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Insulin-like Growth Factor II Signaling in Neoplastic Proliferation Is Blocked by Transgenic Expression of the Metalloproteinase Inhibitor TIMP-1

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Abstract. Insulin-like growth factor (IGF) II is overexpressed in many human cancers and is reactivated by, and crucial for viral oncogene (SV40 T antigen, [TAg])–induced tumorigenesis in several tumor models. Using a double transgenic murine hepatic tumor model, we demonstrate that tissue inhibitor of metalloproteinase 1 (TIMP-1) blocks liver hyperplasia during tumor development, despite TAg-mediated reactivation of IGF-II. Because the activity of IGFs is controlled by IGF-binding proteins (IGFBPs), we investigated whether TIMP-1 overexpression altered the IGFBP status in the transgenic liver. Ligand blotting showed that IGFBP-3 protein levels were increased in TIMP-1–overexpressing double transgenic littermates, whereas IGFBP-3 mRNA levels were not different, suggesting that TIMP-1 affects IGFBP-3 at a posttranscriptional level. IGFBP-3 proteolysis assays demonstrated that IGFBP-3 degradation was lower in TIMP-1–overexpressing livers, and zymography showed that matrix metalloproteinases (MMPs) were present in the liver homogenates and were capable of degrading IGFBP-3. As a consequence of reduced IGFBP-3 proteolysis and elevated IGFBP-3 protein levels, dissociable IGF-II levels were significantly lower in TIMP-1–overexpressing animals. This decrease in bioavailable IGF-II ultimately resulted in diminished IGF-I receptor signaling in vivo as evidenced by diminished receptor kinase activity and decreased tyrosine phosphorylation of the IGF-I receptor downstream effectors, insulin receptor substrate 1 (IRS-1), extracellular signal regulatory kinase (Erk)-1, and Erk-2. Together, these results provide evidence that TIMP-1 inhibits liver hyperplasia, an early event in TAg-mediated tumorigenesis, by reducing the activity of the tumor-inducing mitogen, IGF-II. These data implicate the control of MMP-mediated degradation of IGFBPs as a novel therapy for controlling IGF bioavailability in cancer.

Key words: TIMP-1 • insulin-like growth factor II • signal transduction • extracellular proteolysis • tumor suppression

EXTRACELLULAR matrix (ECM) serves as the immediate microenvironment for interactions with the cell surface, besides providing the structural support for all tissues. The ECM is not static. Rather, it is dynamic in nature with a continuous turnover of its protein constituents and growth factor pools. A major determinant of ECM turnover and integrity is the extracellular proteolytic balance between secreted matrix metalloproteinases (MMPs) and their biological inhibitors (TIMPs) (for reviews see Matrisian, 1992; Denhardt et al., 1993; Mignatti and Rifkin, 1993). The function of extracellular proteolysis extends beyond ECM degradation to the processing of cell surface receptors and ligands and release of protein-bound growth factors (for review see Werb, 1997). Therefore, it is conceivable that extracellular proteolytic activity within the cellular microenvironment can directly impact cell proliferation. Despite transgenic studies showing that cellular proliferation is altered by ectopic expression of MMPs or TIMPs (Symposium et al., 1994; D’Arimiento et al., 1995; Witty et al., 1995; Martin et al.,...
was confirmed by decreased signaling through the IGF-IR signal transduction pathway. To our knowledge, this is the first in vivo example of the modulation of growth factor bioactivity by the regulation of extracellular proteolysis.

**Materials and Methods**

**Transgenic Mice**

Transgenic mice expressing the TIMP-1 (Ts') or TAg (TAg') transgenes in liver were generated and bred as described previously (Martin et al., 1996). Single transgenes were crossed to generate four categories of littermates designated as wild-type controls (TAg/+/Ts'), TIMP-1 controls (Ts'), TAg controls (TAg'), and double transgenic TIMP-1-overexpressing (TAg'/Ts') mice. Female littermates were killed at specified ages, and the liver tissue was processed and embedded or flash frozen for analyses.

**Immunoprecipitation and Western Blotting**

Liver tissue was homogenized in lysis buffer (20 mM Tris-HCl, pH 7.4, 1.0% NP-40, 150 mM NaCl, 0.5 mM PMSF, 1 mM EDTA, 0.1 mM leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin) at 4°C. Samples were centrifuged at 10 min at 16,000 g, the supernatants collected, and the protein content determined by the Bradford assay. Aliquots containing 2.5 mg of protein were added to control for equal loading and transfer of samples.

The liver tissue homogenates were subjected to 10% SDS-PAGE and electroblotted to Hybond N nylon membrane (Amersham Pharmacia Biotech). The membranes were sequentially probed with primary antibodies PAb 108, anti-p53 (clone PAb 108; PharMingen), biotinylated goat anti–mouse IgG (Amersham Pharmacia Biotech), and horseradish peroxidase-conjugated anti–mouse IgG antibodies (Amersham Pharmacia Biotech). The samples were subjected to 10% SDS-PAGE and electrophoresed to Hybond N nylon membrane (Amersham Pharmacia Biotech). The membranes were sequentially probed with primary antibodies PAb 108, anti-p53 (clone PAb 108; PharMingen), biotinylated goat anti–mouse IgG (Amersham Pharmacia Biotech), and horseradish peroxidase-conjugated anti–mouse IgG antibodies (Amersham Pharmacia Biotech).

**Northern Blotting**

Total RNA isolated from individual liver tissue samples (20 μg) was electrophoresed in formaldehyde agarose gels, subjected to Northern blotting and sequential hybridizations with [α-32P]CTP-labeled and random primed cDNA probes for murine IGF-II (cDNA obtained from Dr. G. Bell, University of Chicago, IL), rat IGF-BP-3 (cDNA), obtained from Dr. J. Herrera, Queensland University of Technology, Brisbane, Australia), 18S ribosomal RNA, and glyceraldehyde 6-phosphate dehydrogenase (GAPDH) were performed. The latter two probes were used to control for equal loading and transfer of samples.

