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Review

Post translational activation of latent transforming growth factor beta (L-TGF-ß): clinical implications

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Summary. Transforming growth factor-betas (TGF-ßs) are multifunctional cytokines that exist in 3 isoforms in mammals. The TGF-ßs are ubiquitously expressed and all isoforms are secreted as biologically inactive precursors called latent TGF-ß (L-TGF- ß). L-TGF-ßs are generally not effective molecules because they are unable to interact with their receptors. However, the removal of or conformational change of the precursor protein called the latency associated peptide (LAP) results in the generation of biologically active TGF- ß. *In vitro* active TGF-ß has many biological effects but from a clinical point of view one of the most recognized associations of aberrant TGF-ß production is with diseases characterized by enhanced connective tissue synthesis. Recently a number of observations in the context of fibrotic disorders suggest mechanisms of activation of L-TGF-ß1 *in vivo*. The recognition of mechanisms that activate L-TGF-ß1 *in vivo* offers the possibility of interfering with the activation of L-TGF-ß1 for therapeutic purposes.

Key words: Latency associated protein, Latent TGF-ß, Latent TGF-ß binding protein, Fibrosis, Plasmin, Thrombospondin-1, CD36, M-6-P/IGF-II R, αvß 6

Introduction

Transforming growth factor-betas (TGF-ßs) are members of a supergene family of proteins that regulate numerous biological functions such as embryogenesis, growth and differentiation, connective tissue synthesis, and immune function (Grande, 1997; Letterio and Roberts, 1998; Roberts, 1998; Bonewald, 1999). Three of the 5 isoforms of TGF-ß are expressed in mammals and are called TGF- 1, TGF-ß2, and TGF-ß3 (Grande, 1997; Letterio and Roberts, 1998; Roberts, 1998; Bonewald, 1999). TGF-ß4 is expressed in the chicken (Jakowlew et al., 1988) while TGF-ß5 is found in the *Xenopus* (Kondaiah et al., 1990). Since most cells and cell lines express TGF-ß and its receptors (Grande, 1997; Letterio and Roberts, 1998; Roberts, 1998; Bonewald, 1999) to be biologically effective it is likely that there must be a variety of mechanisms to regulate the biological activity of TGF-ß. In some instances the regulation of TGF-ß could occur by the quantity of TGF-ß produced by changes at the transcriptional (Kelly et al., 1995; Roberts, 1998) or post-transcriptional level (Roberts, 1992, 1998). The production of TGF-ß could be balanced by the expression of proteins that can bind and inactivate or remove TGF-ß from the site of production. Examples of such proteins are decorin, betaglycan, alpha2-macroglobulin, and the latency associated peptide (LAP) (Segarini, 1993). It is also possible that differential expression of TGF-ß signaling receptors could also control TGF-ß activity (Khalil et al., 1997). Despite all of these possibilities, the most important mechanism controlling the effects of TGF-ß is the post-translational activation of a biologically inactive form of TGF-ß called latent TGF-ß (L-TGF-ß) to a biologically active form of TGF-ß called active or mature TGF-ß (Khalil, 1999). It is important to realize that most mechanisms of activation of L-TGF-ß have been described using *in vitro* systems. For this reason it remains largely unknown how L-TGF-ß is activated *in vivo*. However, there is now growing evidence for *in vivo* mechanisms of activation of L-TGF-ß especially in diseases characterized by increased connective tissue synthesis.

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Latent forms of transforming growth factor-beta

All the TGF-ßs are secreted in a biologically latent form called either small latent TGF-ß (L-TGF-ß) or large L-TGF-ß (LL-TGF-ß) (Miyazano et al., 1991; Bonewald, 1999). Neither of the latent forms of TGF-ß can interact with the TGF-ß signaling receptors and thus in most instances are biologically inactive (Miyazano et al., 1991; Grande, 1997; Letterio and Roberts, 1998; Roberts, 1998; Bonewald, 1999). Each isoform of TGF-ß is the product of a separate gene and all isoforms of

TGF-ß are initially synthesized as precursor proteins that are 390-412 amino acids (Grande, 1997; Roberts, 1998). Prior to the secretion of TGF-ß by a cell there are a number of intracellular processing steps (Miyazano et al., 1991; Dubois et al., 1995; McMahon et al., 1996; Roberts, 1998). One of these steps is the proteolytic cleavage of the TGF-ß precursor between amino acids 278 and 279 by furin, an endopeptidase (Dubois et al., 1995). The proteolysis results in a 65-75 kDa dimer of the N-terminal portion of the TGF-ß protein called the latency associated peptide (LAP) and a 25 kDa dimer of the C-terminal portion of the precursor called the mature TGF-ß (Dubois et al., 1995; Wakefield et al., 1988). Although the precursor is initially separated into 2 parts the N-terminal portion becomes non-covalently associated with the rest of the protein (Dubois et al., 1995). The association of the LAP with mature TGF-ß facilitates the transit of the entire TGF-ß protein from the cell (Miyazano et al., 1991). However, the association of LAP with mature TGF-ß covers the site on TGF-ß that interacts with its receptors (Miyazano et al., 1991; Dubois et al., 1995). For this reason the secreted complex of TGF-ß with its LAP is biologically inactive and is called latent-TGF-ß (L-TGF-ß). Once the LAP is removed from the mature TGF-ß the remaining protein is called active or mature TGF-ß.

