

## Invited Review

# Molecular actions of nitric oxide in mesangial cells

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**Summary.** Nitric oxide (NO) is a widely recognized mediator of physiological and pathophysiological signal transmission. Its generation through L-arginine metabolism is relevant in the mesangium of the kidney where NO is produced by constitutive and inducible NO-synthase isoenzymes. Signaling is achieved through target interactions via redox and additive chemistry. In mesangial cells (MC), the outcome of these modifications promote on one side activation of soluble guanylyl cyclase while on the other side cytotoxicity is elicited. These contrasting situations are characterized by: 1) cGMP formation and signal propagation towards myosin light chain kinase, the effector system that regulates F-actin assembly, thereby affecting reversible relaxation/contraction of mesangial cells; and 2) initiation of morphological and biochemical alterations that are reminiscent of apoptosis such as chromatin condensation, p53 or Bax accumulation as well as caspase-3 activation. Off note, NO formation with concomitant initiation of apoptosis is efficiently antagonized by the simultaneous presence of superoxide ( $O_2^-$ ). We will recall the consequences that stem from a diffusion controlled NO/ $O_2^-$  interaction thereby redirecting the apoptotic initiating activity of either NO or  $O_2^-$  towards protection. The crosstalk between cell destructive and protective signaling pathways, their activation or inhibition under the modulatory influence of NO will be discussed. Here we give examples of how NO elicits physiological and pathophysiological signal transmission in rat MC.

**Key words:** Nitric oxide, Superoxide, Apoptosis, Necrosis, p53, Caspases, Bcl-2 family, Cell protection, Actin filaments

### Abbreviations:

MC: mesangial cells; NO: nitric oxide; NOS: nitric oxide synthase; iNOS: inducible nitric oxide synthase; GSH: reduced glutathione; GSNO: S-nitrosoglutathione;

JNK1/2 cJun: N-terminal kinases1/2; sGC: soluble guanylyl cyclase; ROS: reactive oxygen species; RNS: reactive nitrogen species; PDE: phosphodiesterase

### Nitric oxide: Endogenous formation and NO donors

In the late 1980s it was discovered that nitric oxide (NO) participates in regulating vascular, immune, and neurological responses. This motivated intense searches for NO biosynthesis and actions throughout the body. NO is enzymatically produced by NO-synthase (NOS) isoforms in a reaction scheme, involving the five electron oxidation of the terminal guanido nitrogen of the amino acid L-arginine to form NO and stoichiometric amounts of citrulline (Mayer and Hemmens, 1997). The initial NOS nomenclature reflected the early observations that NO synthesis was characteristic upon immunoactivation of inflammatory cells (Moncada et al., 1991; Nathan, 1992). The corresponding enzyme therefore was designated "inducible NOS" (iNOS, also known as NOS2). The iNOS was contrasted to a constitutive NOS activity, "cNOS", that was expressed in characteristic cell types (neuronal cells or endothelial cells, with enzymes known as nNOS or NOS1 and eNOS or NOS3). It is now realized that the level of gene expression of cNOS isoforms may be induced and conversely, that iNOS may function as a "constitutive" enzyme. However, a widely accepted nomenclature describes isoforms as nNOS, iNOS, and eNOS, reflecting the tissue for the original protein and cDNA isolates (Michel and Feron, 1997). Once activated, NOS isoforms not only produce NO, the primary reaction product, but also those species resulting from oxidation, reduction, or adduction of NO in physiological milieu, thereby producing various nitrogen oxides, S-nitrosothiols, peroxynitrite ( $ONOO^-$ ), and transition metal adducts (Stamler et al., 1992). Classification of isoenzyme activity allows to approximate a low versus high output system for endogenously generated NO and a rough correspondence between toxic and homeostatic functions of the molecule. NOS inhibitors such as  $N^G$ -monomethyl-L-arginine (NMMA) or other derivatives of the natural substrate L-arginine are commonly used to intervene pharmacologically in NO production, thus allowing to

trace back individual actions to the NO-signaling system (Moncada et al., 1991). One has to be aware that selective NOS inhibitors that allow to discriminate between isoforms are just beginning to emerge.