**Immunohistochemistry and In Situ Hybridization**

Paraffin sections of formalin-fixed liver tissue were generated as described previously (Martin et al., 1999). Clone PA b 108, biotinylated goat anti–mouse IgG, and streptavidin-peroxidase conjugate (Zymed) were used for TAg immunohistochemistry, and peroxidase-conjugated anti–proliferating cell nuclear antigen (PCNA) antibodies (Dako) were used to detect proliferating cells. The detection of bound antibody was carried out using diaminobenzidine (Kirkegaard & Perry), which forms a reddish-brown pigment at sites of peroxidase activity. Diaminobenzidine-labeled (Boehringer Mannheim) IGF-II riboprobes were synthesized using the rat IGF-II cDNA, which was cloned in Bluescript KS. In situ hybridization was performed as described previously (Martin et al., 1999). Specific signal appears as purple pigment.

**Ligand Blotting**

Liver samples were homogenized as for Western blotting. Equivalent amounts of protein from each sample (40 μg) were subjected to 10% SDS-PAGE followed by electrophoretic transfer to Hybond N. Membranes were blocked by incubating for 1 h in blocking buffer (10% BSA in TBS, 0.1% M Tris-HCl, pH 7.5, 0.15 M NaCl) followed by overnight hybridization with 50,000 cpm/ml 32P-labeled IGF-II (Amersham Pharmacia Biotech) in blocking
buffer. Membranes were washed three times for 15 min with TBS, air dried, then subjected to autoradiography.

**IGFBP-3 Substrate Zymography**

Liver samples were prepared as for Western blotting. Protein from each sample (40 μg) was subjected to SDS-PAGE GE in a 10% polyacrylamide gel containing 1.0 μg/ml recombinant human IGFBP-3 (kindly provided by Dr. C. Mack, Celtrix Pharmaceuticals, Santa Clara, CA.). Following electrophoresis, gels were washed for 30 min with 2.5% Triton X-100. Gels were equilibrated with transfer buffer (50 mM Tris-HCl, pH 8.0, 5 mM CaCl₂), then the proteins were subjected to capillary transfer onto PVDF membranes as described previously (Fowlkes et al., 1997). The transfer was performed either in the absence or presence of 10.0 μg/ml recombinant human TIMP-1 (kindly provided by Dr. H. Nagase, University of Kansas, Kansas City, KS). Transfers took place overnight at 37°C. Under these conditions, only proteolytic fragments of IGFBP-3 transfer from the substrate-gel to the membrane. Intact IGFBP-3 (30 kD) impregnated in the polyacrylamide matrix remains in the gel (Fowlkes et al., 1997). Following transfer, the PVDF membrane was probed with polyclonal antibody against IGFBP-3 (Fowlkes et al., 1997).

**IGFBP-3 Degradation Assay**

Liver tissue was homogenized as for Western blotting, except that NP-40 and proteinase inhibitors were not added. 50 μg of protein from each sample was brought up to a volume of 28 μl with homogenization buffer containing 50 μM CaCl₂, 125I-IGFBP-3 (50,000 cpm/ml; Diagnostic Systems Laboratories) was added, and the samples were incubated for 5 h at 37°C, followed by SDS-PAGE, electroblotting, and autoradiography (Davenport et al., 1993).

**Affinity Chromatography**

Liver protein homogenates were prepared as above. Total levels of IGF-II were quantified according to a published protocol (De Leon and As- meron, 1997). In brief, 10 μl from each sample was dot blotted onto nitrocellulose in triplicate. The membrane was incubated in blocking buffer and then with mAb against IGF-II. The IGF-II concentrations were determined from a standard curve generated with known quantities of purified recombinant IGF-II (kindly provided by E. Lilly and Co.). To assess the relative levels of dissociable IGF-II, equivalent quantities of total IGF-II from each sample (1.2 μg) were incubated with IGFBP-4-conjugated Affigel overnight at 4°C with gentle rocking. Samples were centrifuged for 10 min at 1,000 g and the pellets washed three times with TBS. The pellets were then incubated with 0.5 M acetic acid for 5 min to elute IGF-II bound to the immobilized IGFBP-4. Supernatants were collected and dried under vacuum. Samples were resuspended in TBS and the relative levels of dissociable IGF-II determined using the IGF-II dot blot assay. To validate that increasing the molar ratio of IGFBP-3 to IGF-II affects IGF-II binding to the IGFBP-4 Affigel affinity media, increasing amounts of recombinant IGF-1 (50 μg/ml recombinant IGF-1) were mixed with a constant amount of recombinant IGF-II and the mixtures were subjected to the IGFBP-4 chromatographic separation, as described above.

**Receptor Kinase Assays**

Frozen liver tissue samples were homogenized in TBS containing 1% Triton X-100, 1 mM PM SF, 100 μM Na₃VO₄, 1 mM EDTA, 10 μg/ml peptatin, 10 μg/ml leupeptin. A aliquots from each supernatant containing 5.0 μg of protein were immunoprecipitated as above, using antibodies against the IGF-1R (clone aIR3; Calbiochem). Immunoprecipitates were then incubated with kinase reaction buffer (10 mM Hepes, pH 7.4, 5 mM MnCl₂, 100 μM Na₃VO₄, 50 mM NaF) containing 2.0 μCi [γ-32P]ATP either with or without 50 μg Poly (Glu, Tyr) 4:1 (Sigma Chemical Co.) for 30 min at 37°C. Samples were electrophoresed on 10% SDS-PAGE Gels, dried onto Whatman 3 M Paper, and autoradiographed.

**Phosphorylation Levels**

Liver proteins were isolated as for the receptor kinase assays. For insulin receptor substrate (IRS)-1, 2.5 mg of protein from each sample was immunoprecipitated with polyclonal antibodies against rat IRS-1 (Upstate Biotechnology) as described above. The immunoprecipitates were then Western blotted and the membranes probed sequentially with antiphosphotyrosine (clone 4G10; Upstate Biotechnology) and anti-IRS-1 antibody. For mitogen-activated protein kinase (MAPK), replicate 10% SDS-PAGE Gels were run with 40 μg of protein from each sample loaded on each gel. Following electroblotting, membranes were probed with antibodies specific for phospho-MAPK or MAPK (New England Biolabs).