The structure and function of L-TGF-ß1 is described most extensively. Each LAP-1 contains a number of carbohydrate residues and 2 of the residues have mannose-6-phosphate groups (Purchio et al., 1988) which can bind to mannose-6-phosphate/insulin like growth factor-II receptor (M-6-P/IGF-II R) (Kovacina et al., 1988). The cysteines in position 223 and 225 in the LAP-1 are important for the association of LAP-1 with TGF-ß1 as well as for dimerization of the LAP-1 monomers by disulfide binding (Brunner et al., 1989). In some instances a cysteine in position 33 of the LAP-1 binds to another protein called the latent TGF-ß binding protein-1(LTBP-1) (Saharinen et al., 1996). When L-TGF-ß1 binds to LTBP-1 it is often called large latent TGF-ß1 (LL-TGF-ß1) (Saharinen et al., 1996). There are several isoforms of LTBP and each isoform probably associates with a specific isoform of L-TGF-ß. Since the LTBPs are coded by genes different from those of L-TGF-ß, LTBP is not an obligatory protein of TGF-ß (Saharinen et al., 1996). The LTBPs share some common characteristics such as 15-19 EGF like repeats, several cysteines and homology with fibrillins 1 and 2 (Dallas et al., 1995; Taipale et al., 1996). When LTBP-1 is associated with the L-TGF-ß-1 it may cover the M-6-P residues on the L-TGF-ß1 and thus preventing intracellular interaction with the Golgi apparatus resulting in facilitation of the exit of L-TGF-ß (Purchio et al., 1988; Rifkin et al., 1999). LTBP-1 can associate with the extracellular matrix (ECM) (Taipale et al., 1994) and when L-TGF-ß1 is complexed with LTBP-1 the association of LL-TGF-ß with the ECM may create a reservoir of TGF-ß1 at an extracellular site (Taipale et al., 1994). L-TGF-ß1 in association with LTBP-1 and the ECM can be released by proteases to generate active TGF-ß1 (Dallas et al., 1995; Taipale et al., 1996; Rifkin et al., 1999). The release of TGF-ß1 from its association with the LTBP-1/ECM can be remote from the time that the TGF-ß1 was synthesized and released. This possibility suggests that TGF-ß1 could be available as a biologically effective protein on a chronic basis without *de novo* synthesis.

Post translational activation of latent TGF-ß: plasmin dependent mechanisms

Plasminogen/plasmin proteolytic system

In vitro the LAP from all isoforms of L-TGF-ß can be removed by physiochemical means such as pH of 2 or 8, 100 °C, chaotropic agents, SDS and urea (Brown et al., 1990). More physiological substances described to activate L-TGF-ß are proteases such as plasmin, calpain or endoglycosidase F, sialidase, neuraminidase, cathepsins B and D, and the glycoprotein, thrombospondin-1 (TSP-1) (Miyazano et al., 1991; Schultz-Cherry et al., 1995; Khalil, 1999). Although, it is not always clear which isoform of TGF-ß is susceptible to the above mentioned substances, in those studies where the isoform is identified it is usually TGF- ß1. The activation of L-TGF-ß2 and TGF-ß3 is very poorly understood.

Of all the substances that activate L-TGF-ß1 the most common mediator described in the activation of L-TGF-ß1 is the serine protease, plasmin which removes the LAP from the latent complex, LAP-TGF-ß1 (L-TGF-ß1) (Lyons et al., 1988). Plasmin is derived from plasminogen by the proteolytic actions of urokinase plasminogen activator (uPA) or tissue type plasminogen activator (tPA) (Lijnen, 1996; Reuning et al., 1998). uPA activation of plasminogen to plasmin occurs while uPA is bound to a specific cell surface receptor, urokinase plasminogen activator receptor (uPAR) (Lijnen, 1996; Reuning et al., 1998) (Fig. 1). The formation of plasmin provides an important source of proteolytic activation of L-TGF-ß1. tPA has not been clearly identified as binding to a cell membrane receptor (Allan and Martin, 1995; Lijnen, 1996; Reuning et al., 1998) and the role of t-PA in activation of L-TGF-ß is not defined. tPA and uPA can be inhibited by the actions of plasminogen activator inhibitors (PAI) which exist in at least 4 isoforms (Allan and Martin, 1995; Lijnen, 1996; Reuning et al., 1998). Receptor bound uPA can be inactivated by PAI-1 when the PAI-1/uPAR/uPA complex is internalized (Allan and Martin, 1995). Once cell surface expression of uPA is diminished the generation of plasmin is also reduced (Allan and Martin, 1995).

Plasmin regulates activation of L-TGF-ß in vitro and in vivo

The first reports to describe the activation of cell derived L-TGF-ß by plasmin was in co-cultures of

vascular smooth muscle cells (SMC) which are called pericytes and endothelial cells (Antonelli-Orlidge et al., 1989; Sato et al., 1990). Normally pericytes and endothelial are closely associated with each other and the association appears to be important in generating a substance that prevents endothelial cell proliferation and neovascularization (Antonelli-Orlidge et al., 1989; Sato et al., 1990; Wakui et al., 1997). When capillary pericytes from bovine retinas were co-cultured with adrenal cortex capillary endothelial cells (Antonelli-Orlidge et al., 1989) or bovine aortic endothelial cells (Sato et al., 1990) there was release of biologically active TGF-ß a potent inhibitor of endothelial cell proliferation (Antonelli-Orlidge et al., 1989; Sato et al., 1990). Cultures of the pericytes or endothelial cells alone generated only L-TGF-ß (Antonelli-Orlidge et al., 1989; Sato et al., 1990). The secretion of the active TGF-ß in this system was mediated by plasmin (Sato et al., 1990). Although these are *in vitro* studies the plasminogen/plasmin system may be important for *in vivo* regulation of active TGF-ß1 in vascular biology. Angiogenesis which is an important part of granulation tissue terminates after an initial phase of induction (Wakui et al., 1997). In the early stages of angiogenesis at the time of capillary sprout formation uPA and plasmin immunoreactivity is seen to be present at the capillary sprouts, but no TGF-ß1 expression is observed (Wakui et al., 1997). However, late in the process uPA, plasmin, and TGF-ß1 are all present in regions where endothelial cells and pericytes interdigitation have formed (Wakui et al., 1997). TGF-ß1 is an inhibitor of endothelial cell proliferation and induces endothelial cell differentiation (Wakui et al., 1997). The presence of TGF-ß at sites where endothelial cells and pericytes are

