrophil-activating peptide) and the C-C chemokines MIP-1α (macrophage inflammatory protein-1-alpha), MCP-1 (monocyte/macrophage chemotactic/chemoattractant protein), and RANTES (regulated, upon activation, normal T cell-expressed and secreted).

Chemokine research is a complex field of rapid progress prohibiting speculation on an ultimate role of TAF-derived chemokines on immune, epithelial and endothelial cell function to date. However, because of their role in inflammation and immunity, they are also suspected to be relevant in neoplastic processes (Wang et al., 1998). Brouty-Boyé et al. (2000) were the first to systematically analyze chemokine expression (mRNA/protein) in cultured human fibroblasts from various tissues and pathological settings including tumor stroma. Their data on IL-8, MCP-1, RANTES, MIP1-α, and Eotaxin expression indicate a distinct panel of these chemokines to be produced by fibroblasts depending on both site of origin and pathological condition. However, they also discuss the fact that fibroblasts cultured *in vitro* should be considered permanently activated even as confluent, resting monolayer cells as they maintain some phenotypic characteristics that are specific *in vivo* for activated fibroblasts. For example, all fibroblasts grown *in vitro* by Brouty-Boyé and coworkers expressed CD40, an activating surface molecule for immune cells (Brouty-Boyé et al., 2000). This surface antigen was also shown to be expressed on various bladder, pancreatic, and breast tumor cell lines, and is supposed to mediate tumor-specific T cell responses as ligand binding induced cell surface expression of ICAM-1 (CD54) and FAS, and production of various cytokines in epithelial tumor cells (Alexandroff et al., 2000). In fibroblasts, ligation of CD40 may also induce CD54 expression as documented for dermal fibroblasts (Yellin et al., 1995). In parallel, an up-regulation of CD106 (VCAM-1) and IL-6 and induction of fibroblast proliferative activity was illustrated. The spheroid model with three-dimensional cell-cell and cell-matrix interactions represents a valuable tool to investigate the impact of CD40 expression and ligand binding on the regulation of cytokine/chemokine and cell adhesion molecule expression in an *in-vivo* like tissue environment, not only for epithelial tumor cells but also for cell-cycle arrested fibroblasts.

Considering an autocrine loop of cytokines/chemokines regulating fibroblast activity and expression creates an even more complex and confusing pattern. As an example: MCP-1 is discussed to play a relevant role in tissue fibrosis (Lloyd et al., 1997a,b) and was shown to enhance synthesis of TGF-ß and collagen (Gharaee-Kermani et al., 1996) and also of TIMP-1, and MMP-1 in fibroblasts, the latter one via an autocrine IL-1α loop (Yamamoto et al., 2000). MCP-1 itself is induced in different fibroblast types by various cytokines. In human dermal fibroblasts, autocrine IL-6 was demonstrated to upregulate MCP-1 but not IL-8, with evidence for involvement of an IL-6 – sIL-6Ra – gp130 signal transduction pathway (Sporri et al., 1999). IL-1ß and IFN-γ are potent stimuli of MCP-1 and MCP-2 in human diploid fibroblasts with synergistic or additive effects (Struyf et al., 1998); IL-1ß and TNF-α were capable of inducing MCP-1 and IL-8 secretion in isolated pancreatic periacinar myofibroblasts (Andoh et al., 2000). TNF-α treatment of cultured interstitial renal fibroblasts, which are supposed to be involved in interstitial inflammation, also resulted in an elevated expression of MCP-1 in parallel to IP-10 (interferon-inducible protein 10) (Gonzalez-Cuadrado et al., 1996). Il-1ß and TGF-α also induced MCP-1 production in primary lung fibroblasts (Rolfe et al., 1992). Lung fibroblasts were further investigated with regard to allergic asthma and lung injury as a secondary smoking-