**Densitometry and Quantification**

A utoradiographs were scanned using a Molecular Dynamics Densitometer. A biorbance was quantified using ImageQuant™ software. Statistical significance was determined using t test. For all gel electrophoresis, each lane corresponds to a tissue sample taken from an individual mouse. All samples were obtained from 185-d-old female mice unless indicated otherwise, as we determined previously that TIMP-1 modulation significantly affected TAg-induced neoplastic proliferation at this age (Martin et al., 1999).

**Results**

**TAg Antigen Molecular Interactions Are Maintained in TIMP-1–overexpressing Mice**

We have shown previously that transgenic TIMP-1 expression does not affect TAg oncprotein levels in double transgenic mice that coexpress TAg and TIMP-1 transgenes (TA g+Ts+) (Martin et al., 1996). Equivalent TAg protein levels in both TAg+ and TAg+Ts+ animals are confirmed here in Fig. 1 and 4A (top panel). Since TAg binds to and inactivates the tumor suppressor gene products p53 and Rb to induce hyperplasia (Tan et al., 1986; DeCaprio et al., 1988), we investigated whether TAg interactions with these tumor suppressor proteins are altered by TIMP-1 overexpression. The amount of p53 and Rb that immunoprecipitated with TAg protein was examined by Western blotting and was found to be similar between TAg and TIMP-1–overexpressing (TAg+Ts+) livers, as shown in Fig. 1 and 3A (middle and bottom panels). This indicates that TAg interactions with p53 and Rb proteins are intact, and unaffected by the elevation of TIMP-1.

**Cellular Proliferation Is Inhibited by Transgenic TIMP-1 Despite IGF-II Reactivation**

IGF-II is a fetal mitogen in rodents, and its transcription is normally repressed in adult tissues by p53 (Zhang et al., 1996). However, focal reactivation of IGF-II and its localization to proliferating cells during TAg-induced tumorigenesis have been reported in two independent transgenic tumor models (Schirmacher et al., 1992; Christofori et al., 1994). Cellular proliferation and tumor development have been shown to be profoundly inhibited in one such model when crossed onto an IGF-II–null background (Christofori et al., 1994), revealing the fundamental importance of IGF-II reactivation during TAg-induced tumorigenesis. IGF-II reactivation has been frequently observed during TAg-induced hepatocarcinogenesis (Casola et al., 1995; Hadad and Held, 1997). To investigate whether IGF-II was reactivated in our hepatocellular carcinoma model, we examined its spatiotemporal expression in the livers of experimental and control littermates. IGF-II mRNA was not detected by Northern blot analysis of liver tissue from wild-type mice of all ages, or in the livers of TAg+ mice before 165 d of age. However, from 170 d of age, multiple IGF-II transcripts commonly observed in mouse and human tissues (Daughaday and Rotwein, 1989; Casola et al., 1995) were expressed (Fig. 1, B and data not shown).
IGF-II mRNA levels in the livers of TAg transgene–expressing; TAg littermates. Sequential probing of the blot showed that the levels of p53 and Rb that coimmunoprecipitated with TAg were similar in TAg and TAg littermates at 185 d of age, and was inhibited 3.3-fold in TAg transgenic littermates at this age (Martin et al., 1999). The difference in proliferation was most accentuated at this age, and therefore we anticipated that in vivo analysis of molecular factors in the IGF-II signaling pathway would be most clearly resolved at this point in time. The above observations that IGF-II is reactivated in both TAg and TAg transgenic littermates (Fig. 1 a and Fig. 2, b and e), yet hepatocyte proliferation is only prevalent in TAg tissue at 185 d (Fig. 2, c and f), further supported the use of this age group for IGF-II bioactivity studies.

**IGFBP-3 Levels Are Elevated Due to Reduced Proteolysis**

IGFBPs regulate IGF activity by sequestering free IGFs, thus preventing ligand–receptor interactions. Previous studies from one of our laboratories have demonstrated that MMPs, the primary proteinases inhibited by TIMP-1, can degrade IGFBPs both in vitro and in vivo (Fowlkes et al., 1994a,b; Thrailkill et al., 1995; Fowlkes, 1997). Since the amount of high-affinity, intact IGFBP may be regulated in part by MMP-mediated proteolysis, we compared the levels of intact hepatic IGFBPs using [125I]-IGF-II Western ligand blotting. Of the hepatic IGFBPs that bound [125I]-IGF-II on ligand blots, only IGFBP-3 (42–46-kD doublet) levels were strongly affected by TIMP-1 modulation (Fig. 3 a). The levels of this binding protein, which is also the major serum carrier protein for IGFs (Jones and Clemmons, 1995), were increased by more than twofold in the livers of TAg transgenic littermates (Fig. 3 d, left panel; P < 0.02). TIMP-1 overexpression did not affect the levels of IGFBP-4 (24–26 kD), although there were notable minor elevations of a doublet of proteins at 28–32 kD that, based on molecular mass, likely represent IGFBP-1, -2, and/or -5 (Fig. 3 a).

In contrast to IGFBP-3 protein levels, Northern blot analysis showed that TIMP-1 overexpression did not affect the levels of IGFBP-3 mRNA (Fig. 3 b), attributing the differences in IGFBP-3 protein levels to posttranscriptional events. MMP-1, -2, -3, and -9 have been shown to proteolytically cleave IGFBP-2, -3, and -5, a process inhibited by TIMP-1 in vitro (Fowlkes et al., 1994a,b; Thrailkill et al., 1995; Fowlkes, 1997). Here, IGFBP-3 substrate zymography (Fowlkes et al., 1997) was used to identify...
IGFBP-3–degrading proteases in liver homogenates. We found two IGFBP-3–degrading activities with molecular masses of \( \sim 62 \) and \( \sim 84 \) kD (Fig. 3 c). Both activities were substantially reduced in the presence of recombinant TIMP-1 (Fig. 3 c), demonstrating that the IGFBP-3–degrading proteinases were MMPs. Next, we determined whether a decreased proteolysis of IGFBP-3 is evident in liver tissue obtained from TIMP-1–overexpressing mice. Liver homogenates were analyzed for their ability to degrade \( \text{[125I]} \)–IGFBP-3 into smaller molecular weight species, as described in Materials and Methods. There was significantly less degradation of \( \text{[125I]} \)–IGFBP-3 by liver homogenates from TIMP-1–overexpressing (TA\( g^\dagger \)/Ts\( ^\dagger \)) mice compared with TA\( g^\dagger \) littermates (Fig. 3 d, right panel; \( P < 0.02 \)). These data suggest that TA\( g^\dagger \) can induce MMPs that are capable of degrading IGFBP-3, and that coexpression of TIMP-1 can reduce MMP activity, thereby inhibiting IGFBP-3 degradation. Together, these actions allow for a net increase in tissue IGFBP-3 protein levels.