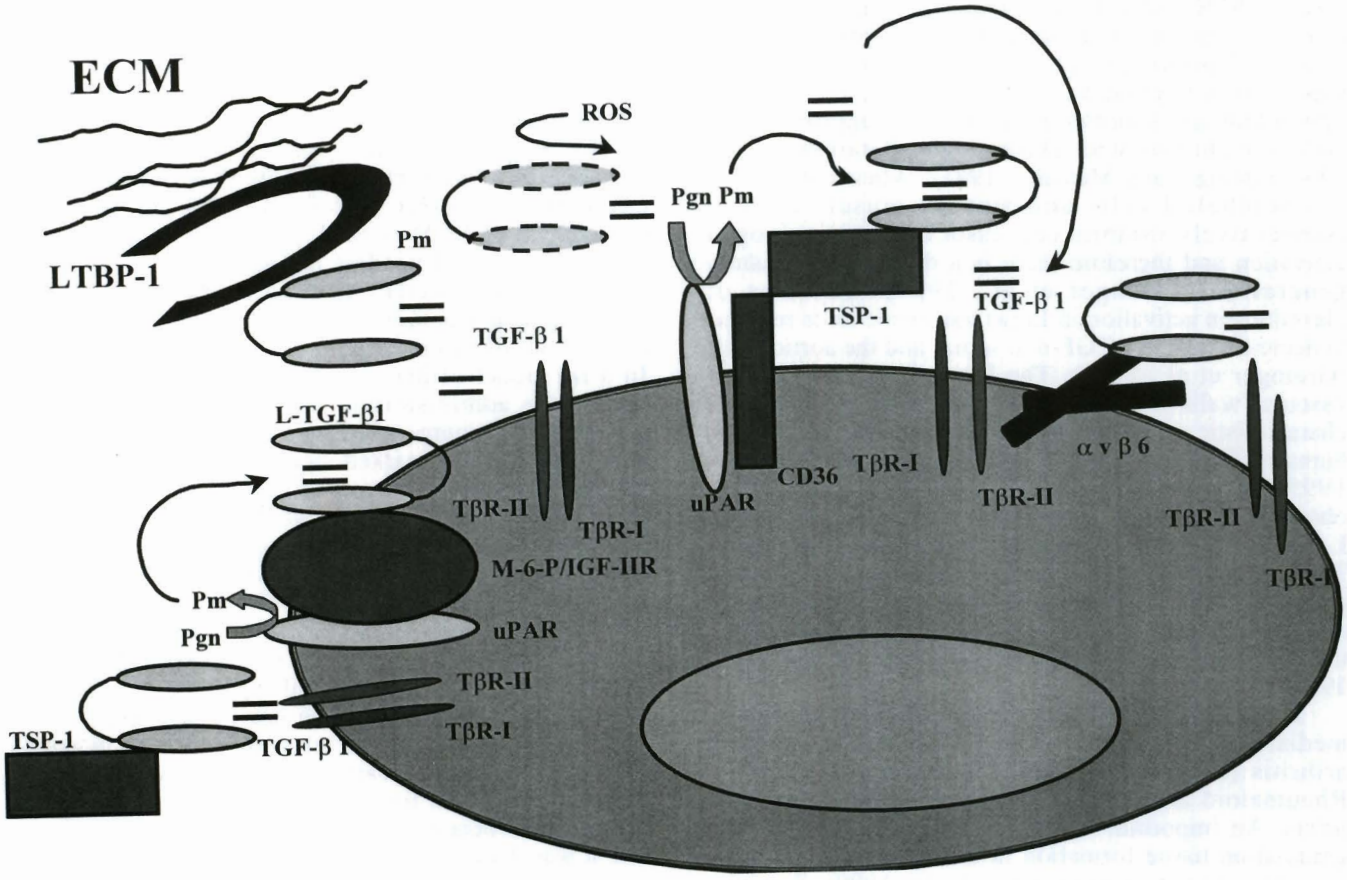

Fig. 1. Activation of L-TGF-ß1. L-TGF-ß can be activated while localized to a cell surface after complexing with L-TGF-ß1/TSP-1 composite interacts with TSP-1 receptor, CD36 prior to the actions of plasmin (Pm). Alternatively, the LAP-1 of the L-TGF-ß 1 may attach to the mannose-6-phosphate/insulin growth factor-II receptor (M-6-P/IGF-II R) prior to release of TGF-ß by Pm. Pm is generated on the cell surface when plasminogen (Pgm) is converted to Pm by the actions of urokinase plasminogen activator (uPA) which is complexed with its receptor urokinase plasminogen activator receptor (uPAR). In other instances cell surface association of the LAP-1 of L-TGF-ß1 may occur by binding to avß6 which results in the interaction of ß6 with actin of the cytoskeleton and sufficient conformational changes such that TGF-ß can bind to the TGF-ß type II receptor (TßR-II). Alternatively, the activation of L-TGF-ß1 by TSP-1 can be independent of cell surface association and plasmin. In these instances the interaction of TSP-1 with the LAP-1 of L-TGF-ß1 may lead to conformational changes sufficient to expose sites on the TGF-ß protein to bind to TßR-II. The effects of ROS on the LAP-1 may cause structural or conformational changes of the LAP-1 that are adequate to expose receptor binding sites on the TGF-ß1. In other circumstances active TGF-ß1 may be released by Pm from its association with the TGF-ß latency binding protein-1 (LTBP-1) which may function to localize L-TGF-ß1 either to a cell surface or to the extracellular matrix (ECM).