The determination of NO (or its oxides) involvement in biology is largely based on the use of compounds that are able to mimic an endogenous response by administration of a NO donor. The pathways leading to NO formation differ significantly among compound classes. Some agents require enzymatic catalysis or a thiol-mediated release reaction, whereas others produce NO nonenzymatically. Furthermore, the influence of pH, oxygen, light, temperature, reduction, oxidation, kinetics of release, and the formation of multiple by-products has to be considered. The term NO donor implies that the compound releases NO but it should be kept in mind that NO donors may be endowed with compound-specific activities and that the extent to which a given chemical will generate NO or related redox-species may depend on ambient conditions and enzymatic profiles of individual cells or tissues used. Examples are organic nitrates/nitrites (nitroglycerin), S-nitrosothiols (S-nitrosoglutathione), N-nitroso compounds, NONOates (spermine-NO), sydnonimines (molsidomine), inorganic NO donors ( $\text{NaNO}_2$ ,  $\text{NOBF}_4$ ,  $\text{ONOO}^-$ ), and transition metal nitrosyls (sodium nitroprusside).

#### Nitric oxide: Pathways of signal transduction

The biological activity of NO is classified by cGMP-dependent and cGMP-independent pathways, both attributed to physiology and pathology (Schmidt and Walter, 1994; Stamler, 1994). NO is a key transducer of the vasodilator message from the endothelium to vascular cells, is a constituent in central and peripheral neuronal transmission, and participates in the nonspecific immune defense. Among the various signaling pathways, activation of soluble guanylyl cyclase (sGC), formation of cGMP, and concomitant protein phosphorylation is considered to be important for physiological signaling. However, during the last years cGMP-independent reactions have gained considerable interest. We appreciate that a variety of effects are achieved through its interactions with targets via redox and additive chemistry, that may promote covalent modifications of proteins as well as oxidation events that do not require attachment of the NO group. The molecular basis of these multiple effects is the reaction of NO with oxygen, superoxide ( $\text{O}_2^-$ ), and transition metals. Studies concerning the role of NO in mammalian organisms has given a picture of complexity. A simplified concept of a protective physiological role of NO was challenged by the discovery of harmful reactions when NO formation is cell destructive or its generation turned out to be detriment to the host. In particular, iNOS turned out to be both friend and foe. On one hand NO conveys protection against bacteria and parasites, fights viral infections, and is a modulator of malignancies, while on the other it may promote tumor

angiogenesis and may facilitate tissue destruction and/or disease states (references in: Nathan, 1997; Bogdan, 1998). Lessons emerging from iNOS "knock-out" mice now provide evidence for a role of NO during infectious disease and inflammation with settings where NO is beneficial or detrimental to the host (references in: Nathan, 1997). The picture is further complicated by the notion that NOS-derived reactive nitrogen species comprise both regulatory and effector functions such as immunosuppressive effects or modulation of cytokine responses as well as pathological effects or immunoprotective activities. There are numerous inflammatory settings in which the capacity to express iNOS appears to signal predominately deleterious effects. Examples are lung damage after LPS intoxication, hemorrhage-induced liver damage which is closely related to NO-evoked activation of proinflammatory transcription factors, or acute rejection of major histocompatibility complex-mismatched cardiac allografts (references in: Nathan, 1997). Many of these disease-related conditions are controlled by macrophages and it is now confirmed that human monocytes or macrophages from patients with a wide rate of infectious or inflammatory diseases express iNOS (references in: MacMicking et al., 1997). As the expression of iNOS in human infectious, autoimmune, and chronically inflammatory diseases as well as several other human disorders such as heart infarction, tumor development, transplantation, or prostheses failure is well established, questions on the signaling properties of NO arise (references in: Kröncke et al., 1998). Under conditions of massive NO formation the various regulatory, cytostatic, and/or toxic consequences of NO may play important roles in the pathophysiology of tissue or cell destruction. Toxic consequences as a result of NO generation seem predictable. However, during infection and inflammation NO generation appears to act both as a direct apoptotic inducer and as a regulator of other effectors. The ability of NO to function as a messenger molecule and to affect signaling pathways of adverse agonists allows to redirect cell destructive pathways into protection. Therefore, the role of NO during cell destruction is ambivalent and NO may function both as an activator and inhibitor of the death program, depending on the biological milieu, i.e. the presence or absence of stimulatory or inhibitory cosignals.

