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(54) Title: METHODS FOR TREATING CONDITIONS ASSOCIATED WITH THE ACCUMULATION OF EXCESS EXTRACELLULAR MATRIX

(57) Abstract: The present invention is methods and compositions for reducing and preventing the excess accumulation of extracellular matrix in a tissue and/or organ or at a wound site using a combination of agents that inhibit TGFβ, or using agents that inhibit TGFβ, or using agents that inhibit TGFβ in combination with agents that degrade excess accumulated extracellular matrix, or at least one agent that degrades excess accumulated extracellular matrix. The compositions and methods of the invention are used to treat conditions such as fibrotic diseases and scarring that result from excess accumulation of extracellular matrix, impairing tissue or organ function or skin appearance in a subject.

METHODS FOR TREATING CONDITIONS ASSOCIATED WITH THE ACCUMULATION OF EXCESS EXTRACELLULAR MATRIX

By, Nancy A. Noble and Wayne A. Border

FIELD OF THE INVENTION

This invention relates to a method for preventing or reducing excess accumulation of extracellular matrix in tissues or organs or at a wound site, and more particularly to the prevention and treatment of conditions resulting from excess accumulation of extracellular matrix, using a combination of agents that inhibit TGFβ, or a combination of agents that inhibit TGFβ and agents that degrade excess accumulated extracellular matrix.

Throughout this application, various publications are referenced within parentheses. The disclosures of these publications are hereby incorporated by reference herein in their entireties.

GOVERNMENT RIGHTS

The work described here was supported, at least in part, by grants from the National Institutes of Health Grants DK 49374 (5R01DK49374), DK 43609 (2R37DK043609) and DK 60508. The United States government may, therefore, have certain rights in the invention. Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

BACKGROUND OF THE INVENTION

Excess deposition and accumulation of extracellular matrix (ECM) is found in diseases such as fibrosis of the kidney or lung. Although the cytokine transforming growth factor Beta (TGFβ) regulates extracellular matrix deposition for tissue repair, overproduction of TGFβ clearly underlies tissue fibrosis caused by excess deposition of extracellular matrix resulting in disease (Border and Ruoslahti, *J. Clin. Invest.* 90:1-7 (1992)). TGFβ's fibrogenic action results from simultaneous stimulation of matrix protein synthesis (Border et al., *Kidney Int* 37:689-695 (1990), inhibition of matrix degradation and turnover and enhanced cell-matrix interactions through modulation of integrin receptors that facilitate ECM assembly. Overproduction of TGFβ has been demonstrated in glomerulonephritis (Okuda et al., *J. Clin. Invest.* 86:453-462 (1990)), diabetic nephropathy and hypertensive glomerular injury and in related fibrotic disorders of the lung, liver, heart, arterial wall, skin, brain, joints and bone marrow (Border and Noble, *N. Eng. J. Med.* 331:1286-1292 (1994)). In addition to the kidney, blocking the action of TGFβ with an agent such as antibody or the proteoglycan decorin has been shown to be therapeutic in fibrosis and scarring of the skin, lung, central nervous system and arterial wall (Border and Noble, *Kidney Int.* 51:1388-1396 (1997)).

Suppression of the production of ECM and prevention of excess accumulation of mesangial matrix in glomeruli of glomerulonephritic rats has been demonstrated by intravenous administration of neutralizing antibodies specific for TGFβ (Border et al., *Nature* 346:371-374 (1990)) or administration of purified decorin, a proteoglycan (Border et al., *Nature* 360:361-364 (1992)) and by introduction of nucleic acid encoding decorin, a TGFβ-inhibitory agent, into a rat model of acute mesangial glomerulonephritis (Isaka et al., *Nature Med.* 2:418-423 (1996)). Inhibition of TGFβ activity, using for example anti-TGFβ antibodies, has been shown to to disrupt TGFβ overproduction (Sharma et al., *Diabetes* 45:522-530 (1996)).

Dermal scarring following dermal injury results from excessive accumulation of fibrous tissue made up of collagen, fibronectin and proteoglycans at a wound site.

Because the fibrous extracellular matrix lacks elasticity, scar tissue can impair essential tissue function as well as result in an undesirable cosmetic appearance. TGFβ is believed to induce the deposition of fibrous matrix at the wound site (Shah et al., *Lancet* 339:213-214 (1992)).

One explanation for persistent TGFβ overexpression in progressive fibrotic kidney disease is that repeated or multiple episodes of tissue injury, such as occurs in chronic diseases such as hypertension, diabetes or immune complex disease lead to continuous overproduction of TGFβ and extracellular matrix resulting in tissue fibrosis (See Border and Noble, *N. Eng. J. Med.* 331:1286-1292 (1994)). Another possible explanation for persistent TGFβ overexpression is the presence of a biologically complex interconnection between TGFβ and the renin-angiotensin system (RAS) in the kidney as part of an emergency system that responds to the threat of tissue injury as discussed further herein.

Renin is an aspartyl proteinase synthesized by juxtaglomerular kidney cells and mesangial cells in humans and rats. (Chansel et al., *Am. J. Physiol.* 252:F32-F38 (1987) and Dzau and Kreisberg, *J. Cardiovasc. Pharmacol.* 8(Suppl 10):S6-S10 (1986)). Renin plays a key role in the regulation of blood pressure and salt balance. Its major source in humans is the kidney where it is initially produced as preprorenin. Signal peptide processing and glycosylation are followed by secretion of prorenin and its enzymatically active form, mature renin. The active enzyme triggers a proteolytic cascade by cleaving angiotensinogen to generate angiotensin I, which is in turn converted to the vasoactive hormone angiotensin II by angiotensin converting enzyme ("ACE").

The sequence of the human renin gene is known (GenBank entry M26901). Recombinant human renin has been synthesized and expressed in various expression systems (Sielecki et al., *Science* 243:1346-1351 (1988), Mathews et al., *Protein Expression and Purification* 7:81-91 (1996)). Inhibitors of renin's enzymatic site are known (Rahuel et al., *J. Struct. Biol.* 107:227-236 (1991); Badasso et al., *J. Mol. Biol.* 223:447-453 (1992); and Dhanaraj et al., *Nature* 357:466-472 (1992)) including an orally

active renin inhibitor in primates, Ro 42-5892 (Fischli et al., *Hypertension* 18:22-31 (1991)). Renin-binding proteins and a cell surface renin receptor on human mesangial cells have been identified (Campbell and Valentijn, *J. Hypertens.* 12:879-890 (1994), Nguyen et al., *Kidney Internat.* 50:1897-1903 (1996) and Sealey et al., *Amer. J. Hyper.* 9:491-502 (1996)).

The renin-angiotensin system (RAS) is a prototypical systemic endocrine network whose actions in the kidney and adrenal glands regulate blood pressure, intravascular volume and electrolyte balance. In contrast, TGFβ is considered to be a prototypical cytokine, a peptide signaling molecule whose multiple actions on cells are mediated in a local or paracrine manner. Recent data however, indicate that there is an intact RAS in many tissues whose actions are entirely paracrine and TGFβ has wide-ranging systemic (endocrine) effects. Moreover, RAS and TGFβ act at various points to regulate the actions of one another.

In a systemic response to an injury such as a wound, the RAS rapidly generates AII that acts by vasoconstriction to maintain blood pressure and later stimulates the secretion of aldosterone, resulting in an increase in intravascular volume. In the wound, TGFβ is rapidly released by degranulating platelets and causes a number of effects including: 1) autoinduction of the production of TGFβ by local cells to amplify biological effects; 2) chemoattraction of monocyte/macrophages that debride and sterilize the wound and fibroblasts that begin synthesis of ECM; 3) causing deposition of new ECM by simultaneously stimulating the synthesis of new ECM, inhibiting the proteases that degrade matrix and modulating the numbers of integrin receptors to facilitate cell adhesion to the newly assembled matrix; 4) suppressing the proinflammatory effects of interleukin-1 and tumor necrosis factor; 5) regulating the action of platelet derived growth factor and fibroblast growth factor so that cell proliferation and angiogenesis are coordinated with matrix deposition; and 6) terminating the process when repair is complete and the wound is closed (Border and Noble, *Scientific Amer. Sci. & Med.* 2:68-77 (1995)).

Interactions between RAS and TGFβ occur at both the systemic and molecular level. It has been shown that the action of TGFβ in causing ECM deposition in a healing wound, is the same action that makes TGFβ a powerful fibrogenic cytokine. (Border and Noble, *New Engl. J. Med.* 331:1286-1292 (1994); and Border and Ruoslahti, *J. Clin. Invest.* 90:107 (1992)). Indeed, it is the failure to terminate the production of TGFβ that distinguishes normal tissue repair from fibrotic disease. RAS and TGFβ co-regulate each other's expression. Thus, both systems may remain active long after an emergency response has been terminated, which can lead to progressive fibrosis. The kidney is particularly susceptible to overexpression of TGFβ. The interrelationship of RAS and TGFβ may explain the susceptibility of the kidney to TGFβ overexpression and why pharmacologic suppression of RAS or inhibition of TGFβ are both therapeutic in fibrotic diseases of the kidney. (Noble and Border, *Sem. Nephrol.*, supra and Border and Noble, *Kidney Int.* 51:1388-1396 (1997)).

Activation of RAS and generation of angiotensin II (AII) are known to play a role in the pathogenesis of hypertension and renal and cardiac fibrosis. TGFβ has been shown to be a powerful fibrogenic cytokine, acting simultaneously to stimulate the synthesis of ECM, inhibit the action of proteases that degrade ECM and increasing the expression of cell surface integrins that interact with matrix components. Through these effects, TGFβ rapidly causes the deposition of excess ECM. AII infusion strongly stimulates the production and activation of TGFβ in the kidney. (Kagami et al., *J. Clin. Invest.* 93:2431-2437 (1994)). Angiotensin II also upregulates TGFβ production and increases activation when added to cultured vascular smooth muscle cells (Gibbons et al, *J. Clin. Invest.* 90:456-461 (1992)) and this increase is independent of pressure (Kagami et al., supra). AII also upregulates TGFβ receptors, even in the presence of exogenously added TGFβ which normally down-regulates its own receptors, leading to enhanced TGFβ signalling and enhanced fibronectin production (Kanai et al., *J. Am. Soc. Nephrol.* 8:518A (1997)). Blockade of AII reduces TGFβ overexpression in kidney and heart, and it is thought that TGFβ mediates renal and cardiac fibrosis associated with activation of RAS (Noble and Border, *Sem. Nephrol.* 17(5):455-466 (1997)), Peters et al., *Kidney International* 54 (1998)). Blockade of AII using inhibitors of ACE slow the progression of renal fibrotic

disease (see, e.g., Anderson et al., *J. Clin. Invest.* 76:612-619 (1985) and Noble and Border, *Sem. Nephrol.* 17(5):455-466 (1997)). What is not clear is whether angiotensin blockade reduces fibrosis solely through controlling glomerular hypertension and thereby glomerular injury, or whether pressure-independent as well as pressure-dependent mechanisms are operating. While ACE inhibitors and AII receptor antagonists have been shown to slow the progress of fibrotic diseases, they do not halt disease and TGFβ levels remain somewhat elevated. (Peters et al., supra).

Thus, RAS and TGFβ can be viewed as powerful effector molecules that interact to preserve systemic and tissue homeostasis. The response to an emergency such as tissue injury is that RAS and TGFβ become activated. Continued activation may result in chronic hypertension and progressive tissue fibrosis leading to organ failure. Because of the interplay between the RAS and TGFβ, and the effects of this interplay on tissue homeostasis, blockade of the RAS may be suboptimal to prevent or treat progressive fibrotic diseases such as diabetic nephropathy.

Components of the renin-angiotensin system act to further stimulate production of TGFβ and plasminogen activator inhibitor leading to rapid ECM accumulation. The protective effect of inhibition of the renin-angiotensin system in experimental and human kidney diseases correlates with the suppression of TGFβ production.(Noble and Border, *Sem. Nephrol.*, supra; and Peters et al., supra).

The renin molecule has been shown to enzymatically cleave angiotensinogen into Angiotensin I. The angiotensin I is then converted by Angiotensin Converting Enzyme ("ACE") to Angiotensin II which acts as an active metabolite and induces TGFβ production. Angiotensin II is an important modulator of systemic blood pressure. It has been thought that if you decrease hypertension by blocking AII's vasoconstrictor effects fibrotic disease is reduced.

In the glomerular endothelium, activation of RAS and TGFβ have been shown to play a role in the pathogenesis of glomerulonephritis and hypertensive injury. Volume

(water) depletion and restriction of potassium have been shown to stimulate both production of renin and TGFβ in the juxtaglomerular apparatus (JGA) of the kidney (Horikoshi et al., *J. Clin. Invest.* 88:2117-2122 (1992) and Ray et al., *Kidney Int.* 44:1006-1013 (1993)). Angiotensin blockade has also been shown to increase the production of renin. TGFβ has been shown to stimulate the release of renin from kidney cortical slices and cultured JG cells (Antonipillai et al., *Am. J. Physiol.* 265:F537-F541 (1993); Ray et al., *Contrib. Nephrol.* 118:238-248 (1996) and Veniant et al., *J. Clin. Invest.* 98:1996-19970 (1996)), suggesting that renin and TGFβ are coregulated. Other interactions between RAS and TGFβ include that AII induces the production of TGFβ in cultured cells and in vivo (Kagami et al., supra) and AII regulates expression of TGFβ receptors (Kanai et al., 1977, supra). It is thus likely that the fibrogenic effects that have been attributed to AII are actually mediated by TGFβ.

Another interplay between RAS and TGFβ is with the production of aldosterone. Aldosterone overproduction has been linked to hypertension and glomerulosclerosis. AII stimulates the production and release of aldosterone from the adrenal gland. In contrast, TGFβ suppresses aldosterone production and blocks the ability of AII to stimulate aldosterone by reducing the number of AII receptors expressed in the adrenal (Gupta et al., *Endocrinol.* 131:631-636 (1992)), and blocks the effects of aldosterone on sodium reabsorption in cultured renal collecting duct cells (Husted et al., *Am. J. Physiol. Renal, Fluid Electrolyte Physiol.* 267:F767-F775 (1994)). Aldosterone may have fibrogenic effects independent of AII, and may upregulate TGFβ expression. The mechanism of aldosterone's pathological effects is unknown but might be due to stimulation of TGFβ production in the kidney (Greene et al., *J. Clin. Invest.* 98:1063-1068 (1996)).

Prorenin or renin may have AII-independent actions to increase fibrotic disease. Prorenin overexpressing rats were found to be normotensive but to develop severe glomerulosclerosis (Veniant et al., *J. Clin. Invest.* 98:1996-1970 (1996)).

Human recombinant renin added to human mesangial cells induces marked upregulation of production of plasminogen activator inhibitors (e.g. PAI-1 and PAI-2)

which block the generation of plasmin, a fibrinolytic enzyme important in the dissolution of clots after wounding generated from plasminogen by two enzymes called plasminogen activators, urokinase (u-PA) and tissue plasminogen activator (t-PA). PAI-1 and 2 regulate U-PA and t-PA in turn. Plasmin appears to be a key mediator of extracellular matrix degradation, carrying out at least three functions important to extracellular matrix degradation. Plasmin directly degrades proteoglycan components of extracellular matrix, proteolytically activates metalloproteinases (MMPs) that, in turn, degrade collagens and other matrix proteins, and enzymatically inactivates tissue inhibitors of MMPs (TIMPs), releasing MMPs from inhibition of TIMPs, allowing them to proteolytically digest matrix proteins. (Baricos et al., *Kidney Int'l.* 47:1039-1047 (1995); Baricos et al., *J. Amer. Soc. Nephrol.* 10:790-795 (1999)). The net generation of active plasmin from the inactive precursor plasminogen results from a balance of the plasminogen activators and PAI-1 and 2, and other factors. PAI-1 binds to vitronectin. (Lawrence et al., *J. Biol. Chem.* 272:7676-7680 (1997)). Mutant PAI-1 molecules have been developed that have enhanced properties for PAI-1 binding to vitronectin molecules, but do not inhibit either t-PA or u-PA activity, resulting in an increase in the amount of the active form of plasmin. (See, WO 97/39028, Lawrence et al.). PAI-1 is increased in response to added TGFβ (Tomooka et al., *Kidney Int.* 42:1462-1469 (1992)).

It has been suggested that TGFβ enhances release of renin from storage granules in the juxtaglomerular apparatus of the kidney (Antonipillai et al., *Am. J. Physiol.* 265:F537-F541 (1993) and Ray et al., *Contrib. Nephrol.* 118:238-248 (1996)).

Thus, the interactions of RAS and TGFβ production form a complex system which impacts fibrotic ECM accumulation and the incidence of fibrotic disease. Various RAS components such as aldosterone, prorenin and renin may be connected with TGFβ production and fibrotic ECM accumulation. Any successful therapeutic regime must take into account these complex relationships to optimize inhibition of TGFβ to prevent and/or reduce ECM accumulation.

The multiple pathways resulting in TGFβ overexpression and fibrosis proposed from in vitro studies are depicted in Figure 1. (See, Kagami et al., *J. Clin. Invest.* 93:2431-2437 (1994); Gibbons et al., *J. Clin. Invest.* 90:456-461 (1992); Abboud, *Kidney Int.* 41:581-583 (1992); Ruiz-Ortega et al., *J. Am. Soc. Nephrol.* 5:683 (1994) abstract; Kim et al., *J. Biol. Chem.* 267:13702-13707 (1992); Ohno et al., *J. Clin. Invest.* 95:1363-1369 (1995); Riser et al, *J. Clin. Invest.* 90:1932-1943 (1992); Riser et al., *J. Am. Soc. Nephrol.* 4:663 (1993); Ziyadeh et al., *J. Clin. Invest.* 93:536-542 (1994); Rocco et al., *Kidney Int.* 41:107-114 (1992); Flaumenhaft et al., *Advan. Pharmacol.* 24:51-76 (1993); Lopez-Armanda et al., *J. Am. Soc. Nepbrol.* 5:812 (1994) abstract; Sahai et al., *J. Am. Soc. Nephrol.* 6:910 (1995); Remuzzi et al., *Kidney Int.* 1:2-15 (1997); and Remuzzi et al., *J. Am. Soc. Nephrol.* 9:1321-1332 (1998)). This diagram shows that a large number of factors implicated in kidney injury are believed to increase the production of TGFβ.

In fibrotic diseases overproduction of TGFβ results in excess accumulation of extracellular matrix which leads to tissue fibrosis and eventually organ failure. Accumulation of mesangial matrix is a histological indication of progressive glomerular diseases that lead to glomerulosclerosis and end-stage kidney disease (Klahr et al., *N. Engl. J. Med.* 318:1657-1666 (1988); Kashgarian and Sterzel, *Kidney Int.* 41:524-529 (1992)). Rats injected with antithymocyte serum are an accepted model of human glomerulonephritis and this model has demonstrated that overproduction of glomerular TGFβ can underlie the development of glomerulosclerosis (Okuda et al., *J. Clin. Invest.* 86:453-462 (1990); Border et al., *Nature (Lond.)* 346:371-374 (1990); Kagami et al., *Lab. Invest.* 69:68-76 (1993); and Isaka et al., *J. Clin. Invest.* 92:2597-2602 (1993)). Using cultured rat mesangial cells where the effects of Angiotensin II on glomerular pressure are not a factor, Angiotensin II has been shown to induce TGFβ production and secretion by mesangial cells, and this in turn has been shown to stimulate extracellular matrix production and deposition (Kagami et al., *J. Clin. Invest.* 93:2431-2437 (1994)). Increases in PAI-1 levels result in decreased degradation of extracellular matrix (Baricos et al., *Kidney Int.* 47:1039-1047 (1995)). Increases in TGFβ result in increased PAI-1 levels (Tomooka et al., *Kidney Int.* 42:1462-1469 (1992)). It has been demonstrated that decreasing TGFβ overexpression in a rat model of glomerulonephritis by in vivo injection

of neutralizing antibodies to TGFβ, reduces TGFβ overexpression (Border et al., *Nature* 346:371-374 (1990)), and reduces PAI-1 deposition into the pathological matrix (Tomooka et al., *Kidney Int.* 42:1462-1469 (1992)). Therefore, decreases in TGFβ levels should result in decreased PAI-1 levels and increased degradation of extracellular matrix to ameliorate organ impairment and fibrotic disease. However, patients present with fibrotic disease that is well advanced in terms of build-up of extra-cellular matrix (ECM). This is because abnormal organ function is undetectable until ECM accumulation is very advanced. For example, in the kidney, standard diagnostic tests do not provide an abnormal reading until about fifty percent of organ function has been lost.

The treatment of conditions associated with excess accumulation of ECM has also focused on decreasing stimuli to disease such as to lower blood pressure or, in the case of diabetic nephropathy to reduce plasma glucose levels. For example, current therapies for treating fibrotic disease in the kidney are limited to AII blockade using ACE inhibitors such as Enalapril or AII receptor antagonists such as Losartan. In addition, patients are encouraged to follow low protein diets since this regimen has some therapeutic value (Rosenberg et al., *J. Clin. Invest.* 85:1144-1149 (1992)). These therapies, at best, prolong organ function by only 1-2 years. This may be because of the multiple pathways that result in TGFβ overexpression or enhanced activity. Moreover, it is likely that current therapeutic strategies to reduce TGFβ overproduction may lead to upregulation of other pathways resulting in continued TGFβ overproduction. For example, when the action of AII is blocked, renin is upregulated which itself increases TGFβ production (see co-pending U.S. patent application, U.S. Serial No. 09/005,255, incorporated in its entirety herein). More recently, treatments aimed to halt the overproduction of TGFβ have been proposed (Border and Noble, *Kidney Internatl.* 54 (1998); and Peters et al., *Kidney Internatl.* 54 (1998)).

Therefore, the most promising therapeutic methods will need to increase ECM degradation to restore organ function as well as decrease TGFβ overproduction and/or activity. Enhanced degradation of excess accumulated ECM can be used to optimize overall reduction in levels of accumulated ECM to restore function to tissues and organs.

Proteases that are able to degrade ECM are known. For example, the serine protease plasmin degrades ECM proteins and activates pro-metalloproteinases, in addition to degrading fibrin (Baricos et al., supra). One goal of therapeutic intervention to increase ECM degradation for treating fibrosis could be increasing plasmin in the region of excess ECM deposition.

There is a need for improved therapies to normalize TGFβ production, that take into account the multiple pathways that stimulate TGFβ production, to prevent or reduce excess accumulation of ECM, to restore function to tissues and organs in which excess ECM has accumulated and/or to reduce scar formation at a wound site.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides methods for preventing or reducing the excess accumulation of extracellular matrix (ECM) associated with fibrotic conditions by inhibiting TGFβ, using a combination of agents that inhibit TGFβ, or by using a combination of agents to inhibit TGFβ and agents that cause the enhanced degradation of excess accumulated ECM.

The methods of the invention contemplate the use of agents that directly or indirectly inhibit TGFβ including direct inhibitors of TGFβ activity such as anti-TGFβ antibodies, proteoglycans such as decorin and ligands for TGFβ receptors, and/or indirect TGFβ inhibitors including aldosterone, inhibitors of aldosterone, inhibitors of angiotensin II, renin inhibitors, ACE inhibitors and AII receptor antagonists which act to decrease TGFβ production.

The methods of the invention also contemplate the use of agents that result in the enhanced degradation of excess accumulated matrix including proteases such as serine proteases including plasmin, metalloproteases, or protease combinations, and agents such as tPA, and PAI-1 mutants that increase the production and/or the activity of proteases such as plasmin

The agents for use in the methods of the invention may be administered as inhibitory compounds in pharmaceutical formulations or as nucleic acid encoding the inhibitors delivered to suitable host cells. The nucleic acid may be directly introduced into a cell in vivo, for example into muscle tissue, or may be first introduced into a cell ex vivo to obtain a cell expressing the inhibitory agent or agents, and the cell then transplanted or grafted into a subject to inhibit or reduce excess accumulation of extracellular matrix.

The invention includes compositions for preventing or reducing the excess accumulation of ECM containing a combination of agents for inhibiting TGFβ or a combination of agents for inhibiting TGFβ and for degrading ECM.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram depicting various pathways resulting in increased TGFβ production.

Figure 2 is a bar graph showing increases in TGFβ production by cultured human mesangial cells in response to renin, as described in Example I, infra,

Figure 3 is a bar graph showing the effect of blocking agents on TGFβ-production by human mesangial cells in response to renin, as described in Example II, infra.

Figure 4A and B are bar graphs showing dose dependent increases in TGFβ (Figure 4A) and Fn production (Figure 4B) with increases in HrRenin as described in Example IV, infra.

Figure 5A and B are bar graphs showing time courses of TGFβ (Figure 5A) and Fn production (Figure 5B) as described in Example IV, infra.

Figure 6A-C are bar graphs showing renin-induced increases in TGFβ, PAI-1 and

Fn mRNAs over time as described in Example IV, infra.

Figure 7 is a bar graph showing the results of inhibitors that block renin's action to increase Angiotensin II, on the renin-induced increase in TGFβ production in adult human mesangial cells as described in Example IV, infra.

Figure 8A and B are photographs depicting the effects of tPA treatment on ECM accumulation in glomeruli as described in Example V, infra.

Figure 9A-D are bar graphs depicting the effects of tPA treatment on amounts of ECM constituents (9A: FN EDA+; 9B:Laminin; 9C:Collagen I and 9D:Collagen IV) as determined by staining, as described in Example V, infra.

Figure 10 is a bar graph showing the effects of tPA on glomerular mRNA expression at day 6, as described in Example V, infra.

Figure 11A and B are bar graphs showing the effects of tPA treatment on glomerular plasmin activity, as described in Example V, infra.

Figure 12 is a bar graph demonstrating that injection of PAI-1 mutant results in increases in plasmin generation of nephritic glomeruli, as described in Example VII, infra.

Figure 13 is a bar graph demonstrating decreased accumulation of Collagen type I after administration of PAI-1 mutant, as described in Example VII, infra.

Figure 14 shows the effect of increasing doses of 1D11, enalapril and combinations of 1D11 and enalapril on the development of glomerulosclerosis (A, normal rats, B disease control rats, C, .01 mg/kg dose of 1D11, D, 0.1 mg/kg dose of 1D11, E, 0.5 mg/kg dose of 1D11, F, 5.0 mg/kg dose of 1D11, G, and enalapril, H, Enal + 0.5 mg/kg 1D11, and I, Enal + 5 mg/kg 1D11, as described in Example IX, infra.

Figure 15 shows the effects of increasing doses of 1D11, enalapril and combinations of 1D11 and enalapril on Matrix score (A), and immunohistochemical staining for fibronectin EDA+ (B), and collagen I (C), as described in Example IX, infra.

Figure 16 is immunofluorence micrographs of fibronectin EDA+, A, normal glomeruli, B, untreated rats, with treatment of 1D11 at C, 0.01 mg/kg, D, 0.1 mg/kg, E, 0.5 mg/kg, F, 5 mg/kg, and G, and enalapril, and combinations of H, Enal + 0.5 mg/kg 1D11, and I Enal + 5 mg/kg, as described in Example IX, infra.

Figure 17 is immunofluorence micrographs of Type I collagen in normal glomeruli (A), untreated disease control rats (B), and using 1D11 treatments at doses of C, 0.01 mg/kg, D, 0.1 mg/kg, E, 0.5 mg/kg, F, 5 mg/kg and G, and enalapril, and H, combination of Enal + 0.5 mg/kg 1D11, and I, combination of Enal + 5 mg/kg 1D11, as described in Example IX, infra.

Figure 18 shows the effects of increasing does of 1D11, enalapril and combinations of 1D11 and enalapril on glomerular production of fibronectin (A), PAI-1(B), and TGF-ß1 (C), as described in Example IX, infra.

Figure 19 is a representative Northern blot showing glomerular mRNA expression of TGF-ß1, PAI-1 collagen I and fibronectin, harvested six days after induction of glomerulonephritis, as described in Example IX, infra.

Figure 20 shows the effects of increasing doses of 1D11, enalapril and combinations of 1D11 and enalapril on glomerular mRNA levels for fibronectin (A), PAI-1 (B), collagen 1 (C) and TGF-ß1 (D), as described in Example IX, infra.

Figure 21 shows the effects of increasing doses of 1D11, enalapril and combinations of 1D11 and enalapril on immunofluorescent staining for the monocyte/macrophage marker ED1, as described in Example IX, infra.

Figure 22 shows the effects of increasing doses of 1D11, enalapril and combinations of 1D11 and enalapril on glomerular p-smad2 protein, as determined by Western blotting, as described in Example IX, infra.