related airway disease showing correlation between presence of inflammatory cells and fibrosis. Here, in addition to TNF-α, TGF-ß was identified as a potential inducer of MCP-1, and bradykinin was shown to augment MCP-1, IL-8, GM-CSF, G-CSF, and TGF-ß production (Koyama et al., 2000a,b); mRNA expression of MCP-1, IL-8, and GM-CSF (Sato et al., 1999) was also enhanced by smoke extracts. Hogaboam and coworkers have intensively investigated mechanisms of lung fibrosis using a mouse model. They examined MCP-1, C-C chemokine receptor CCR2, procollagen I and III, and TGF-ß expression in fibroblasts isolated from normal lung tissue or from Th1- and Th2-type immune reactions, respectively. While all fibroblasts exhibited an MCP-1-dependent TGF-ß synthesis and Th2-type fibroblasts generated twice as much MCP-1 than Th1 or normal fibroblasts, only Th1-type fibroblasts showed an MCP-1-independent regulation of procollagen mRNA expression, indicating that lung fibroblasts are differentially altered in different pulmonary inflammatory processes (Hogaboam et al., 1999).

The data referenced indicate a finely tuned regulation of cytokine/chemokine production and release from fibroblasts of different origin. Since this system is easily manipulated, e.g. by cell-to-cell and cell-to-matrix interactions (Smith et al., 1997), the tumor environment is hypothesized to critically alter the normal fibroblast-type specific expression pattern resulting in a tumor-supportive function of TAF.

Cell matrix receptors and adhesion molecules

Fibroblasts, including TAF, interact with the surrounding ECM mainly via integrins. Integrins are a large family of transmembrane non-covalent-bound αß heterodimeric receptors with a defined but overlapping ligand specificity. Today, at least 23 integrins are decribed consisting of one of sixteen α and seven ß subunits. Five distinct integrins, all containing the ß1 subunit, namely α1ß1, α2ß1, α3ß1, α10ß1, and α11ß1 are described to bind collagens but may partly also interact with other ECM molecules such as laminin (α1ß1) or FN (α3ß1). ß1 integrins are the predominant type on the fibroblast surface. Here, they primarily interact with α1, α2, α3, α4, α5, and/or α6 subunits in normal fibroblasts (Norman and Fine, 1999).

The intriguing review on FN and its integrin receptors in cancer by Erkki Ruoslahti (Ruoslahti, 1999) concentrates on the role of the different integrin subunits in anchorage-dependent epithelial cell growth and prevention of migration, and their involvement in anoikis, i.e. epithelial/endothelial cell apoptosis following cell detachment from ECM. It summarized the state of knowledge of how cancer cells shunt the integrin-signaling pathways to get around the requirement of integrin-mediated control mechanisms but does not discuss the fact that the ECM assembly in and around tumor nodules may be altered by TAF to provide a favorable substrate for cell migration. Also, integrin-ECM interactions in TAF as an outside-in signaling pathway determining fibroblast phenotype and function are not highlighted. In fact, there is evidence that the major integrins present on the fibroblast surface do not only have passive structural functions but do also fulfill specific regulatory tasks such as control of balance between synthesis and degradation of ECM molecules, i.e. shown for procollagens/collagens and collagenases via integrins α1ß1 and α2ß1, respectively (for review: Yamamoto et al., 2000). Here, ECM-integrin-mediated signals are transmitted to defined multiprotein complexes termed focal adhesions that activate intracellular signaling cascades involving several tyrosine kinases and finally alter the activity of specific transcription factors.