#### Cell demise: Apoptosis versus necrosis

Apoptosis is a form of cell death characterized by a series of stereotypic morphological and biochemical features. Nearly every physiological cell death in mammals proceeds by the process of apoptosis, in which the dying cells vanish without a trace, silently sequestered by professional phagocytes or neighboring cells, thereby avoiding any accompanying inflammatory response. In 1972, it was proposed that cell death under normal and some pathological conditions resembles

suicides (Kerr et al., 1972). Cells activate an intracellular death program and kill themselves in a controlled way, a process now known as programmed cell death or apoptosis. In contrast, the accidental, uncontrolled cell death in response to an acute injury is called necrosis. Our knowledge on apoptosis largely became established through genetic studies in the nematode *Caenorhabditis elegans*. Herein genes were identified to control the death program followed by the observations that some of these genes do have mammalian homologous (Evan and Dragovich et al., 1998; Littlewood, 1998). The elements of a core program controlling the proper executive phase of apoptosis seems to be constitutively expressed in virtually every cell. A cell will undergo apoptosis as a result of information received from its environment, interpreted in the context of internal information, e.g. its cell type, state of maturity, developmental history, and state of differentiation (Steller, 1995; Bergmann et al., 1998). Multiple external information triggers apoptosis but they may be influenced by the appearance or disappearance of hormones, growth factors, cytokines, or intercellular and/or cell matrix interactions (Ruoslathi and Reed, 1994). Therefore, a particular external stimulus is only part of the information influencing the decision of self-destruction or survival, and not exclusively controlling apoptosis. Similarly, intracellular signals involved in the induction of apoptosis are often regulators of other cellular responses. Evidence is accumulating that multiple signaling pathways interact, a process known as crosstalk, and therefore a cell response to a given stimulus may alter significantly, not only between different cell types but also within one cell population. Although the great variety of external signals that initiate apoptosis imply multiple signaling systems, there is considerable evidence to suggest that transducing pathways converge to one, or very few, common final executive steps (Peter et al., 1997; Dragovich et al., 1998; Thornberry and Lazebnik, 1998). Regulators such as the tumor suppressor p53, caspases, cytochrome c relocation, or the regulatory role of Bcl-2 family members are consistent with such convergence of activating or antagonistic pathways.

Apoptosis can be triggered by a variety of extrinsic and intrinsic signals, which allows the elimination of cells that have been produced in excess, have developed improperly, or have sustained genetic damage (Thompson, 1995; Fadeel et al., 1999). Among the modulators of apoptosis the molecule nitric oxide has gained considerable attention.

### Kidney mesangial cells

Around 1930 it appeared that the centrolobular or axial space of the glomerulus harbored the mesangium, a structural entity consisting of cells and extracellular matrix. It was noticed that in glomerular diseases the deposition of extracellular matrix occurred in an intercapillary location. In the early sixties the ultrastructure of the mesangium, including mesangial

uptake and trafficking of macromolecular markers, was described in greater detail (references in: Sterzel and Rupprecht, 1997).