Figure 23 is Table 2 showing a determination in rats of the effective dose range of 1D11 antibody in anti-Thy 1 glomerulonephritis, as described in Example VIII, infra.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery that a combination of strategies may be warranted to prevent or treat conditions associated with the excess accumulation of extracellular matrix in tissues or organs, including fibrotic diseases and scarring resulting from TGFβ overproduction and/or activity. As previously reported, TGFβ overproduction may result from multiple pathways and require that more than one pathway be inhibited to achieve any clinically significant reduction in excess accumulation of extracellular matrix and amelioration of disease. For example, as disclosed in co-pending U.S. patent application, U.S. Serial No. 09/005,255, incorporated in its entirety herein, renin stimulates TGFβ production in cells capable of producing TGFβ, in an angiotensin-II and blood pressure-independent manner.

Optimal therapy of disorders associated with excess accumulation of ECM which causes organ impairment and ultimately failure, must take into account the multiple pathways of TGFβ production to effectively combat overproduction of TGFβ. Without such multifactorial strategy, inhibition of one pathway of TGFβ production may be insufficient to block excess accumulation of extracellular matrix and can even result in an increase in the levels of TGFβ production by stimulation of one of the alternative pathways for its production.

While it is now known that multiple stimuli result in TGFβ overexpression and resulting excess accumulation of ECM, therapeutic strategies directly inhibiting TGFβ,

such as the use of anti-TGFβ antibodies or TGFβ receptor antagonists, are being explored. However, because TGFβ has many beneficial actions such as immunosuppressive and immunomodulatory effects, as well as inhibition of epithelial cell growth which retards carcinogenesis (Markowitz, *Science* 268:1336-1338 (1995) and suppression of atherogenesis (Grainger et al., *Nature Med.* 1:74-79 (1995), these therapies may have unacceptable side-effects if administered at doses high enough to successfully stem fibrotic conditions. This has been shown in the TGFβ1 null (knockout) mice which die of overwhelming inflammation at about 6 weeks of age (Letterio et al., *Science* 264:1936-1938 (1994); Kulkami et al, *Proc. Natl. Acad. Sci. USA* 90:770-774 (1993) and Shull et al., *Nature* 359:693-699 (1992)), indicating that TGFβ1 has significant beneficial roles in immune function. Multiple agents, inhibiting TGFβ directly, and/or inhibiting the disease-specific stimuli underlying TGFβ overexpression and/or activity, for example high glucose resulting from diabetes, may be required to adequately reduce TGFβ-associated excess accumulation of ECM, without causing harmful side-effects. Accordingly, it is a goal of the methods of the present invention to accomplish normalization of TGF production without harmful side effects and to prevent or reduce excess accumulation of ECM and ensuing fibrotic conditions.

In addition, degradation of accumulated ECM may be needed to restore tissue or organ function that has been compromised by the presence of the excess accumulated ECM. Prevention or degradation of excess accumulated ECM can also prevent or reduce scar formation at the site of a wound.

The methods of the invention include using multiple agents to reduce the overproduction and/or activity of TGFβ and/or to block alternative pathways of TGFβ production to prevent or reduce excess accumulation of ECM. The methods of the invention further include the use of a combination of agents to reduce TGFβ overproduction and/or activity in combination with agents to enhance the degradation of excess, accumulated ECM. The methods are useful to prevent or reduce excess accumulation of extracellular matrix to ameliorate fibrotic conditions, and to restore or maintain normal tissue or organ function or skin appearance.

As used herein "excess accumulation of extracellular matrix" means the deposition of extracellular matrix components including, collagen, laminin, fibronectin and proteoglycans in tissue to an extent that results in impairment of tissue or organ function and ultimately, organ failure as a result of fibrotic disease. In addition, "excess accumulation of extracellular matrix" means the deposition of extracellular matrix components in the process commonly referred to as "scarring" or "scar formation", e.g. at a wound site. "Reducing the excess accumulation of extracellular matrix" means preventing excess accumulation of extracellular matrix, e.g. in tissue, organs or at a wound site, preventing further deposition of extracellular matrix and/or decreasing the amount of excess accumulated matrix already present, to maintain or restore tissue or organ function or appearance.

A variety of conditions are characterized by excess accumulation of extracellular matrix (collagen, fibronectin and other matrix components). Such conditions include, for example, but are not limited to, glomerulonephritis, adult or acute respiratory distress syndrome (ARDS), diabetes-associated pathologies such as diabetic kidney disease, fibrotic diseases of the liver, lung and post infarction cardiac fibrosis. Also included are fibrocystic diseases such as fibrosclerosis and fibrotic cancers such as cancers of the breast, uterus, pancreas or colon, and including fibroids, fibroma, fibroadenomas and fibrosarcomas.

There are also a number of medical conditions associated with an excess accumulation of extracellular matrix involving increased collagen, fibronectin and other matrix components. Such conditions include, for example, but are not limited to, post myocardial infarction, left ventricular hypertrophy, pulmonary fibrosis, liver cirrhosis, veno-occlusive disease, post-spinal cord injury, post-retinal and glaucoma surgery, post-angioplasty restenosis and renal interstitial fibrosis, arteriovenous graft failure, excessive scarring such as keloid scars and scars resulting from injury, burns or surgery.

As discussed, supra, it is known that TGFβ is indicated in the causation of fibrotic

conditions. During normal tissue repair, TGFβ production is increased to stimulate the process of repair. When repair is complete, TGFβ production is reduced. If not reduced following normal tissue repair, the increased TGFβ overproduction can result in the development of excess extracellular matrix accumulation and fibrotic conditions. Thus, repeated tissue injury or a defect in TGFβ regulation leading to sustained TGF production results in excess accumulation of extracellular matrix.

As used herein "inhibition of TGFβ" includes inhibition of TGFβ activity, for example in causing excess deposition of ECM, as well as inhibition of TGFβ production resulting in overproduction and excess accumulation of ECM, regardless of the mechanism of TGFβ activity or overproduction. This inhibition can be caused directly, e.g. by binding to TGFβ or its receptors, for example by anti-TGFβ antibodies or TGFβ receptor antagonists, or can be caused indirectly, for example by inhibiting a pathway that results in TGFβ production, such as the renin pathway. Inhibition causes a reduction in the ECM producing activity of TGFβ regardless of the exact mechanism of inhibition.

As used herein a "TGFβ inhibitory agent" is an agent that directly or indirectly inhibits TGFβ binding to its receptors, such as a TGFβ-specific inhibitory agent, or an agent that blocks an alternative pathway of TGFβ production. The agent causes a reduction in the ECM producing activity of TGFβ regardless of the mechanism of its action. The agent can be nucleic acid encoding the TGFβ inhibitory agent such as a cDNA, genomic DNA, or an RNA or DNA encoding TGFβ inhibitory activity such as a TGFβ antisense RNA or DNA.

As used herein, a "TGFβ-specific inhibitory agent" means an agent containing TGFβ inhibiting activity, including agents that bind directly to TGFβ such as anti-TGFβ antibodies, or are a ligand for TGFβ which prevents it from binding to its receptors. A TGFβ-specific inhibiting agent also includes a nucleic acid encoding a particular TGFβ-specific inhibitory agent such as a cDNA, genomic DNA or an RNA or DNA encoding TGFβ-specific inhibitory activity such as a TGFβ antisense RNA or DNA.

Agents that bind directly to TGFβ are known and include anti-TGFβ antibodies such as anti-TGFβ1 antibodies (Genzyme, Cambridge, MA) and antibodies which bind both TGFβ1 and TGFβ2 (Dasch et al., U.S. Patent No. 5,571,714), proteoglycans such as decorin, biglycan and fibromodulin, and the nucleic acids encoding such agents.

Antibodies to inhibit TGFβ, renin or other molecules, for use in the present invention, can be prepared according to methods well established in the art, for example by immunization of suitable host animals with the selected antigen, e.g. TGFβ. For descriptions of techniques for obtaining monoclonal antibodies see, e.g. the hybridoma technique of Kohler and Milstein (*Nature* 256:495-497 (1975)), the human B-cell hybridoma technique (Kosbor et al., *Immunol. Today* 4:72 (1983); Cole et al., *Proc. Nat'l. Acad. Sci. USA*, 80:2026-2030 (1983)) and the EBV-hybridoma technique (Cole et al., *Monoclonal antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77096 (1985)). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the monoclonal antibody may be cultivated *in vitro* or *in vivo*. Suitable host animals include, but are not limited to, rabbits, mice, rats, and goats. Various adjuvants may be used to increase the immunological response to the host animal, depending on the host species, including, but not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpit, hemocyanin, dinitrophenol and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and Cornebacterium parvum. Antibodies as used herein includes non-human, chimeric (different species), humanized (see Borrebaeck, Antibody Engineering: A Practical Guide, W.H. Freeman and Co., New York, 1991), human and single-chain antibodies, as well as antibody fragments including but not limited to the $F(ab')_2$ fragments that can be produced by pepsin digestion of antibody molecules and Fab fragments that can be generated by reducing disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed (*Science* 246:1275-1281 (1989)) to permit the rapid and easy identification of monoclonal Fab fragments having the desired specificity.

An indirect TGFβ inhibitor would inhibit the synthesis or secretion of TGFβ or sequester it away from its target cells. Such inhibitors include, but are not limited to, inhibitors of Angiotensin Converting Enzyme ("ACE"), antagonists of the AII receptor such as Losartan^{tm} and Cozar^{tm} (Merck), and aldosterone inhibitors such as Spironolactone^{tm} (Sigma Chemical Co., St. Louis, Mo, Product # S 3378) that would otherwise result in increased TGFβ production.

Also included within the scope of TGFβ inhibitors of the invention are nucleic acids that include antisense oligonucleotides that block the expression of specific genes within cells by binding a complementary messenger RNA (mRNA) and preventing its translation (See review by Wagner, *Nature* 372:332-335 (1994); and Crooke and Lebleu, *Antisense Research and Applications*, CRC Press, Boca Raton (1993)). Gene inhibition may be measured by determining the degradation of the target RNA. Antisense DNA and RNA can be prepared by methods known in the art for synthesis of RNA including chemical synthesis such as solid phase phosphoramidite chemical synthesis or in vitro and in vivo transcription of DNA sequences encoding the antisense RNA molecule. The DNA sequences may be incorporated into vectors with RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly can be introduced into cell lines. The potency of antisense oligonucleotides for inhibiting TGFβ may be enhanced using various methods including 1) addition of polylysine (Leonetti et al., *Bioconj. Biochem.* 1:149-153 (1990)); 2) encapsulation into antibody targeted liposomes (Leonetti et al., *Proc. Natl. Acad. Sci. USA* 87:2448-2451 (1990) and Zelphati et al., *Antisense Research and Development* 3:323-338 (1993)); 3) nanoparticles (Rajaonarivony et al., *J. Pharmaceutical Sciences* 82:912-917 (1993) and Haensler and Szoka, *Bioconj. Chem.* 4:372-379 (1993)), 4) the use of cationic acid liposomes (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417 (1987); Capaccioli et al., *Biochem. Biophys. Res. Commun.* 197:818-825 (1993); Boutorine and Kostina, *Biochimie* 75:35-41 (1993); Zhu et al., *Science* 261:209-211 (1993); Bennett et al., *Molec. Pharmac.* 41:1023-1033 (1992) and Wagner, *Science* 280:1510-1513 (1993)); and 5) Sendai virus derived liposomes (Compagnon et al., *Exper. Cell Res.* 200:333-338 (1992) and Morishita et al., *Proc. Natl.*

Acad. Sci. USA 90:8474-8478 (1993)), to deliver the oligonucleotides into cells. Recent techniques for enhancing delivery include the conjugation of the antisense oligonucleotides to a fusogenic peptide, e.g. derived from an influenza hemagglutinin envelop protein (Bongartz et al., *Nucleic Acids Res.* 22(22):4681-4688 (1994)).

Additional suitable TGFβ inhibitory agents can be readily obtained using methods known in the art to screen candidate agent molecules for binding to TGFβ, such as assays for detecting the ability of a candidate agent to block binding of radiolabeled human TGFβ to cells such as human mesangial cells. Alternatively, candidate compounds may be tested for the ability to inhibit TGFβ production by mesangial cells using an enzyme-linked immunosorbent assay (ELISA), for example using the R & D Systems (Minneapolis, MN) TGFβ ELISA assay kit (Cat. No. DB 100) (for methods see, e.g. Uotila et al., *J. Immunol. Methods* 42:11 (1981)).

Suitable TGFβ-specific inhibitory agents can also be developed by known drug design methods, e.g. using structural analysis of the TGFβ molecule employing methods established in the art, for example, using X-ray crystallography to analyze the structure of the complex formed by TGFβ and one of its known inhibitors (see, e.g. Sielecki et al., supra; Rahuel et al., supra, Badasso et al., supra and Dhanaraj et al., supra.), and/or by modifying known TGFβ antagonists i.e. "lead compounds", to obtain more potent inhibitors and compounds for different modes of administration (i.e. oral vs. intravenous) (see, e.g. Wexler et al., *Amer. J. Hyper.* 5:209S-220S (1992)-development of AII receptor antagonists from Losartan^{tm}). For such procedures large quantities of TGFβ can be generated using recombinant technology or purchased commercially (R & D Systems).

In addition to TGFβ inhibitory agents, agents that result in the degradation of ECM are contemplated for use in the invention. Such agents include serine proteases such as plasmin and metalloproteinases, and protease combinations such as Wobenzym (Mucos Pharma, Geretsried, Germany) . In addition, the present inventors have discovered that agents such as tPA can be used to increase the amount of active proteases in vivo to increase degradation of ECM accumulated in organs and tissues. Tissue

plasmin activator (tPA, Activase, Genentech, S. San Francisco, CA) has been shown to dissolve clots associated with myocardial infarction and stroke. The present inventors theorized that tPA might be helpful in increasing plasmin to reduce accumulated ECM. Shown herein is the use of recombinant tPA (rtPA) to increase the generation of plasmin in vivo to degrade ECM (Example V, infra).

In addition, new proteases or agonists of protease production and/or activity may be discovered or developed using rational drug design and used to degrade ECM according to the methods of the present invention.

The present inventors have also discovered that PAI mutants, such as the PAI-1 mutants disclosed in WO 97/39028 by Lawrence et al., incorporated by reference in its entirety herein, may be used to increase the amount of active plasmin to enhance degradation of ECM accumulated in organs and tissues. These PAI-1 mutants fail to inhibit plasminogen activators, yet retain significant vitronectin binding affinity. Additional PAI-1 mutants for use in the methods of the invention may be obtained and tested for the ability to bind vitronectin while failing to inhibit plasminogen activators (Lawrence et al., *J. Biol. Chem.* 272:7676-7680 (1997)). PAI-1 binding to vitronectin may be determined either functionally (Lawrence et al., *J. Biol. Chem.* 265:20293-20301 (1990)) or in a vitronectin specific ELISA (Lawrence et al., *J. Biol. Chem.* 269:15223-15228 (1994)). The ability of PAI-1 to inhibit plasminogen activators may be evaluated using chromogenic assays as described by Sherman et al., *J. Biol. Chem.* 270:9301-9306 (1995)).

In the methods of the invention, the TGFβ inhibitory agents are administered concurrently or sequentially. For example, an anti-TGFβ antibody is administered with an anti-renin agent. The inhibitory agents will localize at sites of TGFβ overproduction, e.g. organs such as the kidneys. The inhibitory agents may be labelled, using using known radiolabelling methods to detect their localization in a subject after administration. The agents may also be conjugated to targeting molecules such as antibodies to ECM components to improve localization of the agents after administration to the sites of

TGFβ overproduction and/or excess accumulation of ECM in a subject.

In another embodiment of the methods of the invention, TGFβ inhibitory agents are administered concurrently or sequentially with at least one agent that degrades accumulated ECM, for example, a serine protease such as plasmin. Alternatively, an agent that induces protease production, such as tPA, is administered to increase protease production at the site(s) of accumulated ECM. tPA binds fibrin (Rondeau et al., *Clinical Nephrol.* 33:55-60 (1990)) and thus will localize in fibrotic areas where the increased protease production is desired.

In one embodiment of the invention, at least one TGFβ-inhibitory agent is administered to a subject having existing excess accumulation of ECM in tissues or organs, or at high risk for such accumulation to reduce or prevent excess accumulation of ECM. For example, individuals at risk for developing fibrotic conditions, such as a person having or at high risk for diabetes, high blood pressure, autoimmune disease (e.g. lupus) and inflammatory diseases, can be scanned using known medical procedures including tissue biopsies of kidney, lung or liver, to determine whether ECM has accumulated in these organs. If the agent is TGFβ-specific, it binds to circulating TGFβ or tissue TGFβ. If the agent indirectly inhibits TGFβ, for example an anti-renin agent, it reduces the amount of TGFβ produced. As a result of the administration of agents that directly or indirectly inhibits TGFβ, ECM that has accumulated at the time of diagnosis or treatment, as well as further accumulation of ECM is reduced. Moreover, in high risk individuals the methods of the invention for inhibiting TGFβ overproduction with multiple agents can result in prevention of excess accumulation of ECM and the development of fibrotic conditions.

In another embodiment of the methods of the invention, at least one TGFβ inhibitory agent is administered to a subject having an existing excess accumuation of ECM in tissues or organs together with at least one agent to degrade accumulated ECM. The ECM degradation is accomplished using a protease, or an agent that enhances production or the activity of ECM degrading agents such as proteases. As a result of the administration of these agents, excess matrix accumulated at the time of diagnosis or

treatment, as well as further excess accumulation of ECM is reduced.

In addition to the use of molecules such as antibodies and purified compounds such as decorin, nucleic acid encoding the TGFβ inhibitory agents and nucleic acid encoding the agent to directly or indirectly degrade accumulated ECM, are administered to the subject to permit the agents to be expressed and secreted, for inhibiting TGFβ and degrading accumulated ECM. The nucleic acid may be introduced into cells in the subject, for example using a suitable delivery vehicle such as an expression vector or encapsulation unit such as a liposome, or may be introduced directly through the skin, for example in a DNA vaccine.

Alternatively, the nucleic acids encoding the agents are introduced into a cell ex vivo and the cells expressing the nucleic acids are introduced into a subject, e.g. by implantation procedures, to deliver the agents in vivo. Multiple agents can be introduced into a delivery vehicle or in separate vehicles.

Gene Therapy Methods

Methods for obtaining nucleic acids encoding TGFβ inhibitory agents and ECM degrading agents are known in the art. Following is a general description of methods of using the nucleic acids in gene therapy to reduce excess accumulation of ECM.

In one embodiment of the invention, gene therapy is contemplated using nucleic acids encoding the TGFβ inhibitory agents and/or the ECM degradation agent, introduced into cells in a subject to suppress TGFβ overproduction and to degrade accumulated ECM. Gene transfer into cells of these nucleic acids is contemplated in the methods of the invention.

Nucleic Acids

Large amounts of the nucleic acid sequences encoding the TGFβ-inhibiting agents and/or the ECM degradation agents may be obtained using well-established procedures

for molecular cloning and replication of the vector or plasmid carrying the sequences in a suitable host cell. DNA sequences encoding a specific agent can be assembled from cDNA fragments and oligonucleotide linkers, or from a series of oligonucleotides to provide a synthetic inhibitor agent gene and/or ECM degradation gene which can be expressed. Such sequences are preferably provided in an open reading frame uninterrupted by internal non-translated sequences or introns, which are typically present in eukaryotic genes. Genomic DNA containing the relevant sequences can also be used. Sequences of non-translated DNA may be present 5' to 3' from the open reading frame, where such sequences do not interfere with manipulation or expression of the coding regions. Either complete gene sequences or partial sequences encoding the desired agents are employed.

The nucleic acid sequences encoding the agents can also be produced in part or in total by chemical synthesis, e.g. by the phosphoramidite method described by Beaucage and Carruthers, *Tetra Letts.* 22:1859-1862 (1981) or the triester method (Matteucci et al., *J. Am. Chem. Soc.* 103:3185 (1981) and may be performed on commercial automated oligonucleotide synthesizers. A double-stranded fragment may be obtained from the single-stranded product of chemical synthesis either by synthesizing the complementary strand and annealing the strand together under appropriate conditions, or by synthesizing the complementary strand using DNA polymerase with an appropriate primer sequence.

Gene Transfer

For gene transfer, the key steps are 1) to select the mode of delivery, e.g. a proper vector for delivery of the inhibitor genes to the subject, 2) administer the nucleic acid to the subject; and 3) achieve appropriate expression of the transferred gene for satisfactory durations. Methods for gene transfer are known in the art. The methods described below are merely for purposes of illustration and are typical of those that can be used to practice the invention. However, other procedures may also be employed, as is understood in the art. Most of the techniques to construct delivery vehicles such as vectors and the like are widely practiced in the art, and most practitioners are familiar with the standard resource

materials which describe specific conditions, reagents and procedures. The following paragraphs may serve as a guideline.

Techniques for nucleic acid manipulation are well known. (See, e.g. *Annual Rev. of Biochem.* 61:131-156 (1992)). Reagents useful in applying such techniques, such as restriction enzymes and the like, are widely known in the art and commerically available from a number of vendors.

The natural or synthetic nucleic acid coding for the inhibitors for expression in a subject may be incorporated into vectors capable of introduction into and replication in the subject. In general, nucleic acid encoding the selected inhibitor molecules and/or ECM degradation molecules are inserted using standard recombinant techniques into a vector containing appropriate transcription and translation control sequences, including initiation sequences operably linked to the gene sequence to result in expression of the recombinant genes in the recipient host cells. "Operably linked" means that the components are in a physical and functional relationship permitting them to function in their intended manner. For example, a promoter is operably linked to a coding sequence if the promoter effects its transcription or expression.

Sequences encoding selected inhibitor and/or degradation genes will include at least a portion of the coding sequence sufficient to provide the TGFβ inhibitory or ECM degradation activity in the expressed molecule. For example, in the case of a renin inhibitor, a portion of the coding sequence that enables the inhibitor to bind to renin can be used. Methods for determining such portions or domains, including binding domains of molecules, are known in the art (See, e.g., Linsley et al., *Proc. Natl. Acad. Sci. USA* 87:5031-5035 (1990)). It is possible that it may be necessary to block both the renin enzymatic site and the renin-cell binding domain in order to effectively prevent the stimulus to TGFβ overproduction by renin. In such case, renin antisense molecules can be prepared using standard methods to accomplish complete blockade.

The selected nucleic acid sequences are inserted into a single vector or separate

vectors. More than one gene encoding a selected agent, or portion thereof containing the desired activity, may be inserted into a single vector or into separate vectors for introduction into the host cells. Alternatively, these sequences can be administered as naked nucleic acid sequences or as part of a complex with other molecules, e.g. liposomes.

A variety of expression vectors and gene transfer methods useful for obtaining expression of selected molecule in recipient cells are well known in the art, and can be constructed using standard ligation and restriction techniques (see, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989; Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York (1982), Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (W.H. Freeman and Co., New York, NY 1990) and Wu, *Methods in Enzymol.* (Academic Press, New York, NY 1993), each of which is incorporated by reference herein). The choice of vector or method depends on several factors such as the particular molecule to be expressed.

Suitable vectors may be plasmid or viral vectors (Kaufman, in *Gene Expression Technology,* Goeddel (Ed.) (1991)) including baculoviruses, adenoviruses, poxviruses (Moss, *Current Opin. Biotech.* 3:518-522 (1993)), retrotransposon vectors (Cook et al., *Bio/Technology* 9:748-751 (1991) and Chakraborty et al., *FASEB J.* 7:971-977 (1993)) adeno-associated viruses (AAV) (Yei et al., *Gene Therapy* 1:192-200 (1994) and Smith et al., *Nat. Genet.* 5:397-402 (1993)), herpes virus and retrovirus vectors (Price et al., *Proc. Natl. Acad. Sci. USA* 84:156-160 (1987); Naviaux and Verma, *Current Opinion in Biotechnol.* 3:540-547 (1992); Hodgson and Chakraborty, *Curr. Opin. Thera. Patients* 3:223-235 (1993)) such as the MMLV based replication incompetent vector pMV-7 (Kirschmeier et al., *DNA* 7:219-225 (1988)), as well as human and yeast artificial chromosomes (HACs and YACs) (Huxley, *Gene Therapy* 1:7-12 (1994) and Huxley et al., *Bio/Technology* 12:586-590 (1994)). Plasmid expression vectors include plasmids including pBR322, pUC or Bluescript^{tm} (Stratagene, San Diego, CA).

Vectors containing the nucleic acid encoding the selected agents are preferably recombinant expression vectors in which high levels of gene expression may occur, and which contain appropriate regulatory sequences for transcription and translation of the inserted nucleic acid sequence. Regulatory sequences refer to those sequences normally associated (e.g. within 50 kb) of the coding region of a locus which affect the expression of the gene (including transcription, translation, splicing, stability or the like, of the messenger RNA). A transcriptional regulatory region encompasses all the elements necessary for transcription, including the promoter sequence, enhancer sequence and transcription factor binding sites. Regulatory sequences also include, inter alia, splice sites and polyadenylation sites. An internal ribosome entry site (IRES) sequence may be placed between recombinant coding sequences to permit expression of more than one coding sequence with a single promoter.

Transcriptional control regions include: the SV40 early promoter region, the cytomegalovirus (CMV) promoter (human CMV IE94 promoter region (Boshart et al., *Cell* 41:521-530 (1985)); the promoter contained in the 3' long terminal repeat of Rous Sarcoma Virus or other retroviruses; the herpes thymidine kinase promoter; the regulatory sequences of the methallothionein gene; regions from the human IL-2 gene (Fujita et al., *Cell* 46:401-407 (1986)); regions from the human IFN gene (Ciccarone et al., *J. Immunol.* 144:725-730 (1990); regions from the human IFN gene (Shoemaker et al., *Proc. Natl. Acad. Sci. USA* 87:9650-9654 (1990); regions from the human IL-4 gene (Arai et al., *J. Immunol.* 142:274-282 (1989)); regions from the human lymphotoxin gene (Nedwin et al., *Nucl. Acids. Res.* 13:6361-6373 (1985)); regions from the human granulocyte-macrophage CSF gene (GM-CSF) (Miyatake et al., *EMBO J.* 4:2561-2568 (1985)) and others. When viral vectors are used, recombinant coding sequences may be positioned in the vector so that their expression is regulated by regulatory sequences such as promoters naturally residing in the viral vector.

Operational elements for obtaining expression may include leader sequences, termination codons and other sequences needed or preferred for the appropriate transcription and translation of the inserted nucleic acid sequences. Secretion signals

may also be included whether from the native inhibitor or from other secreted polypeptides, which permit the molecule to enter cell membranes and attain a functional conformation. It will be understood by one skilled in the art that the correction type and combination of expression control elements depends on the recipient host cells chosen to express the molecules ex vivo. The expression vector should contain additional elements needed for the transfer and subsequent replication of the expression vector containing the inserted nucleic acid sequences in the host cells. Examples of such elements include, but are not limited to, origins of replication and selectable markers. Additionally, elements such as enhancer sequences, for example CMV enhancer sequences, may be used to increase the level of therapeutic gene expression (Armelor. *Proc. Natl. Acad. Sci. USA* 70:2702 (1973)).

The vector may contain at least one positive marker that enables the selection of cells carrying the inserted nucleic acids. The selectable molecule may be a gene which, upon introduction into the host cell, expresses a dominant phenotype permitting positive selection of cells carrying the gene ex vivo. Genes of this type are known in the art and include, for example, drug resistance genes such as hygromycin-B phosphotransferase (hph) which confers resistance to the antibiotic G418; the aminoglycoside phosphotransferase gene (*neo* or *aph*) from Tn5 which codes for resistance to the antibiotic G418; the dihydrofolate reductase (DHRF) gene; the adenosine deaminase gene (ADA) and the multi-drug resistance (MDR) gene.

Recombinant viral vectors are introduced into host cells using standard techniques. Infection techniques have been developed which use recombinant infectious virus particles for gene delivery into cells. Viral vectors used in this way include vectors derived from simian virus 40 (SV40; Karlsson et al., *Proc. Natl. Acad. Sci. USA* 82:158 (1985)); adenoviruses (Karlsson et al., *EMBO J.* 5:2377 (1986)); vaccinia virus (Moss et al., *Vaccine* 6:161-3 (1988)); and retroviruses (Coffin, in Weiss et al. (Eds.), RNA Tumor Viruses, 2nd Ed., Vol. 2, Cold Spring Laboratory, NY, pp. 17-71 (1985)).

Nonreplicating viral vectors can be produced in packaging cell lines which

produce virus particles which are infectious but replication defective, rendering them useful vectors for introduction of nucleic acid into a cell lacking complementary genetic information enabling encapsidation (Mann et al., *Cell* 33:153 (1983); Miller and Buttimore, *Mol. Cell. Biol.* 6:2895 (PA317, ATCC CRL9078). Packaging cell lines which contain amphotrophic packaging genes able to transduce cells of human and other species origin are preferred.