**Dissociable IGF-II Levels Are Reduced by Transgenic TIMP-1**

We determined whether the increased IGFBP-3 levels in the TIMP-1–overexpressing liver tissue affected the amount of dissociable IGF-II. Fig. 4 a shows that using a dot blot procedure developed to quantify total IGF-II (bound and unbound; De Leon and A verom, 1997), recombinant IGF-II can be measured linearly over a range of concentrations (0.4–3.2 \( \mu \)g). Next, dissociable IGF-II was measured by IGFBP-4–conjugated Affigel affinity chromatography. Using recombinant IGF-II and recombinant IGFBP-3 in different molar ratios, we were able to confirm that increasing molar ratios of IGFBP-3 reduced...
the amount of IGF-II that bound to the Affigel (Fig. 4b). To measure difference in levels of dissociable IGF-II in liver samples, we first quantified total IGF-II using the dot blot assay. Aliquots containing equivalent amounts of total IGF-II were subjected to the affinity chromatography procedure. TIMP-1 overexpression resulted in a sixfold decrease in dissociable IGF-II levels in the livers of TAg1/Ts1 mice compared with their control TAg1 littermates (Fig. 4c; P, 0.02). This demonstrates that despite an equivalent extent of IGF-II reactivation, the level of dissociable or bioavailable IGF-II is reduced in TIMP-1–overexpressing animals.

Reduced Signaling through the IGF-IR
A reduction in the levels of dissociable or bioavailable IGF-II should result in decreased signaling through the IGF-IR pathway in the livers of TAg1/Ts1 mice. Unlike postnatal IGF-II inactivation that occurs in normal rodent liver, the IGF-IR is expressed at constitutive levels in adult
mouse liver, and IGF-II exerts its mitogenic effect through this receptor (Osborne et al., 1989; Rubin and Baserga, 1995). Therefore, IGF-IR kinase activity, as well as the phosphorylation status of the downstream signaling effectors, IRS-1 and MAPK, were assessed. IGF-IR immunoprecipitated from TA g'/Ts+ liver tissue exhibited lower autophosphorylation (data not shown) and showed reduced kinase activity on an exogenous substrate (Fig. 5 a), compared with receptor from TA g+ controls. Moreover, tyrsoine phosphorylation of IRS-1, which binds to IGF-IR following activation, was also reduced in TIMP-1-overexpressing livers (Fig. 5 b, top panel). Sequential probing of the same blot with an antibody against nonphosphorylated IRS-1 showed that IRS-1 protein levels were not altered in TIMP-1-overexpressing animals (Fig. 5 b, bottom panel). Phosphorylation of the downstream signaling molecules, the MAPKs, extracellular signal regulatory kinase (Erk)-1 and Erk-2, was also reduced in TIMP-1-overexpressing liver tissue (Fig. 5 c, top panel), whereas the absolute levels of these proteins were unaffected (Fig. 5 c, bottom panel). These data provide direct evidence that the protein levels of the IGF-IR downstream signaling mediators were not altered, but that signaling from the IGF-IR was attenuated in TIMP-1-overexpressing transgenic tissue.

**Discussion**

**Regulation of the IGF-II Pathway by TIMP-1**

IGFs are critical growth factors involved in growth, transformation, and tumorigenesis and act through the IGF-IR (for reviews see Jones and Clemmons, 1995; Rubin and Baserga, 1995, Werner and Le Roith, 1997). Studies have shown that cells null for the IGF-IR do not display the normal increase in proliferation in response to growth factors or serum as seen in normal cells, and that all phases of the growth cycle are prolonged (for review see Resnicoff and Baserga, 1998). Indeed, in several cell lines, abrogation of the IGF-IR has resulted in enhanced apoptosis. Furthermore, IGF-IR-null cells cannot be transformed by TAg (Sell et al., 1993), activated Ha-ras, a combination of both, or by the overexpression of other growth factor receptors. And finally, a number of studies have shown that ablation of this receptor in tumor cell lines significantly
decreases their tumorigenic potential in vivo. Together, these data demonstrate that interruption of the ligand-receptor interaction between IGFs and the IGF-IR effectively disrupts several aspects of the tumorigenic process.

In nature, under homeostatic circumstances, little or no IGFs are present in the free or bioavailable form due to their sequestration by one or more of the six known high-affinity IGFBPs. Because IGFBPs demonstrate equal or higher affinities for IGFs than does the IGF-IR, little or no IGFs are normally available to interact with receptors. Recent studies have begun to elucidate mechanisms by which IGFs can be released from IGFBPs so they may interact with cell-surface receptors and exert their mitogenic and metabolic effects. These studies have demonstrated that a primary phenomenon invoked to release IGFs from IGFBPs is through decreasing the affinities of IGFBPs for IGFs. The best-characterized mechanism involved in decreasing the affinities of IGFBPs is proteolytic degradation (for review see Fowlkes, 1997).