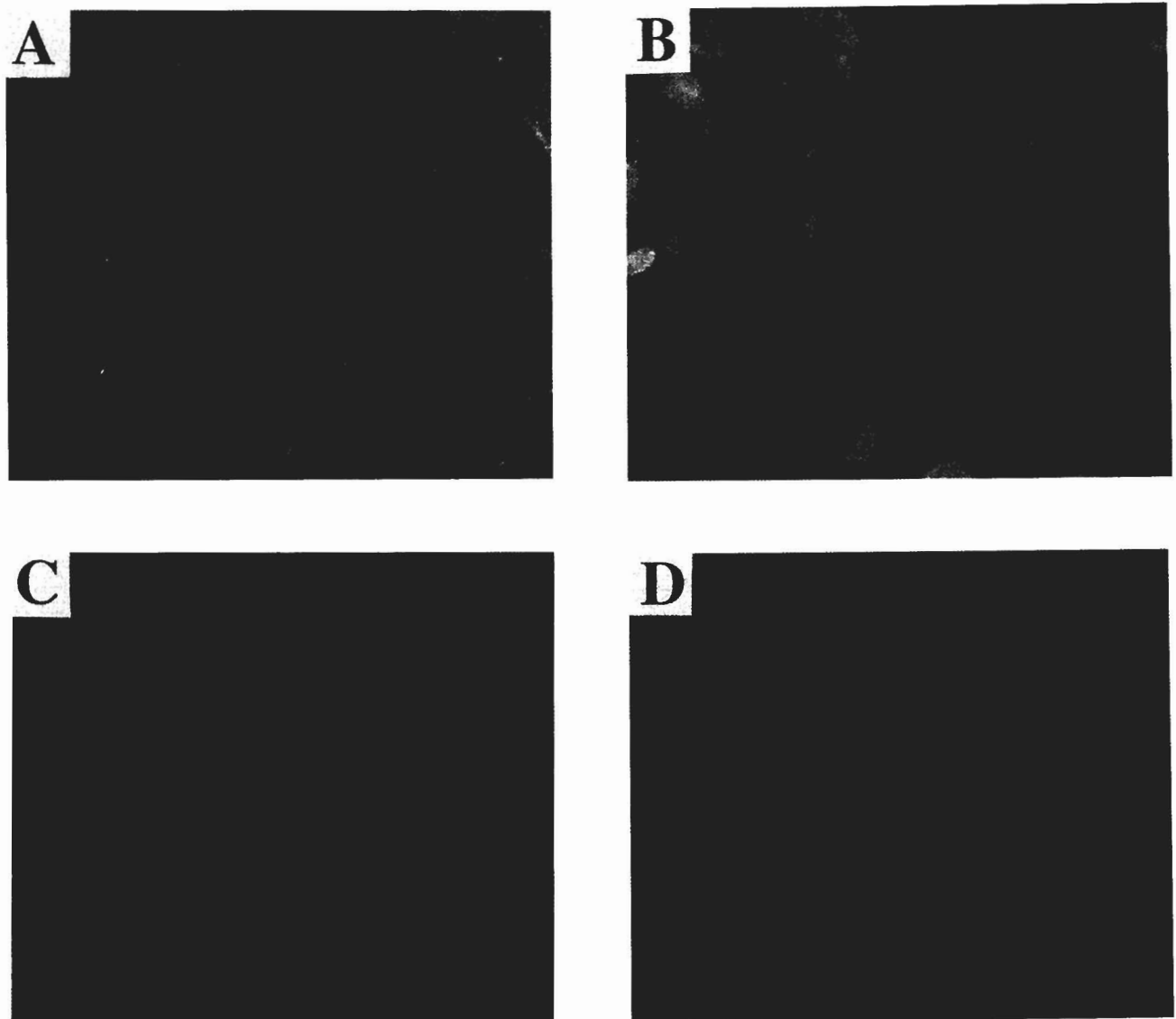
The mesangium, containing mesangial cells (MC) and matrix, occupies the central region of a glomerular lobule. The capillary endothelium is in direct contact with the mesangium because the glomerular basement membrane is not developed at those specific sites (Latta, 1992). MC represent one third of 600-750 cells in a rat glomerulus, with the notion that the turnover rate of MC *in vivo* is low. MC resemble a vascular smooth muscle-like phenotype, containing numerous microfilament bundles. In culture they stain with antibodies to smooth muscle  $\alpha$ -actin and myosin and contain actomyosin bundles that span its length and insert at the cell membrane. These stress fibers maintain the basic cellular tone and are responsible for contractile responses which can be elicited by vasopressors *in vitro* (Ausiello et al., 1980; Mene et al., 1992). Contraction and relaxation of MC have been observed in response to a variety of agents with the notion that contractile MC contribute to the regulation of glomerular ultrafiltration. Several vasoactive compounds which contract MC reduce the filtration rate. Some of these factors are produced by MC in an autocrine loop, while vasodilators may function in a negative feedback manner to attenuate the contractile action of vasoconstrictors. Vasoactive agents decrease filtration by reducing blood flow to selected capillary loops which may be accomplished by MC contraction. Moreover, MC are located at the glomerular hillus, where they surround glomerular capillaries at their branching points and are well suited to regulate the number of perfused glomerular capillaries. Moreover, functional properties of mesangial cells encompass a response to local injury to promote proliferation, formation of soluble regulator molecules with autocrine and/or paracrine effects, as well as formation, breakdown, and remodeling of mesangial matrix (references in: Sterzel and Rupprecht, 1997). The mesangium is the target of different types of injury. Primary injuries may encounter immunological, metabolic, toxic, infectious, mechanical, or other insults such as shear stretch, ischemia/hypoxia, or radiation (Hawkins et al., 1990). Secondary injury may arise due to inflammatory responses/regulators that act as autocrine factors released by MC or paracrine factors that stem from macrophages, granulocytes, platelets, or endothelial cells. Compounds are grouped as cytokines, growth factors, enzymes, vasoactive factors, autacoids, and radicals ( $O_2^-$ , NO) (references in: Savill, 1999). In addition, the loss/inhibition of anti-inflammatory factors (growth factors, cAMP or cGMP-elevating maneuvers) or the three-dimensional matrix context may contribute to mesangial cell injury. Injury usually attracts bone marrow-derived inflammatory cells in addition to changes to the proliferative and secretory MC phenotype. An acute and transitory injury is followed by a phase of resolution (Sterzel et al., 1982). However, if the injury is prolonged it becomes chronic and may