Vectors containing the inserted inhibitor genes or coding sequences are introduced into host cell using standard methods of transfection including electroporation, liposomal preparations, Ca-PH-DNA gels, DEAE-dextran, nucleic acid particle "guns" and other suitable methods.

In additional to various vectors including viral vectors, other delivery systems may be used including, but not limited to, microinjection (DePamphilis et al., *BioTechnique* 6:662-680 (1988)); liposomal mediated transfection (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417 (1987); Felgner and Holm, *Focus* 11:21-25 (1989) and Felgner et al., *Proc. West. Pharmacol. Soc.* 32:115-121 (1989)); use of naked or particle mediated DNA transfer and other methods known in the art. Recently, cationic liposomes have been used to enhance transfection (Felgner et al., *Nature* 349:351 (1991); Zhu et al., *Science* 261:209 (1993)).

Suitable host cells for gene transfer consist of vertebrate cells such as fibroblasts, keratinocytes, muscle cells, mesangial cells (see, Kitamura et al., *Kidney Int.* 48:1747-1757 (1995)), and any other suitable host cell including so-called universal host cells, i.e. cells obtained from a different donor than the recipient subject but genetically modified to inhibit rejection by the subject. Autologous cells are preferred, but heterologous cells are encompassed within the scope of the invention.

Expression of the selected TGFβ inhibitor genes after introduction into the host cells is confirmed using standard methods. For example, expression of TGFβ-specific inhibitory agents can be determined by assaying for the ability of the supernatant from

transfected cells to inhibit the binding of radiolabeled TGFβ to human mesangial cells using Fluorescent Activated Cell Sorting (FACS) or ELISA. Expression from host cells of an agent that inhibits TGFβ indirectly, such as Losartan, can be confirmed by detecting a decrease in fibronectin production by mesangial cells exposed to supernatant from transfected cells, relative to controls. Expression of genes encoding ECM degrading agents can be determined using, for example, an in vitro system using mesangial cells cultured on a ECM substrate such as Matrigel™ (Collaborative Research, Inc., Bedford, MA) that contains the major components of the mesangial matrix, including laminin, type IV collagen, entactin and heparan sulfate proteoglycan, as described by Baricos et al., *Kidney Internatl.* 47:1039-1047 (1995)). The ECM substrate is radiolabeled. and ECM degradation by the product of an expressed gene from transfected host cells is determined by measuring the release of radioactivity from the ECM into serum-free medium. These assay systems may also be employed to screen candidate TGFβ inhibiting and ECM degrading agents.

Administration of TGFβ Inhibitory Agents and Agents Degrading Accumulated ECM

Agents for inhibiting TGFβ and agents for degrading accumulated ECM are suspended in physiologically compatible pharmaceutical carriers, such as physiological saline, phosphate-buffered saline, or the like to form physiologically acceptable aqueous pharmaceutical compositions for administration to a subject. Parenteral vehicles include sodium chloride solution, Ringer's desctrose, dextrose and sodium chloride and lactated Ringer's solution. Other substances may be added a desired, such as antimicrobials.

The TGFβ inhibiting and ECM degrading agents may be administered together or apart, simultaneously or sequentially, to carry out the methods of the invention.

Modes of administration of the TGFβ inhibitory agents and ECM degrading agents are those known in the art for therapeutic agents and include parenteral, for example, intravenous (e.g. for antibody inhibitors or proteases), intraperitoneal, intramuscular, intradermal, and epidermal including subcutaneous and intradermal, oral (e.g. small molecule renin and TGFβ antagonists), or applied to mucosal surfaces, e.g. by

intranasal administration using inhalation of aerosol suspensions, and by implanting to muscle or other tissue in the subject (e.g. for gene transfer of nucleic acid expressing renin and/or TGFβ inhibitors). Suppositories and topical preparations are also contemplated.

The TGFβ inhibitory and ECM degrading agents are introduced in amounts sufficient to prevent or reduce excess accumulation of extracellular matrix in susceptible tissues and organs including, but not limited to, lung and kidney tissue. Before or after administration, if necessary to prevent or inhibit the subject's immune response to the vehicles carrying the inhibitors, immunosuppressant agents may be used. Alternatively, the vehicles carrying the TGFβ inhibitory and ECM degrading agents can be encapsulated.

The most effective mode of administration and dosage regimen for the TGFβ inhibitory and ECM degrading agents for use in the methods of the present invention depend on the extent of TGFβ overproduction, the severity of the accumulation of extracellular matrix and resulting impairment of tissue or organ function, the subject's health, previous medical history, age, weight, height, sex and response to treatment and the judgment of the treating physician. Therefore, the amount of TGFβ inhibitory and ECM degrading agents to be administered, as well as the number and timing of subsequent administrations, are determined by a medical professional conducting therapy based on the response of the individual subject. Initially, such parameters are readily determined by skilled practitioners using appropriate testing in animal models for safety and efficacy, and in human subjects during clinical trials of candidate therapeutic formulations. Suitable animal models of human fibrotic conditions are known (see, e.g. Border and Noble, *New Eng. J. Med.* 331:1286-1292 (1994), incorporated by reference herein).

After administration, the efficacy of the therapy using the methods of the invention is assessed by various methods including biopsy of kidney, lung or liver or other tissue to detect the amount of extracellular matrix accumulated. An absence of

significant excess accumulation of ECM, or a decrease in the amount or expansion of ECM in the tissue or organ will indicate the desired therapeutic response in the subject. Preferably, a non-invasive procedure is used to detect a therapeutic response. For example, changes in TGFβ activity can be measured in plasma samples taken before and after treatment with an inhibitor (see, Eltayeb et al., *J. Am. Soc. Nephrol.* 8:110A (1997)), and biopsy tissue can be used to individually isolate diseased glomeruli which are then used for RNA isolation. mRNA transcripts for TGFβ, and extracellular matrix components (e.g. collagen) are then determined using reverse transcriptase-polymerase chain reaction (RT-PCR) (Peten et al., *J. Exp. Med.* 176:1571-1576 (1992)).

Advantages of the Invention

The invention provides improved treatment and prevention of fibrotic conditions associated with overproduction of TGFβ and excess accumulation of ECM in tissues and/or organs resulting in impaired function, or scarring, by reducing TGFβ overproduction directly and that resulting from multiple biological pathways, to effectively inhibit the TGFβ induced component of extracellular matrix deposition, and by increased degradation of ECM using degrading agents.

The therapeutic effects of the invention result from a reduction in or prevention of the TGFβ-induced excess accumulation of extracellular matrix in tissues and/or organs, and when combined with ECM degrading agents, from the increased degradation of ECM over time.

The following examples are presented to demonstrate the methods of the present invention and to assist one of ordinary skill in using the same. The examples are not intended in any way to otherwise limit the scope of the disclosure of the protection granted by Letters Patent granted hereon.

EXAMPLE I

DEMONSTRATION THAT RENIN UPREGULATES TGFβ IN HUMAN MESANGIAL CELLS

Normal fetal human mesangial cells (Clonetics Corp., Clonetics, Walkersville, MD) passaged 5 to 8 times, were plated (3,000 cell/cm^2) in 12 well plates in 2ml of medium (Mesangial Basal Medium (Clonetics Corp.) containing 5% FCS, 10 μg/ml penicillin and 100 μg/ml streptomycin) and allowed to grow to confluence for 48 hours at 37EC, 5% CO_2. Cultures were washed three times using sterile phosphate buffered saline at room temperature and then 2 ml/well of serum free MBM medium to induce quiescence. After 48 hours, the serum-free medium was removed and 2 ml/well of fresh serum-free medium was added. Human recombinant renin (Hoffman-La Roche Ltd., Basel, Switzerland) in concentrations from 10^{-6} to 10^{-12} M was added to each well. A blank and 5 ng/ml of TGFβ (R & D Systems, Minneapolis, MN) were used as controls. Cells and supernatants were harvested by centrifugation after 24 hrs of culture and frozen at ¯70EC until analysis. The total production and release of TGFβ into the culture supernatant was measured using an ELISA kit (R & D Systems). Induction of PAI-1 and fibronectin in the supernatant are also measured using anti-PAI-1 and anti-fibronectin antibodies in an ELISA to provide further confirmation of the inhibition of TGFβ. TGFβ, fibronectin and PAI-1 mRNA are measured using semi-quantitative RT-PCR.

(1) Determination of Dose Dependency of Renin Induction of TGFβ

As shown in Figure 2, renin increases the TGFβ production by cultured human mesangial cells in a dose-dependent manner.

EXAMPLE II

DEMONSTRATION OF THE EFFECT OF INHIBITING RENIN ON TGFβ PRODUCTION BY HUMAN MESANGIAL CELLS

Renin inhibitor Ro42-5892 (Hoffman-LaRoche, Basel, Switzerland), Losartan^{tm} (Merck Pharmaceuticals, West Point, PA), Enalapril^{tm} (Sigma Chemical Co., St. Louis, MO, Prod. No. E6888), or TGFβ1 neutralizing antibody (R & D Systems) were added in the amounts indicated below to separate wells in triplicate to block the renin cascade at different sites after stimulation by renin:

10^{-5} M Renin Inhibitor R042-5892 (Hoffman-LaRoche)
30 ng/ml Anti-TGFβ1 antibody (R & D Systems, #AB 101 NA)
30 ng/ml Chicken IgG (control for anti-TGFβ1 antibody, R & D Systems, # AB 101 C)
10^{-5} M Enalapril^{tm} (Sigma Chemical Co., St. Louis, MO)
10^{-5} M Losartan^{tm} (Merck Pharmaceuticals, West Point, PA)

These inhibitors were added at zero time with 10^{-7} M human recombinant renin (Hoffman-LaRoche).

As shown in Figure 3, use of inhibitors that block renin's action to increase Angiotensin II, i.e. blocking Angiotensin I production from Angiotensinogen (Ro 42-5892), blocking Angiotensin I conversion to Angiotensin II (Enalapril^{tm}) and blocking binding of Angiotensin II to its type I receptor (Losartan^{tm}), does not reduce the renin-induced increase in TGFβ production. These results demonstrate for the first time an alternative pathway in which TGFβ production is stimulated by renin.

EXAMPLE III

DEMONSTRATION OF INHIBITION OF TGFβ BY BLOCKING RENIN IN VIVO IN THE PRESENCE OF AN ANTI-FIBROTIC DRUG

In this example, a known fibrotic disease drug, Enalapril[tm] which inhibits the production of Angiotensin II, is combined with an inhibitor of renin, antisense renin oligonucleotide, to obtain an enhanced therapeutic effect on fibrotic disease in an animal model.

Rats are administered Enalapril[tm] in their drinking water prior to anti-thymocyte serum

injection, e.g. three (3) days prior to injection. Anti-thymocyte antibody, e.g. OX-7, is injected intravenously into the rats at day three to produce fibrotic disease. (Bagchus et al., Lab. Invest. 55:680-687 (1986)). Renin antisense oligonucleotides are administered one hour following administration of OX-7 by introducing the oligonucleotides into a suitable vehicle, such as HVJ liposomes, and injecting the formulations into the left renal artery of Sprague Dawley rats as described for renin genes by Arai et al., *Biochem. And Biophys. Res. Comm.* 206(2):525-532 (1995), incorporated by reference herein. A control consisting of nonsense encoding oligonucleotides (e.g. derived from the renin antisense gene sequence) is also injected into the left renal artery of additional rats. The renin antisense localizes in the juxtaglomerular apparatus of the glomerulus where renin is produced blocking renin production.

Animals are sacrificed on day 7 and kidney tissue samples are taken for analysis of levels of TGFβ in the glomeruli. Glomeruli are sieved individually from each rat and placed in culture in suitable medium for three days. At the end of culture, culture supernatant is harvested by centrifugation and TGFβ, fibronectin and PAI-1 production are determined as markers of fibrotic renal disease severity. Other glomeruli are pooled and used to isolate RNA. RNA is used by standard methods to quantitate expression of mRNAs of interest, including TGFβ, fibronectin and collagens.

Glomeruli are also examined histologically for phenotypical changes, e.g. changes resulting from deposition for ECM. Phenotypic changes are associated with pathological alteration of glomeruli indicative of fibrotic disease. Such changes include expansion of extracellular matrix in the mesangial area of the kidney in animal models and the presence of activated mesangial cells which have acquired the characteristics of fibroblasts, e.g. expressing α-smooth muscle actin and interstitial collagen, indicating progressive glomerular injury (Johnson et al., *J. Am. Soc. Nephrol.* 2:S190-S197 (1992)). Tissue for light microscopy is fixed in formaldehyde, then dehydrated in graded ethanol and embedded in paraffin. Sections are cut at 3 μm thickness and are stained with with the periodic Schiff reagent. The paraformaldehyde-fixed renal section of the rats are also incubated with mouse anti-human renin monoclonal antibody (Kaiichi Radioisotope Labs, Ltd., Tokyo, Japan), mouse anti-α-smooth muscle actin monoclonal antibody (Immunotech S. A. (Marseille, France) and rabbit anti-collagen antibodies (Chemicon, Temicula, CA, prod. No. AB755). The sections are further processed using Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, CA).

Results of antibody binding indicate the extent of glomerular injury and the effects of inhibition of renin on such injury.

EXAMPLE IV

ADDITIONAL DEMONSTRATION THAT RENIN UPREGULATES TGFβ IN HUMAN MESANGIAL CELLS

Primary cultures of adult human mesangial cells were grown from human nephrectomy tissues using standard methods. Cells were passaged 4-7 times and then plated (3,000 cell/cm^2) in 12 well plates in 2ml of medium (Mesangial Basal Medium (Clonetics Corp.) containing 5% FCS, 10 μg/ml penicillin and 100 μg/ml streptomycin) and allowed to grow to 70% confluency for 48 hours at 37EC, 5% CO_2. Cultures were washed three times using sterile phosphate buffered saline at room temperature and then

2 ml/well of serum free MBM medium to induce quiescence. After 48 hours, the serum-free medium was removed and 2 ml/well of fresh serum-free medium was added for 24 hours. Human recombinant renin (HrRenin, Hoffman-La Roche Ltd., Basel, Switzerland) in concentrations from 10^{-6} to 10^{-12} M was added to each well for 24 hours. A blank (no HrRenin) was used as a control. Cells and supernatants were harvested by centrifugation after 24 hrs of culture and frozen at ¯70EC until analysis.

The total production and release of TGFβ into the culture supernatant was measured using an ELISA kit (R & D Systems). Induction of the matrix protein fibronectin (Fn) in the supernatant was measured using anti-fibronectin antibodies in an ELISA to provide further confirmation of induction of TGFβ. Renin-induced induction of TGFβ, fibronectin and PAI-1 mRNA were measured over time using semi-quantitative RT-PCR in a multiplex system where multiple cDNAs are amplified simultaneously according to Dostal et al., *Anal. Biochem.* 223:239-250 (1994), incorporated by reference herein Determinations were done in triplicate mesangial cell cultures.

(1) Determination of Dose Dependency of Renin Induction of TGFβ

As shown in Figure 4, statistically significant ($p < 0.05$) dose dependent increases in TGFβ (Figure 4A) and Fn production (Figure 4B) were observed, peaking with 2- and 1.4-fold increases at 10^{-6}M HrRenin, respectively. Time course experiments using 10^{-7}M HrRenin revealed significant increases in TGFβ and Fn production at 24 and 48 hours ($p<0.03$ (Figure 5A and B). As shown in Figure 6A-C, renin-induced increases in TGFβ, PAI-1 and Fn mRNAs peaked at 4 hours with increases from 1.5- to 2-fold.

(2) Demonstration that Renin Upregulation of TGFβ is not mediated through Renin Enzymatic Activity or Angiotensin II

Renin inhibitor Ro42-5892 (Hoffman-LaRoche, Basel, Switzerland), Losartan^{tm} (Merck Pharmaceuticals, West Point, PA), Enalapril^{tm} (Sigma Chemical Co., St. Louis, MO, Prod. No. E6888), or TGFβ1 neutralizing antibody (R & D Systems) were added in

the amounts indicated below to separate wells in triplicate to block the renin cascade at different
sites after stimulation by renin:
10^{-5} M Renin Inhibitor R042-5892 (Hoffman-LaRoche)
10^{-5} M Enalapril^{tm} (Sigma Chemical Co., St. Louis, MO)
10^{-5} M Losartan^{tm} (Merck Pharmaceuticals, West Point, PA)
Controls included neutralizing antibody to TGFβ (ATG) and control IgG (TgG)

These inhibitors were added at zero time with 10^{-7} M human recombinant renin (Hoffman-LaRoche).

As shown in Figure 7, use of inhibitors that block renin's action to increase Angiotensin II, i.e. blocking Angiotensin I production from Angiotensinogen (RO 42-5892), blocking Angiotensin I conversion to Angiotensin II (Enalapril^{tm}) and blocking binding of Angiotensin II to its type I receptor (Losartan^{tm}), does not reduce the renin-induced increase in TGFβ production.

These results provide additional evidence that renin upregulates TGFβ production by human mesangial cells through a mechanism which is independent of renin's enzymatic action to convert angiotensin to Angiotensin I, and independent of Angiotensin II generation. These results may have profound implications for progression of fibrotic renal disease, particularly in states of high plasma renin as are observed with therapeutic Angiotensin II blockade. Thus, the use of therapeutic agents such as Enalapril^{tm} or Losartan^{tm} for Angiotensin blockade may not be optimal as treatment agents because of resulting high renin levels, preventing a therapeutic reduction in TGFβ. In addition, antagonists developed to block the site on renin that acts in the Angiotensin II pathway, would not be expected to block the action of renin that is independent of this pathway. Therefore, effective therapy of fibrotic diseases must take these multiple pathways for TGFβ increase into consideration.

EXAMPLE V

DEMONSTRATION OF THE ABILITY OF tPA TO INCREASE PLASMIN DEGRADATION OF ACCUMULATED ECM IN VIVO

In this Example, recombinant tissue type plasminogen activator (rtPA) was shown to promote generation of the protease plasmin in nephritic glomeruli and to degrade pathological ECM proteins leading to a therapeutic reduction in matrix accumulation.

Six Sprague-Dawley rats with were injected with phosphate buffered saline (PBS, as a control) and 18 rats were injected with 300 ug of mouse monoclonal OX7 antibody produced in the laboratory using commercially obtained hybridoma cells (American Type Culture Collecton (Rockville, MD, USA; Peters et al., *Kidney Internatl.* 54:1570-1580 (1998)) on day 1 to induce anti-Thy-1 nephritis. Injection of the anti rat-thymocyte antibody intravenously causes binding to an epitope in rat glomerular mesangial cells call Thy 1.1. The complement-mediated mesangial cell lysis that follows initiates a cascade of tissue injury, followed by a repair process that involves induction of TGFβ-driven production and deposition of ECM components. In addition, the plasmin protease system is altered such that PA is decreased and PAI-1 is markedly increased. These alterations favor decreased plasmin generation which decreases matrix turnover and enhances matrix accumulation. Plasmin is the key to mesangial cell matrix turnover (Baricos et al, Kidney Int. 47:1037-1047 (1995)).

Three days after the initial injection, rtPA (Genentech, Inc., San Francisco, CA) in a formulation designed for rodent intravenous injection (GenBank EO8757) or PBS was injected intravenously. Injections were repeated twice a day from day 3 to day 5. RtPA was injected i.v. at a dose of 1 mg/kg BW (n'6). Controls received saline (n'6). Glomerular staining for ECM matrix proteins (collagen type I and III, fibronection EDA+ and tenascin) and glomerular mRNA levels of TGFβ1, fibronectin and PAI-1 were evaluated at day 6. Localization of rtPA in nephritic glomeruli and the effect of rtPA on glomerular plasmin were investigated. Rats were sacrificed at day 6 and kidney tissues excised, fixed in formalin and frozen for histological analysis.

Table 1

Groups of Six Rats	Treatment
Group 1-Normal controls	300 ug of PBS on day 1, then 300 ug PBS 2X
Group 2- Disease control	300 ug of OX7 on day 1, then 300 ug PBS 2X
Group 3 - Disease + Dose 1	300 ug of OX7 on day 1, then 0.25 mg/day rtPA 2X/day on days 3, 4 and 5

Kidney tissue sections were stained for extracellular matrix using Periodic Acid Schiff (PAS) using standard procedures and were stained for specific relevant matrix proteins such as Collagen I, Collagen IV, Fibronectin EDA and tenascin using standard immunohistochemical staining procedures. Matrix proteins were scored by image analysis of 30 glomeruli per rat.

Figure 8A (control) and B (tPA) show an overall decrease in matrix accumulated as a result of tPA treatment. Compared to the untreated, disease control group (Figure 9A-D), the percentage of the glomerular area with positive staining was significantly lower in the rtPA treated group at day 6 for fibronectin EDA+(FN) (19 ± 2 vs. 14 ± 1, $p<0.01$), laminin (35 ± 2 vs. 25 ± 2, $p<0.001$), type I collagen 33 ± 1 vs. 21 ± 3, $p<0.001$) and type IV collagen (27 ± 2 vs. 23 ± 1, $p<0.01$). Glomerular levels of TGFβ1, FN and PAI-1 mRNA were unchanged (Figure 10). rtPA co-localized with fibrin along the glomerular capillary loops and in the mesangium.

rtPA was injected into nephritic rats 10, 20 and 30 minutes before sacrifice. At sacrifice, glomeruli were isolated and placed in culture with a chromogenic substrate for tPA. Plasmin generation by nephritic glomeruli, as shown in Figure 11, was significantly elevated in tPA treated nephritic glomeruli compared to nephritic gomeruli from disease control rats.

This example demonstrates that injected rtPA binds fibrin in nephritic glomeruli where it increases plasmin generation and promotes pathological ECM degradation. rtPA may thus be used in the methods of the invention as an ECM degrading agent.

EXAMPLE VI

EFFECT OF ADMINISTRATION OF TGFβ INHIBITORY AGENTS AND AGENTS THAT PROMOTE DEGRADATION OF ECM

In this example, at least one agent that inhibits TGFβ, anti-TGFβ antibody or decorin, is administered in combination with an ECM degrading agent, such as rtPA to reduce excess ECM accumulation and degrade accumulated ECM in an animal model of glomerulonephritis.

Sprague-Dawley rats are treated as described in the above Examples to induce nephritis. Groups of six (6) rats each include untreated disease controls, rats treated with tPA alone as in Example V, above, rats treated with Enalapril^{tm} alone (200 mg/day) in drinking water and rats treated with both intravenous rtPA and Enalapril^{tm} in drinking water. On day 6 rats are sacrificed and kidney sections are excised, fixed in formalin and frozen for histological analysis. Glomeruli are isolated and used for in vitro analysis of production of TGFβ, fibronectin and PAI-1 using ELISA assays of culture supernatants and for isolation of RNA for Northern analysis of message levels of TGFβ, fibronectin and PAI-1. Tissue samples are stained for ECM proteins and glomerular mRNA levels of TGFβ1, fibronectin and PAI-1.

It is expected that the results of treatments with both anti-TGFβ antibody and rtPA treatment are significantly lower positive staining both in PAS stained tissue and in glomeruli stained for specific matrix components, as shown in Example V, compared with groups treated with either agent alone or in the control disease group.

EXAMPLE VII

DEMONSTRATION OF THE EFECTS OF ADMINISTRATION OF A PAI-1 MUTANT ON EXTRACELLULAR MATRIX DEGRADATION

The human PAI-1 mutant used in this experiment (see WO 97/39028) was constructed on the wild-type PAI-1 background (Ginsburg et al., *J. Clin. Invest.* 78:1673-1680 (1986)), and disabled by the introduction of two Arg residues at positions 333 and 335 of the mature protein, which are also referred to as residues P14 and P12 of the reactive center loop (Lawrence, *Adv. Exp. Med. Biol.* 425:99-108 (1997)). Upon interaction with a proteinase, these substitutions greatly retard the insertion of the reactive center loop into β-sheet A and prevent the mutant from adopting the latent conformation. Since loop insertion results in loss of vitronectin affinity (Lawrence et al., 1997, supra), the PAI-1 mutant retains significant vitronectin activity while failing to inhibit all plasminogen activators.

Four to six week old male Sprague-Dawley rats (Sasco, Inc., Omaha, NE) were treated as described in the above Examples to induce anti-thy-1 nephritis by intravenous injection of the monoclonal anti-thymocyte antibody OX-7 350 mg/200 g body weight. Groups of six (6) rats included a normal control group (injected with saline), an untreated disease control group (injected with PBS), and a group treated with 1 mg/Kg PAI-1 mutant injected once a day beginning 24 hours after induction of ATS nephritis and ending at day 5. Two additional groups of rats were treated with 1) 100 mg/liter of Enalapril (in drinking water) with a loading dose of Enalapril given by gavage 24 hr after disease induction followed by 100 mg/liter of Enalapril in drinking water, and 2) a 6% low protein diet (Teklad, Madison, WI, diet number TD86551) started 24 hours following disease induction.

Rats were sacrificed at day 6 and kidney tissues excised, fixed in formalin and frozen for histological analysis. Kidneys were perfused in situ with cold buffered saline (PBS) at pH 7.4, and then excised. Pieces of cortex were removed and either snap frozen

in 2-methylbutane that had been cooled in liquid nitrogen or fixed in 10% neutralized formalin for immunohistologic examination. The capsules were removed and the cortical tissue dissected out and minced with a razor blade prior to isolation of glomeruli by standard graded seiving. Kidney tissue sections were stained for extracellular matrix using Periodic Acid Schiff (PAS) using standard procedures and were stained for specific relevant matrix proteins such as Collagen I, Collagen IV, Fibronectin EDA and tenascin using standard immunohistochemical staining procedures. Matrix proteins were scored by a blinded observer. 20 glomeruli per rat were evaluated. Isolated glomeruli were also used to determine glomerular mRNA levels of TGFβ1, fibronectin and PAI-1 at day 6.

Reagents to measure plasmin activity, including plasminogen, low molecular weight u-PA and H-D-Val-Leu-Lys-p-nitroanilide (S-2251) were obtained from KabiVitrum (Franklin, OH). PAI-1 activity was assayed by measuring the hydrolysis of synthetic substrate by formed plasmin in the presence of plasminogen (Marshall et al., *J. Biol. Chem.* 265:9198-8204 (1990)). Assays were performed in polyvinyl chloride microtiter plates. The total volume of 125 µl was comprised of the following: sample, H-D-Val-Leu-Lys-P-nitroanilide (0.01 µM) and plasminogen (0.03 µM) in 0.5% Triton X-100, 0.1 M Tris, at pH 8.0. The amount of p-nitroaniline released was measured at 410 nm with a Thermomax microplate reader (Molecular Devices, Menlo Park, CA). A standard curve was generated with each assay using low molecular weight human u-PA. Each sample was also assayed without plasminogen to establish the plasminogen-dependence of the enzyme activity. The plasmin activity in culture supernatant or cell lysate was expressed as IU/1000 glomeruli.

Figure 12 shows an increase in plasmin generation of glomeruli in culture as a result of injection of the PAI-1 mutant. Compared to the untreated, disease control group, the glomerular plasmin activity was significantly higher in the PAI-1 treated group, being approximately halfway between the activity of disease controls and normal glomeruli. Notably, the significant increase in glomerular plasmin activity in nephritic glomeruli was observed with the PAI-1 mutant 24 hours following the final injection.

In addition, treatment with the PAI-1 mutant resulted in decreased accumulation of Collagen Type I, relative to diseases controls (Figure 13), while glomerular levels of TGFβ1, FN, PAI-1 mRNA and Collagen I mRNA were not significantly altered. The decreased accumulation of Collagen Type I together with the fact that the Collagen I mRNA does not significantly decrease suggests enhanced extracellular matrix degradation rather than decreased production of Collagen I.

These results suggest that the increase in glomerular plasmin activity with a PAI-1 mutant can be titrated to avoid large increases in plasmin generation that may lead to hemorrhaging. Thus, the dose of the PAI-1 mutant may be altered, for example by doubling the dose, to increase glomerular plasmin activity to normal, but not excessive, levels to decrease deleterious accumulation of extracellular matrix. In addition, the time of treatment may be extended, for example to 10 days to obtain desired degradation.

EXAMPLE VIII

Enhanced Anti-Fibrotic Effects Obtained by Combining TGF-ß Inhibition and Angiotensin II Blockade

A mouse model of acute glomerulonephritis induced by injection of an anti-Thy1 antibody was used to investigate the anti-fibrotic potential of different doses of the mouse anti-TGF--ß1, ß2 and ß3 monoclonal antibody, 1D11, to reduce disease. We then compared the maximally effective dose of 1D11 with the previously determined maximally effective dose of the ACEI, enalapril. Finally, we determined whether combination of the antibody and enalapril at maximally therapeutic doses could further reduce disease.

