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Invited Review

Growth factors in mechanisms of malignancy: roles for TGF-ß and FGF

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Summary. Malignant progression is a complex process involving the accumulation of multiple genetic alterations leading to changes in many specialized cell functions. Important in this process is the loss of growth control which is frequently associated with modifications in growth factor production, and growth factor response pathways. Indeed, oncogenes have been characterized that code for polypeptide growth factors or their receptors, and many tumor cell populations release potently mitogenic growth factors which contribute to the malignant properties of tumor cells. In this review, the importance of growth factors in mechanisms of malignant progression is emphasized, using as examples the transforming growth factor-beta (TGF-ß) and fibroblast growth factor (FGF) families. We describe many of the properties and biological activities of these two families of growth factors, focusing on mechanisms of autocrine and intracrine mitogenic stimulation of tumor cell proliferation and malignant progression. The discussion includes evidence for altered growth factor expression in tumor cells, and the relationship between these changes in growth factors and alterations in the regulation of DNA synthesis, cell proliferation, protease production and cell motility required for invasion and metastasis. Recent studies are described that show that aberrant expression of TGF-$ß_1$, bFGF or K-FGF results in dramatic changes in the genetic stability of cells, leading to increased rates of spontaneous gene amplification and the generation of drug resistant variants. These findings describe new malignancy relevant functions for altered growth factor expression.

Key words: TGF-$ß_1$, bFGF, K-FGF, Tumor progression, Metastasis, Gene amplification, Cell proliferation

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Introduction

Growth factors are fundamentally involved in cellular processes that are important in the progression of malignant disease, so it is not surprising to find that alterations in the regulation of growth factors and their signal pathways are frequently observed in tumor cells (Sporn and Todaro, 1980; Heldin and Westermark, 1984; Wright et al., 1990c; Sporn and Roberts, 1991; Halaban and Moellmann, 1991; Cross and Dexter, 1991). These regulatory molecules play a critical role, either directly or indirectly, in mechanisms of tumorigenesis and metastasis. Although certain growth factors are present in the circulation and may function as systemic agents, they are frequently synthesized by the specific tissues in which they perform as local modifiers, and may either affect the cell of origin by an autocrine mechanism or interact with other cells by a paracrine mechanism of action (Sporn and Todaro, 1980; Heldin and Westermark, 1984; Sporn and Roberts, 1991). The two growth factor families, transforming growth factor-ß (TGF-ß) and fibroblast growth factor (FGF) are good models for analyzing the involvement of growth factors in mechanisms of malignant progression, therefore this review focuses on their roles in the control of cellular events important in mechanisms of malignant progression, including gene amplification.

Transforming growth factor-ß

Reviews of the structure, function and regulation of the TGF-ß family have been published, and we refer the reader to these articles (e.g. Massagué, 1990; Sporn and Roberts, 1990; Laiho and Keski-Oja, 1992; Sieweke and Bissell, 1994). TGF-ß is found in three highly homologous isoforms in mammals, TGF-$ß_1$, TGF-$ß_2$ and TGF-$ß_3$, and each exists as a 25-kDa homodimer. The molecule is secreted in association with N-terminal amino acid sequences, which are unique to each isoform and maintain the TGF-ß in a latent state so that receptor-

ligand interaction is blocked. Although pH and proteolysis can activate TGF-ß *in vitro*, the physiological activation of TGF-ß by tumors is not well understood. A role for proteinases such as plasmin and cathepsin D has been suggested in the extracellular activation of latent TGF-ß from endothelial cell and smooth muscle cell cocultures (Sato and Rifkin, 1989; Lyons et al., 1990; Sato et al., 1990). Once the 25-kDa dimer has been dissociated from the amino terminal glycopeptide, it is able to interact with the TGF-ß receptors. The receptors were initially identified by cross-linking to radiolabelled TGF-ß, and exist in three forms of 55-65, 85-95 and approximately 300 kDa named types, I, II and III. The type I and type II, which are the lower molecular-weight forms, appear to be necessary for TGF-ß action (Massagué, 1990). The type III form, also called betaglycan, may regulate the ligand binding ability or surface expression of the type II receptor (López-Casillas et al., 1991; Wang et al., 1991). All three receptors have been cloned, and interestingly the type I and II forms appear to possess intracellular serine/threonine kinase domains (Lin et al., 1992; Franzen et al., 1993). Recent studies indicate that type I and II receptors form a heterodimeric serine/threonine receptor complex in which the type I receptor requires the type II receptor to bind growth factor, and the type II receptor needs the type I receptor binding to generate signal transduction (Wrana et al., 1992; Moutsakas et al., 1993; Bassing et al., 1994). Interesting stoichimetric interactions of these receptors with each other have been described, as well as with the different TGF-ß isoforms (Yamashita et al., 1994). All three TGF-ß isoforms are produced by fibroblast and epithelial cells maintained in cell culture. Many tissues have detectable TGF-ß mRNA levels, although the isoforms expressed in any given tissue may differ significantly (Sporn and Roberts, 1990). The reasons for this are unclear but suggest novel effects of the isoforms; however, surprisingly little difference has been noted in the action of each isoform. In contrast, regulation of isoform expression is quite distinct. For example, TGF-ß secretion during embryogenesis is regulated and isoforms are expressed in specific tissues during particular periods of differentiation, as detected by immunohistochemistry and *in situ* hybridization (Heine et al., 1987; Pelton et al., 1989). The differential expression of these isoforms is likely the result of unique 5'-regulatory regions for each gene, which contains different response elements. For example, TGF-$ß_1$ regulates its own mRNA by specific AP-1 sites on its promoter (Kim et al., 1989a,b, 1990a), whereas the TGF-$ß_2$ and $ß_3$ promoters have cyclic adenosine monophosphate responsive elements (Lafyatis, 1990). Furthermore, complex interactions between the isoforms appear to be regulatory for mRNA expression (Bascom et al., 1989).

Elevated levels of TGF-ß mRNA and enhanced secretion of TGF-ß have been observed in many human and rodent tumors (Anzano et al., 1985; Derynck et al., 1987; Jakowlew et al., 1988; Braun et al., 1989; Jasani et al., 1990; Mizukami et al., 1990; Kim et al., 1990b; Schwarz et al., 1990; Gomm et al., 1991; Ito et al., 1991), although not all tumors exhibit increased expression. Experimental rodent systems have shown that high levels of secretion are detected in metastatic variants of H-*ras*-transformed fibrosarcomas (Schwarz et al., 1990) and Lewis lung carcinoma (3LL) (Perotti et al., 1991), and this can be detected both *in vitro* and *in vivo*. Also, we found that some tumors did not secrete more TGF-ß, but they exhibited a much higher proportion of the TGF-ß in the active rather than latent form (Schwarz et al., 1990); this activation will effectively increase TGF-ß action without modifying mRNA or total protein levels. The posttranscriptional control of TGF-ß through processing of the latent form has been suggested as an important regulator of its *in vivo* effects (Wakefield et al., 1989; Lyons et al., 1990). Activation can be mediated directly by the TGF-ß secreting cells as well as by a paracrine mechanism. The latter has been observed in pericytes that activate latent TGF-ß secreted by endothelial cells through plasmin secretion (Sato and Rifkin, 1989).