never lead to a phase of resolution which then results in replacement of the delicate glomerular capillary tuft with inert scar tissue. This will promote partial or complete glomerular sclerosis and may initiate additional injury due to hyperfiltration and hypertension in remaining glomerular tufts and loops as well as ischemic changes.

Among the factors that achieved considerable interest in affecting physiological as well as pathophysiological entities of the mesangium are oxygen radicals ( $O_2^-$ ) and NO which emerged of primary interest.

Several studies have shown, directly or indirectly by

infusion of radical scavengers, that ROS formation is found in association with the development of glomerular injury (Shah, 1989). Infiltrating phagocytes are a major source of ROS but also MC are capable of their production. Soluble and insoluble immune complexes, stimulated phagocytosis, and cytokine-treatment markedly stimulate ROS formation. The role of ROS in mediating glomerular injury has been established in experimental models of immune complex glomerulonephritis (Narita et al., 1995). In MC, ROS generation is considered to stem from NAD(P)H oxidases, that are blocked by the pharmacological antagonist DPI



**Fig. 1.** NO-induced F-actin dissolution is mediated via cGMP and cAMP. Quiescent cultured rat MC ( $10^4$  cells/chamber) were kept one day before and during the experiments in medium without FCS. Afterwards, cells were exposed to vehicle (A), 250  $\mu$ M GSNO (B), 250  $\mu$ M GSNO and 5  $\mu$ M of the guanylyl cyclase inhibitor NS 2028 (C), or 250  $\mu$ M GSNO in combination with 1 mM of the protein kinase A type I inhibitor 8-chloroadenosine-3',5'-cyclic monophosphorothioate (D). NS 2028 and 8-chloroadenosine-3',5'-cyclic monophosphorothioate were preincubated for 15 min. F-actin was visualized with phalloidin-TRITC staining 30 min after agonist addition. The experiments were performed at least three times and representative pictures were taken with a Leica fluorescence microscope and camera. For further details see text.