closely associated could be considered to emulate the *in vitro* co-culture system described above (Antonelli-Orlidge et al., 1989; Sato et al., 1990). The presence of plasmin in regions of interdigitation could lead to the activation of L-TGF-ß1 by plasmin. Once activated, the TGF-ß1 would then inhibit endothelial cell proliferation and terminate further neovascularization (Wakui et al., 1997). In addition, Herbert and Carmeliet demonstrated that when L-TGF-ß1 was added to serum starved vascular SMC it prevented their apoptosis (Herbert and Carmeliet, 1997). However, L-TGF-ß1 added to vascular SMC from mice that were obtained with the phenotypes uPA-/-, tPA-/-, uPAR-/-, and plasminogen-/- there was no effect of L-TGF-ß1 on vascular SMC apoptosis (Herbert and Carmeliet, 1997). These findings suggest that when vascular SMC express uPA, uPAR and plasminogen their presence activates L-TGF-ß leading to vascular SMC apoptosis and thus regulation of the bioavailable vascular SMC mass. Equally important are observations made by Grainger et al. using transgenic mice that over express lipoprotein (a) (Grainger et al., 1994). Lipoprotein (Lp(a)) has 2 components, low density lipoprotein and apoprotein (a). Lp(a) is approximately 80% homologous with plasminogen (Grainger et al., 1994; Grainger and Metcalfe, 1995). When Lp(a) binds to endothelial cells and smooth muscle cells it competitively inhibits cell associated plasminogen activation and therefore there is a decrease in plasmin generation (Grainger et al., 1994). The loss of plasminogen activation in Lp(a) transgenic mice resulted in decrease in active TGF-ß in serum and the aortic walls (Grainger et al., 1994). The loss of active TGF-ß in vascular walls was associated with SMC proliferation characteristic of atherosclerotic lesions that are seen in humans (Grainger et al., 1994; Grainger and Metcalfe, 1995; Harpel et al., 1995). Collectively these observations suggest that plasmin mediated activation of L-TGF-ß1 is critical in regulating vascular SMC mass. The loss of TGF-ß1 activity as a consequence of plasmin deficiency may be important in vascular disorders such as atherosclerosis and restenosis after angioplasty where there is an increase in vascular SMC mass (Harpel et al., 1995).

In another example of a clinical disorder that may be mediated by plasmin activated TGF-ß is rheumatoid arthritis (Fava et al., 1989; Hamilton et al., 1991). Rheumatoid arthritis is a progressive disease of the joints. An important feature of rheumatoid arthritis is granulation tissue formation in the synovium, which is the lining found in joints (Breedveld, 1999). Synovial fluid contains increased quantities of uPA and active TGF-ß1 (Fava et al., 1989; Hamilton et al., 1991). The findings suggest that the presence of uPA may generate plasmin which in turn would lead to an increase in the active form of TGF-ß1 that could then regulate the granulation tissue in the synovium. In yet another example, using tissues from patients with chronic pancreatitis which is characterized by inflammation and fibrosis, immunohistochemical distribution of uPA, uPAR and TGF-ß1 were all increased (Freiss et al., 1997). There was co-localization of uPA, uPAR and TGF-ß1 leading one to speculate that uPA/plasminogen interaction could generate plasmin and thus activation of L-TGF-ß1 and subsequent increase in inflammation and extracellular matrix synthesis that is observed in chronic pancreatitis (Freiss et al., 1997).

The effect on activation of L-TGF-ß by alterations of the plaminogen/plasmin system

Retinoic acid (RA) is a metabolite of vitamin A and has been demonstrated to induce the expression of uPA and plasmin on the cell membrane of bovine endothelial cells (BEC) *in vitro* (Roberts and Sporn, 1992; Kojima and Rifkin, 1993). The induction of uPA and plasmin was demonstrated to release active TGF-ß by BECs (Kojima and Rifkin, 1993). The importance of retinoic acid in TGF-ß mediated pathogenesis of hepatic fibrosis has been described (Knook et al., 1995; Okuro et al., 1997). Hepatic stellate cells are the major source of connective tissue proteins and nodule formation in cirrhosis or fibrosis of the liver (Knook et al., 1995; Okuro et al., 1997). *In vitro* TGF-ß induces hepatic stellate cells to differentiate into myofibroblasts, increase extracellular matrix proteins (ECM) and diminish degradation of ECM (Knook et al., 1995; Okuro et al., 1997). In a dose dependent fashion the presence of RA induced hepatic stellate cells to increase cell surface expression of plasmin and release plasmin as well as active TGF-ß1 (Knook et al., 1995; Okuro et al., 1997). In a rat model of hepatic fibrosis induced by porcine serum, the administration of RA concomitantly with porcine serum compared to porcine serum administration alone resulted in a marked increase in total liver TGF-ß content and hepatic fibrosis (Knook et al., 1995; Okuro et al., 1997). These findings would suggest that *in vivo* excessive RA may increase the generation of plasmin, conversion of L-TGF-ß to active TGF-ß which would in turn lead to enhanced hepatic fibrosis (Knook et al., 1995; Okuro et al., 1997). Recently another interesting clinical observation has been made that supports the importance of plasmin mediated activation of L-TGF-ß1 *in vivo* (Cai et al., 1997). The use of corticosteroids in renal transplant patients is associated with an increased incidence of Kaposi's sarcoma while the withdrawal of corticosteroids can result in spontaneous regression of Kaposi's sarcoma (Cai et al., 1997). Using AID-KS cell line it was demonstrated that the generation of TGF-ß by AIDS-KS cells inhibits proliferation of these cells in an autocrine manner (Cai et al., 1997). However, in AID-KS cells cultured with corticosteroids PAI-1 is increased while uPAR is decreased, both events that would diminish plasmin generation. The loss of plasmin may be important in abrogating the generation of active TGF-ß thus preventing the proliferation of AIDS-KS cells. These findings would suggest that the lack of active TGF-ß in the presence of corticosteroids may be pathogenically associated with manifestation of Kaposi's sarcoma.