The postulated interactions of fibroblasts with the ECM and the role of integrins and focal adhesions have been reviewed in recent articles that focus on fibrosis and alterations in the ECM-integrin signaling system associated with the fibrotic process (Dogic et al., 1999; Norman and Fine, 1999; Yamamoto et al., 2000). Considering that desmoplasia represents a type of fibrotic response to malignant cells, some if not all of the observations may also apply to TAF. Norman and Fine (1999), for example, reference a number of publications showing that interstitial fibrosis is associated with enhanced expression of α1, α2, α5, αv, and ß1 integrins, with ligation of α5 integrin promoting myofibroblastic differentiation, and ß1 implicated in cell contraction. ECM molecules upregulated in interstitial fibrosis include laminin, ED-A fibronectin and hyaluronan. All of these characteristics are also described in the desmoplastic reaction and in scirrhous regions of solid tumors, indicating that TAF may show fibrosis-type expression and dimerization pattern of integrin subunits with an analogous activation of intracellular signaling pathways.

As mentioned earlier, CD40 ligation in fibroblasts may result in an enhanced ICAM-1 and VCAM-1 expression (Yellin et al., 1995). In fact, numerous reports have described an induction of ICAM-1 *in vitro* in fibroblasts of different normal and pathological origins by various stimuli such as TNF-α, IL1-α, IL1-ß, IFN-ß, IFN-γ, prostaglandin F2, or LPS indicating a potentially enhanced expression of ICAM-1 in tumor-stromal fibroblasts to result from and to be involved in multiple paracrine/autocrine regulatory mechanisms (e.g. Vogetseder et al., 1989; Pang et al., 1994; Banner et al., 1995). Elevated levels of VCAM-1 expression are also thought to be a consequence of the presence of inflammatory mediators, in particular IL-1ß and TNF-α. Also, IL-4 was documented to affect expression of cell-adhesion molecules, but the data are inconsistent probably due to the different origin of fibroblasts, e.g. dermal fibroblasts were shown to express elevated ICAM-1 levels on the cell surface following treatment (Piela-Smith et al., 1992), while a systematic examination of lung fibroblasts to study asthmatic

inflammatory responses verified that the Th2 cytokine IL-4 only induced VCAM-1 expression while the Th1 cytokine IFN-γ solely stimulated ICAM-1 synthesis (Spoelstra et al., 1999). In parallel, a TNF-α- and IFN-γ-induced expression of soluble ICAM-1 (sICAM-1) has also been documented in lung fibroblasts as a negative feedback mechanism (Leung, 1999). Vice versa, ligation of ICAM-1 may further increase the synthesis of ICAM-1 and may also lead to an enhanced expression of VCAM-1 in fibroblasts (Clayton et al., 1998). Recapitulation of the literature data, however, indicates that the expression and regulation of ICAM-1, sICAM, and VCAM-1 in TAF of different tumor entities is not adequately characterized to be able to create a general and coherent picture. The same is true for other diverse cell-adhesion molecules such as the cadherin/catenin system. The existence of N-cadherin/catenin complexes as well as of a non-cadherin-associated ß-catenin in fibroblasts and myofibroblasts has been documented earlier (Van Hoorde et al., 1999) with its function and relevance in tumor progression still incompletely understood.

Conclusion

This article emphasizes the phenotypic and functional characteristics of TAF as opposed to normal fibroblasts. Fetal-like and myofibroblastic phenotypes are described with respect to the desmoplastic reaction in epithelial tumors and are discussed with regard to new data on genetic alterations in tumor-adjacent stroma. Reports describing the impact of tumor cell-fibroblast interactions on the expression profile of fibroblasts are reviewed, indicating that the expression of diverse ECM components, ECM-modulating molecules, ECM receptors and cell-cell adhesion molecules, as well as of autocrine/paracrine peptide growth factors and cytokines/chemokines is profoundly altered in TAF in spite of the organ- and site-specific normal phenotypic and functional diversity. TAF inevitably affect the behavior of tumor cells and other stromal host cells via direct and indirect mechanisms, some of which are depicted in the second issue of this review article series. As a consequence, TAF are to be considered important therapeutic targets.

Acknowledgements. Our work with 3-D spheroid cocultures is supported by the Deutsche Forschungsgemeinschaft and by the Bayerische Staatsministerium für Wissenschaft, Forschung und Kunst.

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Accepted December 28, 2001