(diphenyliodonium). RNS production is facilitated by NOS-isoenzymes, all of which are found in the glomerulus. Endothelial cells express eNOS, the macula densa is bNOS positive, whereas iNOS can be induced in MC or is found in infiltrating immune cells (Cattell and Cook, 1995). MC are competent in expressing iNOS in response to various cytokines or activation of the cAMP-stimulatory pathway (Pfeilschifter, 1994). The production of NO may act via cGMP formation, thus participating in vasomotion and additional physiological responses. In contrast, higher amounts of NO (most likely iNOS derived) may signal non-specific cytotoxic effects of macrophages, thus providing a local host defence. However, at the same time NO is equipped with a toxic, pro-inflammatory potential, e.g. in glomerulonephritis and other types of immune-mediated diseases. Available evidence shows that NO is endowed with injurious, proinflammatory as well as antiinflammatory, cytoprotective abilities. The balance of cellular damage versus cell protection as a result of NO delivery appears to depend not only on NO concentrations and duration of NO release but also on the oxidation-reduction milieu of the microenvironment. Only if we understand these multiple regulatory components we will be able to intervene and thus open avenues for therapeutical interventions. The following sections will deal with reactions of NO that exemplify physiological as well as pathophysiological signal transducing steps in mesangial cells.

### Physiological actions of nitric oxide in the mesangium

In the glomerulus, MC may be affected either by exogenously produced NO, released from endothelial cells or infiltrated macrophages or by mesangial iNOS activation when NO acts in an autocrine manner. Independent of the source, MC start to change their phenotype as soon as they face NO. In analogy to smooth muscle cells the phenotype of MC is mainly based on F-actin, assembled in so-called stress fibers. They can be nicely visualized by phalloidin-TRITC staining. When MC cope with NO, most stress fibers vanish and only a few contacts to neighboring cells remain to be seen. This phenomenon is fully reversible and does not result in detachment of the cells. The mechanism leading to F-actin dissolution is very sensitive toward NO as 500 nM or 1  $\mu$ M of a NO donor such as S-nitrosoglutathione is sufficient to achieve these alterations. Under these conditions, F-actin disruption occurs within 10 to 30 min but appears normal again after 4 to 6 hours. MC stimulation with cytokines evoke F-actin breakdown after 12 to 16 hours which correlates with the endogenous production of NO. Although NO generation residues, as seen by increasing nitrite production, MC structure is rebuilt after 24 hours, thus resembling a reversible mechanism. Further analysis revealed that the NO-inducible process can be blocked by the selective soluble guanylyl cyclase inhibitor NS

2028 which indicates a cGMP-dependent mechanism. Similar effects are known for the cardiovascular system where endothelium-derived NO provokes vasorelaxation of smooth muscle cells in a cGMP-dependent way (Moncada et al., 1991). But in contrast to smooth muscle cells, MC relaxation is unrelated to protein kinase G activation as protein kinase G inhibitors are ineffective in attenuating NO alterations.