Proteolytic cleavage has been demonstrated for at least five IGFBPs, IGFBP-2 to IGFBP-6, and occurs in several physiologic as well as pathologic circumstances (Jones and Clemmons, 1995; Fowlkes, 1997), yet little is known of the identity of these IGFBP-degrading proteinases in vivo, or the mechanisms that regulate the proteolytic cleavage. In vitro, we and others have provided evidence that production of proteinases, a common feature of transformed cells (Alexander and Werb, 1989), can result in degradation of IGFBP-IGF complexes, releasing IGFs to interact with cell surface receptors, thereby triggering proliferation of target cells. We have also shown recently that in vitro, MMPs function as IGFBP-3- and IGFBP-5-degrading proteinases (Fowlkes et al., 1994a,b; Thrailkill et al., 1995). In addition to degrading IGFBPs, extensive data also support the role of MMPs in tumorigenesis, angiogenesis, and metastasis (Alexander and Werb, 1989; Flaimenhauf and Rifkin, 1991; Liotta et al., 1991; Werb, 1997). In the current studies, we now demonstrate the importance of MMP-mediated IGFBP degradation in neoplastic proliferation in vivo, and a means of controlling this degradation.

We have used a double transgenic, TA g-based tumor model to determine directly whether TIMP-1 inhibits IGF bioactivity in vivo to suppress hepatocyte proliferation that leads to tumorigenesis. We selected this model as it provided important features: (a) IGFs are crucial mitogens for TA g-induced transformation, proliferation, and tumorigenesis (Schirmacher et al., 1992; Sell et al., 1993; Christofori et al., 1994; Casola et al., 1995; Haddad and Held, 1997); and (b) transgenic TIMP-1 overexpression in this model substantially inhibits TA g-induced proliferation and hepatocellular carcinoma (Martin et al., 1996, 1999), making it possible to measure in vivo differences in molecular factors and IGF-IR signal transduction in transgenic tissue. A systematic analysis has now been undertaken to explore connections that might exist between TA g expression and IGF-II bioactivity on cell proliferation to define the molecular mechanisms behind TIMP-1-mediated tumor suppression in vivo. The events explored herein and the results are summarized in Fig. 6, and are discussed below.

First, an examination of p53 and Rb in these mice showed that TIMP did not interfere with the molecular interactions between TA g and these tumor suppressor proteins. Next, we found that IGF-II expression was indeed reactivated at the onset of cell proliferation, similar to the

Figure 6. The molecular mechanism underlying TIMP-1-mediated suppression of hepatocarcinogenesis. The schematic highlights the molecular events that were examined here to uncover the mechanism of TIMP-1-mediated inhibition of hepatocyte proliferation. The primary event in the pathway between TA g-induced IGF-II reactivation and hepatocyte proliferation affected by TIMP-1 overexpression was an increase in IGFBP-3 levels, due to the inhibition of MMP-mediated proteolysis of IGFBP-3 by TIMP-1. The direct result of increased IGFBP-3 levels was decreased dissociable IGF-II levels concomitant with decreased signaling through the IGF-IR and decreased hepatocyte proliferation.
model of TAg-induced pancreatic tumorigenesis (Christofori et al., 1994), indicating a key mitogenic role for IGF-II in our tumor model. IGF-II reactivation is frequent in TAg-induced liver tumorigenesis (Casola et al., 1995; Hadadd and Held, 1997), as well as in liver tumor formation induced by oncogene or growth factors (Liu et al., 1997; Harriss et al., 1998). Furthermore, our data showing that TIMP-1 significantly inhibited hepatocellular proliferation despite IGF-II reactivation suggested that TIMP-1 might directly act at a posttranscriptional level to modulate IGF-II activity. Our investigations reveal the novel finding that hepatic TIMP-1 overexpression specifically inhibits IGBP-3 proteolysis, leading to a significant elevation of hepatic IGBP-3 levels. We have determined that MMPs induced during TAg tumorigenesis (Martin et al., 1999) appear to function in degrading IGBP-3. Furthermore, we demonstrate that dissociable IGF-II levels are decreased in TAg-overexpressing hepatic tissue. The physiologic consequence of reducing dissociable IGF-II levels (e.g., a reduction in IGF-II bioactivity) was confirmed by demonstrating a significantly reduced signal transduction from the IGF-I, as measured by reduced IGF-I, kinase activity and tyrosine phosphorylation levels of IRS-1 and MAPK. Thus, despite TAg-induced reactivation of IGF-II in the liver, the transgenic TIMP-1-mediated increase of IGBP-3 levels blocks TAg-induced hepatocyte proliferation by effectively reducing bioavailable IGF-II levels. Together, our data provide direct evidence that the inhibition of extracellular proteolysis by TIMP-1 attenuates the bioactivity of the tumor-inducing growth factor IGF-II.

Transgenic TIMP or MMP Modulation and Effects on Early Tumor Development

Based on previous studies in transgenic systems, a relationship has begun to emerge between TIMP/MMP expression within a tissue and the tissue’s susceptibility to tumor development. We have demonstrated that overexpression of TIMP-1 in the liver inhibits hepatocellular carcinoma (Martin et al., 1996), and its elevation in the skin compromises the ability of transplanted lymphoma cells to grow as a primary tumor (Krüger et al., 1997), whereas a reduction of TIMP-1 in these tissues augments tumor development (Martin et al., 1996; Krüger et al., 1997). Consistent with these observations, the overexpression of MMP-3 (stromelysin-1) in mammary tissue leads to spontaneous mammary tumor development (Sympon et al., 1995) and the transgenic expression of MMP-1 (type I collagenase) in skin augments carcinogenesis (D’Armiento et al., 1995). In addition, the ablation of MMP-7 (matrilysin) in skin augments carcinogenesis (D’Armiento et al., 1995). In many of these studies, ectopic TIMP or MMP expression results in altered cellular proliferation. For example, hepatic TIMP-1 overexpression inhibits hepatocyte hyperplasia (Martin et al., 1999), MMP-3 overexpression leads to mammary epithelial hyperplasia (Sympon et al., 1994; Witty et al., 1995), and MMP-1 overexpression to epidermal hyperproliferation (D’Armiento et al., 1995). Despite the many reports indicating that shifts in the extracellular proteolytic balance have a strong influence on early tumor development and on cell proliferation within the afflicted organ, the molecular mechanisms for these effects have remained elusive. Having previously excluded the effects of TIMP-1 on hepatocyte apoptosis (Martin et al., 1999), our present investigation provides in vivo evidence of a link between the inhibition of extracellular proteolysis, reduced growth factor bioavailability, and reduced cellular proliferation. We have shown previously that this reduction in cellular proliferation precedes suppressed tumor development in this model (Martin et al., 1996).