Materials

The anti-TGF-ß antibody, 1D11, which neutralizes isoforms TGF-ß1, ß2 and ß3, was provided by Cambridge Antibody Technology (Granta Park CB1 6GH,

Cambridgeshire, UK) and Genzyme Corporation (Cambridge, MA, USA).

Unless otherwise indicated, materials, chemicals or culture media were purchased from Sigma Chemical Co (St. Louis, MO, USA).

Animals

These experiments were performed on male Sprague Dawley rats (200-250 g) obtained from the Sasco colony of Charles River Laboratories (Wilmington, MA, USA). Animal housing and care were in accordance with the NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985. Animals were fed a normal protein diet (22% protein, Teklad No. 86 550, Teklad Premier Laboratory Diets, Madison, WI, USA). Glomerulonephritis was induced by tail vein injection of 2.25 mg/Kg of the monoclonal anti-Thy 1.1 antibody OX-7 (NCCC, Biovest International Inc, Minneapolis, MN, USA). OX-7 binds to a Thy 1-like epitope on the surface of mesangial cells causing complement-dependent cell lysis followed by fibrotic tissue repair (Bagchus et al., Lab Invest. 55:680-687 (1986)). Control animals were injected with similar volumes of phosphate-buffered saline (PBS).

Methods

An experiment was carried out to determine the effective dose range of 1D11 antibody in anti-Thy 1 glomerulonephritis. Doses of 1D11 from 0.05 - 5mg/Kg were administered to 10 groups of 4 rats. Based on the results a larger experiment was carried out.

Groups of 8 rats were assigned and treated as outlined in Table 2. Doses of

enalapril above 100mg/ml in drinking water are maximally effective in this model (Peters et al., Kidney Int. 54:1570-1580 (1998)). A dose of 200mg/ml enalapril was used here. Enalapril treatment began 24 hours after disease induction. At that time, based on an average water intake of 40 ml per day, 60% of the daily dose was administered by gavage as described previously (Peters et al., supra). The results indicated that the dose response was in the range 0.01 – 5mg/kg; 1D11 doses of 0.5 and 5.0 mg/kg maximally reduced ECM accumulation so these doses were used in combination with enalapril, in groups 8 and 9.

Six days after induction of glomerulonephritis, the animals were anesthetized with isoflurane. Following a midline abdominal incision, 5-10 ml blood was drawn from the lower abdominal aorta and kidneys were subsequently perfused with 30 ml ice-cold PBS. For histological examination cortical tissue was snap frozen and fixed in 10% neutral buffered formalin. Glomeruli from individual rats were isolated by a graded sieving technique (150, 125, and 75 μm mesh metal sieves) as described previously (Okuda et al., J.Clin.Invest. 86:453-462 (1990)), and resuspended at 5000 glomeruli/ml/well in RPMI supplemented with 0.1 U/ml insulin, 100 U/ml penicillin, 100 μg/ml streptomycin and 25 mM HEPES buffer. After a 48 hour incubation at 37°C/ 5% CO_2 the supernatant was harvested and stored at -70°C until analysis of glomerular production of fibronectin (FN), plasminogen activator inhibitor-type 1 (PAI-1) and TGF-ß1.

Measurement of Fibronectin, PAI-1and TGF-ß1

Fibronectin and PAI-1 synthesis were measured with modified ELISA assays according to published methods (Rennard e al., Anal Biochem 104:205-214 (1980)). TGF-ß1 production of cultured glomeruli was measured after acid-activation using a commercially available ELISA kit (DuoSet®, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Three samples from each rat were analyzed.

Light microscopy

All microscopic examinations were performed in a blinded fashion. Three µm sections of paraffin-embedded tissue were stained with periodic acid Schiff (PAS) and glomerular matrix expansion was evaluated as previously described (Okuda et al., supra). Briefly, in 30 glomeruli from each rat, the percentage of mesangial matrix occupying each glomerulus was rated as 0 ' 0%, 1 ' 25%, 2 ' 50%, 3 ' 75% and 4 ' 100%.

Immunofluorescent staining

Goat anti-human collagen I antibody was obtained from Southern Biotechnology Associates Inc. (Birmingham, AL, USA). Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat IgG (DAKO Corporation, Carpinteria, CA, USA) was used as secondary antibody. Monoclonal Mouse anti-cellular fibronectin EDA^+, was obtained from Harlan Sera-Lab LTD (Loughborough, LE12 9TE, England). FITC-rat $F(ab')_2$ anti-mouse IgG(H+L) was used as secondary antibody (Jackson Immunoresearch, West Grove, PA, USA). FITC-conjugated mouse anti-rat monocyte/macrophage antibody (ED1) was obtained from Serotec (Oxford, UK).

Renal tissue was snap-frozen in 2-methylbutane that had been pre-cooled at -80°C. Four micrometer sections were obtained using a cryostat (Leica CM 1800, E LICHT Company, Denver, CO, USA). Tissues were air dried and fixed in alcohol, washed in PBS, pH 7.4, incubated with the primary antibodies, washed with PBS, incubated with the appropriate FITC-conjugated secondary antibodies, washed again and mounted with cover glasses using Fluoromount-G (Southern Biotechnology Associates Inc, Birmingham, AL, USA).

The intensity of glomerular staining of collagen I and fibronectin was evaluated according to a 0 to 4 scale, which has been described in detail previously (Yamamoto et al., Kidney Int. 49:461-469 (1996)). The number of cells per glomerulus, staining positive for the monocyte/macrophage marker ED1, were counted. Thirty glomeruli per sample were evaluated. The mean values with standard error values were calculated.

RNA-Preparation and Northern Hybridization.

Total RNA was extracted by a guanidinium isothiocyanate method using Trizol® Reagent according to the manufacturer's instructions. RNA from 8 rats of each group was pooled for further examination. For Northern analysis, RNA was denatured and fractionated by electrophoresis through a 1.0% agarose gel (30 µg/lane) and transferred to a nylon membrane (BrightStar™-Plus, Ambion Inc., Austin, TX, USA). Nucleic acids were immobilized by UV irradiation (Stratagene, La Jolla, CA, USA). Membranes were prehybridized with ULTRAhyb™ buffer (Ambion Inc.) and hybridized with DNA probes labeled with ^{32}P-dATP by random oligonucleotide

priming (Strip-EZ DNA™, Ambion Inc.). The blots were washed in 2 X SSC, 0.1% SDS at room temperature for 10 minutes and in 0.1 X SSC, 0.1% SDS at 42 °C for 15 minutes 2 times. DNA probes used were: 1) mouse 18S (ATCC, Manassas, VA, USA), 2) rat procollagen α_1 cDNA (provided by Dr. D. Rowe (Genovese et al., Biochem. 23:6210-6216 (1984)), 3) Fibronectin-EDA cDNA (provided by Dr. R.O. Hynes) (Schwarzbauer et al., Cell 35:421-431 (1983)), 4) PAI-1 cDNA (provided by Dr. T. D. Gelehrter) (Zeheb et al., Gene 73:459-468 (1988)), and 5) TGF-ß1 cDNA (provided by Dr. H. Moses), (Derynck et al., J. Biol. Chem. 261:4377-4379 (1986)). Three blots were performed for each probe. Autoradiographic signals obtained with 18S cDNA probe served as controls for equal loading of the gel. Autoradiographs were scanned on a BIO-RAD GS-700 imaging densitometer (BIO-RAD Laboratories Inc, Hercules, CA, USA). Changes in mRNA levels were determined by first correcting for the densitometric intensity of 18S for each sample. For comparison, this ratio was set at unity for normal control samples and other lanes on the same gel were expressed as fold increases over this value.

Western blot

Glomeruli from individual rats were isolated and resuspended at $2x10^4$ glomeruli/ml in RIPA buffer (50mM Tris/HCl, pH 7.5, 150mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS and 1 tablet/5ml protease inhibitor Mix [Complete, mini; Roche Diagnostics Corp., Indianapolis, Indiana, USA], 50µl/5ml phosphatase inhibitor cocktail II [Sigma]). Glomeruli were homogenized two times on ice by sonication. Each 15-second sonication was followed by a 15-second cool down. After two centrifugations at 10,000 X g for 10 min at 4°C, the supernatant was stored at –

70°C until analysis. 40µl of each supernatant was separated by 10% Tris-Glycine gel electrophoresis and transferred to a 0.45µm nitrocellulose membrane (Millipore, Bedford, MA, USA). Non-specific binding was blocked by 10% non-fat milk powder in Tris-buffered saline (TBS) for 1 hour at room temperature followed by 4°C overnight incubation with primary antibody (Anti-Phospho-Smad2, Upstate, Lake placid, NY, USA, diluted 1:500 in 5% BSA in TBS/0.1%Tween-20 with 0.02% NaN_3; or Anti-ß-actin, Sigma, diluted 1:10,000). The blot was washed three times for 10 minutes in TBS/0.1%Tween-20. The second antibody, goat anti-rabbit horse-radish peroxidase or goat anti-mouse horse-radish peroxidase (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), was incubated at a dilution of 1:2,000 for an additional hour at room temperature followed by three washes as described above. Bound antibodies were detected by developing the blot in ECL™ Western blotting detection reagents (Amersham Pharmacia Biotech) for 1 min. Quantitation of the bands on autoradiograms was performed using a BIO-RAD GS-700 imaging densitometer. Changes in p-Smad2 levels were determined by correcting for the densitometric intensity of ß-actin for each sample.

Statistical Analysis and Calculation of Percent Reduction in Disease Severity.

Data are expressed as mean ± SEM. Statistical analysis of differences between the groups was performed either by ANOVA and subsequent Student-Newman-Keuls or Dunnett testing for multiple comparison or, in the case of PAS scoring, collagen I, and fibronectin immunoflouresence staining, differences between the groups were tested using analysis of ranks by Kruskal-Wallis with Dunn's post analysis. Mean or median values were considered significantly different where $P<0.05$.

The disease-induced increase in a variable was defined as the mean value for the disease control group minus the mean value of the normal control group. The percent reduction in disease severity in a treated group was calculated as:

$$\left[\frac{\text{Disease control group mean - Treated control group mean}}{\text{Disease control group mean - Normal control group mean}} \times 100\right]$$

Results

Three questions were asked in these experiments. First, is 1D11 effective in this model of nephritis and can the maximally effective dose of 1D11 be determined? Second, how does this dose compare with effects seen with maximal doses of enalapril? And third, can additivity of enalapril and 1D11 be observed, when both are administered at maximally effective doses?

Effect of 1D11

The induction of disease by the administration of OX-7 produced a rapid accumulation of extracellular matrix in glomeruli as measured by PAS staining. Representative glomeruli from this study are shown in Figure 14. The disease-induced increase in extracellular matrix is seen when Figure 14A (normal control) and 14B (disease control) are compared. Increasing doses of 1D11 (0.05mg/Kg – 5.0 mg/Kg), shown in Figure 14C - 14F, reveal a clear decrease in PAS positive material with increasing doses of 1D11. To quantify this histological effect of 1D11 on the accumulation of extracellular matrix, 30 glomeruli per animal were scored for PAS

staining, scores were averaged for each animal and then for each group. This analysis, presented graphically in Figure 15A, indicates that 1D11 produced a dose-dependent reduction in PAS staining. A moderate but significant ($p<0.05$) decrease of approximately 56% was seen for PAS staining in rats treated with 0.5mg/kg 1D11 compared to disease control animals.

In order to determine the contribution of specific matrix proteins to the PAS positive material immunofluorescent staining for specific glomerular proteins was performed. Representative glomeruli stained for fibronectin EDA^+ and type I collagen are shown in Figures 16 and 17. Again a dramatic increase in staining for matrix proteins was seen in disease control animals (Figures 16B and 17B) (up to 13- fold for collagen and 3.6-fold for fibronectin) compared to normal control animals (Figures 16A and 17A). Treatment with 1D11 produced dose-dependent decreases the in accumulation of fibronectin EDA^+ and collagen I in glomeruli which are clearly seen when disease control glomeruli (Figures 16B and 17B) are compared with increasing doses of 1D11 as shown in Figures 16C-F and 17C-F. As with PAS scores, all staining scores were averaged and are presented graphically in Figures 15B and 15C. Doses of 0.5mg/kg and 5mg/kg 1D11, reduced both fibronectin EDA^+ and collagen I deposition between 32% and 36%, however these changes were not significant.

Glomerular synthesis of proteins of interest was determined by ELISA on culture supernatants from isolated glomeruli cultured for 48h. Again, as shown in Figure 18, 1D11 treatment caused a dose-related reduction of these markers of disease. At a 1D11 dose of 5mg/kg, FN, PAI-1 and TGF-ß1 levels were reduced by

54%, 115% and 67% respectively ($P<0.05$).

Glomerular mRNA expression of fibronectin EDA^+, collagen I, PAI-1 and TGF-ß1 is shown in Figures 19 and 20. Blots for one gel are shown in Figure 19. The mean ± S.E. of densitometry values obtained from scans of three gels are shown graphically in Figure 20. 1D11 treatment reduced gene expression of fibronectin EDA^+ and PAI-1, Collagen I and TGF-ß1 mRNA in a dose-dependent manner. However, the maximal effect for the inhibition of fibronectin EDA+, collagen I and TGF-ß1 expression was achieved at a dose of 0.5mg/kg, with 5mg/kg appearing to be less effective. 1D11 maximally reduced fibronectin EDA^+ expression by 63%, PAI-1 by 58%, collagen I by 96% and TGF-ß1 by 48% (Figure 20A-D respectively).

Treatment with 1D11 also reduced proteinuria in a dose-dependent manner. 1D11 at 5mg/kg maximally reduced proteinuria giving mean urinary proteins of 17.7±2.3 mg/24h, compared to the disease control group (35.1±8.5mg/24h). However, for this readout, the control antibody 13C4 mouse IgG1 (5mg/kg) also reduced proteinuria to 10.2+/-0.9mg/24h. Hence, it is difficult to interpret the effect of 1D11 on this variable. The control antibody, 13C4, had no significant effect on any other measure of disease (compared to the disease control).

Comparison of the Effect of 1D11 and Enalapril

From data presented above, it is clear that either 0.5 or 5.0mg/Kg 1D11 show maximal therapeutic effects in this model. Also, from our previous dose-response study with enalapril (Peters et al., Kidney Int. 54:1570-1580 (1998)) , we know that

enalapril, at doses greater than 100 mg/liter in drinking water, produces the maximally obtainable therapeutic response in this model. Thus, 200 mg/liter enalapril alone or in combination with 0.5 or 5 mg/kg 1D11 were used to determine the effect of combining these two treatments.

The 0.5mg/Kg dose of 1D11 reduced matrix score by 56% whereas enalapril alone reduced it by 59% (Figures 14G and 15A). This similarity in disease reduction was seen for most measures of disease. Enalapril or maximally effective 1D11 (either 0.5mg/kg or 5mg/kg) reduced fibronectin immunostaining by 36%, and 36% (Figure 16E and 16G, Figure 2B), collagen I immunostaining by 33%, and 34% (Figure 17E and 17G, Figure 15C) and fibronectin production by 53%, and 54%, respectively (Figure 18A).

In contrast, the therapeutic effect of 1D11 on PAI-1 production by cultured glomeruli (Figure 18B) and on PAI-1 mRNA (Figure 19 and Figure 20B) appears to be somewhat greater than that seen for enalapril although statistical tests did not reach significance for comparisons between these groups. Similarly, the mRNA analysis suggested that 1D11 was much more effective in reducing collagen I mRNA than is enalapril ($P<0.05$, Figure 19 and 20C).

Combination of 1D11 and Enalapril

It is clear from the data that combining maximally effective doses of enalapril and

1D11 confers additional therapeutic benefits compared to either drug given alone.

Combination of enalapril with 1D11 reduced glomerular matrix accumulation, as measured by PAS staining, from approximately 59% enalapril alone or 1D11 alone to 80% for enalapril plus 5.0mg/Kg 1D11 ($p<0.001$ compared to disease control animals) (Figures 14E-I and 15A). The enhanced therapeutic effect of combined treatment with both agents was also seen for fibronectin (Figures 16 and 15B) and collagen I (Figures 17 and 15C) immunostaining, as well as glomerular production of fibronectin and PAI-1 (Figure 18A and 18B). Addition of 1D11 (0.5mg/kg) to enalapril further reduced fibronectin production by 25% to a total inhibition of 77%. In terms of proteinuria, monotherapy reduced disease-induced increases in urinary protein by 78%, 74% and 62% for enalapril, 0.5 1D11 and 5 1D11, respectively. Combination treatment further reduced proteinuria to 98% and 99% for 0.5 and 5.0 mg/Kg 1D11 respectively, values that did not differ from normal. Combination treatment had no further effect on PAI-1 production. However, this variable was reduced to levels lower than those of the normal control group, as with 1D11 alone (Figure 18B). In contrast, as shown in Figure 18C, the treatment related reduction in TGF-ß1 production by cultured glomeruli was similar across all treatment groups, indicating no additive effects of combination on this readout.

The results obtained for glomerular mRNA levels for fibronectin, collagen I, TGF-ß1 and PAI-1 produced a more mixed picture than other variables and one that did not entirely agree with the results of protein production by cultured glomeruli (Figures 19 and 20). Fibronectin mRNA levels were similarly reduced whether enalapril or 1D11 were used alone and the maximum inhibition of fibronectin mRNA

was achieved with 0.5mg/kg 1D11, with 5mg/kg being less effective. However, the combination of enalapril with 1D11 5mg/kg appeared to reverse this apparent reduction in efficacy of 1D11 restoring the inhibition of fibronectin mRNA production (Figure 20A). Interestingly, while the therapeutic effect of enalapril or 1D11 alone resulted in very similar reductions in PAI-1 mRNA, combination of these two drugs appeared to have reversed the effect for PAI-1 message (Figure 20B). Compared to the protein levels measured in the supernatant, the data suggest that the increase in PAI-1 mRNA was not translated into PAI-1 protein production and secretion into culture supernatant. Collagen I mRNA was reduced to control levels by 1D11 treatment alone at both 0.5 and 5mg/kg, therefore, it is not surprising that combination of 1D11 and enalapril had no further effect. (Figure 20C). Finally, the picture for TGF-ß1 mRNA levels is also somewhat mixed (Figure 20D). While enalapril or 0.5mg/Kg 1D11 reduced TGF-ß1 mRNA by 29% and 48%, respectively, high-dose antibody or combinations entirely reversed this effect bringing levels essentially to those of the disease control group (Figure 20D). As with PAI-1 mRNA, this finding was seen only for TGF-ß1 mRNA and not for TGF-ß1 secreted into culture supernatant by glomeruli. The significance of these mRNA data is therefore not fully supportive of the observed protein changes. The reason for this is unknown.

Mechanisms of the Additivity of Enalapril and 1D11.

Infiltration of macrophages into injured glomeruli is thought to contribute to disease in anti-Thy1 nephritis (Westerhuis, Am. J. Path. 156:303-310 (2000)). The results of staining for ED1+ cells of the monocyte/macrophage lineage are shown in Figure 21. At the three low doses of antibody a clear dose-response is seen, with the

number of ED1+ cells being significantly ($P<0.05$) reduced by 71% with the 0.5mg/Kg dose of 1D11. However, at the highest dose of 1D11, 5mg/kg was less effective, such that the reduction was only 23% (Figure 21). Enalapril alone also significantly ($P<0.05$) reduced ED1+ cells by 62%. Interestingly, as with fibronectin mRNA, combination of both drugs appeared to prevent the rebound seen with high-dose 1D11 and the reduction in $ED1^+$ cells was sustained at 70% and 69% in the two combination groups (Figure 21).

To study intracellular TGF-ß signal transduction, activation of Smad2 was analyzed using

an antibody specifically detecting phosphorylated Smad2 (Figure 22). Diseased glomeruli have dramatically increased intracellular p-Smad2 compared to normal control. 1D11 dose dependently reduced Smad2 activation, with the maximal reduction of 75% at the highest dose. Enalapril also reduced Smad2 activation by 36%. Adding Enalapril to 1D11 resulted in a further reduction. 5mg/Kg 1D11 plus Enalapril nearly normalized p-Smad2 levels.

Discussion

The data presented in this example, show that increasing doses of the TGF-ß1, ß2 and ß3 neutralizing antibody 1D11 result in increasing therapeutic efficacy. In this model of acute renal fibrosis, a dose dependant reduction of fibrotic disease is observed until the maximal effect at 0.5 mg/Kg 1D11, after which, efficacy appears to plateau. Since some of the measures we employed to assess efficacy were actually less

effectively blocked in the group receiving 5.0mg/Kg 1D11, it is likely that the true maximally therapeutic dose is between 0.5 and 5.0mg/Kg. The data suggest that the maximal disease reduction possible with 1D11 is close to 50% and that the disease reduction of 1D11 given alone is comparable to enalapril given alone.

We have shown that a combination of TGF-ß inhibition with Angiotensin II blockade resulted in an enhanced disease reduction. Thus, the combination of Ang II blockade and TGF-ß inhibition could represent a major step forward in efforts to halt disease in humans. While we have previously shown additivity in anti-Thy1 nephritis with L-arginine supplementation and low protein diet and also with angiotensin blockade and low protein diet (Peters et al., Kidney Int. 57:992-1001, 2000; Peters et al., Kidney Intl 57:1493-1501 2000), both the degree of additivity and the feasibility of the combination of Ang blockade and TGF-ß inhibition are clearly superior to the previously used combinations.

A series of studies in the anti-Thy 1 model have shown that monotherapy with enalapril, losartan, 1% L-arginine in drinking water or 6% low protein diet all reduced markers of fibrotic disease about 50% (Peters et al., Kidney 54:1570-1580, 1998, Peters et al., Kidney Int. 57:992-1001, 2000). The discovery of the increased reduction of fibrosis using a combination of inhibitors of TGF- ß1, ß2 and ß3, with Ang blockade, is particularly important, because of the many molecules involved in fibrosis, none has been so consistently or thoroughly implicated as overexpression of TGF-ß. Here, even with the potent anti-TGF-ß neutralizing antibody 1D11, we see only a maximum 50% reduction in disease severity. The fact that no single treatment

is able to prevent fibrotic disease suggests, as we propose, that either different pathways are mediating disease, or that the therapy itself stimulates back-up mechanisms to operate. Interestingly, the severity of fibrosis in the rat model of unilateral ureteral obstruction was also decreased about 50% in animals, where both angiotensinogen genes were disrupted (Fern et al., J. Clin. Invest. 103:39-46 (1999)).

It should be noted that a number of studies have shown additivity of sub-optimal doses of various treatments including ACEi and AT_1RA (Peters et al., Kidney Intl 57:1493-1501 (2000), Benigni et al., Kidney Int. 54:353-359 (1998), Remuzzi et al., J Am Soc Nephrol 10:1542-1549 (1999), Amann et al., J Am Soc Nephrol 12:2572-2584 (2001), Benigni et al., J Am Soc Nephrol 14:1816-1824 (2003)). While combinations of sub-optimal doses of two drugs may be clinically useful to reduce side effects of high doses of one drug, true additivity, or synergism, can only be shown when maximally effective doses of at least one of two drugs are given. This is the first demonstration of true additivity of ACE inhibition and TGF-ß antibodies. The ability of 1D11 and enalapril to show additivity, when given at maximally therapeutic doses, suggests that these drugs act, at least in part, through different pathways. This is in contrast to results when enalapril and losartan were combined and no additivity was seen (Peters et al., Kidney Int 54:1570-1580 (1998)), suggesting that these drugs work though a common pathway.

An interesting finding in the study presented here is that the 1D11 dose response is modulated by the addition of enalapril. Reversal of the therapeutic effect of 1D11 on matrix score (Figure 15A), fibronectin mRNA (Figure 20A), collagen I

mRNA (Figure 20C) and TGF-ß1 mRNA (Figure 20D) were seen at the highest dose of 1D11 (5.0mg/Kg). While this reversal was often marginal for all but fibronectin, the results do suggest that more complete inhibition of TGF-ß may be less effective at treating disease. This is most clearly seen in Figure 8, where the highest dose of 1D11, 5mg/kg, is less effective at reducing ED1+ cell influx than a lower dose of this agent. Interestingly, a similar effect on monocyte/macrophage cells with the 5.0mg/Kg dose of 1D11 was recently reported in the puromycin nephropathy model (Ma L.J.- et al. J Am Soc Nephrol 12:2001:819A). In the present study, the therapeutic effect was also reduced with this dose of 1D11. Both Ang II and TGF-ß play important roles in recruitment of activated macrophages to the site of injury. These activated macrophages are thought to release chemotactic and profibrotic factors. TGF-ß, however, has dual effects on macrophages. It strongly attracts macrophages and at the same time inhibits activated macrophage function by reducing the production and release of other chemotactic and inflammatory factorsLetterio et al., Annu Rev Immunol 16:137-161 (1998), Pawluczyk et al., Kidney Int. 60:533-542 (2001), Suto et al., J Immunol 159:2476-2483 (1997)). It is possible that increasing doses of 1D11 decrease macrophage infiltration initially, but as more TGF-ß is neutralized, escape from TGF-ß inhibition may lead to release of agents that further recruit macrophages. While the reduction of effect at higher doses of 1D11 and the possible role of macrophages in this observation requires further study, it is very interesting that combination of high-dose 1D11 with enalapril appears to reverse this tendency to rebound for all variables, except for TGF-ß1 mRNA. This supports the notion that combination of Ang II blockade with TGF-ß antibodies will not only provide greater therapeutic benefit than currently available therapies but that any

potential negative consequences may be ameliorated by this drug combination.

It is noteworthy that TGF-ß antibody treatment did not cause any noticeable side effects over the 6 days of the experiment. Other long-term studies with TGF-ß antibody, including four publications with the mouse monoclonal antibody, 1D11 used here (Ziyadeh et al.Proc natl Acad Sci USA 97:8015-8020 (2000), Miyajima et al., Kidney Int 58:2301-2313 (2000),), Dahly et al. Am J Physiol Regul Integr Comp Physiol 283:R757-767, (2002), Benigni et al., J Am Soc Nephrol 14:1816-1824 (2003), Islam et al., Kidney Int. 59:498-506 (2001), Ling et al. J Am Soc Nephrol 14:377-388 (2003)), reported no safety problems with this therapy.

In summary, the study presented here demonstrates a clear dose-dependant therapeutic response for the TGF-ß1, ß2 and ß3 neutralizing antibody, 1D11. The maximal therapeutic effect of monotherapy is close to 50% disease reduction, whether the therapy is Ang II blockade, or TGF-ß inhibition. Finally, and most importantly, the data show an additional reduction in disease severity when maximally effective doses of enalapril are combined with TGF-ß antibody.

Various publications are cited herein that are hereby incorporated by reference in their entirety. As will be apparent to those skilled in the art in which the invention is addressed, the present invention may be embodied in forms other than those specifically disclosed without departing from the spirit or potential characteristics of the invention. Particular embodiments of the present invention described above are therefore to be considered in all respects as illustrative and not restrictive. The scope of the invention is as set forth in the appended claims and equivalents thereof rather than being limited to the examples contained in the foregoing description.

WHAT IS CLAIMED IS:

1. A method for reducing excess accumulation of extracellular matrix associated with TGFß overproduction and/or activity, in a tissue and/or organ, or at a dermal wound site, by administering a combination of agents that inhibit TGFß, in an amount sufficient to inhibit TGFß overproduction and/or activity, the administration of said combination of agents resulting in greater reduction in extracellular matrix, than when each agent is administered separately, whereby the accumulation of extracellular matrix in said tissue and/or organ or wound site is reduced from the level existing at the time of administration of the agents.

2. The method of claim 1, wherein said agents that inhibit TGFβ are selected from the group consisting of inhibitors of aldosterone, inhibitors of angiotensin II, anti-TGFβ antibodies, renin, ACE inhibitors, AII receptor antagonists, proteoglycans and ligands for the TGFβ receptor.

3. The method of claim 2, wherein said agent is a proteoglycan selected from the group consisting of decorin, biglycan, fibromodulin, lumican, betaglycan and endoglin.

4. The method of claim 2, wherein said ACE inhibitor is Enalapril^{tm}.

5. The method of claim 2, wherein said AII receptor antagonist is Losartan^{tm}.

6. The method of claim 2, wherein said anti-TGFß antibody is 1D11.

7. The method of claim 2, wherein one agent of the combination is an anti-TGFß antibody and another agent is an inhibitor of angiotensin II.

8. The method of claim 7, wherein the anti-TGFß antibody is 1D11.

9. The method of claim 8, wherein the inhibitor of angiotensin II is enalapril.

10. The method of claim 7, wherein the inhibitor of angiotensin II is enalapril.

11. The method of claim 1 wherein said reducing the accumulation of extracellular matrix associated with TGFβ activity, comprises contacting renin with an anti-renin agent.

12. The method of claim 1, further comprising the step of degrading excess accumulated extracellular matrix in said tissue and/or organ or wound site.