Growth in tissue culture on plastic or even on extracellular matrix does not necessarily reflect the natural environment, therefore evidence for *in vivo* TGF-ß expression and secretion by tumors is important. TGF-ß has been detected immunohistochemically in invasive breast carcinoma (Mizukami et al., 1990) and malignant thyroid tumors (Jasani et al., 1990). In an experimental metastasis model, we have found that H-*ras* transformed fibroblasts progressively increase TGF-$ß_1$ mRNA expression and TGF$ß_1$ secretion following lung implantation and growth (Schwarz et al., 1990). Immunohistochemical evidence of enhanced secretion utilized an anti-TGF-$ß_1$ peptide antibody that binds TGF-ß only after it is secreted and associated with extracellular matrix (Schwarz et al., 1990) (Fig. 1). Other similarly have found that TGF-$ß_1$ mRNA expression in metastases was elevated when compared with the primary tumor of a poorly metastatic 3LL Lewis lung carcinoma (Perotti et al., 1991).

It has been suggested that the most appropriate description for the action of TGF-ß is as a biological switch; that is, there is no well-defined action of the molecule which can be used to describe its primary function, but that it is best characterized in relationship to the cell and its environment. This type of description leads to a consideration of the action of TGF-ß within different environments as well as during stages of cell differentiation. This concept has profound implications for malignancy where transformation permanently alters both the differentiation state of the cell and the interaction of the neoplastic cell with its environment. The potential importance of the environment milieu affecting TGF-ß responses can best be demonstrated during tumor metastasis, when tumor cells are released from the primary tissue site and move into entirely novel environments in distant organs. This could create conditions in which the action of TGF-ß could be

modified dramatically. Possible examples of the influences of the extracellular environment on the TGF-ß response include matrix/proteoglycan interaction with TGF-ß, which may inhibit or prolong the action of the polypeptide, and proliferative and secretory responses to TGF-ß, which may be influenced by interaction of the responding cells with extracellular matrix of tissues could affect the presentation of TGF-ß to cells for receptor activation as well as modify the cellular response to TGF-ß. Adherence of cytokines to matrix protein is well known (Nathan and Sporn, 1991). When the active TGF-ß dimer is secreted, it can interact with proteins in its environment that either restrict or enhance its range of action. Most notable for inhibiting TGF-ß action are α2-macroglobulin (O'Connor-McCourt and Wakefield, 1987) and the proteoglycan decorin (Yamaguchi et al., 1990). Interestingly, TGF-ß induces decorin, therefore restricting its own action. In contrast, others have reported that when the protein thrombospondin binds to TGF-ß it remains in an active form (Murphy-Ullrich et al., 1992), even when confronted with decorin and α2-macroglobulin. Thrombospondin-complexed TGF-ß appears to be the active form in which it is released from platelets (Murphy-Ullrich et al., 1992). Thus, TGF-ß may exist in either active or inactive forms following secretion into the surrounding milieu, depending on the proteoglycan or matrix interaction it encounters. TGF-$ß_1$ regulates its own secretion through autocrine induction of the TGF-$ß_1$ gene (Van Obberghen-Schilling et al., 1987). Regulation between TGF-ß isoforms has also been described. The matrix or proteoglycan complex in which TGF-ß exists in its environment will directly affect its ability to regulate its own secretion. Cellular responses to TGF-ß are also dependent on matrix interaction. NRK rat fibroblast monolayers, which are growth inhibited by TGF-ß, are growth stimulated in soft agar through their ability to produce extracellular matrix proteins (Roberts and Sporn, 1991). Increased expression of bioactive TGF-$ß_1$ in EIA-transformed kidney fibroblast cells results in

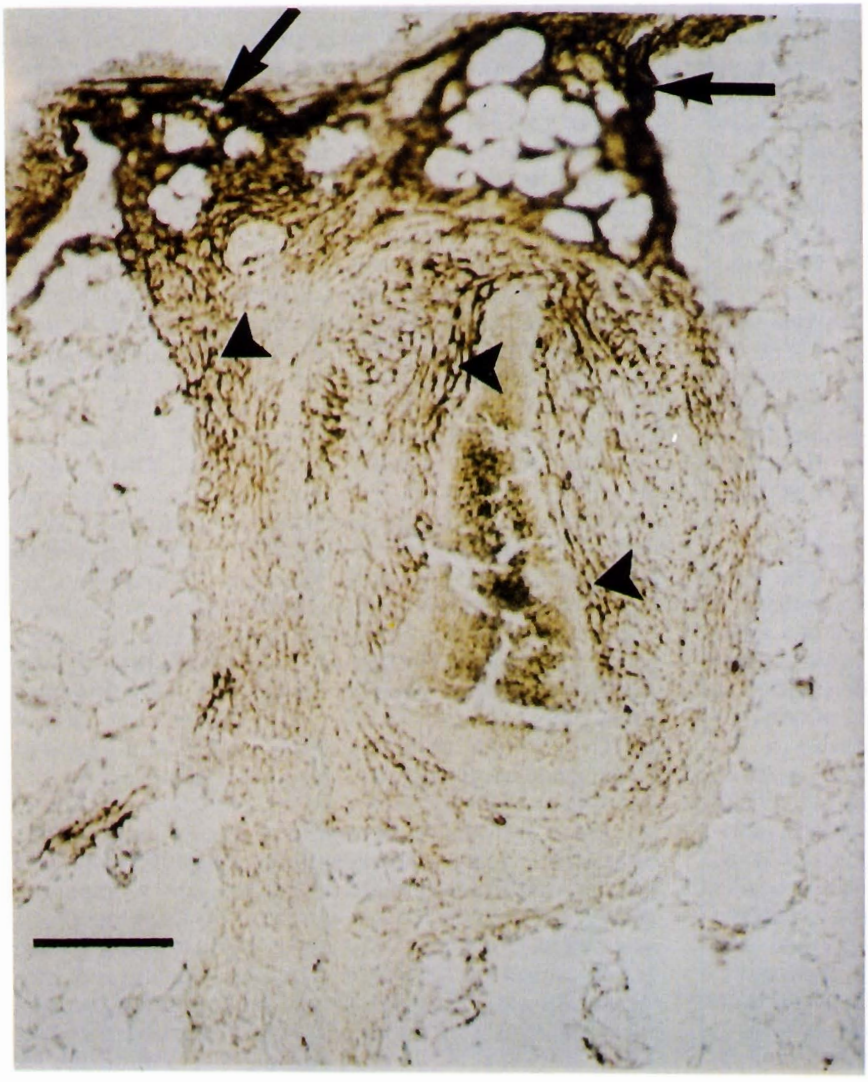

Fig. 1. Immunolocalization of TGF-ß in metastatic lung tumors 21 days following intravenous injection of mouse tumor cells into the tail veins of syngeneic C3H/HeN mice. The immunochemical staining procedure has been described (Khalil et al., 1989; Schwarz et al., 1990). In brief, 5 µm sections were incubated overnight at 4 °C in the presence of anti-TGF-ß IgG. Following washing, the sections were blocked with normal goat serum, then presented with affinity purified goat anti-rabbit IgG with avidin-biotin-peroxidase complex. Visualization was accomplished by incubating with 3'3-diamino-benzidine HCl. Controls were performed with normal rabbit IgG instead of anti-TGF-ß or blocking with a TGF-ß peptide as described by Khalil et al., 1989. In the figure, antibody to TGF-ß localizes to intracellular spaces between tumor cells as shown by the arrowheads, and to subepithelial matrix as indicated by the arrows. Bar: 50 µm. For further experimental details see Schwarz et al., 1990.