The importance of L-TGF-ß association to the cell membrane for activation

In several instances it has been demonstrated that although plasmin may be critical in releasing TGF-ß from its association with LAP, that this effect of plasmin occurs when L-TGF-ß is localized to the cell surface (Sato et al., 1990, 1993; Dennis and Rifkin, 1991; Miyazano et al., 1991; Kojima and Rifkin, 1993; Khalil et al., 1996: Nunes et al., 1997; Wakui et al., 1997). In the co-culture model of endothelial cells and pericytes the interaction of the mannose-6-phosphate (M-6-P) moieties on the LAP with the M-6-P/IGF-II Rs present on the cells in the co-culture is necessary for the activation of L-TGF-ß (Dennis and Rifkin, 1991; Bonewald, 1999). In monocytes M-6-P/IGF-II acts directly with the lysine residues of uPAR (Godar et al., 1999). The M-6-P/IGF-II R/uPAR complex then recruits several ligands such as uPA, plasminogen and L-TGF-ß1 (Godar et al., 1999). In this manner M-6-P/IGF-II R regulates uPA mediated conversion of plasminogen to plasmin (Fig. 1). The plasmin generated remains associated with M-6-P/IGF-II R and is protected from inactivation by alpha2-antiplasmin. The cell surface association of L-TGF-ß1 via the M-6-P/IGF-II R would bring the L-TGF-ß spatially closer to the plasminogen/ plasmin system and to a limited surface area on the cell where plasmin is also present (Rifkin Det al., 1999) (Fig. 1). The plasmin bound to M-6-P/IGF-II R is then likely to be more efficient in the release of active TGF-ß1 from L-TGF-ß which is complexed with M-6-P/IGF-II R. It is unknown if cells other than monocytes that express M-6-P/IGF-II R also form complexes with uPAR, uPA, plasminogen and L-TGF-ß1.

The importance of the *in vivo* relevance of activation of L-TGF-ß1 by M-6-P/IGF-II R can be speculated by a number of reported observations. In a rat model of common bile duct ligation there is biliary fibrosis and an increase in expression of M-6-P/IGF-II R and L-TGF-ß1 (Saperstein et al., 1993). These findings suggest that if plasmin were present in the periductal space the L-TGF-ß1 associated with the M-6-P/IGF-II R could be activated and regulate the increase in connective tissue synthesis that was observed in this model. Radiotherapy to the intestine results in mucosal ulceration, inflammation, and intestinal wall fibrosis (Wang et al., 1999). Wang et al. demonstrated that normal intestinal epithelial cells express M-6-P/IGF-II R but after irradiation there is shift of M-6-P/IGF-II R expression to intestinal fibroblasts. The expression of M-6-P/IGF-II R on intestinal fibroblasts corresponds to a time of maximal connective tissue synthesis (Wang et al., 1999). These observations suggest that L-TGF-ß1 that is released by cells in the vicinity of fibroblasts may associate with the M-6-P/IGF-II R on intestinal fibroblasts. In this manner the induction of M-6-P/IGF-II R on the fibroblasts may be involved in the activation of L-TGF-ß1 *in vivo* and the pathogenesis of radiation induced intestinal fibrosis (Wang et al., 1999). Another example for the role of M-6-P/IGF-II R in the activation of L-TGF-ß1 *in vivo* are the findings of Sue et al. (1995). They demonstrated that there was a 58% decrease in expression of M-6-P/IGF-II R mRNA in hepatocellular tumor cells compared to the normal liver surrounding the tumor. Using immunohistochemistry normal liver had homogeneous co-localized staining with M-6-P/IGF-II R and TGF-ß1 (Sue et al., 1995). However, the hepatocellular carcinoma had significantly lower levels of both M-6-P/IGF-II R than the normal surrounding tissue (Sue et al., 1995). Similar findings were observed in phenobarbitol induced hepatic carcinoma (Jirtle et al., 1994). Liver carcinomas induced by phenobarbitol were partially or completely negative for M-6-P/IGF-II R and TGF-ß1 compared to the surrounding normal liver tissue (Jirtle et al., 1994). Since TGF-ß1 is a potent inhibitor of epithelial cell proliferation it is speculated that one of the steps in carcinogenesis may be the loss of the ability of the epithelial cells to release and respond to the antiproliferative effects of TGF-ß1 leading to autonomous proliferation. The findings from these studies suggest that cells unable to activate L-TGF-ß lose the endogenous antiproliferative effect of TGF-ß1 resulting in uncontrolled proliferation.

The necessity for L-TGF-ß1 to associate with the cell membrane has been described in another fibrotic disorder (Yehaulaeshet et al., 1999). Using a rat model of pulmonary fibrosis induced by the antineoplastic antibiotic, bleomycin (Yehaulaeshet et al., 1999), alveolar macrophages obtained 7 days after bleomycin administration released maximal quantities of L-TGF-ß1 associated with TSP-1 (Yehaulaeshet et al., 1999). The TSP-1/L-TGF-ß1 complex was demonstrated to interact with the alveolar macrophage cell membrane by the TSP-1 receptor, CD36 prior to the proteolytic effects of plasmin (Fig. 1). TSP-1 interacts with CD36 between amino acids 93-110 on the ectodomain of CD36 (Lueng et al., 1993). A synthetic peptide mimicking this region of CD36 prevented the activation of L-TGF-ß1 *in vitro* (Yehaulaeshet et al., 1999) and further confirmed that localizing L-TGF-ß to the cell surface is necessary for activation of L-TGF-ß1. It is of interest that in the rat the administration of the CD36 synthetic peptide 93-110 concomitantly with bleomycin compared to bleomycin administration alone resulted in reducing the quantity of active TGF-ß1 released by alveolar macrophages and decreased inflammation and connective tissue synthesis (Yehaulaeshet et al., 2000). These findings suggest that the association of L-TGF-ß to alveolar macrophages *in vivo* may be necessary in generating active TGF-ß1 (Yehaulaeshet et al., 1999, 2000). Lastly Using co-cultures of endothelial cells and pericytes Nunes et al. described that LTBP-1/L-TGF-ß1 association with the cell was also important in activation of L-TGF-ß by plasmin (Nunes et al., 1997). In addition it was also observed that the interaction of LTBP-1/ L-TGF-ß 1 with the ECM required stabilization of cross linking by transglutaminases prior to plasmin mediated release of the L-TGF-ß that was associated with LTBP-1

(Flaumenhaft et al., 1993).