13. The method of claim 1, wherein said excess accumulation of extracellular matrix is associated with a fibrotic condition.

14. The method of claim 13, wherein said fibrotic condition is selected from the group consisting of glomerulonephritis, adult or acute respiratory distress syndrome (ARDS), diabetes, diabetic kidney disease, liver fibrosis, kidney fibrosis, lung fibrosis, post infarction cardiac fibrosis, fibrocystic diseases, fibrotic cancer, post myocardial infarction, left ventricular hypertrophy, pulmonary fibrosis, liver cirrhosis, veno-occlusive disease, post-spinal cord injury, post-retinal and glaucoma surgery, post-angioplasty restenosis, renal interstitial fibrosis, arteriovenous graft failure and scarring.

15. The method of claim 1, wherein said tissue or organ is selected from the group consisting of kidney, lung, liver, heart, arteries, skin and the central nervous system.

16. The method of claim 1, wherein said condition associated with the excess accumulation of extracellular matrix is scarring.

17. The method of claim 1, wherein said agents that inhibit TGFβ, are nucleic acids encoding the agents.

18. The method of claim 13, wherein said protease is nucleic acid encoding a protease.

19. The method of claim 1, wherein the agents are administered concurrently.

20. The method of claim 1, wherein the agents are administered sequentially.

21. A composition for reducing the excess accumulation of extracellular matrix in a tissue and/or organ, or at a dermal wound site, comprising two or more agents that increases the amount of active protease present in the tissue and/or organ or at the wound site in a pharmaceutically acceptable carrier.

22. The composition of claim 21, wherein said protease is plasmin and said agent is tPA..

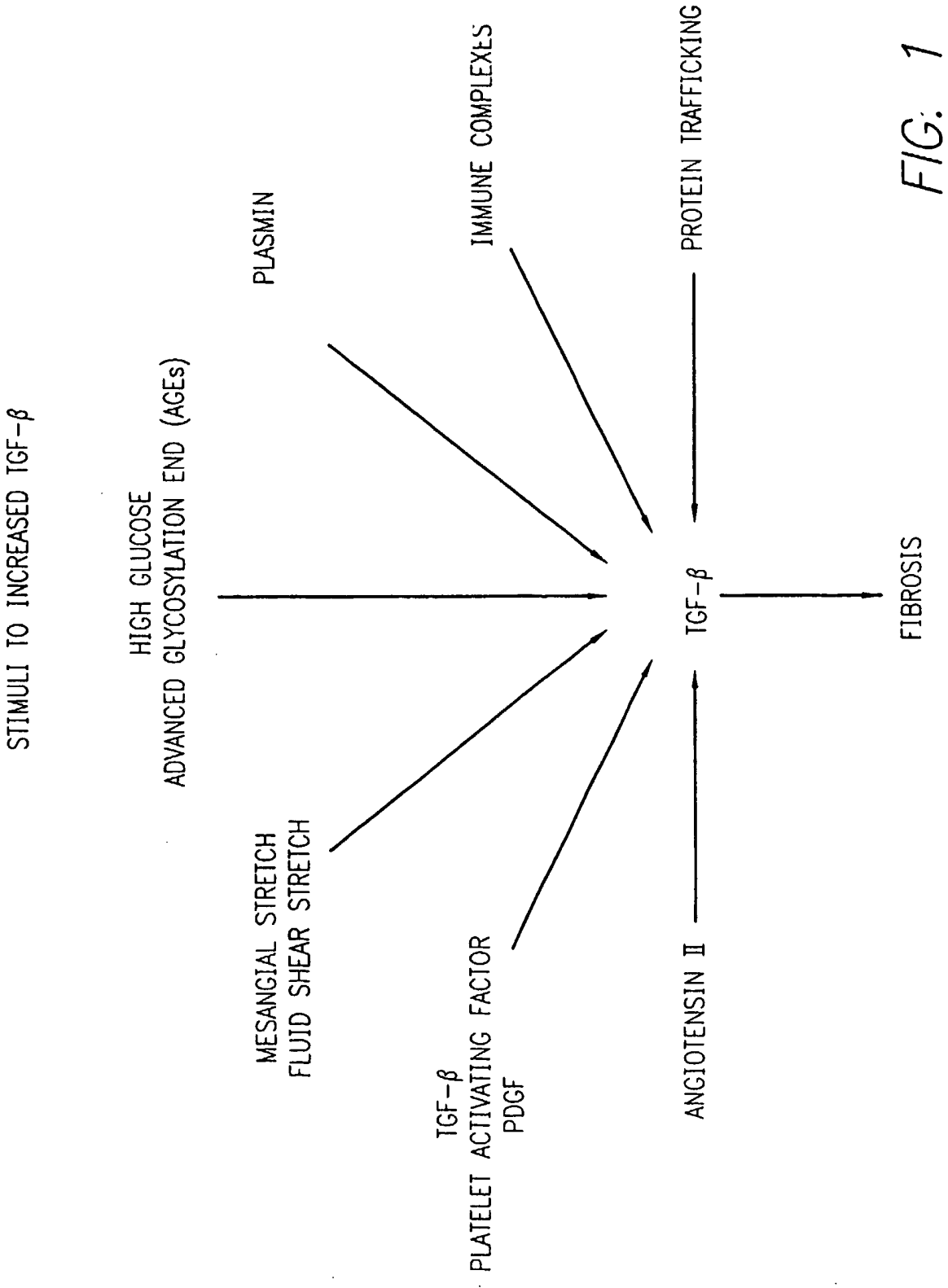

STIMULI TO INCREASED TGF-β
HIGH GLUCOSE
ADVANCED GLYCOSYLATION END (AGEs)
PLASMIN
IMMUNE COMPLEXES
PROTEIN TRAFFICKING
MESANGIAL STRETCH
FLUID SHEAR STRETCH
TGF-β
PLATELET ACTIVATING FACTOR
PDGF
ANGIOTENSIN II
TGF-β
FIBROSIS

FIG. 1

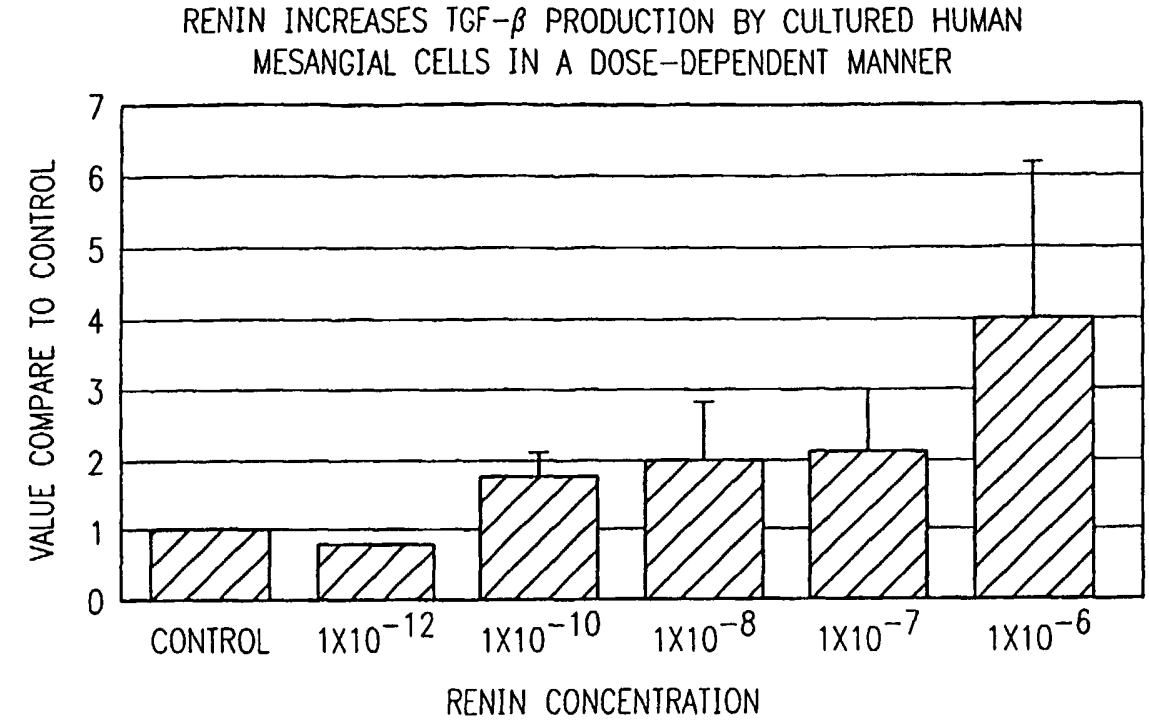

RENIN INCREASES TGF-β PRODUCTION BY CULTURED HUMAN MESANGIAL CELLS IN A DOSE-DEPENDENT MANNER
VALUE COMPARE TO CONTROL
0
1
2
3
4
5
6
7
CONTROL
1X10-12
1X10-10
1X10-8
1X10-7
1X10-6
RENIN CONCENTRATION

FIG. 2

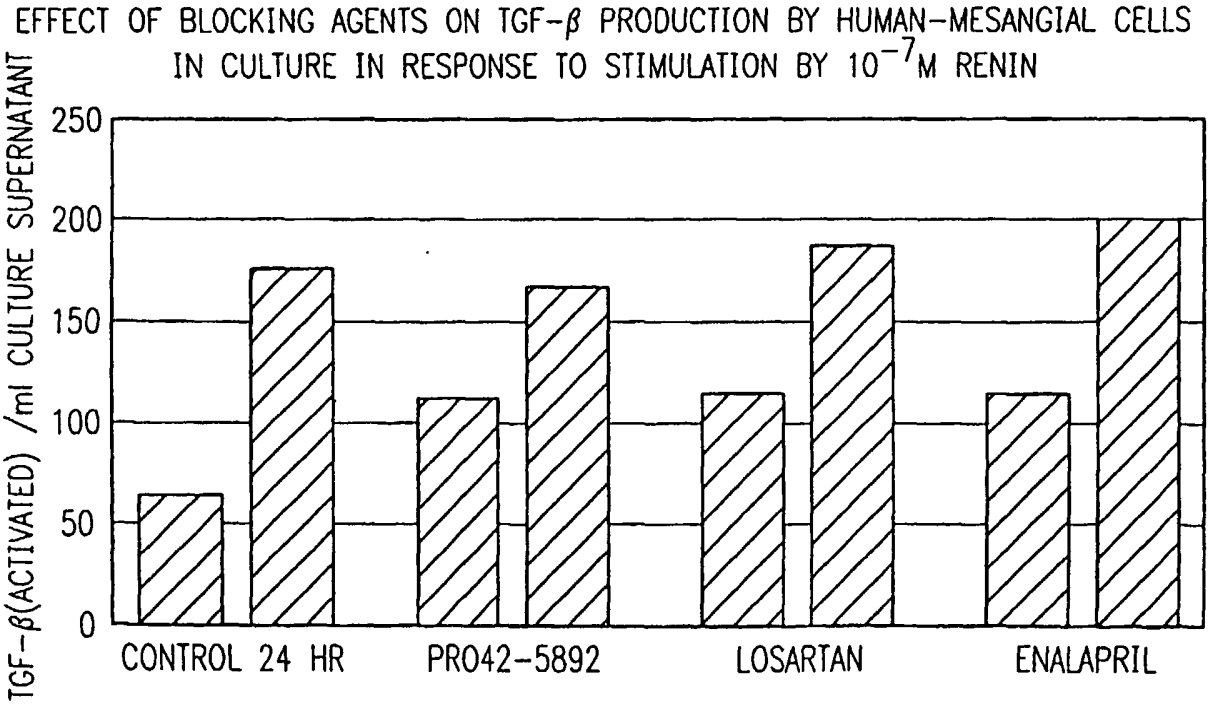

EFFECT OF BLOCKING AGENTS ON TGF-β PRODUCTION BY HUMAN-MESANGIAL CELLS IN CULTURE IN RESPONSE TO STIMULATION BY 10-7M RENIN
TGF-β(ACTIVATED) /ml CULTURE SUPERNATANT
0
50
100
150
200
250
CONTROL 24 HR
PRO42-5892
LOSARTAN
ENALAPRIL

FIG. 3

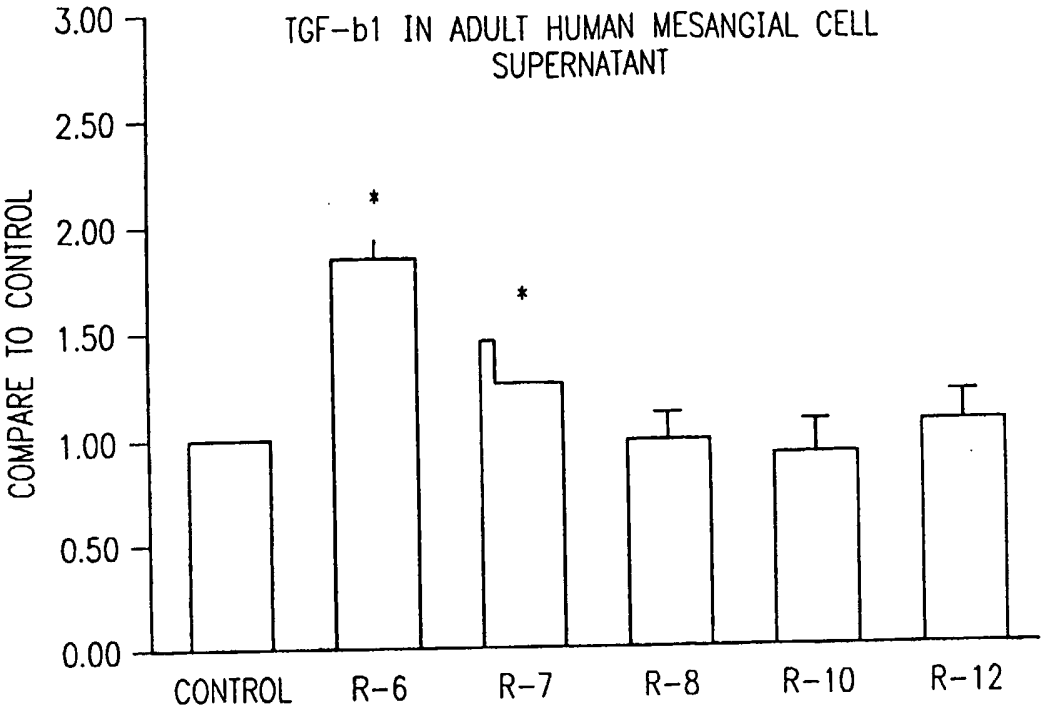


FIG. 4A

* = p < 0.05

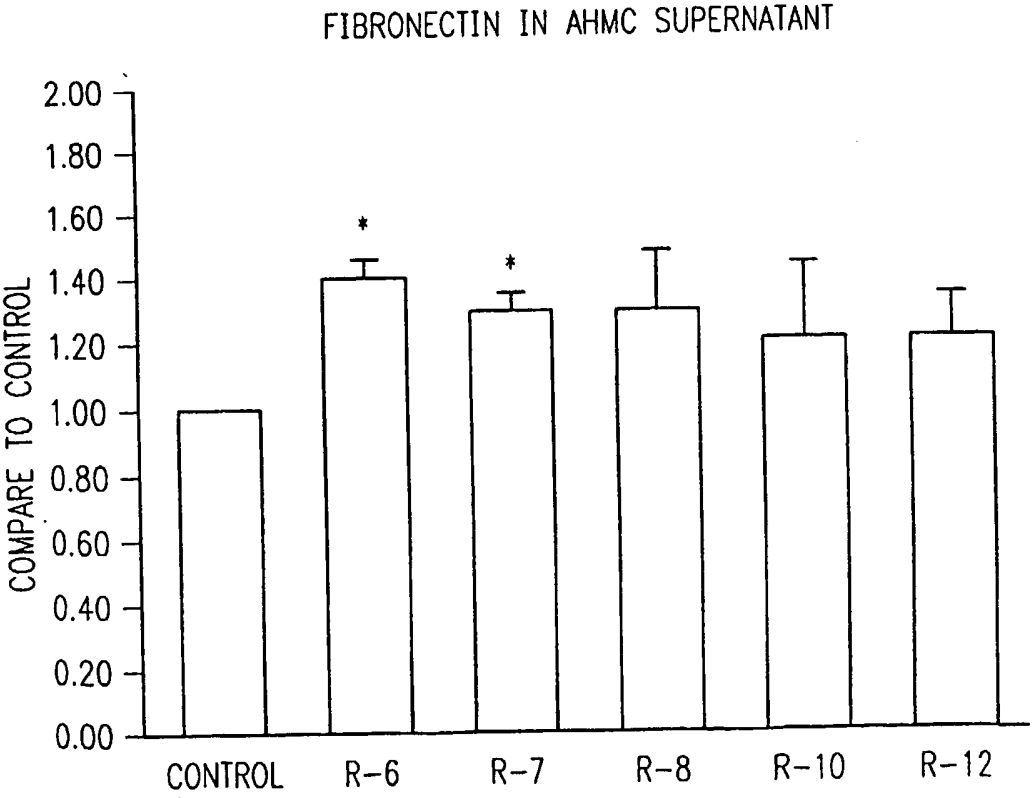


FIG. 4B

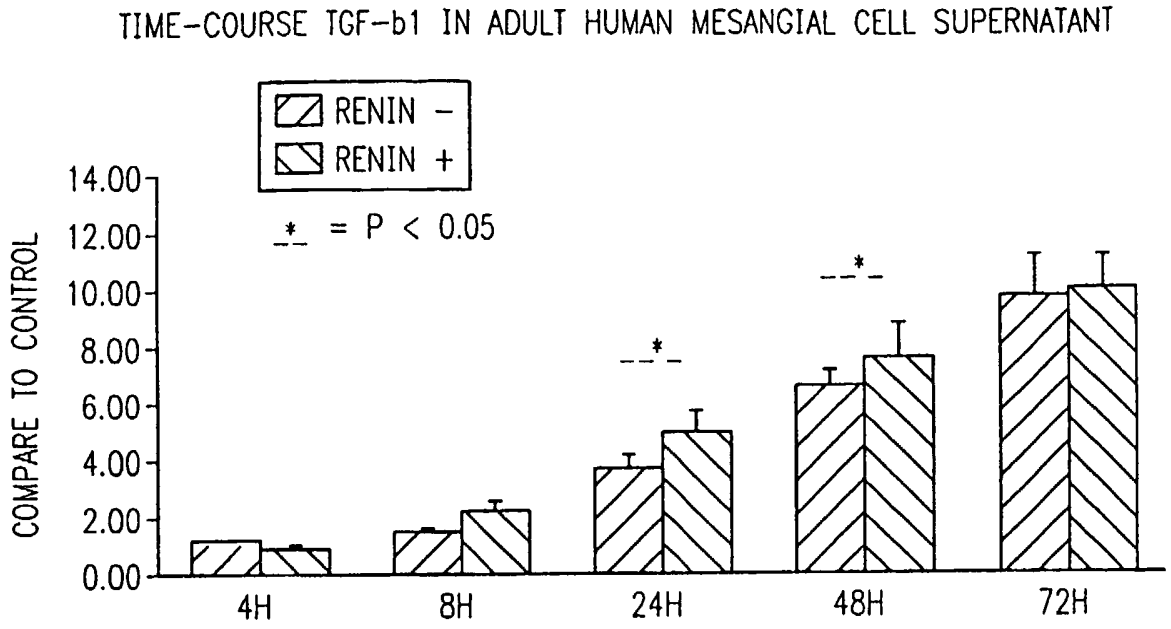

TIME-COURSE TGF-b1 IN ADULT HUMAN MESANGIAL CELL SUPERNATANT
RENIN -
RENIN +
* = P < 0.05
COMPARE TO CONTROL
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
4H
8H
24H
48H
72H

FIG. 5A

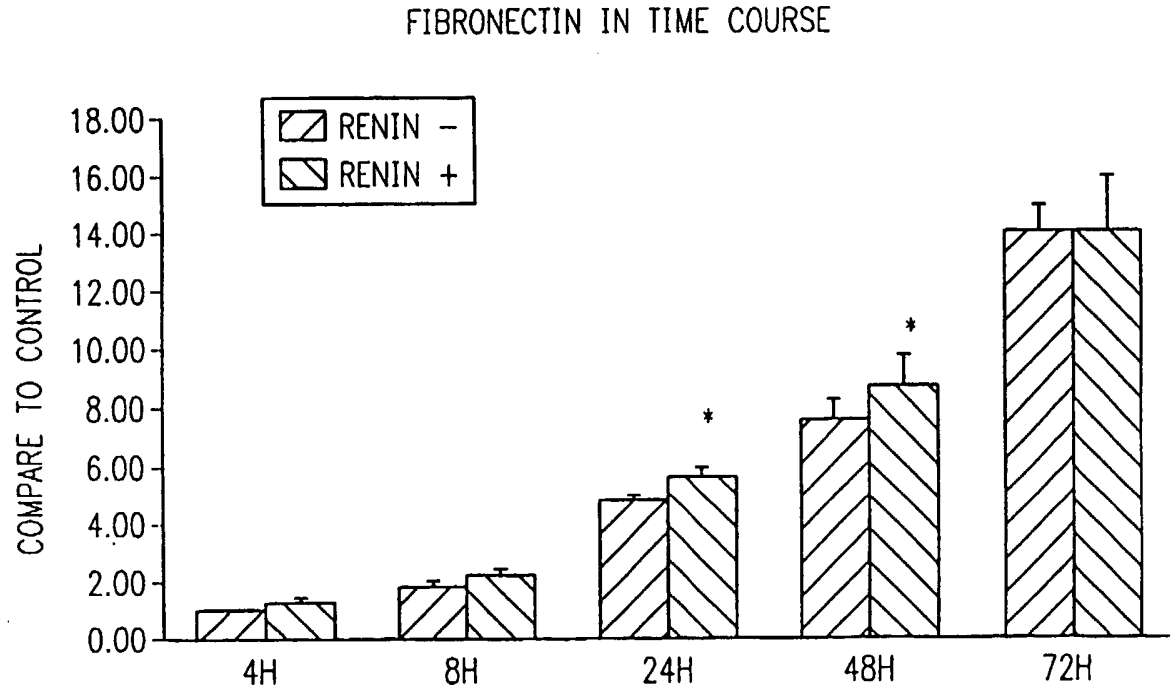

FIBRONECTIN IN TIME COURSE
RENIN -
RENIN +
COMPARE TO CONTROL
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
4H
8H
24H
48H
72H

FIG. 5B

FIG. 6A

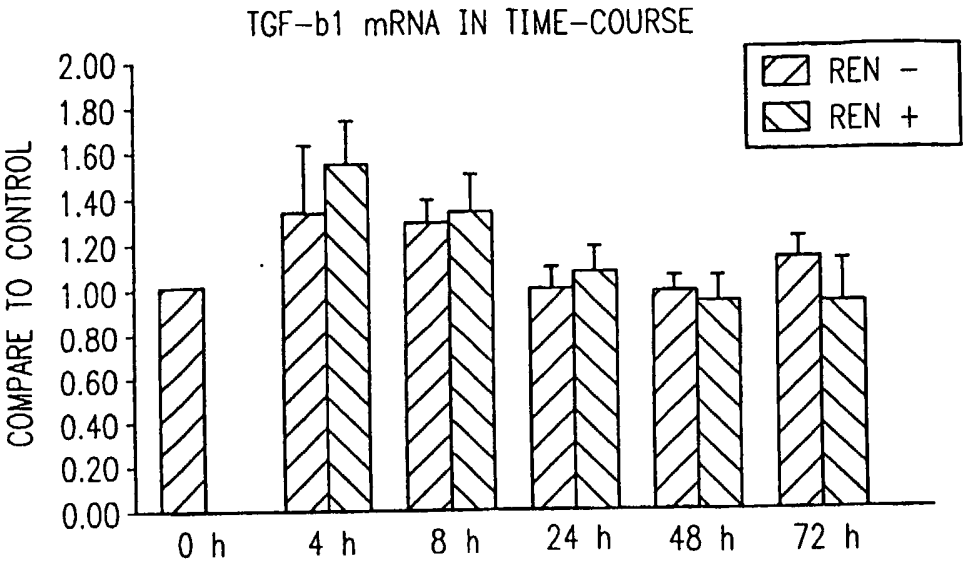


FIG. 6B

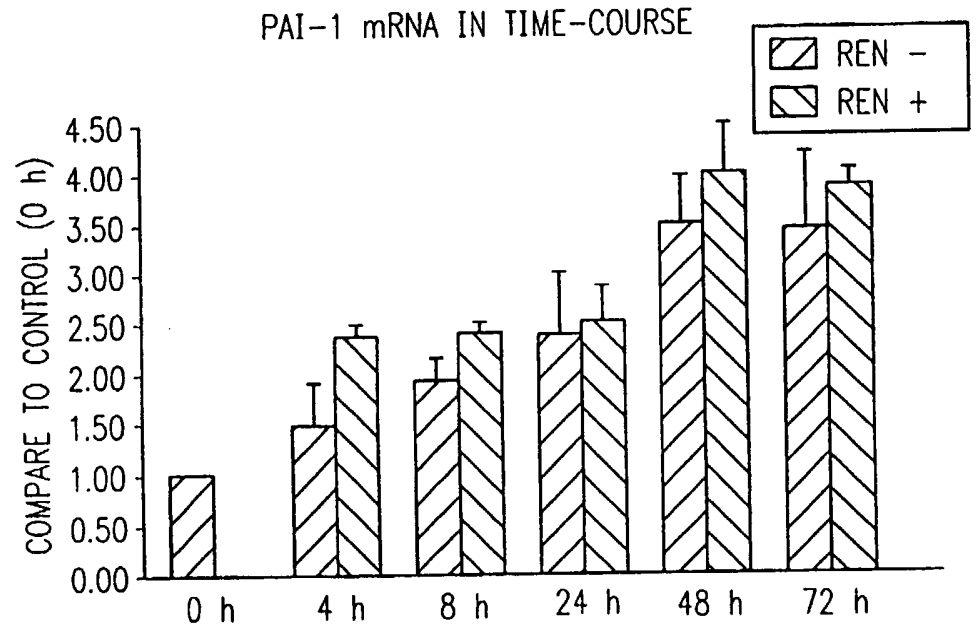
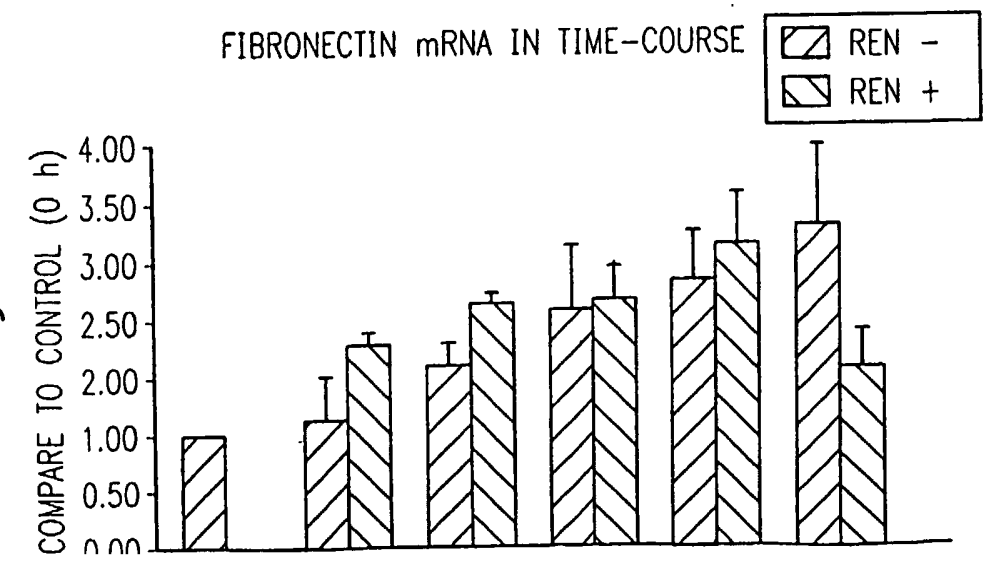