increased adhesiveness that is associated with more rapid growth rates *in vivo* (Arrick et al., 1992). Another example is endothelial cell monolayers, which are growth inhibited by TGF-ß but are induced to form complex branching tubes resembling capillaries when grown in extracellular matrix (Madri et al., 1988). The effect of matrix interaction on cellular responses to many cytokines, including TNF-α, IFN-λ, PDGF, IL-1 and FGF, has been observed (Nathan and Sporn, 1991). The response to cytokines of metastatic cells implanting in a secondary organ is dependent upon the matrix of the new environment. This type of matrix interaction could account for the preferential growth of metastases in certain organ sites that may contain the appropriate matrix for cytokine induction of the genes that are necessary for cell growth.

The observation that TGF-ß can be either an inhibitor or a stimulator of cell proliferation has intrigued the field since these observations were first described approximately a decade ago (Tucker et al., 1984; Roberts et al., 1985). TGF-ß can stimulate growth of some mesenchymal cell types, although normal epithelial and most mesenchymal cells are growth inhibited. We have observed that the responsiveness of fibrosarcoma tumor cells to the growth-suppressive effects of TGF-ß changes dramatically with metastatic potential (Schwarz et al., 1988). Similar observations have been made using a mammary adenocarcinoma, and it has been suggested that this might account for clonal dominance of tumors (Kerbel, 1990). Others have also observed alterations in response to TGF-$ß_1$ following transfection with *ras* and *myc* and immortalization of fibroblasts (Leof et al., 1987; Sorrentino and Brandyopodhyay, 1989). Of particular interest is the observation that transfection and expression of the metastasis-related gene nm23 reduced both metastatic potential of a murine melanoma and TGF-ß stimulation of soft agar colonization, although no effect on anchorage-dependent growth was observed (Leone et al., 1991).

The mechanism by which TGF-ß mediates its antiproliferative signal is under intense study. The most interesting cellular genes that appear to be involved in TGF-ß regulation are c-*myc* and Rb (Laiho et al., 1990; Pietenpol et al., 1990). At least in mouse skin keratinocytes, reduction of c-*myc* expression appears to be important in the TGF-$ß_1$-induced inhibition of DNA synthesis, and a link between a block in Rb phosphorylation in late G_1 and TGF-$ß_1$ growth inhibition has been indicated (Pietenpol et al., 1990). Neither appears to be a completely satisfactory explanation, as *myc* suppression is not observed in all cells in which TGF-ß inhibits growth, and growth inhibition has been observed in cells without a functional Rb protein (Smeland et al., 1987; Chambard and Pouyssegur, 1988; Ong et al., 1991). However, interesting relationships between cyclin dependent kinases and Rb phosphorylation have been described, in which cells treated with TGF-ß were found to contain normal levels of cyclin E and cyclin dependent protein kinase 2 (cdk2), but were unable to assemble cyclin E-cdk2 complexes or cyclin E associated kinase activity. These cyclin dependent effects correlated with the inhibition of Rb phosphorylation, demonstrating a cell cycle dependent mechanism responsible for regulating the negative growth effects observed with TGF-ß (Koff et al., 1993).

Due to the potent growth inhibitory action of TGF-ß on most cell types, it has often been stated that for transformation to occur, the negative regulatory signal must be abrogated. It has also been suggested (Misssero et al., 1991), that the suppression of activated H-*ras* transformation by normal dermal fibroblasts was due to the release of a TGF-ß like molecule. With the exception of tumors such as retinoblastoma, which have lost all of the TGF-ß receptors, most cells that resist the growth-inhibitory effect of TGF-ß apparently do so through some mechanism that is downstream of the receptor-ligand interaction. This is best exemplified by human foreskin keratinocytes transformed by either HPV-16 E7, AD5 E1A, or SV40 Tag (Pietenpol et al., 1990), in which TGF-ß down-regulation of c-*myc* is lost when sequences that bind the retinoblastoma gene product are deleted.

The mechanism of TGF-ß growth stimulation is not well understood. Some cells are directly stimulated (Roberts et al., 1985; Leof et al., 1987), whereas the effects on others are at least partly mediated through the actions of other growth factors such as PDGF (Leof et al., 1986) or EGF (Newman et al., 1986; Nugent et al., 1989). TGF-ß mitogenesis may be important during tumor progression, promoting the growth of malignant cells (Roberts et al., 1985; Leof et al., 1986; Schwarz et al., 1988; Sorrentino and Brandyopodhyay, 1989; Hurta et al., 1991, 1992; Roberts and Sporn, 1991). This stimulation may also occur indirectly through the induction of other mitogens (Roberts et al., 1985; Leof et al., 1987), or by promoting growth *in vivo* through stimulation of mitogen production in adjacent cells. It has also been proposed that TGF-ß may indirectly stimulate mitogenesis through promotion of vascularization on extracellular matrix production (Roberts and Sporn, 1991). Our proposal that TGF-ß is a direct mitogen was based on observations that TGF-$ß_1$ inhibition of DNA synthesis in the C3H $10^1/_2$ fibroblast line was converted to stimulation by H-*ras* transformation to malignancy, and that malignant transformation also leads to elevated rates of secretion of activated TGF-$ß_1$ (Schwarz et al., 1988, 1990). If autocrine stimulation is important to proliferation, this hypothesis predicts that metastatic cell populations will exhibit significant alterations in expression of important genes involved in the coordination of DNA synthesis and cell proliferation. We have recently tested this idea by investigating the highly regulated expression of two genes (R1 and R2) that code for a rate-limiting enzyme of DNA synthesis, ribonucleotide reductase (Wright, 1989; Wright et al., 1990a). A series of radiation and H-