MMP Substrates of Importance other than ECM during Tumorigenesis

TIMPs have traditionally been considered regulators of cell invasion and motility by virtue of their ability to inhibit MMP-mediated ECM degradation. Now it is recognized that in addition to the effects on ECM structural proteins, extracellular proteolysis has the potential to control the release of growth factors tethered to the ECM (Dallas et al., 1995; Whitelock et al., 1996), the processing of soluble growth factor binding proteins, i.e., IGBP3s (Foulkes, 1997), and the processing of cell surface molecules such as membrane-bound TNF-α, the Notch receptor in Drosophila (Blobel, 1997), and the FGF receptor 1 (Levi et al., 1996). Although these and other studies (Kimura et al., 1998) support the overall concept that extracellular proteolysis can broadly influence cell proliferation, behavior, and fate, the observations were made in vitro systems. To our knowledge, our data are the first in vivo evidence to substantiate the importance of proteolytic degradation of a tethering or binding/carrier protein for a growth factor in the pathological development of cancer, and of its regulation by the natural inhibitor, TIMP-1. Our data demonstrate that the dynamics of IGF-II bioactivity can be altered in vivo by proteolytic modulation, and emphasize that factors other than ECM proteins constitute important physiological targets of MMP-mediated cleavage within the extracellular microenvironment.

Role of IGF-II in Tumorigenesis and Hepatocarcinogenesis

The importance of IGFs in cellular transformation has been demonstrated by findings that cells which do not express IGF-I, cannot be transformed by any of a number of dominant oncogenes (for review see Werner and Le Roith, 1997). Indeed, TAg was unable to transform IGF-I–null fibroblasts, despite the presence of various growth factors present in serum (Sell et al., 1993). Thus, IGF-I is necessary for TAg-mediated transformation to take place. Consistent with these findings, TAg expression has been shown to reactivate IGF-II gene transcription in vivo. IGF-II reactivation occurs in pancreatic beta cells transgenically expressing TAg (Christofori et al., 1994), and here we show that IGF-II reactivation occurs in hepatocytes expressing TAg transgene coincident with the onset of hepatocyte hyperplasia. Similar to our finding, other investigators have reported that TAg expression in the liver tissue coincides with reactivation of IGF-II gene transcription and this reactivation has been associated with neoplastic transformation (Casola et al., 1995; Hadadd and Held, 1997). Such reactivation of IGF-II has also been re-
ported during c-myc- and TGF-α-induced hepatocellular carcinomas in transgenic mice (Liu et al., 1997; Harris et al., 1998). The consequence of IGF-II reactivation as it relates to neoplastic transformation has only been addressed recently in TAg models of pancreatic cancer and hepatocellular carcinoma. In the former study, the investigators bred TAg transgenics into an IGF-II-null background (Christofori et al., 1994), whereas in the latter TAg transgenics were bred into an IGF2 (+/-) background (Haddad and Held, 1997). In both instances, tumor size and incidence were decreased, strongly suggesting a role for IGF-II in the pathogenesis of these two tumor types. Furthermore, several transgenic lines overexpressing IGF-II demonstrate that IGF-II is associated with tumor formation, including mammary tumors, lymphomas, and hepatocellular carcinomas (Rogler et al., 1994; Bates et al., 1995; for review see Wolf et al., 1998). Athough these studies provide compelling support for IGF-II as a causal mitogen in the tumorigenesis evidenced by the current animal model, final resolution of this hypothesis must await studies examining our findings in models that are either null or up-regulated for IGF-II. Such studies are currently being pursued in our laboratories.

**Significance of Regulated IGF Bioavailability in Clinical Medicine**

Both IGF-I and IGF-II are widely implicated in promoting several human cancers, including liver, prostate, and breast cancer, and their expression correlates with poorer prognosis (Osborne et al., 1989; Tengnatt et al., 1996; Werner and LER oth, 1996; Sohda et al., 1997; Chan et al., 1998; Harkin et al., 1998). Furthermore, in transgenic models, IGF-I and IGF-II are causally implicated in tumorigenesis (Rogler et al., 1994; Bates et al., 1995; Haddell et al., 1996; for review see Wolf et al., 1998). These studies point to IGF bioactivity as a target for cancer therapeutics. Our results indicate that TIMP-1-like biomolecules or synthetic MMP inhibitors may be promising candidates for the therapeutic modulation of IGF dosage in novel clinical strategies. Alternatively, strategies to alter specific IGFBP levels or the production of proteinase-resistant IGFBPs (Chernausek et al., 1995; Conover et al., 1995; Imai et al., 1998; Rees et al., 1998) may prove to be effective therapeutic interventions. A distinct feature of all of these approaches will be to target the bioavailability rather than the production of a growth factor.

The results presented here provide compelling evidence for a novel mechanism by which endogenous TIMPs contribute to the cellular microenvironment. We demonstrate that the inhibition of extracellular proteolysis in vivo impairs the activity of a specific growth factor responsible for hyperplasia during TAg-induced tumorigenesis. Because TIMPs are also capable of inhibiting invasion, metastasis, and angiogenesis (Kokha et al., 1989; DeClerck et al., 1992; Kokha, 1994; A nand-A pte et al., 1997; K rüger et al., 1997, 1998; Wang et al., 1997; Martin et al., 1999), all of which are promoted by IGF action (Bae et al., 1998; D unn et al., 1998), the combined outcome of TIMP-1 elevation may be to suppress multiple stages of tumor development, maintenance, and progression.

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**References**

Alexander, C. M., and Z. Werb. 1989. Proteinaseas and extracellular matrix re-modeling. Curr. Opin. Cell Biol. 1:974–982.

A nand-A pte, B., M. S. Pepper, E. V oest, R. M. ontanesano, B. Olsen, G. Murphy, S. S. A pte, and B. Z etter. 1997. Inhibition of angiogenesis by tissue inhibitor of metalloproteinases-3. Invest. Ophthalmol. Vis. Sci. 38:817–823.