FIG. 6C



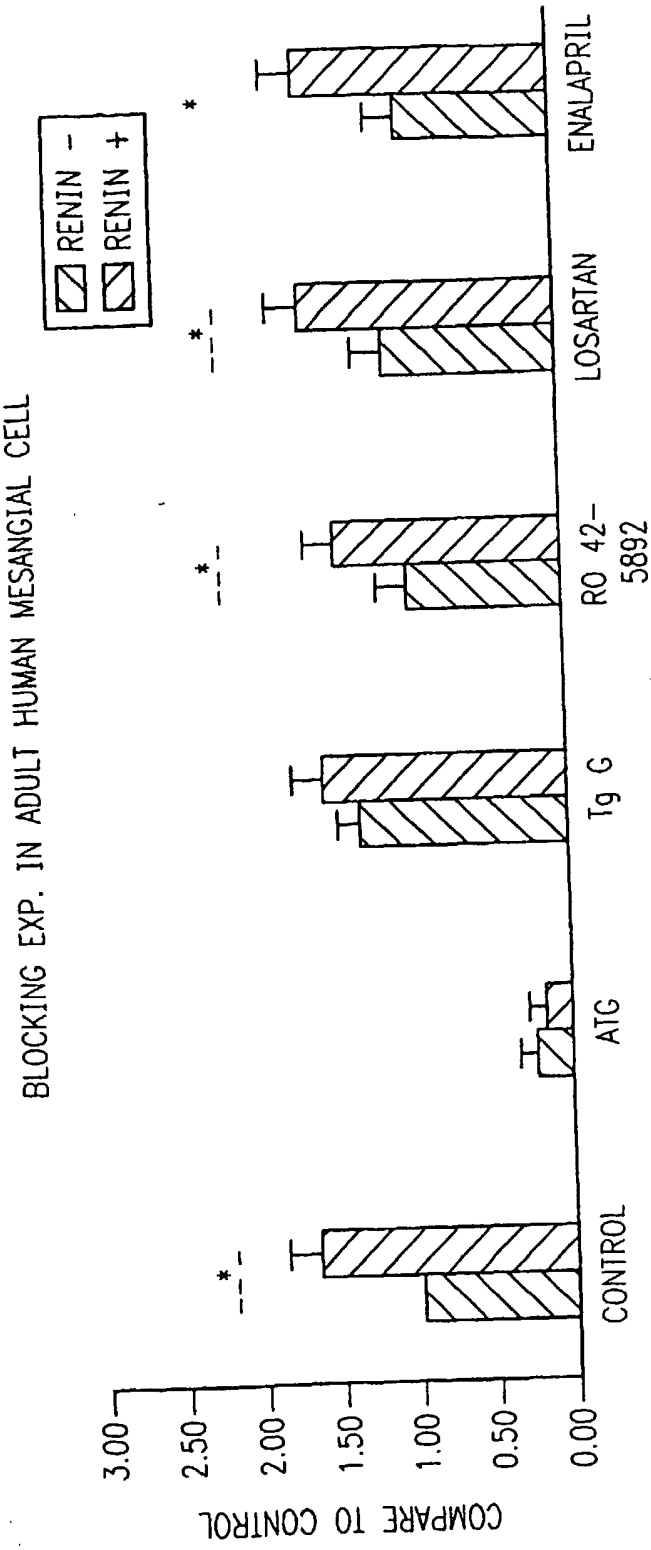

BLOCKING EXP. IN ADULT HUMAN MESANGIAL CELL
RENIN -
RENIN +
COMPARE TO CONTROL
3.00
2.50
2.00
1.50
1.00
0.50
0.00
CONTROL
ATG
Tg G
RO 42-5892
LOSARTAN
ENALAPRIL
*
*
*

FIG. 7

EFFECT OF tPA ON ANTI-THY-1 NEPHRITIS AT DAY 6

OX-7 DISEASE CONTROL

tPA TREATED (DAY 3 - DAY 5)

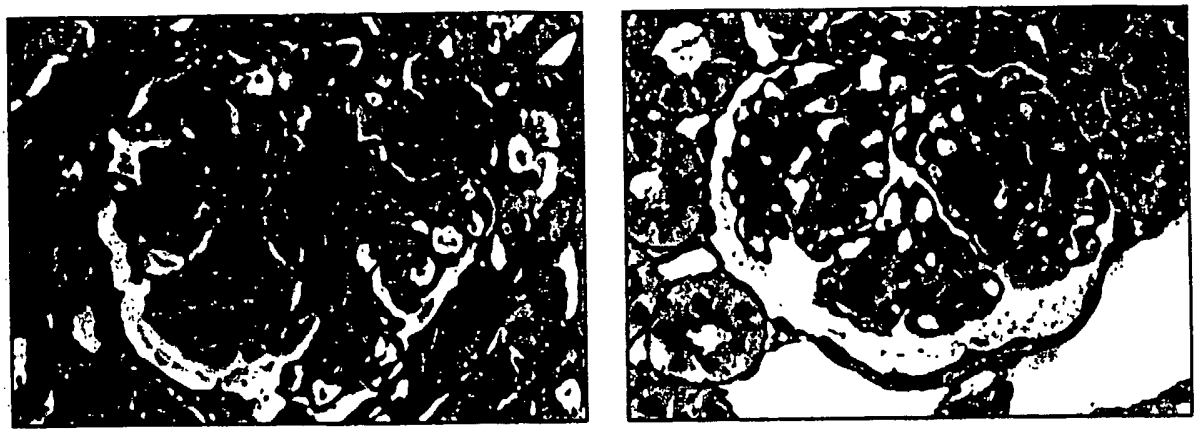


FIG. 8

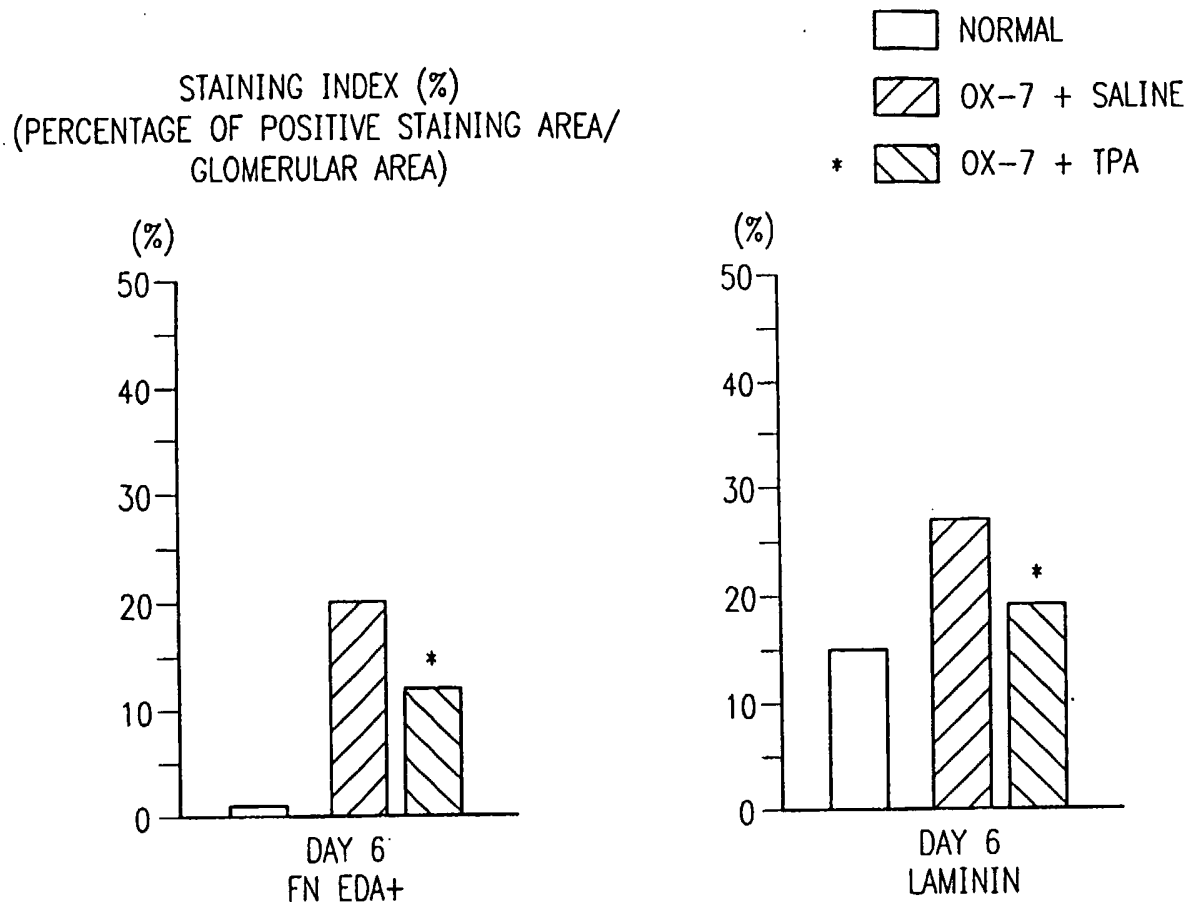


FIG. 9A

FIG. 9B

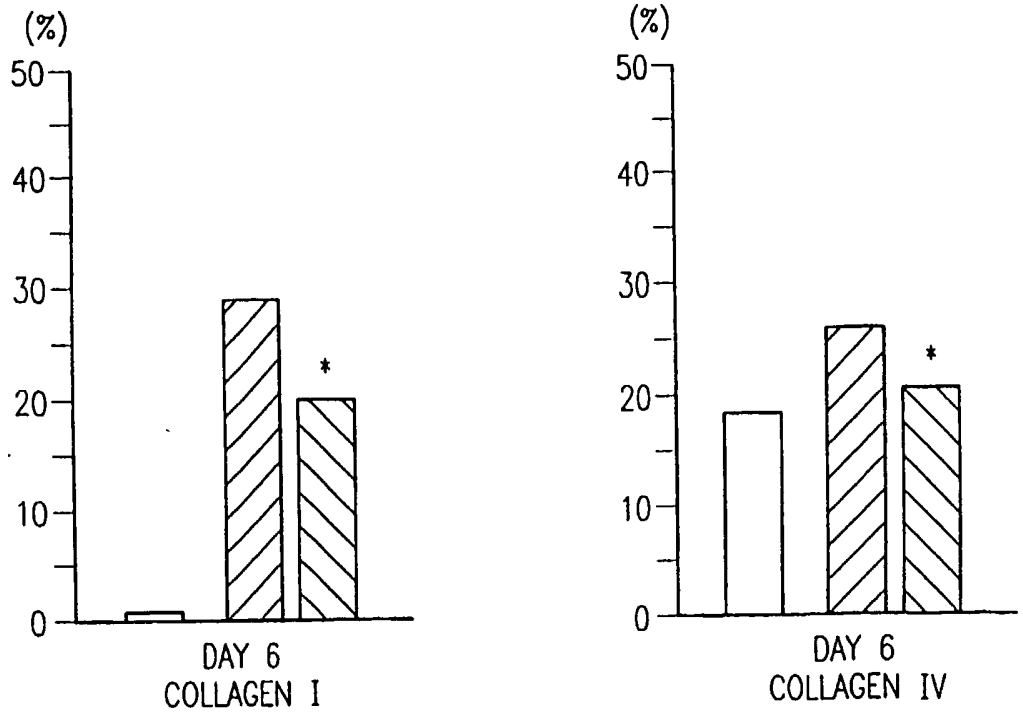


FIG. 9C

FIG. 9D

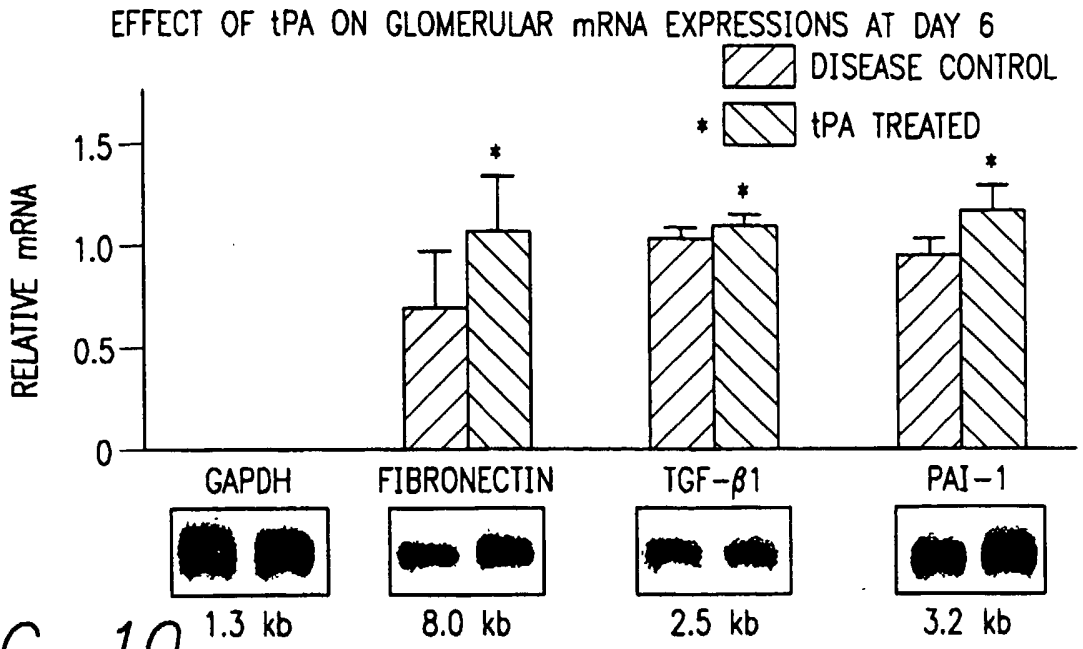

EFFECT OF tPA ON GLOMERULAR mRNA EXPRESSIONS AT DAY 6
DISEASE CONTROL
* tPA TREATED
RELATIVE mRNA
1.5
1.0
0.5
0
GAPDH
FIBRONECTIN
TGF-β1
PAI-1
1.3 kb
8.0 kb
2.5 kb
3.2 kb

FIG. 10

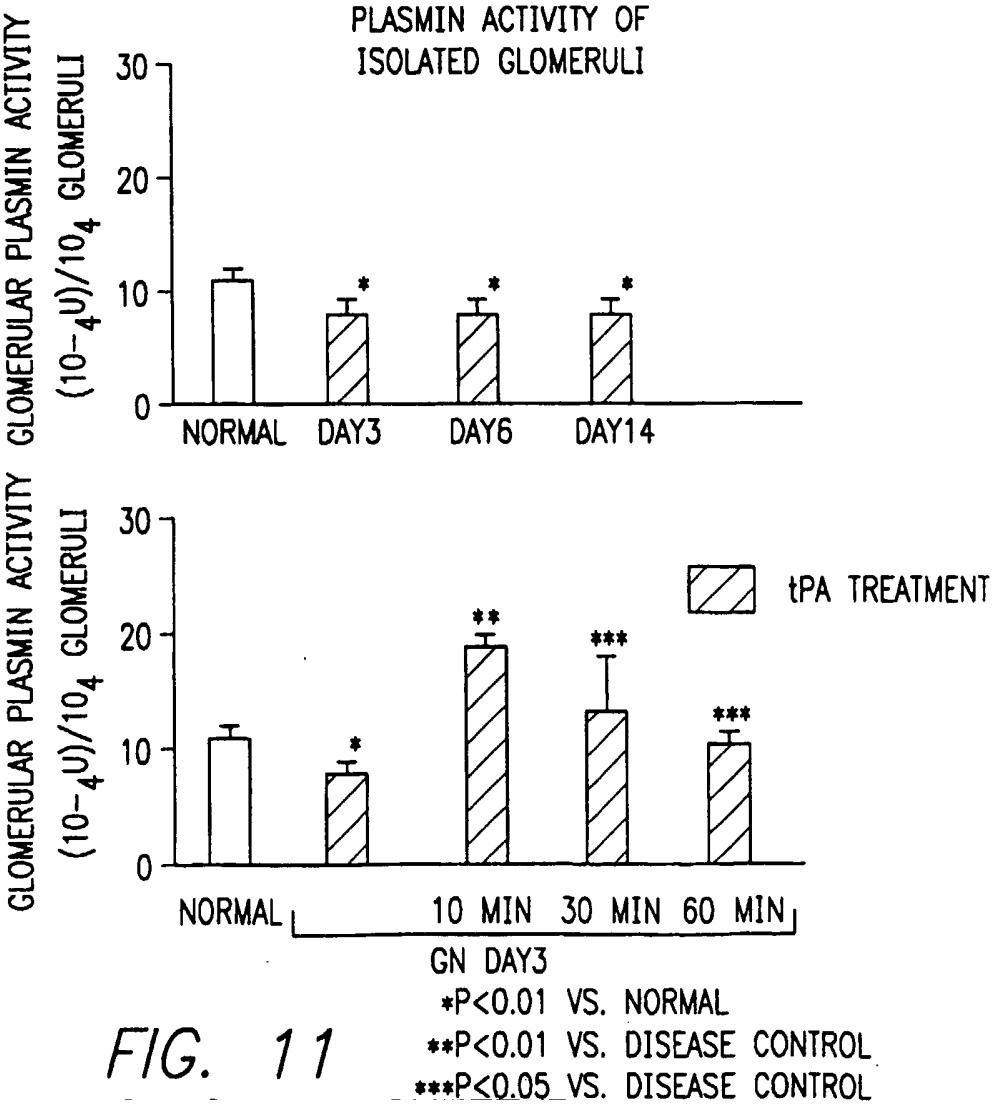

PLASMIN ACTIVITY OF ISOLATED GLOMERULI
GLOMERULAR PLASMIN ACTIVITY (10-4U)/10^4 GLOMERULI
30
20
10
0
NORMAL
DAY3
DAY6
DAY14
tPA TREATMENT
NORMAL
10 MIN
30 MIN
60 MIN
GN DAY3
*P<0.01 VS. NORMAL
**P<0.01 VS. DISEASE CONTROL
***P<0.05 VS. DISEASE CONTROL

FIG. 11

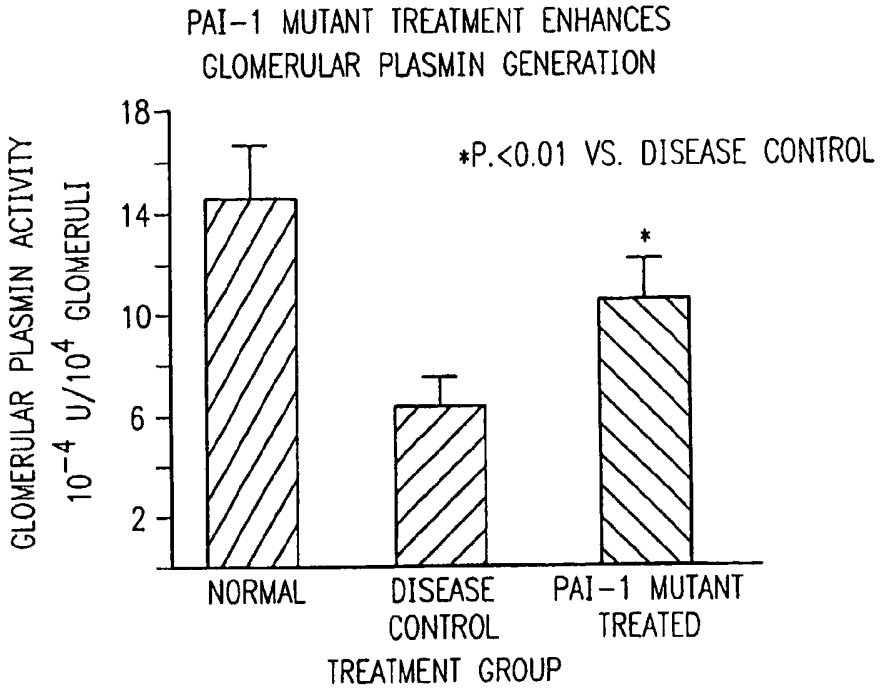

PAI-1 MUTANT TREATMENT ENHANCES
GLOMERULAR PLASMIN GENERATION
*P.<0.01 VS. DISEASE CONTROL
GLOMERULAR PLASMIN ACTIVITY
10^{-4} U/10^4 GLOMERULI
18
14
10
6
2
*
NORMAL
DISEASE CONTROL
PAI-1 MUTANT TREATED
TREATMENT GROUP

FIG. 12

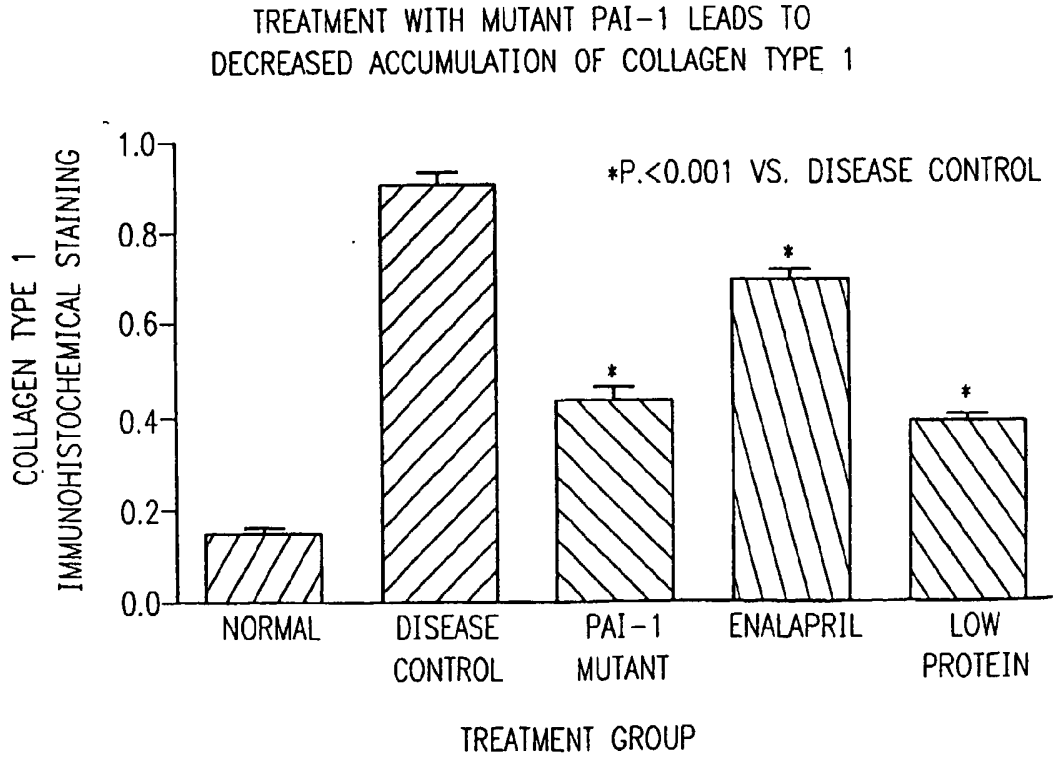

TREATMENT WITH MUTANT PAI-1 LEADS TO
DECREASED ACCUMULATION OF COLLAGEN TYPE 1
*P.<0.001 VS. DISEASE CONTROL
COLLAGEN TYPE 1
IMMUNOHISTOCHEMICAL STAINING
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
NORMAL
DISEASE CONTROL
PAI-1 MUTANT
ENALAPRIL
LOW PROTEIN
TREATMENT GROUP

FIG 13

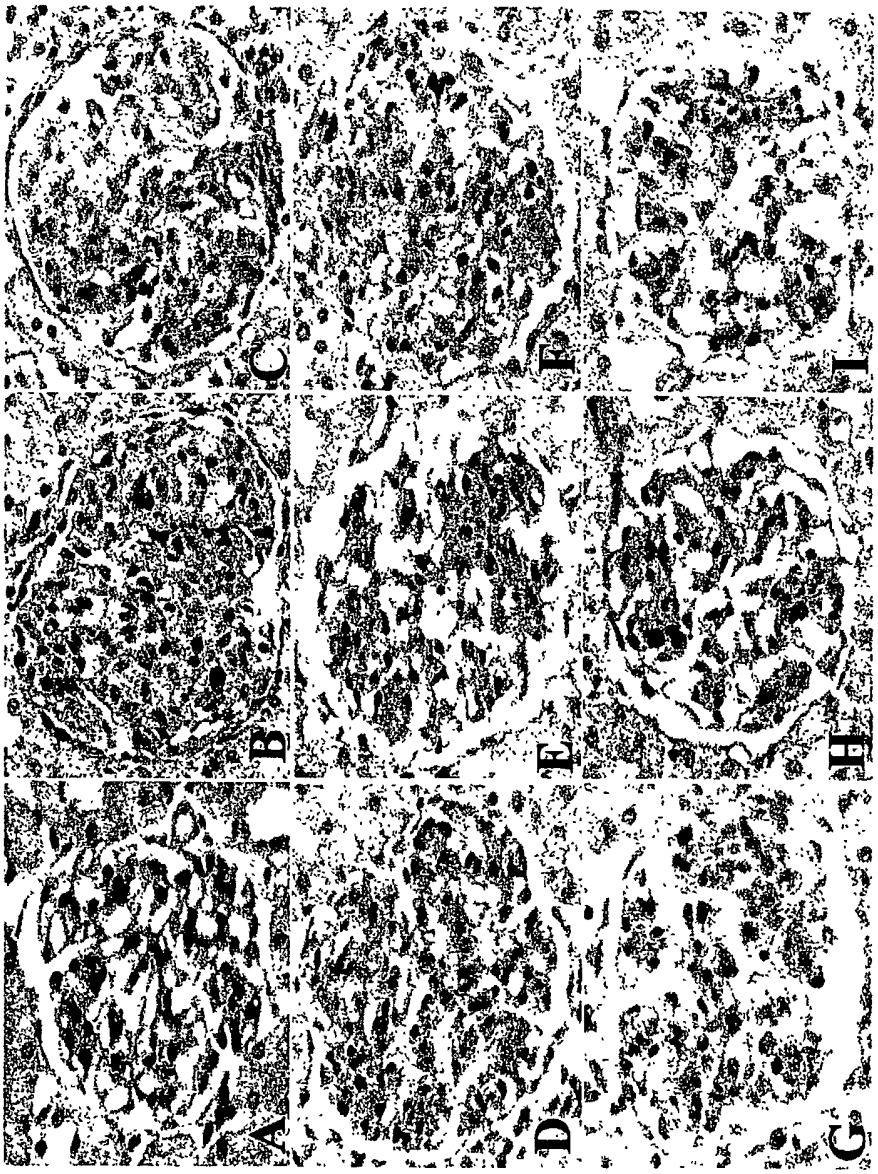


FIG. 14

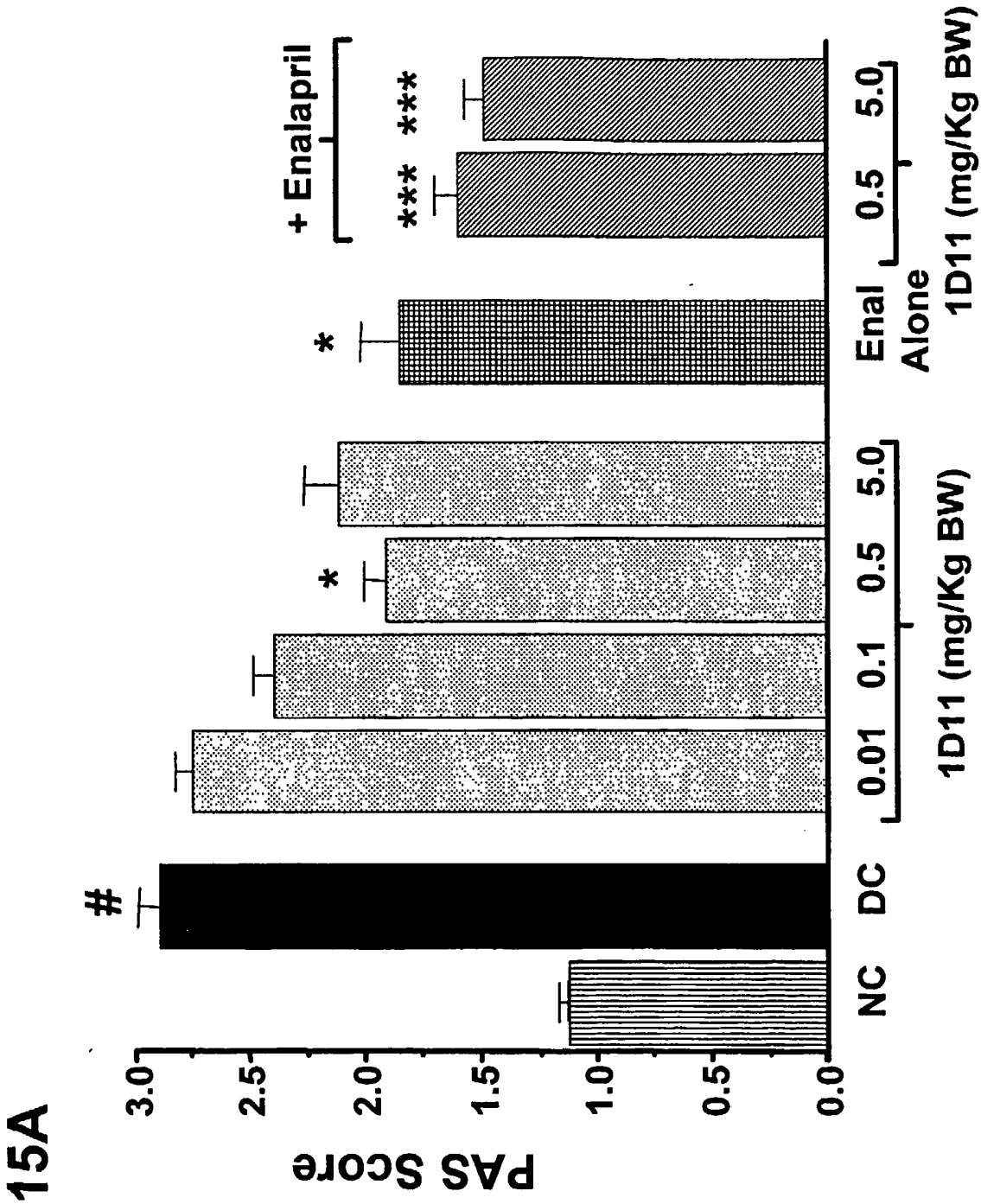

PAS Score
3.0
2.5
2.0
1.5
1.0
0.5
0.0
NC
DC
0.01
0.1
0.5
5.0
1D11 (mg/Kg BW)
#
*
*
Enal Alone
0.5
5.0
1D11 (mg/Kg BW)
*
***
***
+ Enalapril