ras-transformed $10T^1/_2$ cell lines exhibiting increasing malignant potential was evaluated for TGF-ß induced alterations in ribonucleotide reductase R1 and R2 gene expression (Hurta et al., 1991). Interestingly, early increases in R1 and/or R2 message and protein levels were observed only in the highly malignant cell lines and not in normal fibroblasts or benign fibromas. Although previous studies have shown that the activity of ribonucleotide reductase correlates more closely with DNA synthesis than any other biosynthetic activity (Weber, 1983), we observed that the TGF-$ß_1$-induced changes in R1 and/or R2 gene expression in malignant cell lines occurred prior to any detectable changes in the rates of DNA synthesis. This rapid increase in expression, within 1 h, suggests that it occurs before mitogenic cytokines would be synthesized and secreted by these cells, in keeping with a model of direct activation by TGF-$ß_1$. A more direct test of the autocrine hypothesis was performed by placing the TGF-$ß_1$ gene under the control of a zinc-sensitive metallothionein promoter and transfecting it into an H-*ras*-transformed $10T^1/_2$ fibrosarcoma line (Hurta et al., 1991). When these cells were induced by $ZnSO_4$, TGF-$ß_1$ message was observed within 1 h, quickly followed by R1 and R2 genes. At least part of the mechanism responsible for TGF-$ß_1$ mediated elevations in ribonucleotide reductase activity has recently been shown to occur through unique *cis*-trans interactions at the R2 mRNA, and involves the binding of a TGF-$ß_1$ responsive protein to a *cis*-element in the 3'-untranslated region of the message (Amara et al., 1995a). This link between TGF-$ß_1$ mitogenic stimulation of malignant cells and alterations in the expression of ribonucleotide reductase supports a mechanism of autocrine stimulation of abnormal proliferation of malignant cells by TGF-$ß_1$. This hypothesis has also been tested with another rate limiting activity for cell growth. Ornithine decarboxylase is rate limiting for the synthesis of polyamines that are required for the growth of all cells (Pegg, 1988). Interestingly, ornithine decarboxylase gene expression and activity is also quickly elevated in response to TGF-$ß_1$ treatment of malignant cells, and at least part of the mechanism responsible involves TGF-$ß_1$ alterations in ornithine decarboxylase message stability characteristics (Hurta et al., 1993). The switch from growth suppressive to stimulatory signals indicates that a novel pathway is activated that leads to the enhanced expression of ribonucleotide reductase and other key enzymes like ornithine decarboxylase, that are essential for the synthesis of DNA and cell proliferation.

Cell motility is an important property of malignant cells, and it has been shown that TGF-ß can regulate the motile properties of cells (Postlethwaite, 1987; Samuel et al., 1992), but the mechanism through which TGF-ß alters cell locomotion is not yet well understood. In neutrophils TGF-$ß_1$ enhances protein synthesis and actin assembly required for chemotaxis but unlike most chemoattractants, it does not appear to act through common signal transduction pathways. In many cell types TGF-ß increases hyaluronan (HA) synthesis, which is known to be involved in fibrosarcoma cell locomotion after H-*ras* transformation, and also in mammary carcinoma cell locomotion (Schor et al., 1989; Toole et al., 1989; Turley et al., 1991). Furthermore, increased synthesis of HA correlates with increased invasiveness and metastatic ability of melanoma, carcinoma and fibrosarcoma cell lines (Toole et al., 1979; Kimata et al., 1983; Turley and Tretiak, 1985). Several HA binding proteins have been described, and two cell membrane receptors, CD44 and RHAMM, have been cloned (Turley et al., 1991). Recently, RHAMM has been shown to act as a transforming gene (Hall et al., 1995), and to play a key role in TGF-ß stimulation of tumor cell locomotion (Samuel et al., 1993). The evidence to date indicates that TGF-$ß_1$ can regulate malignant cell motility characteristics by promoting the synthesis of both HA and one of its cell membrane receptors, RHAMM (Samuel et al., 1992, 1993). As in the case with TGF-$ß_1$ induction of ribonucleotide reductase and ornithine decarboxylase gene expression discussed earlier, RHAMM gene expression is TGF-$ß_1$ responsive, at least partly, through a mechanism that involves changes in RHAMM mRNA stability, and includes a recently described *cis-trans* interaction between cytosolic protein and a unique *cis*-sequence within the 3'-untranslated region of the RHAMM message (Amara et al., 1995b).

In normal cells, the action of TGF-ß generally enhances adhesion and this is a result of a combination of increased matrix production and decreased proteolysis. TGF-ß suppresses the expression of protease (Laiho et al., 1986; Matrisian et al., 1986; Edwards et al., 1987; Samuel et al., 1992), while increasing proteases inhibitors in normal tissue (Laiho et al., 1986; Edwards et al., 1987; Overall et al., 1989; Stetler-Stevenson, 1990), although there have also been observations of enhanced protease production (Laiho et al., 1986), indicating cell type-specific effects. Proteolytic modification is limited in time and strictly regulated at many levels in normal cells. Tumor cells use proteolysis coupled with motility to achieve invasion and progression but in circumstances that would be inappropriate for normal cells. It has often been observed that highly malignant and invasive cells show increased protease activity when compared with normal and poorly invasive cells (Matrisian et al., 1986; Sloane et al., 1986; Stetler-Stevenson, 1990). Therefore, the strict regulation of proteolytic activity may be absent in aggressive tumor cells, thus suggesting a defective response to those regulatory factors that normally limited proteolysis such as TGF-ß. In keeping with this idea, expression of collagenases (Edwards et al., 1987; Overall et al., 1989) and transin/stromelysin (Matrisian et al., 1986) are suppressed by TGF-$ß_1$ in normal fibroblasts, and this is in striking contrast to the response of tumors (Keski-Oja et al., 1988; Welch et al., 1990; Samuel et al., 1992). Compared with parental fibro-

blasts, the 92-kDa collagenase IV and the procathepsin L transcription in H-*ras*-transformed fibrosarcomas is markedly stimulated by TGF-$ß_1$ (Samuel et al., 1992). Stimulation of plasminogen activator and gelatinolytic activity by TGF-ß has also been observed in murine mammary adenocarcinoma and human lung carcinoma A549 cells (Keski-Oja et al., 1988; Welch et al., 1990).

Following transformation, proteases are often constitutively expressed at high levels. The observation that TGF-$ß_1$ stimulates fibrosarcomas and adenocarcinomas to express proteases at high levels and that malignant fibrosarcomas secrete high levels of TGF-ß (Schwarz et al., 1990), raises the possibility that these tumors may be maintaining increased protease production through TGF-ß autocrine effects. The hypothesis has been directly tested in our laboratory with a plasmid used to show a regulatory link between TGF-$ß_1$ stimulation of DNA synthesis and altered ribonucleotide reductase and ornithine decarboxylase gene expression (Hurta et al., 1991). When induced, TGF-$ß_1$, secreted by the tumor cells enhanced protease transcription and secretion in an autocrine manner, and promoted invasion of collagen gels (Samuel et al., 1992). Zinc-stimulated cells showed increases in the activity of many proteases by exhibiting gelatinolytic activity in bands varying in molecular weights from 12 kDa to 105 kDa. The parental cell line, which does not contain the zine inducible TGF-$ß_1$ gene, was unaffected by identical treatment (Samuel et al., 1992). Two of the protease bands at the molecular weights of about 92 and 29 kDa were similar to that determined for collagenase IV and cathepsin L, and marked elevations in collagenase IV and procathepsin L message levels were detected.