Ba e, M. H., M. J. Le e, S. K. B ae, O. H. Lee, Y. M. Lee, B. C. Park, and K. W. Kim. 1998. Insulin-like growth factor II (IGF-II) secreted from HepG2 human hepatocellular carcinoma cells shows angiogenic activity. Cancer Lett. 128: 41–46.

Bates, P., R. Fisher, L. Ward, L. Richardson, D. J. Hill, and C. F. Graham. 1995. Mammary cancer in transgenic mice expressing insulin-like growth factor II (IGF-II). Br. J. Cancer. 72:1189–1193.

Blobel, C. P. 1997. Metalloprotease-disintegrins: links to cell adhesion and cleavage of TNF alpha and Notch. Cell. 90:589–592.

Bozyczko-Coyne, D. M., A. M. Glickman, J. E. Pratner, B. McKenna, T. Conners, and F. Friedman. 1993. IGF-I supports the survival and/or differentiation of multiple types of central nervous system neurons. Ann. N.Y. Acad. Sci. 692: 311–313.

Casola, S., P. U ngaro, P. V. Pedone, D. Lazzaro, E. Fattori, G. Ciliberto, R. Zarrilli, C. B. Bruno, and A. R. Riccio. 1995. Loss of heterozygosity of imprinted genes in SV 40/T antigen-induced hepatocellular carcinomas. Oncogene. 11: 711–721.

Chan, J. M., M. J. Stingper, E. Giovannucci, P. H. Gann, J. M. A. Wilkinson, C. H. Hennekens, and M. Pollak. 1998. Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. Science. 279:563–566.

Chernausek, S. D., C. E. Smith, K. L. Duffin, W. H. Busby, G. Wright, and D. R. Clemmons. 1995. Proteolytic cleavage of insulin-like growth factor binding protein 4 (IGF-BP-4). Localization of cleavage site to non-homologous region of native IGF-BP-4. J. Biol. Chem. 270:11377–11382.

Christofori, G., P. Naik, and D. Hanahan. 1994. A second signal supplied by inulin-like growth factor II in oncogene-induced tumorigenesis. Nature. 369: 414–418.

Cohen, P. H., C. Graves, D. M. Peeth, M. Kamare, L. C. Giudice, and R. G. Rosenfeld. 1992. Prostate-specific antigen (PSA) is an insulin-like growth factor protein-2 tested found in seminal plasma. J. Clin. Endocri nom. Metab. 75:1046–1050.

Conover, C. A., and D. D. De Leon. 1994. A cid-activated insulin-like growth factor binding protein-3 proteolysis in normal and transformed cells. Role of cathepsin D. J. Biol. Chem. 269:7076–7080.

Conover, C. A., S. K. Durham, J. Z affer, C. E. Huse, M. E. Schenkel, and M. Pollak. 1995. Cleavage analysis of insulin-like growth factor (IGF)-dependent IGF binding-protein 4 proteolysis and expression of protease-resistant IGF-binding-protein 4 mutants. J. Biol. Chem. 270:4395–4400.

Dallas, S. L., K. M iyazato, T. M. Skerry, G. R. Mundy, and L. F. Bonevald. 1995. Dual role for the latent transforming growth factor-beta binding protein in storage of latent TGF-beta in the extracellular matrix and as a structural matrix protein. J. Cell Biol. 131:539–549.

D'A mmiento, J., T. D. Colandrea, S. S. Dalal, Y. Okada, M. T. Huang, A. H. Conney, and K. Chada. 1995. Collagenase expression in transgenic mouse skin causes hyperkeratosis and acanthosis and increases susceptibility to tumorigenesis. Mol. Cell. Biol. 15:5732–5739.

Daugaday, W. H., and P. Robeinf. 1989. Insulin-like growth factors I and II. Pesticide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. Endocr. Rev. 10:68–91.

Davenport, M. L., J. Puclowska, D. R. Clemmons, R. L. Underwood, and J. A. Spencer, and L. E. Underwood. 1991. Tissue-specific expression of IGF-BP-3 protease activity during rat pregnancy. Endocrinology. 130:2505–2512.

De Leon, D. D., and Y. A. A meron. 1997. Quantification of insulin-like growth factor I (IGF-I) without interference by IGF-1 binding proteins. Endocrinology. 138:2199–2202.

DeClerck, Y. E., N. Perez, H. Shimada, T. C. Boone, K. E. Langley, and S. M. Taylor. 1989. Inhibition of invasion and metastasis in cells transfected with an inhibitor of metalloproteases. Cancer Res. 52:701–708.