Null mutations of the plasminogen/plasmin system and L-TGF-ß1

The plasminogen/plasmin system has been either demonstrated to directly activate L-TGF-ß in several models or is implied to be important in activating L-TGF-ß1 in an indirect manner. This leads to the puzzling observation that mice that have null mutations for plasminogen, uPA, uPAR, or PAI-1 do not have lesions characteristic of TGF-ß1 null mice (Kulkarni et al., 1993). One could speculate that for physiological functions of TGF-ß adequate quantities of L-TGF-ß are converted to active TGF-ß by another protease or a mechanism independent of proteases. However, it is possible that in the event of an injury these mice may demonstrate an impaired response to repair due to lack of active TGF-ß1. For example, mice deficient in both uPA and tPA developed cutaneous wounds that healed poorly (Herbert and Carmeliet, 1997) and mice deficient in plasminogen expression have inadequate repair of arterial injury (Herbert and Carmeliet, 1997). Unfortunately, none of these studies determined if the impairment was due to a lack of biologically active TGF-ß1. It is of interest that in mice that over expressed PAI-1 which would be expected to result in diminished plasmin production and thus active TGF-ß1 had an increased quantity of collagen after bleomycin administration (Eitzman et al., 1996). Since plasmin degrades collagen (Eitzman et al., 1996) it is possible that the collagen synthesis mediated by other cytokines albeit less than that observed with TGF-ß1 would accumulate in the absence of plasmin. The increase in collagen deposition in these mice could then be independent of the level of active TGF-ß1.

Post translational activation of latent TGF-ß: plasmin independent mechanisms

The activation of L-TGF-ß1 by TSP-1

TSP is a trimeric glycoprotein that exists in 5 isoforms (Lawler et al., 1993). Of these isoforms the RFK sequence of TSP-1 has been described by Murphy-Ullrich's laboratory to interact with the LAP of L-TGF-1 derived from bovine aortic endothelial (BAE) cells (Schultz-Cherry et al., 1995). This interaction leads to a conformational change that exposes the TGF-ß receptor binding site on the L-TGF-ß1 protein resulting in TGF-ß1 that can bind to its receptor without releasing the LAP (Schultz-Cherry et al., 1995; Ribeiro et al., 1999). Unlike the previously discussed mechanisms of activation of L-TGF-ß1, in Murphy-Ullrich's model the activation of L-TGF-ß1 did not require the presence of the plasminogen/plasmin system or cell surface localization. More recently an association of TSP-1 with TGF-ß1 has been described in cells other than bovine endothelial cells or alveolar macrophages (Schultz-Cherry et al., 1995; Ribeiro et al., 1999; Yehaulaeshet et al., 1999). *In vitro* high concentrations of glucose in cultures of renal mesangial cells induces TSP-1 expression (Tada and Isogai, 1998). The induction of TSP-1 by cultures of mesangial cells leads to the release of active TGF-ß1 which in an autocrine manner regulates fibronectin synthesis (Tada and Isogai, 1998). In an animal model of glomerulonephritis where there is an increase in interstitial fibrosis active TGF-ß1 and TSP-1 were co-localized, but not TSP-1 and L-TGF-ß1 (Hugo et al., 1998). The *in vivo* role of TSP-1 in activating L-TGF-ß1 was suggested in congenital hepatic fibrosis (El-Youssef et al., 1999). Using immunohistochemistry TGF-ß1 and TSP-1 were present in perisinusoidal regions in the location of hepatic stellate cells which are the major source of connective tissue proteins in the liver (El-Youssef et al., 1999). One could speculate that the presence of both TSP-1 and L-TGF-ß1 in the hepatic perisinusoidal region or in glomerulonephritis could generate active TGF-ß1and thus regulate increased connective tissue synthesis. The activation of L-TGF-ß1 by TSP-1 in more physiological conditions may also occur. This possibility is supported by the observation that TSP-1 null mice (Crawford et al., 1998) have inflammatory infiltrates in the lungs and pancreas similar to those described in TGF-ß1 null mice (Kulkarni et al., 1993). However, when the TSP-1 null mice were treated systemically with injections of KRFK peptide, a region of TSP-1 responsible for activating L-TGF-ß1 there was significant abrogation of the inflammatory lesions in the lungs and pancreas suggesting that critical regions of the TSP-1 protein activate L-TGF-ß in vivo (Crawford et al., 1998).

Angiotensin II (AngII) activation of L-TGF-ß1

Angiotensin II is an octapeptide and a major effector molecule in the rennin-angiotensin-system (Motojima et al., 1999). Originally, AngII was described to regulate systemic blood pressure by a direct or indirect vasopressive effect (Wolfe and Ziyadeh, 1997). However, more recent findings have demonstrated that AngII promotes cellular proliferation and ECM accumulation in vascular SMC and glomerular mesangial cells leading to glomerulosclerosis and cardiovascular disease (Wolfe and Ziyadeh, 1997). AngII is activated by the actions of angiotensin converting enzyme (ACE) which can be inhibited by a number of ACE inhibitors (Wolfe and Ziyadeh, 1997). AngII is elevated in several models of renal disease where there is evidence of hypertrophy of glomerular and tubular structures, thickening of glomerular and tubular basement membrane that result in obliteration of glomerular capillaries, tubulointerstitial fibrosis, renal arteriosclerosis, and ultimately renal failure (Border and Noble, 1997). AngII induces renal mesangial and renal tubular cells to release active TGF-ß (Kagami et al., 1994; Border and Noble, 1997). The release of TGF-ß1 results in increased connective tissue synthesis by the