The ability of tumor cells to use TGF-ß to elevate protease gene expression and promote invasion also suggests that it could enhance metastatic potential. This appears to be correct since, for example, treatment of rat mammary adenocarcinoma with TGF-$ß_1$ *in vitro* enhanced lung tumor formation when injected intravenously (Welch et al., 1990). We have shown that an antisense oligonucleotide sequence that specifically targets the TGF-$ß_1$ message to reduce TGF-$ß_1$ synthesis also significantly reduced the invasive and metastatic properties of fibrosarcoma cells, indicating an important role for altered TGF-$ß_1$ gene expression in the regulation of malignant cell proliferation, invasion and metastasis (Spearman et al., 1994).

Tumor progression usually proceeds with the accumulation of multiple genetic changes, and it has

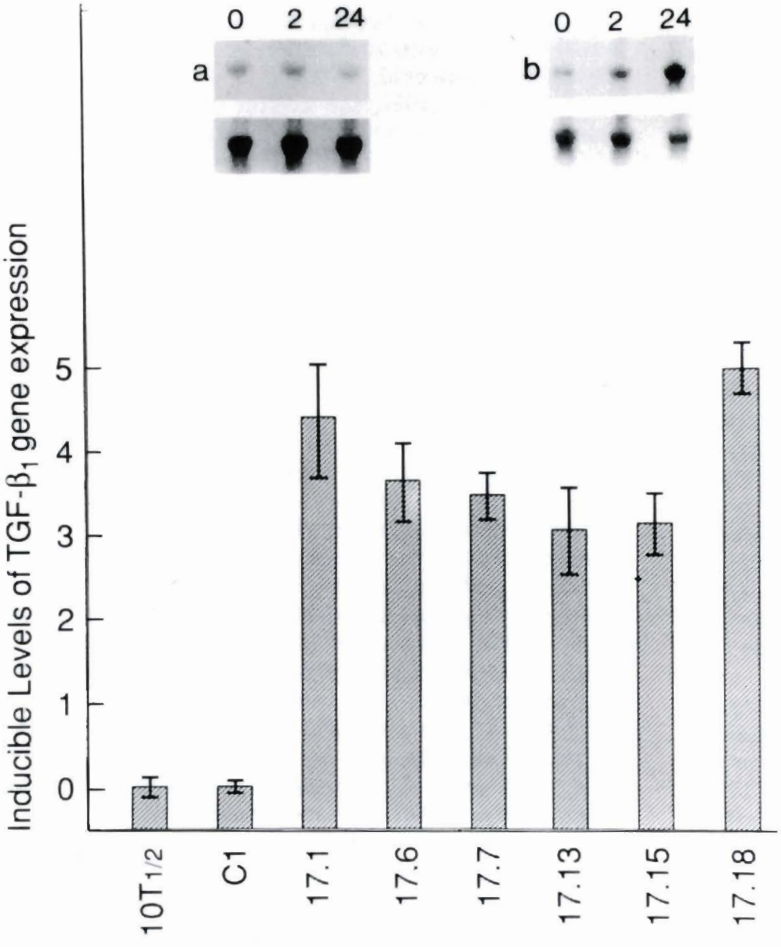

Fig. 2. Relative TGF-$ß_1$ gene expression in cells cultured in the presence of zinc sulfate compared to gene expression in the absence of zinc sulfate. $10T^1/_2$ cells are parental wild type mouse cells used to obtain the H-*ras* transformed C1 cell line (Egan et al., 1987). The C1 cell line was transfected with a plasmid containing the TGF-$ß_1$ gene sequence under the control of the zinc sensitive metallothionein promoter (Huang et al., 1995), and 17.1, 17.6, 17.7, 17.13, 17.15 and 17.18 are independently selected cell lines. The TGF-$ß_1$ sequence was mutated so that only active TGF-$ß_1$ was produced from the plasmid. The figure shows Northern blot analyses performed with cells cultured in the presence or absence of zinc sulfate for 24 h. The results are from 3 independent experiments for each cell line. Bars: S.E. Inset: examples of Northern blot results indicating the levels of TGF-$ß_1$ mRNA in $10T^1/_2$ cells (a) or in 17.1 cells (b) cultured in the absence (0) or the presence of zinc sulfate for 2 h (2) and 24 h (24). 28s rRNA loading controls are shown below each experimental result. For further experimental details see Huang et al. (1995).

been suggested that these changes involve mutations, including DNA amplification, resulting from a decrease in the genetic stability of the genome (Nowell, 1986, 1993; Stark et al., 1990; Tlsty, 1990; Wright et al., 1990b). The frequent observation of chromosomal abnormalities in preneoplastic as well as in highly malignant cells supports this view (Nowell, 1993). The concept that has arisen from these findings is that alterations in genetic stability, including changes in DNA amplification potential, linked to selection pressures exerted by the cellular environment, play a key role in generating populations of variant cells with different malignant properties. This is consistent with the observation that DNA amplification is rare, if it occurs at all, in normal mammalian diploid cells (Tlsty, 1990; Wright et al., 1990b), but it is a commonly used mechanism for the overexpression of oncogenes in malignant cells (Schwab, 1990; Brison, 1993), and for the elevation of gene products that are important in determining the drug sensitivities of cells (Stark et al., 1990; Wright et al., 1990a). The strong relationship between modifications in growth factor regulation and mechanisms of malignant progression as outlined in this review, has prompted us to investigate whether or not a relationship exists between altered growth factor expression and genetic stability as determined by gene amplification potential. To carry out this study, we chose to determine the gene amplification properties of a series of mouse $10T^1/_2$ cell lines transfected with sequences coding for TGF-$ß_1$ under the control of the zinc-sensitive metallothionein promoter (Fig. 2). The only mechanism that has been observed for resistance to the chemotherapeutic compound, N-(phosphonacetyl)-L-aspartate (PALA) involves the amplification of the gene