FIG. 15A

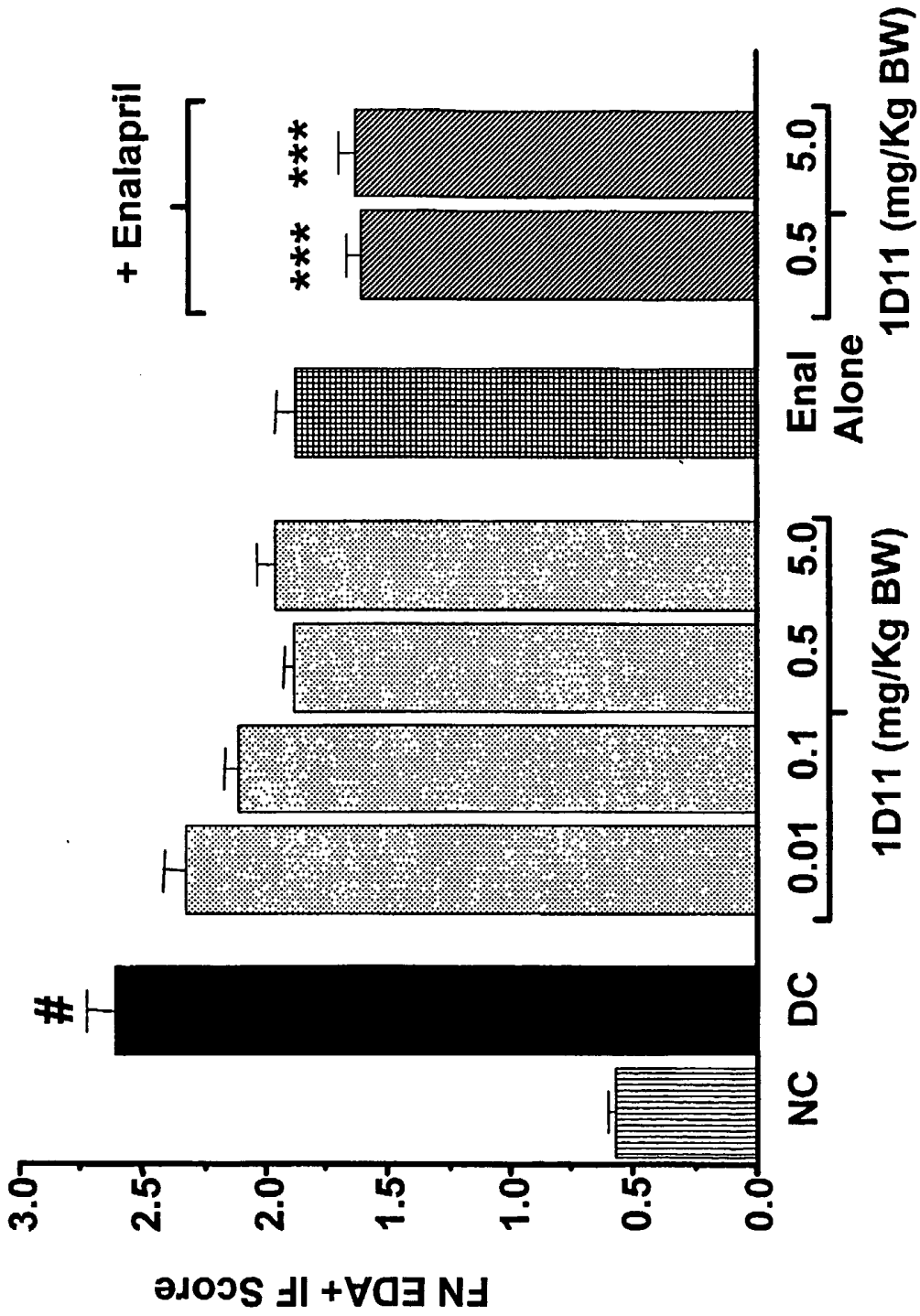

FN EDA+ IF Score
3.0
2.5
2.0
1.5
1.0
0.5
0.0
#
NC
DC
0.01
0.1
0.5
5.0
1D11 (mg/Kg BW)
Enal Alone
0.5
5.0
1D11 (mg/Kg BW)
+ Enalapril
***
***

FIG. 15B

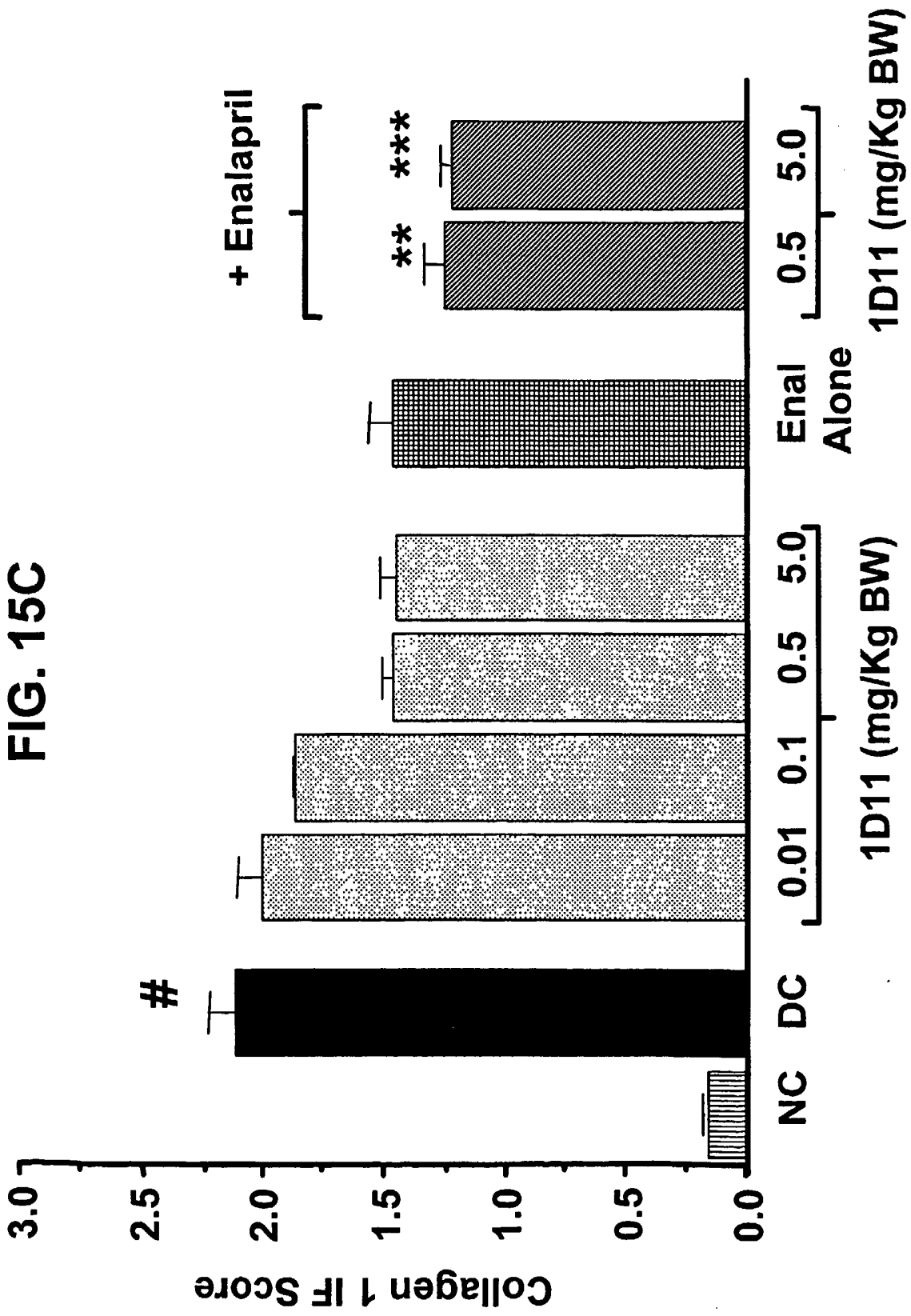


FIG. 15C
Collagen 1 IF Score
3.0
2.5
2.0
1.5
1.0
0.5
0.0
#
**
***
+ Enalapril
NC
DC
0.01
0.1
0.5
5.0
1D11 (mg/Kg BW)
Enal Alone
0.5
5.0
1D11 (mg/Kg BW)

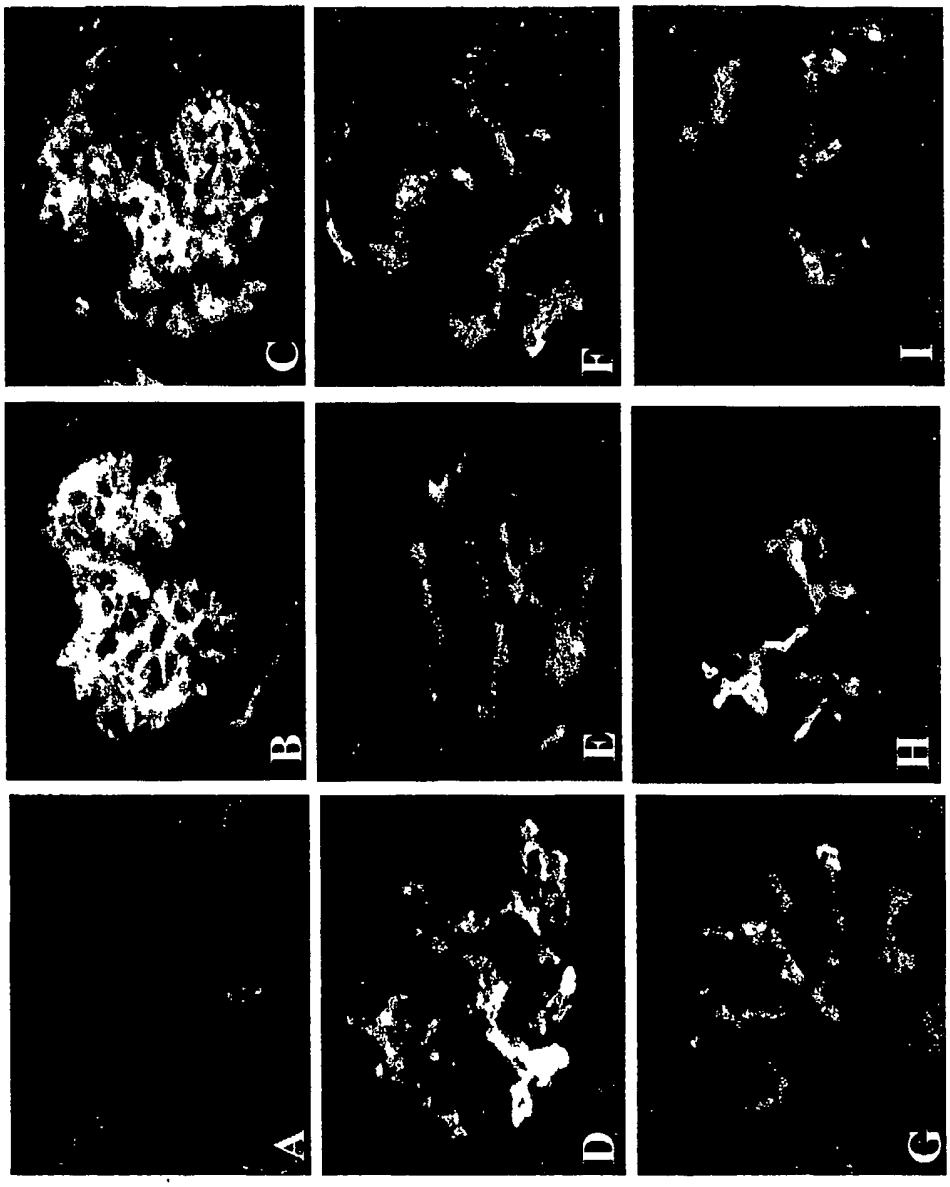


FIG. 16

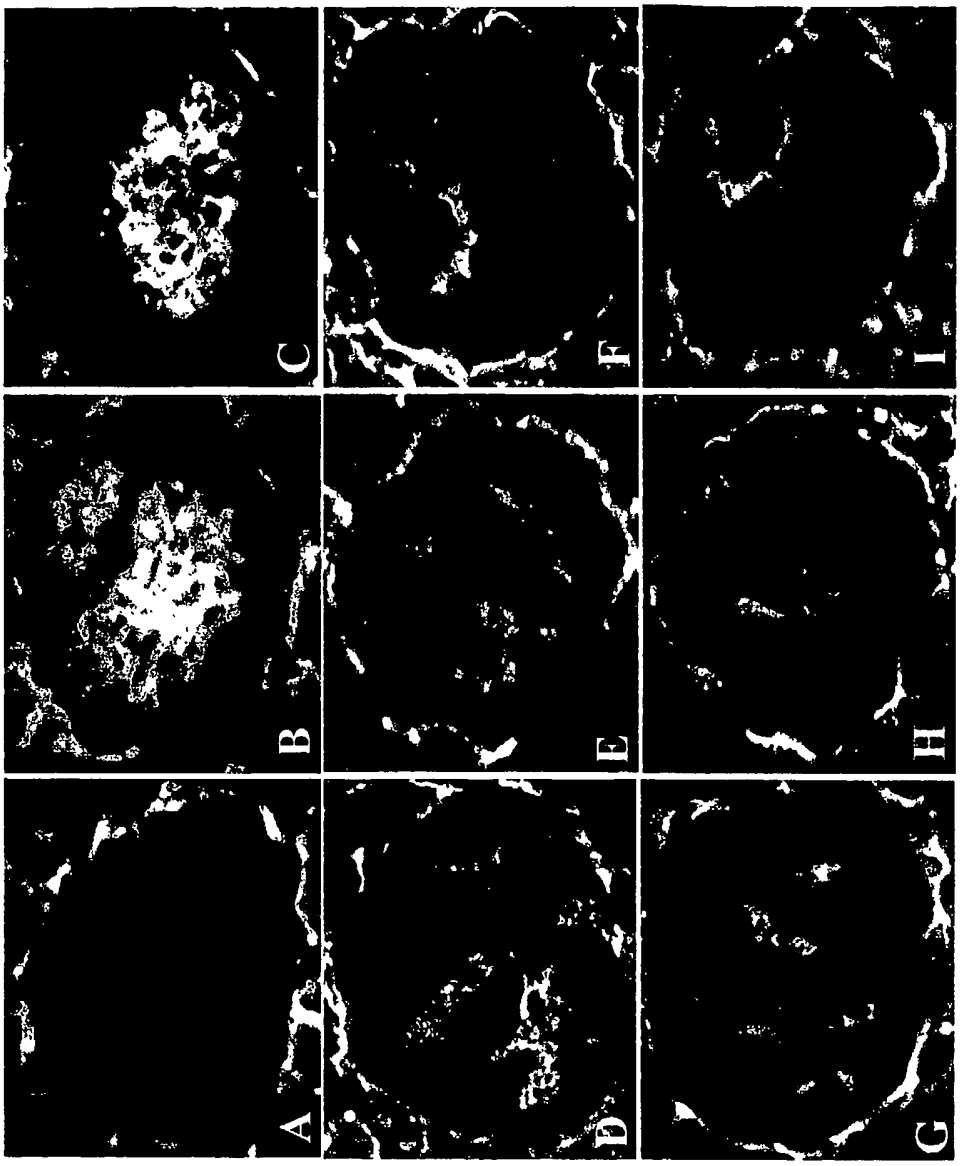
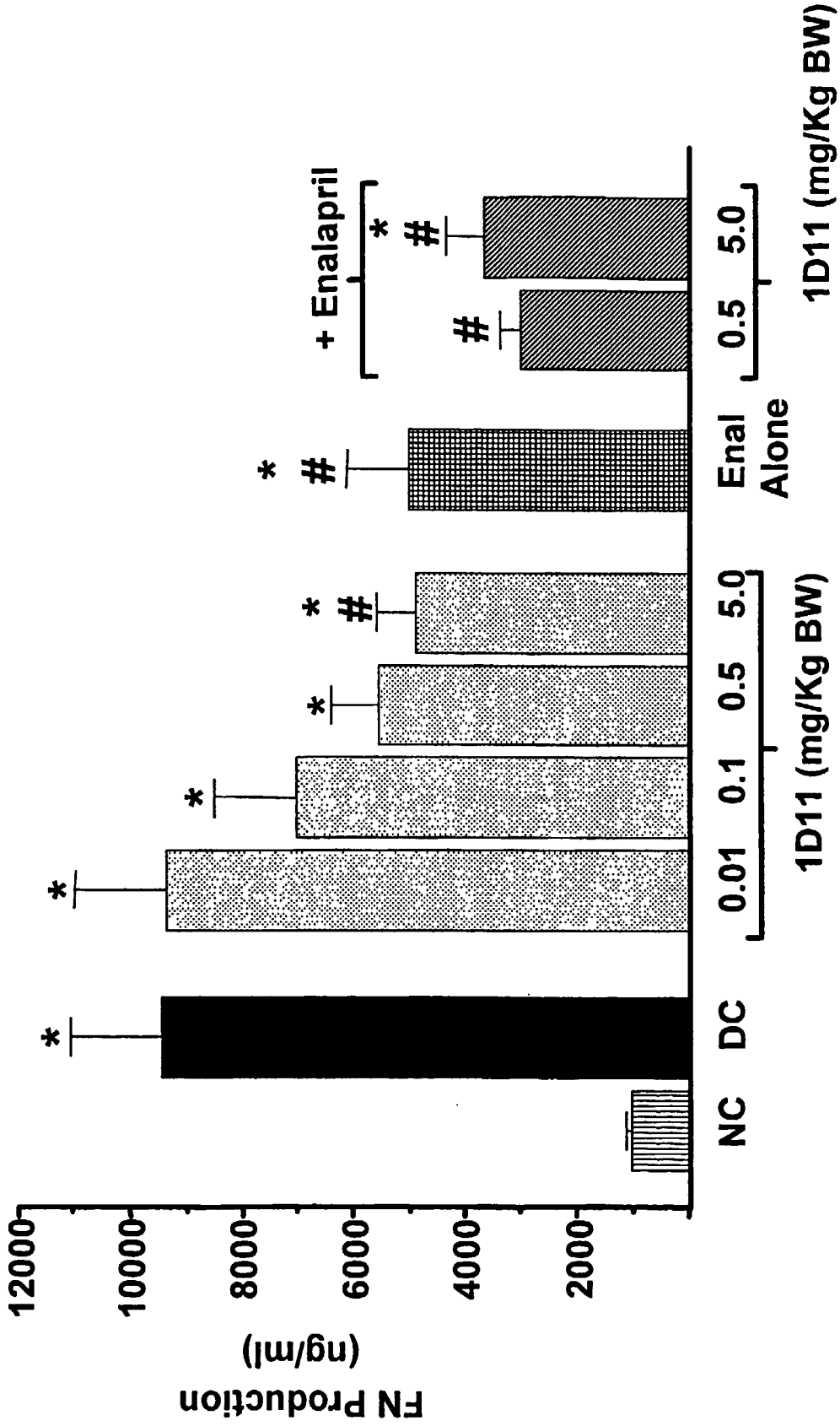


FIG. 17

FIG. 18A


FN Production
(ng/ml)
12000
10000
8000
6000
4000
2000
NC
DC
0.01
0.1
0.5
5.0
1D11 (mg/Kg BW)
Enal
Alone
0.5
5.0
1D11 (mg/Kg BW)
+ Enalapril
*
#

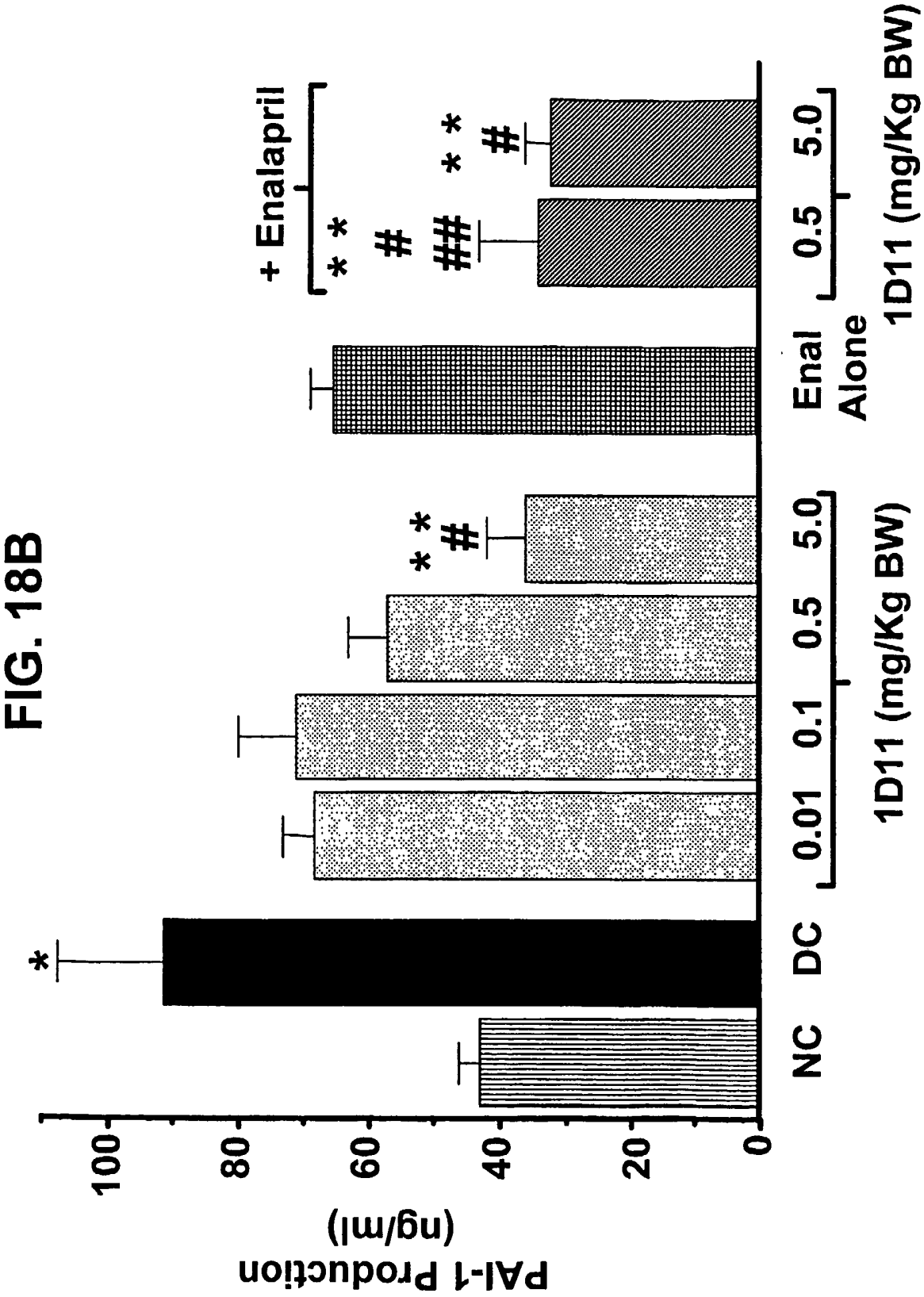


FIG. 18B
PAI-1 Production (ng/ml)
100
80
60
40
20
0
NC
DC
0.01
0.1
0.5
5.0
1D11 (mg/Kg BW)
Enal Alone
0.5
5.0
1D11 (mg/Kg BW)
+ Enalapril
*
* *
#
##
* *
#

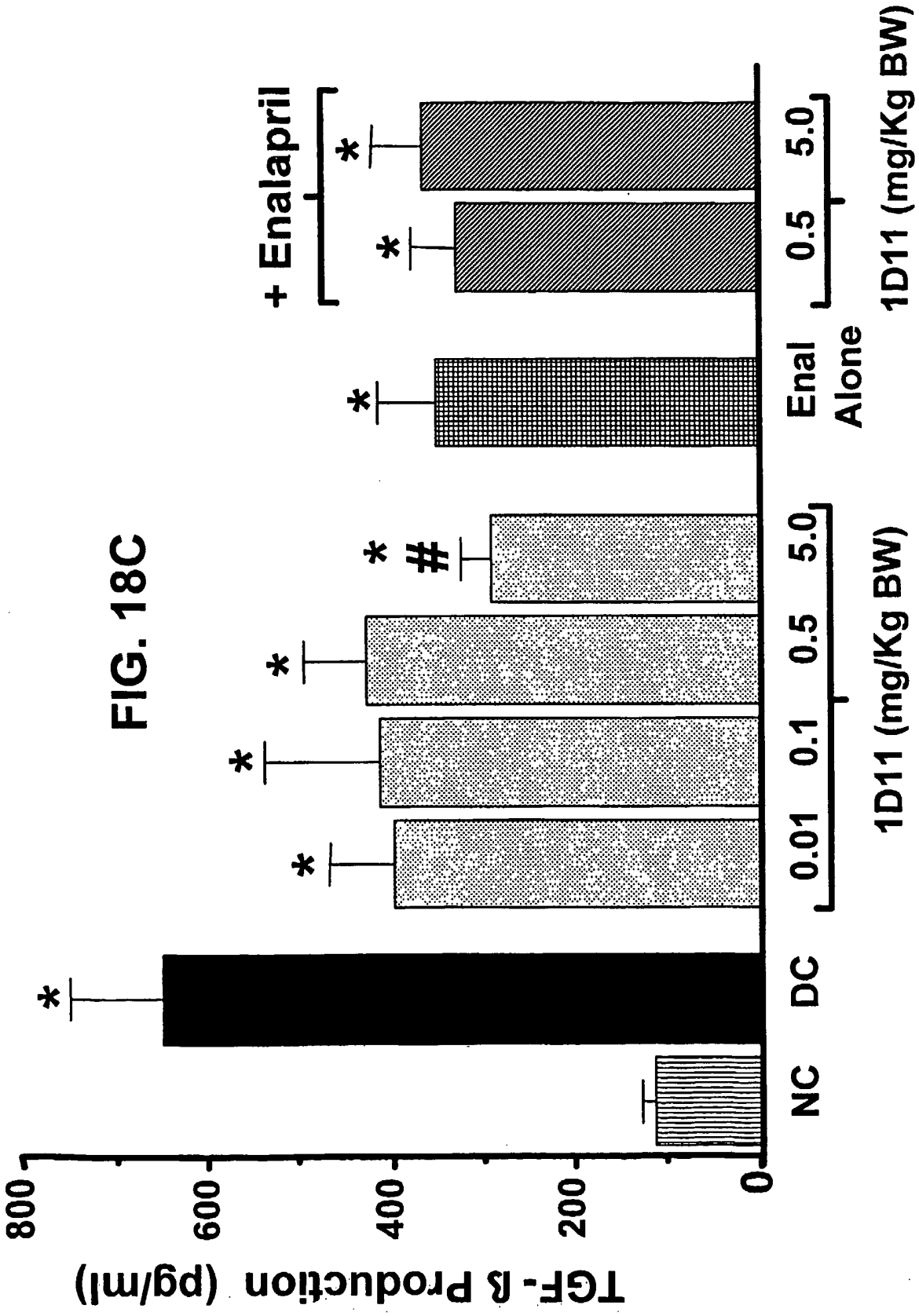
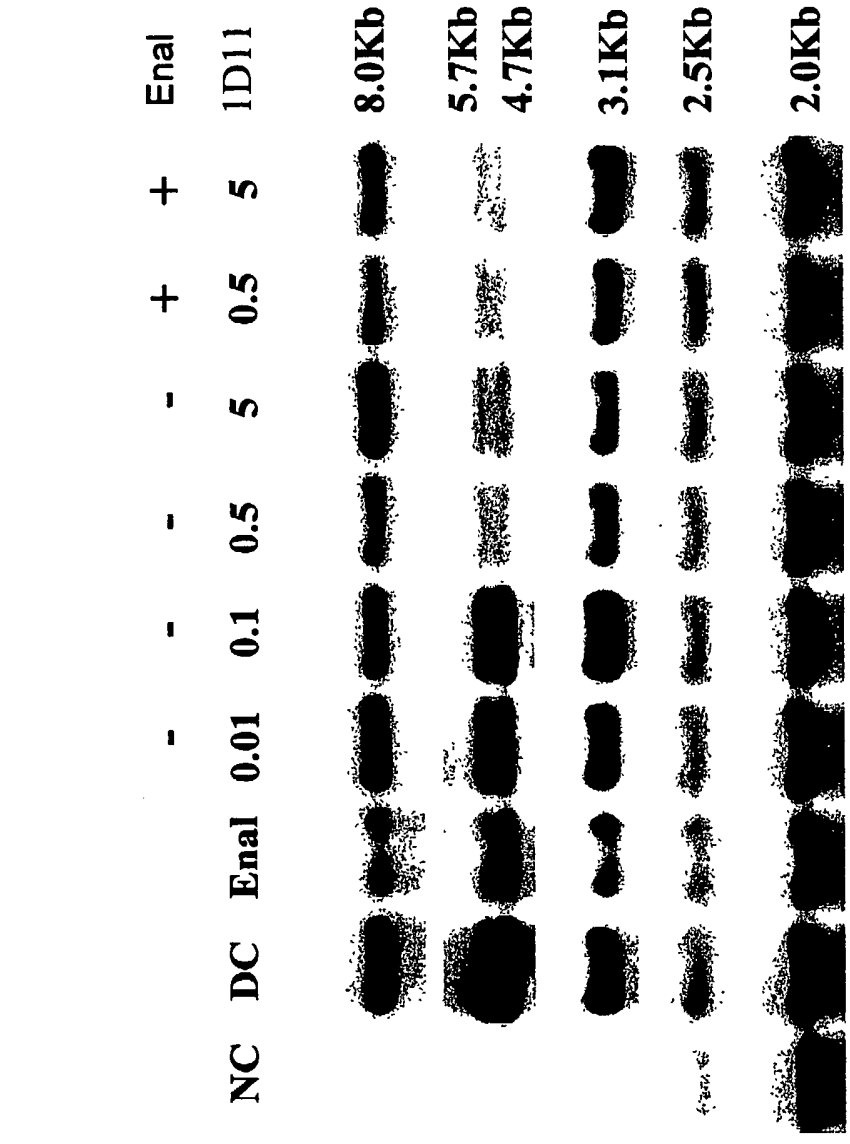