mesangial cells in an autocrine fashion (Kagami et al., 1994). Continuous *in vivo* administration of AngII to normal rats increased glomerular mRNA of TGF-ß1 and collagen I (Kagami et al., 1994). These effects of AngII were inactivated by ACE inhibitors, AngII receptor antagonists and antibodies to TGF-ß1 (Kagami et al., 1994; Wolfe and Ziyadeh, 1997). Taken together these observations suggest that AngII induces the release of active TGF-ß which in turn results in induction of connective tissue proteins in the kidney. The mechanisms by which AngII activates L-TGF-ß1 has not been clearly defined. However, since AngII increases plasminogen activator and decreases plasminogen activator inhibitor-1 it could lead to an induction of plasmin and the conversion of L-TGF-ß to its active form (Fogo, 2000). The induction of uPA and plasmin by AngII may occur in the kidney, but this has not yet been described.

Activation of TGF-ß by hyperglycemia

Although AngII has been described to be important in renal fibrosis, it has also been demonstrated that hyperglycemia leads to similar fibrotic lesions in the kidney as described above (Sharma and Ziyadeh, 1995). In the presence of high glucose concentrations cultured mesangial cells (Hoffman et al., 1998), proximal tubular cells (Wolf and Ziyadeh, 1999), and glomerular epithelial cells (van Det et al., 1997) lead to induction of TGF-ß mRNA and the release of biologically active TGF-ß (van Det et al., 1997; Hoffman et al., 1998; Wolf and Ziyadeh, 1999). It is of interest that the renal proximal tubular cells from TGF-ß K/O mice do not release TGF-ß or synthesize collagen in response to hyperglycemia as do their normal counterparts (Wolf and Ziyadeh, 1999). Furthermore, in streptozotocin (STZ) induced diabetic mice there is increase in renal mRNA TGF-ß1 and ECM proteins (Sharma and Ziyadeh, 1995). Treatment of STZ-diabetic mice with insulin reduced renal hypertension, and decreased mRNA expression of TGF-ß, collagen IV and fibronectin synthesis (Sharma and Ziyadeh, 1995). Additionally, in patients with established diabetic nephropathy there is increased expression of TGF-ß protein (Sharma and Ziyadeh, 1995; vanDet et al., 1997; Hoffman et al., 1998; Wolf and Ziyadeh, 1999). Based on the *in vitro* data and animal models of renal fibrosis or glomerulosclerosis one can speculate that hyperglycemia induces active TGF-ß1 release that is then important in the increased expression of ECM proteins seen in lesions of glomerulosclerosis. The mechanism by which hyperglycemia releases active TGF-ß is unknown. Hyperglycemia induces protein kinase C and oxidative metabolites (Struder et al., 1997). In the presence of anti-oxidants such as α-tocopherol, taurine, and N-acetylcysteine there is inhibition of the release of active TGF-ß and TGF-ß mediated synthesis of the ECM in response to hyperglycemia (Struder et al., 1997). These observations would suggest that hyperglycemia may generate reactive oxygen species (ROS) that lead to activation of L-TGF-ß1.

Reactive oxygen species activate L-TGF-ß1

When recombinant native human L-TGF-ß (rL-TGF-ß1) is irradiated with 50-200 Gy of $^{60}Co\gamma$ in iron containing saline there is generation of active TGF-ß1 (Barellos-Hoff et al., 1996). To determine if ROS released by gamma radiation could activate L-TGF-ß, ROS were generated by oxidation of ascorbate catalyzed by Fe^{+3}/EDTA or Ca^{+2} in a solution with rL-TGF-ß1 which resulted in activation of the rL-TGF-ß1 (Barcellos-Hoff et al., 1996). The mechanism by which ROS could activate L-TGF-ß1 may be due to the effects of ROS on the LAP-1. Cysteine and methionine located on the LAP are susceptible to oxidation which could lead to sufficient changes in the LAP-1 that the association of LAP-1 with L-TGF-ß could become very unstable. Such instability could either release TGF-ß1 or undergo conformational change to expose the TGF-ß receptor binding site (Barcellos-Hoff et al., 1996). The biological significance of these findings were confirmed by the same group (Barcellos-Hoff et al., 1994). The immunohistochemical distribution of active and L-TGF-ß1 in mammary gland tissue of mice after irradiation were examined and compared to normal mammary gland tissue (Barcellos-Hoff et al., 1994). The antibody described as anti-LAP-1 detects L-TGF-ß1 while the antibody designated as anti-LC-1-30 recognizes an epitope of TGF-ß1 only when the LAP-1 is not present and thus anti-LC-1-30 recognizes biologically active TGF-ß1 (Barcellos-Hoff et al., 1995). Barcellos-Hoff et. al. demonstrated in normal epithelium that there was little staining with the anti-LC-1-30 antibody yet after irradiation there was prominent staining with the anti-LC antibody and the staining co-localized with collagen (Barcellos-Hoff et al., 1994). These findings are clinically significant because TGF-ß1 is a potent inducer of connective tissue synthesis (Grande, 1997; Letterio and Roberts, 1998; Roberts, 1998; Bonewald, 1999) and it is well recognized that irradiation of human tissue can lead to fibrosis (Barcellos-Hoff et al., 1994). It is then possible that L-TGF-ß1 activated by ROS generated from radiotherapy then induces connective tissue synthesis.