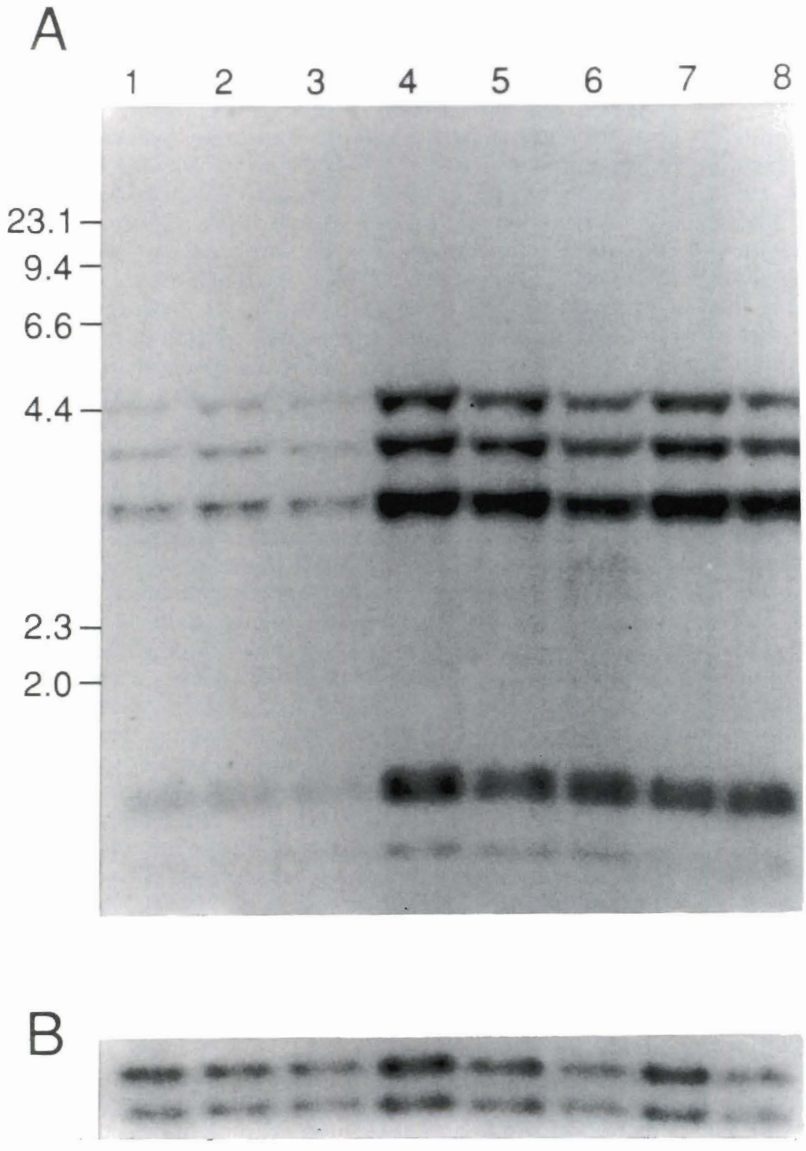

Fig. 3. Relative CAD gene copy number determined by Southern blot analyses. **A.** 20 µg of genomic DNA digested to completion with Pst 1. DNA size markers are shown on the left side. Lane 1, $10T^1/_2$ mouse cells; Lane 2, C1 cells; lane 3, 17.6 cells cultured in the absence of PALA; lanes 4 to 8, 17.6 cells obtained from independent colonies selected in the presence of 40, 50, 50 and 50 µM PALA, respectively. The increases in CAD gene copy numbers indicated in lanes 4 to 8 were 4.0, 4.5, 5.6, 4.6 and 5.1, respectively, as estimated by densitometric analysis (Huang et al., 1995). **B.** DNA loading controls performed with glutathione peroxidase cDNA. For further experimental details, see Huang et al., 1995.

that codes for the protein target of PALA, CAD (a multifunctional polypeptide containing carbamyl phosphate synthase, aspartate transcarbamylase and dihydroorotase). Therefore, resistance to PALA is taken as strong evidence that CAD gene amplification has occurred. In all cases, sensitivity to PALA cytotoxic effects were significantly reduced when cells were cultured under conditions leading to low basal levels of TGF-$ß_1$ gene expression. Furthermore, significantly higher rates of PALA resistance/cell/generation were observed in cell populations expressing high levels of TGF-$ß_1$ than in the same cells containing relatively low levels of this growth factor. Southern blot analyses confirmed that CAD gene amplification occurred in colonies that survived PALA treatment (Fig. 3). In total these studies demonstrated a novel malignancy related function for TGF-$ß_1$ alterations in malignant cells, and suggested a new role for aberrant expression of this growth factor in mechanisms of drug resistance and tumor progression (Huang et al., 1995).

Fibroblast growth factors

Communication between cells is mediated by growth factors and a large number of different growth factors have been described (Bradshaw and Cavanaugh, 1990; Cross and Dexter, 1991), so the hypothesis that TGF-ß may be important in promoting invasion and metastasis is consistent with the observation that other growth factors also appear to be fundamentally involved in this process. Several growth factors possess potent transforming potential, and some oncogenes encode proteins that are directly involved in growth factor regulation (Aaronson, 1991; Cross and Dexter, 1991). The fibroblast growth factor (FGF) family is a particularly good example, and therefore, some pertinent observations relating to these growth factors are described. Some excellent reviews detailing structure, function and regulation of members of this growth factor family have been published (e.g. Bascilico et al., 1989; Rifkin and Moscatelli, 1989; Baird and Bohlen, 1990; Klagsbrun and Baird, 1991; Fernig and Callagher, 1994; Cockerill et al., 1995).

It was shown quite some time ago that several different proteins can provide growth stimulatory properties associated with FGF (Trowell et al., 1939; Hoffman, 1940; Gospodarowicz, 1974). For example, basic FGF (bFGF) has a basic pI and elutes from heparin-sepharose in the presence of 1.5M NaCl, whereas a second protein with similar properties, acidic FGF (aFGF) has an acidic pI and elutes from heparin-sepharose at 1M NaCl (Maciaq et al., 1984; Thomas et al., 1984; Cross and Dexter, 1991). Protein sequence data demonstrated that there was significant structural homology (about 55%) between the two polypeptides (Esch et al., 1985). It is known that bFGF and aFGF are members of a heparin-binding family of at least seven different but related gene products. This family includes the K-*fgf* (or hst) proto-oncogene, first detected by its ability to induce the transformation of NIH-3T3 fibroblasts in a focus assay with tumor DNA from several human sources (Sakamoto et al., 1986; Delli Bovi and Basilico, 1987; Nakagama et al., 1987), the *int*.2 gene, which is homologous to a mouse *int*.2 sequence found to be activated by insertion of mammary tumor virus DNA (Peters et al., 1983), the *fg.f*5 gene, detected by the transfer of DNA from human tumor cell lines into NIH-3T3 fibroblasts followed by selection for transformed cells in a growth factor-deficient medium (Zhan et al., 1987), the *fgf*.6 gene, identified by screening a mouse cosmid library with K-*fgf* (hst) probe under low stringency conditions (Marics et al., 1989), and the keratinocyte growth factor (KGF), which shows high specificity for mitogenic activity with epithelial cells (Rubin et al., 1989).