TGF- ß Production (pg/ml)
800
600
400
200
0
NC
DC
0.01
0.1
0.5
5.0
1D11 (mg/Kg BW)
Enal Alone
0.5
5.0
1D11 (mg/Kg BW)
+ Enalapril
*
#

FIG. 18C

FIG. 19


NC
DC
Enal 0.01
- 0.1
- 0.5
- 5
+ 0.5
+ 5
Enal 1D11
FN
8.0Kb
Collagen I
5.7Kb
4.7Kb
PAI-1
3.1Kb
TGFβ1
2.5Kb
18S rRNA
2.0Kb

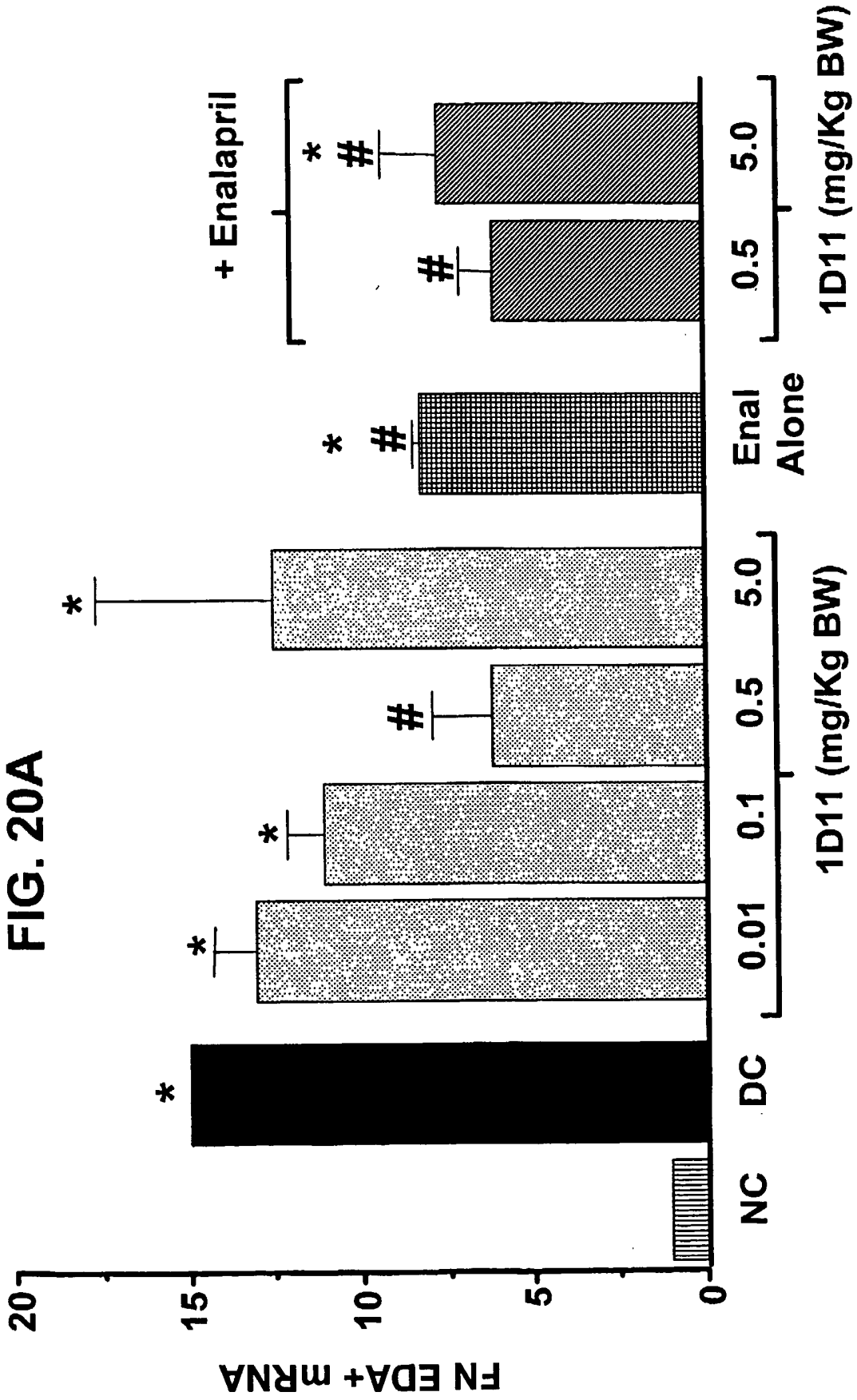


FIG. 20A
FN EDA+ mRNA
20
15
10
5
0
NC
DC
0.01
0.1
0.5
5.0
1D11 (mg/Kg BW)
Enal Alone
0.5
5.0
1D11 (mg/Kg BW)
+ Enalapril
*
#

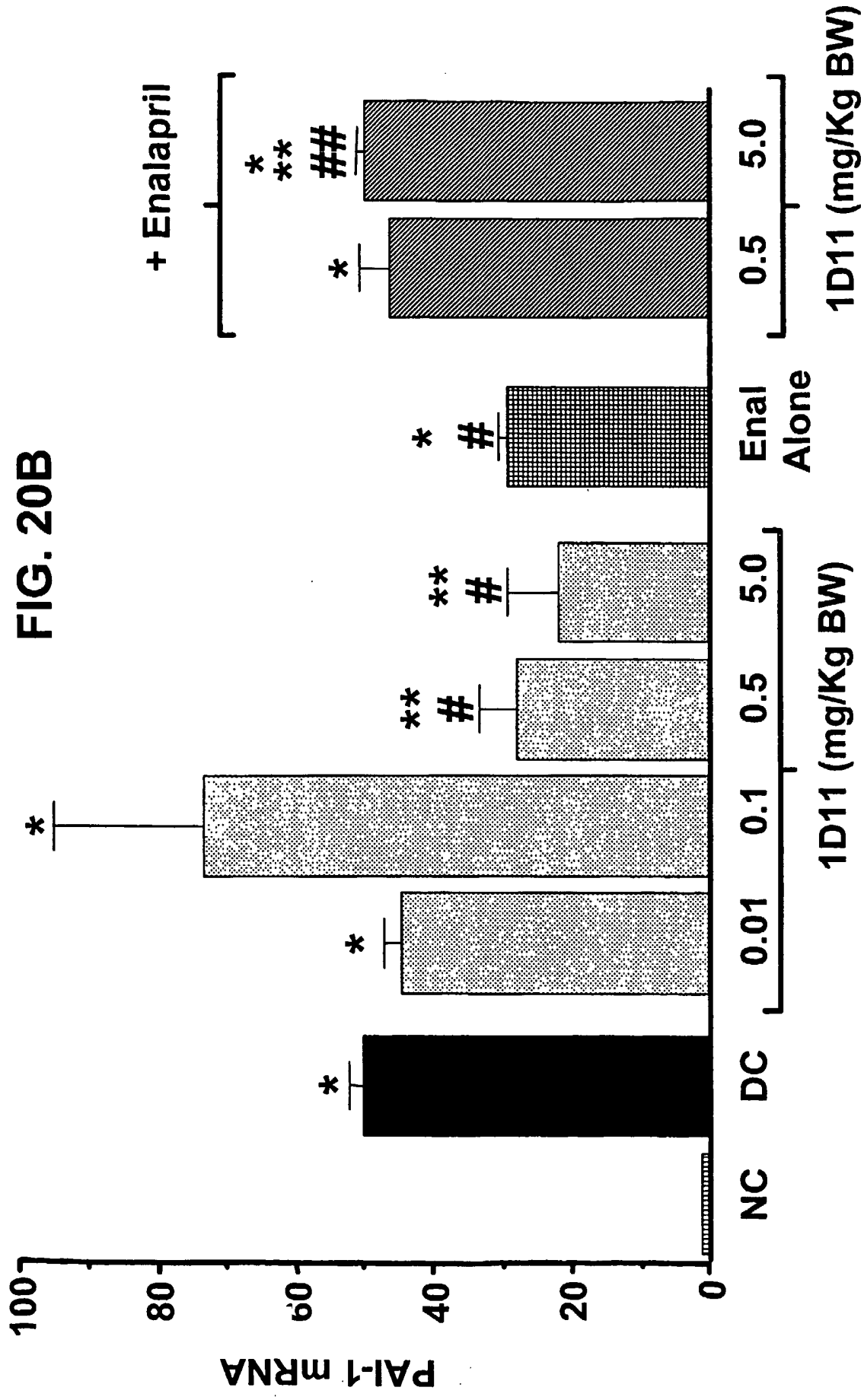


FIG. 20B
PAI-1 mRNA
100
80
60
40
20
0
NC
DC
0.01
0.1
0.5
5.0
1D11 (mg/Kg BW)
Enal Alone
+ Enalapril
0.5
5.0
1D11 (mg/Kg BW)

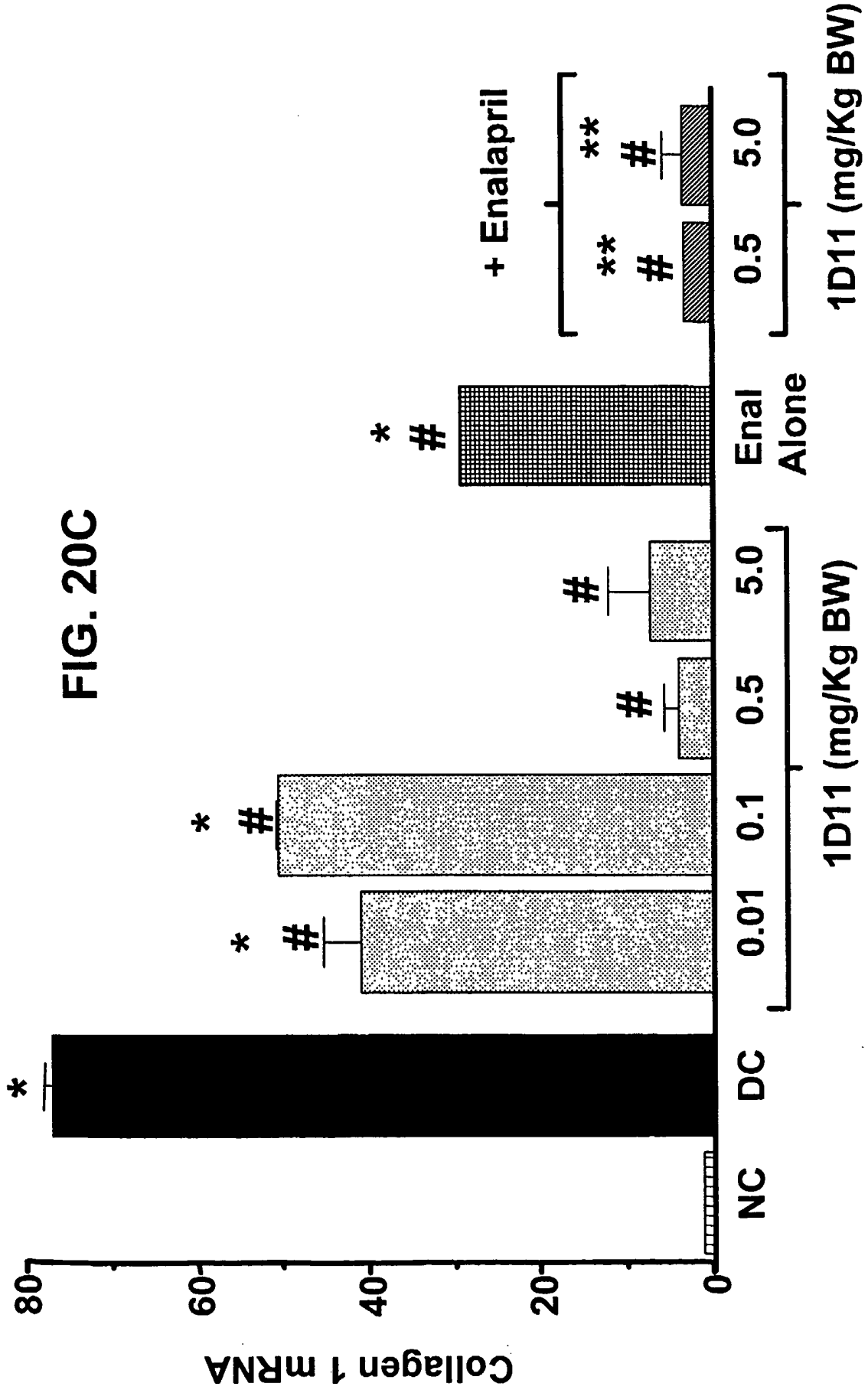

Collagen 1 mRNA
80
60
40
20
0
NC
DC
0.01
0.1
0.5
5.0
1D11 (mg/Kg BW)
Enal Alone
0.5
5.0
1D11 (mg/Kg BW)
+ Enalapril

FIG. 20C

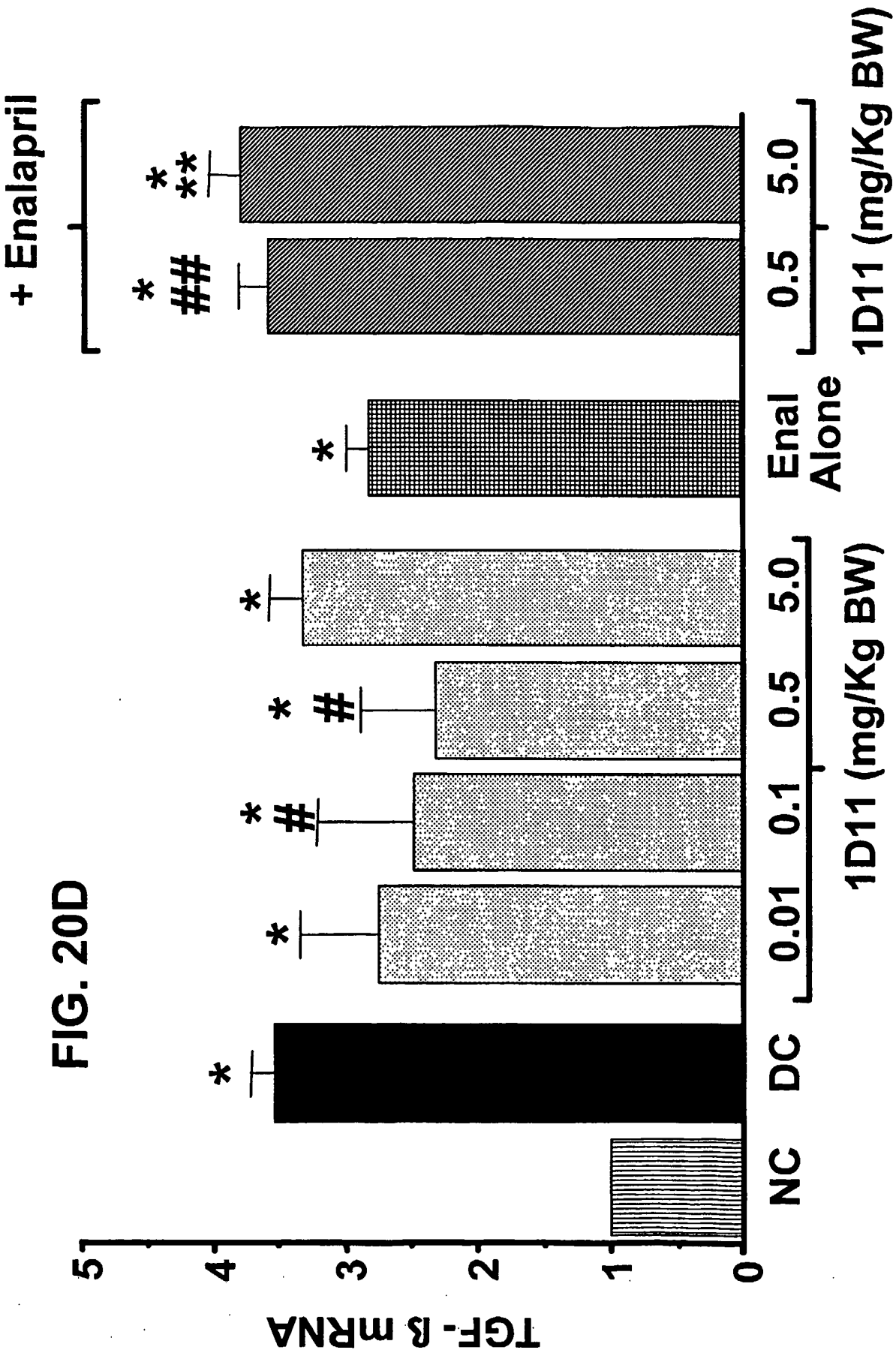

TGF- β mRNA
0
1
2
3
4
5
NC
DC
0.01
0.1
0.5
5.0
1D11 (mg/Kg BW)
Enal Alone
0.5
5.0
1D11 (mg/Kg BW)
+ Enalapril
*
*
*
*
*
*
#
*
#
*
**
*
##

FIG. 20D

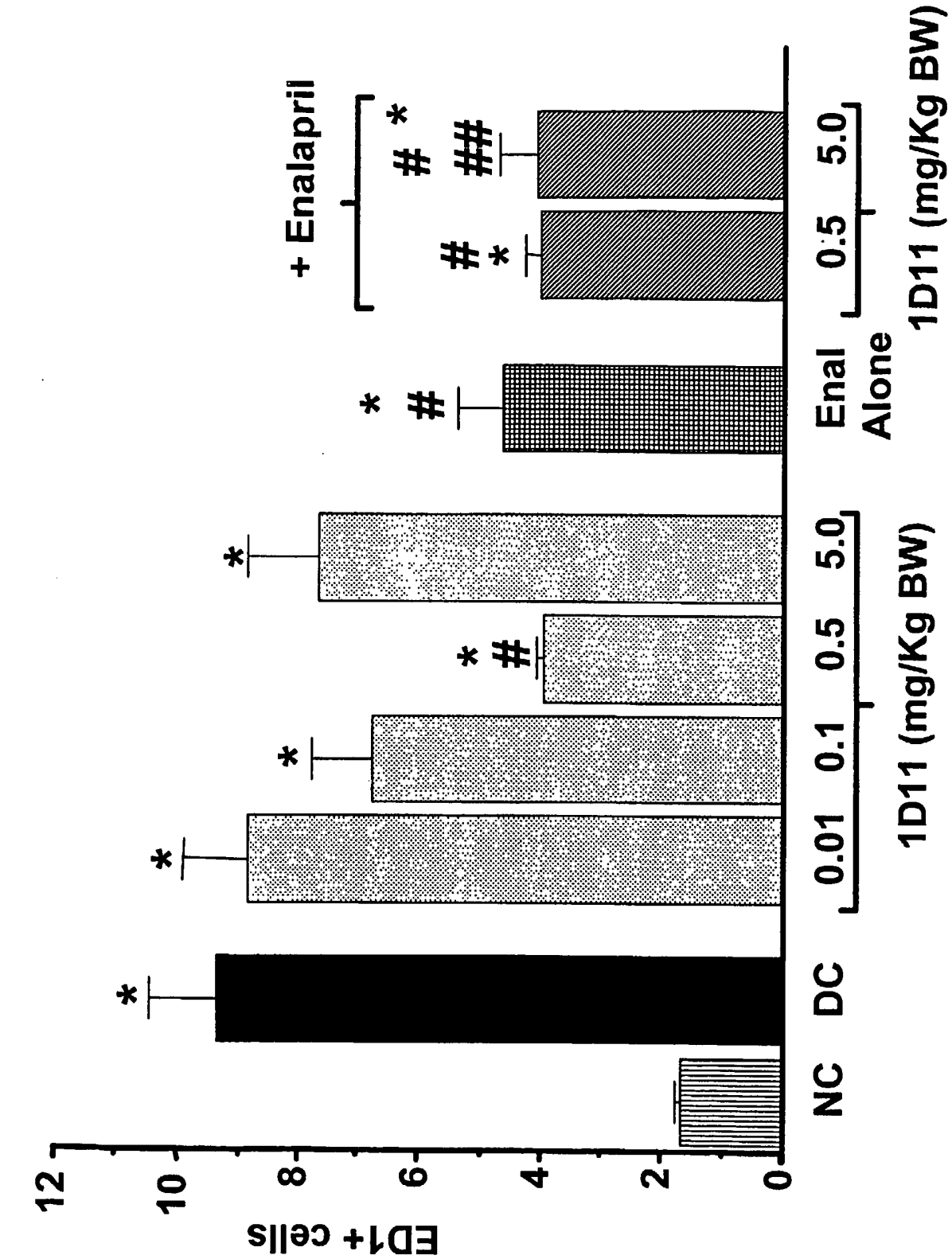

ED1+ cells
0
2
4
6
8
10
12
NC
DC
0.01
0.1
0.5
5.0
1D11 (mg/Kg BW)
Enal Alone
0.5
5.0
1D11 (mg/Kg BW)
+ Enalapril
*
#
##

FIG. 21

FIG. 22

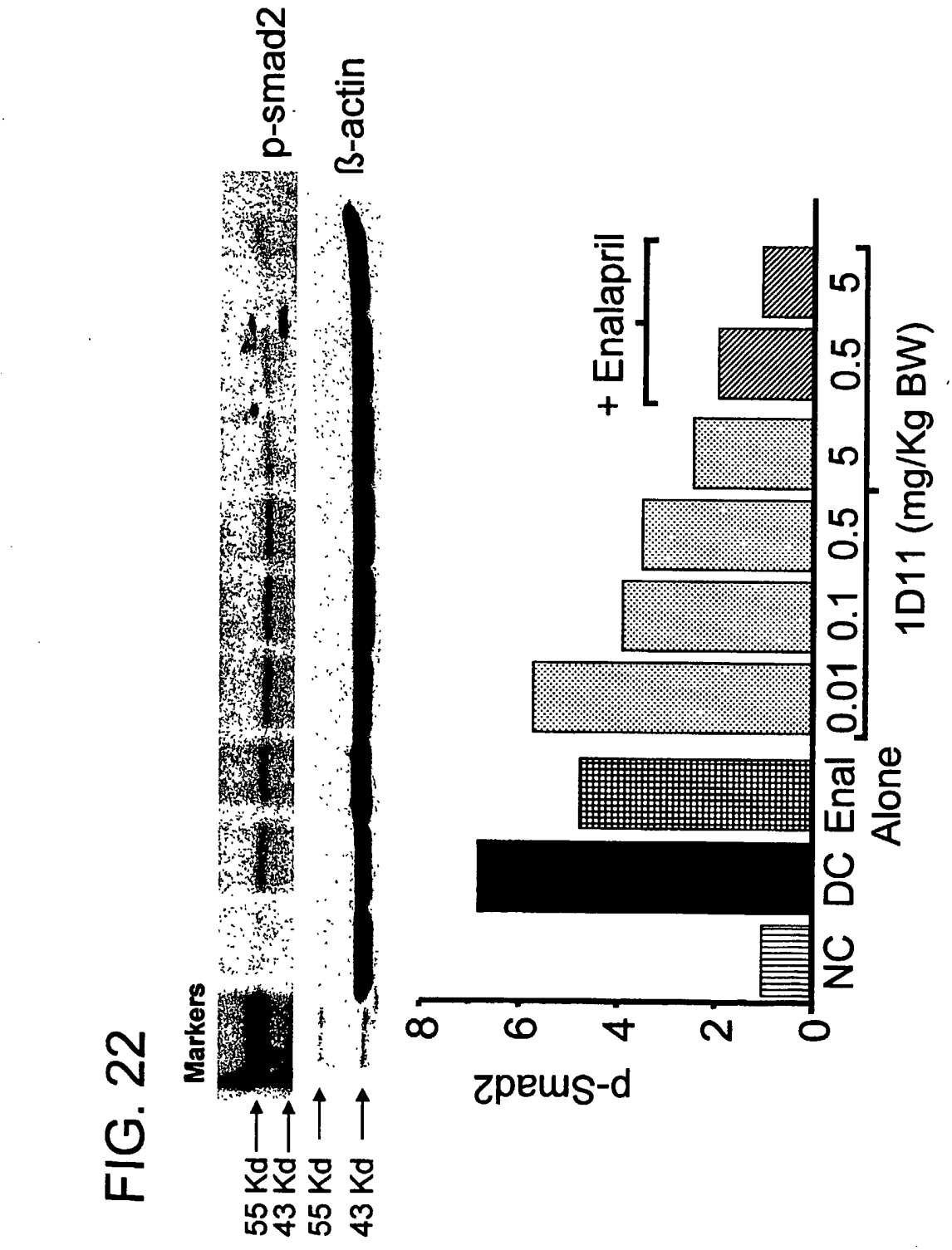

Markers
p-smad2
β-actin
55 Kd
43 Kd
55 Kd
43 Kd
p-Smad2
0
2
4
6
8
NC
DC
Enal
0.01
0.1
0.5
5
0.5
5
Alone
+ Enalapril
1D11 (mg/Kg BW)

FIG. 23

TABLE 2

GROUP	TREATMENT AND (DAYS OF TREATMENT)	DESIGNATION
1	PBS IV (0)	Normal control (NC)
2	OX7 IV (0), PBS IP (1, 3, and 5)	Disease control (DC)
3	OX7 IV (0), 1D11, 0.01mg/kg IP (1, 3 and 5)	Ab 0.01
4	OX7 IV (0), 1D11, 0.1mg/kg IP (1, 3 and 5)	Ab 0.1
5	OX7 IV (0), 1D11, 0.5 mg/kg IP (1, 3 and 5)	Ab 0.5
6	OX7 IV (0), 1D11, 5mg/kg IP (1, 3 and 5)	Ab 5.0
7	OX7 IV (0), enalapril 200mg/l in the drinking water (1-6)	Enal
8	OX7 IV (0), enalapril 200mg/l in the drinking water (1-6), 1D11 0.5mg/kg IP (1, 3 and 5)	Enal + Ab 0.5
9	OX7 IV (0), enalapril 200mg/l in the drinking water, 1D11 5mg/kg IP (1, 3 and 5)	Enal + Ab 5.0
10	OX7 IV (0), 13C4 (Control antibody), 5mg/kg IP (1, 3 and 5)	Control Ab