The integrin αvß6 activates L-TGF-ß1

Munger et al. described that the RGD (arginine-glycine-aspartic acid) sequences present in the LAP-1 of L-TGF-ß1 complexes with the integrin αvß6 located on epithelial cells (Munger et al., 1999). Once L-TGF-ß1 interacts with the αvß6 on epithelial cells the ß6 cytoplasmic domain associates with the actin in the cytoskeleton of the cell. This interaction of L-TGF-ß1 with αvß6 leads to a conformational change in the L-TGF-ß1 still anchored to the cell membrane such that the mature TGF-ß1 interacts with the TGF-ß receptor type II located in close vicinity of the L-TGF-ß1-αvß6 complex

(Munger et al., 1999) (Fig. 1). These findings suggest that the presence of L-TGF-ß by epithelial cells that express αvß6 the L-TGF-ß1 can have a biological effect without being released from its association with LAP or requiring a protease (Munger et al., 1999). The association of L-TGF-ß with αvß6 located on epithelial cells may be important *in vivo*. For example, when mice with an αvß6 K/O defect are given bleomycin there was markedly less pulmonary fibrosis than when compared to bleomycin administration to the wild type (Munger et al., 1999). Another example of a role for αvß6 in regulating L-TGF-ß is in cutaneous wounding. One of the most important events in cutaneous wound closure is the formation of an epithelial tongue of migrating keratinocytes at the edge of the wound (Zambruno et al., 1995). The keratinocytes at the wound edge are highly migratory, do not proliferate and express αvß6 (Zambruno et al., 1995). TGF-ß is present in increased quantities in the wounds and induces αvß6 expression by keratinocytes (Zambruno et al., 1995). TGF-ß enhances motility of cells and is antiproliferative to epithelial cells suggesting that TGF-ß which is abundant at sites of wounding could associate with αvß6 on migrating keratinocytes, become activated, and induce migration of these cells while maintaining their low proliferative index.

Mechanical stretching activates L-TGF-ß

In normal renal function mesangial cells serve as part of a support structure for the glomerular loop (Ingram et al., 1999). *in vivo* mesangial cells are consistently exposed to cyclic stretch and relaxation (Ingram et al., 1999). The stretching of mesangial cells is particularly likely to occur in situations of glomerular hypertension which is associated with glomerulosclerosis. Explanted mesangial cells or those obtained from a cell line when exposed to mechanical stretching were induced to increase the expression of TGF-ß on the surface of the mesangial cell (Hirakata et al., 1997) and release active TGF-ß into the conditional media (Ingram et al., 1999). The release of active TGF-ß by the mesangial cells in an autocrine manner led to connective tissue synthesis (Ingram et al., 1999). The mechanism by which stretching induces TGF-ß is not well understood, but stretching induces protein kinase C (PKC) and inhibitors of PKC can lead to attenuation of the stretch induced mediated induction of TGF-ß (Hirakata et al., 1997). However, it remains unclear how stretching of mesangial cells leads to the increased release of active TGF-ß. One can speculate that since vascular smooth muscle injury leads to induction of plasmin generation by an undefined mechanism it is conceivable that stretching of the mesangial cells may be important in the generation of plasmin which would lead to the activation of L-TGF-ß. There is another suggestion of how stretching may lead to the release of L-TGF-ß. Stretching deformity of mesangial cells also induces the release of LTBP-1 (Hori et al., 1998) which has been reported to localize TGF-ß to the cell surface prior to plasmin mediated activation of L-TGF-ß (Flaumenhaft et al., 1993). It is then conceivable that the process of stretching may mediate the activation of L-TGF-ß by induction of LTBP-1.

Summary and conclusion

Even though many observations support the importance of the plasminogen/plasmin system for activation of L-TGF-ß1 *in vitro* and *in vivo* there is sufficient evidence to demonstrate that one universal mechanism of activation of L-TGF-ß is unlikely. Just as the biological functions of TGF-ß are highly context dependent it seems probable that the activation of L-TGF-ß is also very context dependent. For example in the liver activation of L-TGF-ß1 seems to be supported by data suggesting that the expression of M-6-P/IGF-II R has a major role in L-TGF-ß1 activation (Saperstein et al., 1993; Jirtle et al., 1994; Sue et al., 1995; Godar et al., 1999). In the kidney AngII, mechanical stretching, TSP-1 and hyperglycemia appear to activate L-TGF-ß1 (Kagamami et al., 1994; Sharma and Ziyadeh, 1995; Border and Noble, 1997; van Det, 1997; Struder et al., 1997; Wolf and Ziyadeh, 1997, 1999; Hoffman et al., 1998; Motojima et al., 1999). In the lung the expression of cell surface adhesion proteins like αvß6 and CD36 have been demonstrated to be necessary for the activation of L-TGF-ß1 (Munger et al., 1999; Yehaulaeshet et al., 1999). Whereas, the activation of L-TGF-ß1 by TSP-1 and ROS may not be organ specific. It is also possible that during the course of repair by fibrosis or in disorders characterized by enhanced connective tissue synthesis that more than one mechanism of activation of L-TGF-ß1 may be involved. For example early in the course of a fibrotic disorder infiltrating macrophages may be a prominent source of active TGF-ß1 (Yehaulaeshet et al., 1999). In this early stage the activation may involve the CD36-TSP-1/L-TGF-ß1 or L-TGF-ß1/M-6-P-IGF-II R complexes and plasmin (Godar et al., 1999; Yehaulaeshet et al., 1999). However, later in the course of the disease structural cells such as epithelial or endothelial cells may be important in activation of L-TGF-ß by the interaction of L-TGF-ß1 with M-6-P/IGF-II R (Sato et al., 1990) or αvß6 and be independent of plasmin (Munger et al., 1999). Currently a number of progressive diseases have been associated with the aberrant expression of TGF-ß1 (Border and Noble, 1994). Some examples are idiopathic pulmonary fibrosis, scleroderma, cirrhosis of the liver, glomerulosclerosis, and post-lung transplant bronchiolitis obliterans (Border and Noble, 1994; Bergman et al., 1998). These diseases rarely if at all respond to the standard therapy using immuno-suppressive agents. Understanding the mechanisms of activation of L-TGF-ß1 in these disease processes may then direct the development of therapeutic agents that could retard or prevent further progression of fibrosis resulting in reduced morbidity and mortality.

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