Since basic and acidic FGFs have been investigated for a longer period of time than other members of the FGF family, much more information about their biological properties is available. For example, bFGF is synthesized by a variety of cultured normal and tumor cells (Rifkin and Moscatello, 1989; Baird and Bohlen, 1990), and the function of this growth factor *in vivo* is under intense investigation (Baird and Bohlen, 1990; Klagsbrun and Bairn, 1991). From *in vitro* studies, it is clear that bFGF exhibits a broad spectrum of biological activities with a variety of cell types. These include growth stimulation and, in some cases, inhibition (Schweigerer et al., 1987; Rodeck et al., 1991), induction of proteases such as collagenases, plasminogen activator and transin (Presta et al., 1986; Buckley-Sturrock et al., 1989; Machida et al., 1989; Mignatti et al., 1989; Peppers et al., 1990) and modification of cell motility and chemotaxis characteristics (Presta et al., 1986; Sato and Rifkin, 1988). For example, we have analyzed the motility rates of K-*fgf* and bFGF transfected cell lines as a function of malignant potential. A significant correlation between cell locomotion and malignant potential was observed, supporting the view that growth factor mediated motility is important in mechanisms that promote tumor cell dissemination (Taylor et al., 1993). Furthermore, exogenous administration of bFGF induces a variety of dramatic effects, including neurovascularization in cornea, kidney capsule or skin, indicating that it may have a major role to play in neurovascularization or wound repair (Folkman and Klagsbrun, 1987; Finklestein et al., 1988; Rifkin and Moscatello, 1989; Fina et al., 1991). There is also evidence that bFGF may be an important inducer of differentiation during embryonic development (Slack et al., 1987). Therefore, similar to the TGF-ß family, the FGFs appear to be multifunctional and possess the potential to participate in a variety of interesting biological activities. Indeed, synergistic interactions between FGF and TGF-ß have been observed (Rizzino et al., 1986; Peppers et al., 1990).

A major obstacle in understanding the mechanism of action of bFGF and aFGF and their biological activities

is that these proteins do not contain the conventional signal sequence for passage through the secretory pathway leading to eventual release from the cell (Abraham et al., 1986). This is in contrast to several other members of this growth factor family. For example, K-FGF, KGF and FGF.5 proteins contain a hydrophobic N-terminal region that appears to be the signal sequence responsible for their secretion (Finch et al., 1989). Although basic and acidic FGF molecules do not contain a conventional secretory signal sequence, they can be detected extracellularly, and it has been demonstrated that many cell types contain cell-surface FGF receptors. Interestingly, there is evidence that several members of the FGF family, bFGF, aFGF and K-FGF can bind to a common cell-surface receptor with high affinity (Neufeld and Gospodarowic, 1986; Moscatelli and Quarto, 1989; Mansukhani et al., 1990; Damen et al., 1991). High- and low-affinity classes of FGF receptors have been cloned; it has been shown that the high-affinity receptors are tyrosine protein kinases of 125 and 145 kDa, and sequence data indicate that these receptors belong to the immunoglobulin superfamily of proteins (Neufeld and Gospodarowicz, 1985; Imanura et al., 1988; Friesel et al., 1989; Lee et al., 1989; Ruta et al., 1989; Dionne et al., 1990 Reid et al., 1990; Hou et al., 1991; Miki et al., 1991). Binding of growth factor to high affinity sites leads to autophosphorylation of 125- and 145-kDa receptor proteins and several other intracellular proteins, including phospholipase Cγ (Coughlin et al., 1988; Friesel et al., 1989; Burgess et al., 1990; Cuadrado and Molloy, 1990). This enzyme hydrolyzes the membrane phospholipid phosphatidyl-inositol-4,5-bisphosphate to produce the second messenger 1,2-diacylglycerol, an activator of protein kinase C (Cuadrado and Molloy, 1990), and inositol triphosphate, which regulates the intracellular release of calcium (Berridge, 1987). The acidic and basic forms of FGF have also been detected in the nucleus and there is some evidence that they may have a function at this level as well (Baldin et al., 1990; Tessler and Neufeld, 1990; Florkiewicz et al., 1991). For example, mutant forms of aFGF, which could not localize to the nucleus, were unable to initiate DNA synthesis and NIH-3T3 cell proliferation, even though the protein could bind to cell-surface receptors, stimulate the phosphorylation of membrane-bound and cytosolic proteins, and elevate *c-fos* expression (Imamura et al., 1990). The finding that members of the FGF family have a strong affinity for heparin implies that an important aspect of their extracellular mechanism of action may involve interactions with constituents of the extracellular matrix such as the heparin sulfate proteoglycans. This suggests that secretion of K-FGF, KGF, or FGF.5 proteins, probably through a conventional secretion pathway, or the low release of basic or acidic FGF by cell death (or by some undefined secretory process) may provide for the storage of these growth factors through extracellular matrix interactions. They would then be mobilized when required, by remodeling of the basement membrane or extracellular matrix through the action of hydrolases (Vlodavsky et al., 1987). Clearly, the intracellular and extracellular pathways involved in mediating the biological effects of FGF proteins appear to be complex.

Earlier observations of high levels of bFGF activity associated with cultured tumor cells has been taken as evidence of a possible role for FGFs in mechanisms of malignant transformation as mitogenic stimulators of tumor cell proliferation (Rifkin and Moscatelli, 1989; Baird and Bohlen, 1990; Cross and Dexter, 1991; Rodeck et al., 1991). Furthermore, it has been suggested that aberrant expression of FGF proteins by tumor cells may contribute to subsequent angiogenesis and tumor vascularization (Mignatti et al., 1989; Peppers et al., 1990). The discovery of additional sequence-related gene family members on the basis of transforming capabilities has stressed the potential importance of this class of growth factors in mechanisms of tumorigenicity. It is now clear that k-*fgf*, *int.2*, *fgf*.5, and *fgf*.6 can transform cultured fibroblasts to tumorigenic populations (Zhan et al., 1987; Marics et al., 1989; Damen et al., 1991; Goldfarb et al., 1991), and alterations in expression of several of these genes appear to play a potentially important role in the development of some human cancers (Adelaide et al., 1988; Hatada et al., 1988; Neufeld et al., 1988; Yoshida et al., 1988; Ali et al., 1989; Theillet et al., 1989; Tiesman and Rizzino, 1989).

The lack of a secretion peptide in acidic and basic FGF proteins, in contrast to other members of the FGF family, has led to questions about the overall significance of elevated levels of these growth factors in tumor cells. Therefore, several studies have been carried out to test directly the transforming potential of the acidic and basic FGF genes. For example, a human bFGF cDNA expression vector has been introduced into baby hamster kidney fibroblasts to isolate a cell line that accumulated high intracellular concentrations of bFGF (Neufeld et al., 1988). This cell line grew in serum-free medium and exhibited a loss of anchorage-dependent growth. Similar experiments have been carried out with mouse BALB/c3T3 cells that produced very high levels of bFGF (Sasada et al., 1988). The transfected cells appeared morphologically transformed, exhibited increased saturation densities in serum-free medium, and their phenotypic alterations were reduced by the addition of anti-human bFGF antibodies. It is apparent that only cell lines with high levels of growth factor expression exhibit changes in transformation-related properties (Quarto et al., 1989). Transfection experiments have also been performed with recombinant plasmids containing human aFGF sequence and Swiss 3T3 cells (Jaye et al., 1988) or NBT-II epithelial carcinoma cells (Jouanneau et al., 1991). In general, changes in cell morphology, cell density, enzyme activity, and cell motility were observed in these studies. The Swiss 3T3 aFGF-producing cells also formed small nonprogressive tumors in nude mice (Jaye et al., 1988).