Denhardt, D. T., B. Fong, D. R. Edwards, E. T. Cocuzza, and U. M. Malyankar.
1993. Tissue inhibitor of metalloproteinases (TIMP, aka EAPA): structure, function, and regulation of expression and biological functions. Pharmacol. Ther. 59:309–341.
D’Errico, A., W.F. Grigioni, M. Fiorentino, P. Baccarin, E. Lamas, S. De Mtri, G. Gozetti, A.M. Mancini, and C. Brechot. 1994. Expression of insulin-like growth factor I (IGF-I-II) in human hepatocellular carcinomas: an immunohistological study. Lab. In vest. 141:131–137.
Dunn, S.E., M. Ehrlich, N.J. Sharp, K. Reiss, G. Solomon, R. Hawkins, R. Baserga, and J.C. Barereet. 1998. A dominant negative mutant of the insulin-like growth factor-I receptor inhibits the attachment, invasion, and metastasis of breast cancer cells in vitro. Cancer Res. 58:3353–3360.
Flaumenhaft, R., and D.B. Rifkin. 1991. Extracellular matrix regulation of growth factor and protease activity. Curr. Opin. Cell. Biol. 3:817–823.
Fowlkes, J.L. 1997. Insulin-like growth factor-binding protein proteolysis: an emerging paradigm in insulin-like growth factor physiology. Trends Endocr. Mol. Biol. 8:299–306.
Fowlkes, J.L., J.J. Enghild, K. Suzuki, and H. Nagase. 1994a. Matrix metalloproteinases degrade insulin-like growth factor-binding protein-3 in dermal fibroblast cultures. J. Biol. Chem. 269:25762–25767.
Fowlkes, J.L., K. Suzuki, H. Nagase, and K.M. Thraillik. 1994b. Proteolysis of insulin-like growth factor binding protein-3 during rat pregnancy: a role for matrix metalloproteinases. Endocrinology. 135:2810–2813.
Fowlkes, J.L., K.M. Thraillik, D.G. Serra, and H. Nagase. 1997. Insulin-like growth factor binding protein (IGFBP) substate zymography. A new tool to identify and characterize IGFBP-degrading proteases. Endocrinology. 7:336–339.
Gargosky, S.E., H.M. Pham, K.F. Wilson, F. Liu, L.C. Giudice, and R.G. Mignatti. 1991. Identification and characterization of the P53 protein involved in formation of the simian virus 40 large T-antigen-p53 protein complex. J. Biol. Chem. 266:422–429.
Gargosky, S.E., W.E. Willetta, G.A. Colditz, D.J. Hunter, D.S. Michaud, B. Deero, B. Rossner, F.E. Speizer, and M. Pollak. 1998. Circulating concentrations of insulin-like growth factor I and risk of breast cancer. Lancet. 351:1393–1396.
Harris, T.M., L.E. Rogler, and C.E. Rogler. 1998. Reactivation of the maternally imprinted IGF2 allele in TGalpha induced hepatocellular carcinomas in mice. Oncogene. 16:203–209.
Imai, Y., W.H. Busby, J.R., C.E. Smith, J.B. Clarke, A.J. Garmong, G.D. Horwitz, C. Rees, and D.R. Clemmons. 1998. Insulin-like growth factor-I receptor action on porcine smooth muscle cells in culture. J. Clin. Invest. 100:2596–2605.
Jones, J.I., and D.R. Clemmons. 1995. Insulin-like growth factors and their binding proteins: biologic actions. Endocr. Rev. 16:3–6.
Khokha, R. 1998. Suppression of the tumorigenic and metastatic abilities of insulin-like growth factor-I receptor action. Oncogene. 13:131–137.
Khokha, R. 1994. Suppression of the tumorigenic and metastatic abilities of insulin-like growth factor-I receptor action on porcine smooth muscle cells in culture. J. Clin. Invest. 100:2596–2605.
Khokha, R., M. Schirmacher, P., W.A. Held, D. Yang, L. Rossetti, J. Donohoe, E. Alt, C.J. Chang, R. Rosenfeld, K. Neely, and R. Hintz. 1994. Tissue inhibitor of metalloproteinase-1. Oncogene. 9:2813–2815.
Khokha, R., M. Schirmacher, P., W.A. Held, D. Yang, L. Rossetti, J. Donohoe, E. Alt, C.J. Chang, R. Rosenfeld, K. Neely, and R. Hintz. 1994. Tissue inhibitor of metalloproteinase-1. Oncogene. 9:2813–2815.
Khokha, R., M. Schirmacher, P., W.A. Held, D. Yang, L. Rossetti, J. Donohoe, E. Alt, C.J. Chang, R. Rosenfeld, K. Neely, and R. Hintz. 1994. Tissue inhibitor of metalloproteinase-1. Oncogene. 9:2813–2815.
Khokha, R., M. Schirmacher, P., W.A. Held, D. Yang, L. Rossetti, J. Donohoe, E. Alt, C.J. Chang, R. Rosenfeld, K. Neely, and R. Hintz. 1994. Tissue inhibitor of metalloproteinase-1. Oncogene. 9:2813–2815.
Khokha, R., M. Schirmacher, P., W.A. Held, D. Yang, L. Rossetti, J. Donohoe, E. Alt, C.J. Chang, R. Rosenfeld, K. Neely, and R. Hintz. 1994. Tissue inhibitor of metalloproteinase-1. Oncogene. 9:2813–2815.
Khokha, R., M. Schirmacher, P., W.A. Held, D. Yang, L. Rossetti, J. Donohoe, E. Alt, C.J. Chang, R. Rosenfeld, K. Neely, and R. Hintz. 1994. Tissue inhibitor of metalloproteinase-1. Oncogene. 9:2813–2815.
Khokha, R., M. Schirmacher, P., W.A. Held, D. Yang, L. Rossetti, J. Donohoe, E. Alt, C.J. Chang, R. Rosenfeld, K. Neely, and R. Hintz. 1994. Tissue inhibitor of metalloproteinase-1. Oncogene. 9:2813–2815.
Khokha, R., M. Schirmacher, P., W.A. Held, D. Yang, L. Rossetti, J. Donohoe, E. Alt, C.J. Chang, R. Rosenfeld, K. Neely, and R. Hintz. 1994. Tissue inhibitor of metalloproteinase-1. Oncogene. 9:2813–2815.
Whitelock, J. M., A. D. Murdoch, R. V. Iozzo, and P. A. Underwood. 1996. The degradation of human endothelial cell-derived perlecan and release of bound basic fibroblast growth factor by stromelysin, collagenase, plasmin, and heparanases. J. Biol. Chem. 271:10079–10086.

Wilson, C. I., K. J. Heppner, P. A. Laboski, B. L. Hogan, and M. L. Matrisian. 1997. Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin. Proc. Natl. Acad. Sci. USA. 94:1402–1407.

Witty, J. P., J. H. Wright, and L. M. Matrisian. 1995. Matrix metalloproteinases are expressed during ductal alveolar mammary morphogenesis, and misregulation of stromelysin-1 in transgenic mice induces unscheduled mammary alveolar development. Mol. Biol. Cell. 6:1287–1303.

Wolf, E., A. Hoeflich, and H. Lahm. 1998. What is the function of IGF-II in postnatal life? Answers from transgenic mouse models. Growth Horm. IGF Res. 8:185–193.

Zhang, L., F. Kashanchi, Q. Zhan, S. Zhan, J. N. Brady, A. J. Fornace, P. Seth, and L. J. Helman. 1996. Regulation of insulin-like growth factor II P3 promoter by p53: a potential mechanism for tumorigenesis. Cancer Res. 56:1367–1373.