Although the experiments described demonstrated

that bFGF and aFGF, without a secretory signal sequence, were capable of modifying several cellular properties relevant to the transformed phenotype, they appeared to have only limited oncogenic potential. Therefore, several laboratories have designed experiments to determine whether the forced secretion, through the addition of a secretion signal sequence, would lead to further alterations in the malignant properties of cells. In a very interesting study, the immunoglobulin signal sequence for secretion was fused to bovine bFGF cDNA and found to confer altered morphology and tumorigenic properties to NIH-3T3 cells, which were not observed when the bFGF gene alone was tested in transfection experiments (Rogelj et al., 1988). Consistent with these studies was our observation that cell lines containing the immunoglobulin signal sequence-bFGF gene, but not the bFGF gene alone, exhibited highly aggressive metastatic properties in nude mice lung colonization assays (Egan et al., 1990). A complication in the interpretation of these studies is the finding that bFGF protein could be detected in cell extracts but not in growth medium from cells containing an immunoglobulin signal sequence fused to bFGF (Rogelj et al., 1988; Egan et al., 1990). This suggests that either bFGF protein was transferred to the cellular membrane where it was immediately associated with receptors to activate a mitogenic pathway for autocrine stimulation or that transformation by bFGF occurred with the protein acting intracellularly. An intracellular mode of action has also been suggested for several other growth-stimulating factors (Keating and Williams, 1988; Bejcek et al., 1989; Dunbar et al., 1989). In another study, the bFGF coding region was coupled to the human growth hormone signal sequence for secretion and was found to induce foci of transformation in NIH-3T3 cells at high frequency (Blam et al., 1988). The transformed cells grew in soft agar and were tumorigenic in nude mice. In this investigation a majority of the immunoreactive bFGF protein synthesized by the transformed cells was found in growth medium, and unexpectedly, the secreted growth factor appeared to be posttranslationally modified and had low mitogenic activity. The authors argued that this suggested that the interaction of bFGF with its receptor occurs while the fusion protein is being processed along the secretory pathway (Blam et al., 1988). In studies of other growth-stimulating factors known to be secreted from the cell, it has also been reported that stimulation may require, at least in part, an interaction with intracellular receptors (Keating and Williams, 1988; Bejcek et al., 1989). For example, in cells transformed by v-*sis*, tyrosine phosphorylation of immature derivatives of platelet-derived growth factor receptors has been observed, suggesting that important interactions between a growth factor and its receptor can occur prior to the growth factor reaching the cell surface (Keating and Williams, 1988; Bejcek et al., 1989). In total, the experiments with chimeric forms of bFGF-signal peptide molecules indicate that when the bFGF gene is linked to a secretion signal, it has potent transforming potential, but it is still unclear from these experiments whether induction of the malignant phenotype is due to extracellular or intracellular activation of the mitogenic pathway or whether both mechanisms are involved in this process.

The important question of how members of the FGF family stimulate cell proliferation has been approached in interesting studies carried out with the K-*fgf* oncogene. Activation of this oncogene occurs through an unregulated expression of the normal gene product (Delli-Bovi et al., 1988; Talarico and Bascilico, 1991) and physiological expression of the gene appears to be restricted to early stages of animal development (Velcich et al., 1989; Herbert et al., 1990). We have shown through analysis of NIH-3T3 cells transfected with K-*fgf* that expression of the protoncogene in a single step induces both tumorigenic and metastatic characteristics (Damen et al., 1991), as determined in soft agar cloning experiments, and in tumorigenicity and experimental lung metastasis assays in nude mice. Transfection and subsequent amplification of the K-*fgf* gene leads to the induction of highly malignant and aggressive metastatic cell lines (Damen et al., 1991), and this is consistent with the finding that the K-*fgf* gene is found amplified in a variety of human tumors (Adelaide et al., 1988; Hatada et al., 1988; Ali et al., 1989; Theillet et al., 1989; Tiesman and Rizzino, 1989).

There is good evidence to support the idea that transformation by the K-*fgf* oncogene occurs through an autocrine growth mechanism that requires activation of the mitogenic pathway at the cell surface. In agreement with this option is the ability of suramin or anti-K-FGF neutralizing antibodies to inhibit the growth of K-*fgf*-transformed cells in soft agar or in serum-free medium (Wellstein et al., 1990; Talarico and Bascilico, 1991). Furthermore, deletion of sequences encoding the signal peptide for secretion suppresses the ability of K-*fgf* to induce transformed foci in NIH-3T3 cells, and addition of a sequence encoding the KDEL endoplasmic reticulum and Golgi retention signal to K-*fgf* leads to accumulation of K-FGF in the cell and reduces the foci forming ability of the oncogene (Talarico and Bascilico, 1991). Although recognition and binding to the K-FGF receptor may still occur in intracellular compartments during processing, these observations indicate that a significant component of the K-FGF signal transduction pathway requires the activation of constituents available only at the cell surface.

As with TGF-ß described earlier, we have investigated the drug resistance and gene amplification potential of cells transfected with sequences coding for K-FGF or bFGF (Huang and Wright, 1994; Huang et al., 1994). Resistance to methotrexate, PALA and hydroxyurea was observed with K-*fgf* transfectants due to amplification of dihydrofolate reductase, CAD or ribonucleotide reductase genes, respectively. Consistent with the gene amplification frequency, cells transfected with the K-*fgf* gene also exhibited a marked increase in

CAD gene amplification rate, as determined by fluctuation analysis in the presence of PALA. Cells transfected with bFGF encoding cDNA also exhibited a significant elevation in PALA resistance, and CAD gene amplification potential. Treatment with suramin, which interferes with the interaction of FGFs with their cell surface receptors (LaRocca et al., 1990), did not reduce the drug resistance properties of K-*fgf* transfected cells. These findings with suramin and with bFGF, which lacks a conventional signal sequence for secretion, suggests that the growth factor-mediated effects on drug resistance and gene amplification occur through an intracellular as opposed to autocrine mode of action. Therefore, as with TGF-ß (Huang et al., 1995), aberrant expression of members of the FGF family dramatically alters the genetic stability of cells as indicated by changes in drug resistance characteristics and gene amplification potential, and demonstrates a new function for altered growth factor gene expression in mechanisms of tumor progression (Huang and Wright, 1994; Huang et al., 1994).

In summary, many experimental results support the hypothesis that basic and acidic forms of FGF contribute to the malignant properties of tumor cells, especially when expressed at high levels, perhaps through activation of intracellular components of a mitogenesis transduction pathway. Results obtained in experiments performed with chimeric forms of bFGF indicate the enormous potential for bFGF to act directly as an oncogene, particularly if it could be shown that chromosomal rearrangements placing the bFGF (or aFGF) gene next to a secretion-signal sequence can actually occur *in vivo*. It is clear that K-*fgf*, which represents FGF family members normally containing sequence information for secretion, is a powerful transforming gene, capable of activating mitogenesis by an autocrine mechanism involving cell-surface components. These types of observations demonstrate both the potential and the actual importance of members of the FGF family of growth factors in mechanisms of tumorigenicity and metastasis.

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