Interestingly, MC display high activity of two different cyclic nucleotide phosphodiesterase (PDE) isoforms, namely PDE-1 and PDE-3, and a moderate activity of PDE-4. PDE-1 preferably degrades cGMP whereas PDE-4 and to a lesser extent PDE-3 are specific for cAMP (references in: Schudt et al., 1999). As PDE-3 is the most prominent cAMP-hydrolyzing PDE in MC and it is defined as cGMP-inhibitable, an endogenous cGMP increase, as generated by NO donors, would result in a cAMP increase. Therefore, it is not surprising that inhibition of PDE-3 by motapizone in combination with RP 73401, a specific PDE-4 inhibitor, as well as the unspecific PDE inhibitor isobutyl-1-methyl-xanthine evoke F-actin dissolution in rat MC similar to NO. The picture emerges that NO-initiated F-actin breakdown is mediated by cGMP, thus blocking different PDE isoforms which then results in a cAMP increase. In line, F-actin alterations can be reproduced by lipophilic cAMP analogs with the notion that the subsequent response is triggered by protein kinase A, type I. Therefore, it appears logical that 8-chloroadenosine-3',5'-cyclic monophosphorothioate, a protein kinase A type I inhibitor, blocked F-actin changes evoked by NO, PDE inhibition, or lipophilic cAMP analogs. For other cell types, it is established that protein kinase A phosphorylates myosin light chain kinase, thereby attenuating its activity (Lamb et al., 1988). Concomitant with myosin light chain kinase inhibition, phosphorylation of myosin light chain decreases which is responsible for stimulating the actomyosin contractile apparatus (Tan et al., 1992). Phosphorylated myosin light chain stabilizes F-actin, and therefore the phosphorylation and dephosphorylation cycle of myosin light chain regulates stress fiber cell contraction and relaxation. In MC, it has been shown that elevation of cAMP decreases phosphorylation of myosin light chain (Kreisberg et al., 1985) whereas enhanced myosin light chain phosphorylation in association with myosin light chain kinase activation have been described for angiotension II, endothelin-1, or vasopressin (Hiraoka-Yoshimoto et al., 1991).

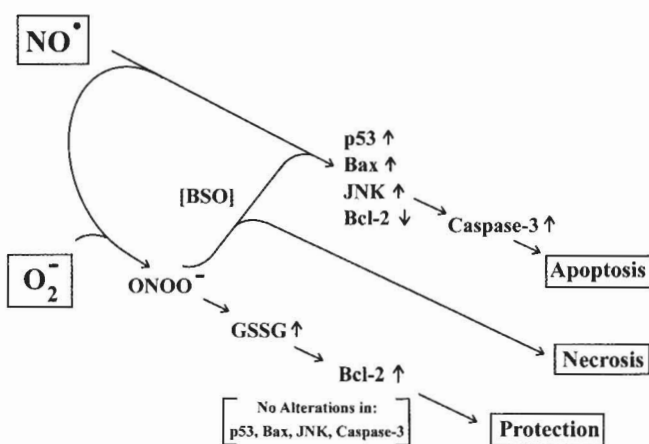
In conclusion, small concentrations of NO are sufficient to activate soluble guanylyl cyclase in MC thereby elevating intracellular cGMP. Either cGMP transfers the NO impulse by inhibition of cAMP-hydrolyzing PDEs, thus initiating a cAMP increase and protein kinase A activation, or cGMP directly activates protein kinase A, a possibility which presently cannot be excluded (Cornwell et al., 1994). In any case, protein kinase A activation results in dephosphorylation of myosin light chain and disruption of F-actin.

Despite the notion that this signal transduction mechanism evoked by NO has been studied in cultured MC, it can be assumed that NO will act in a similar way *in vivo*, thus provoking physiological hemodynamic changes within the glomerulus as described.

### Pathophysiological actions of nitric oxide in the mesangium

Using various NO donors such as GSNO or spermine-NO we established a proapoptotic action in cultured rat MC. Apoptosis was elicited in a time- and concentration-dependent manner and was characterized by morphological (chromatin condensation) or biochemical markers (DNA laddering, p53 accumulation, caspase-3 activation, alterations in the expression of Bcl-2 and Bax) (Mühl et al., 1996; Sandau et al., 1997b). In addition, generation of  $O_2^-$  by application of a redox cyler (DMNQ, 2,3-dimethoxy-1,4-napthoquinone) promoted apoptosis as well. This is in line with the apoptotic action of NO and/or  $O_2^-$  in several other systems. Moreover, it appears that the proapoptotic action of NO in MC is transmitted at least in part by activation of the cJun N-terminal kinases1/2 (JNK1/2) (Sandau et al., 1999). NO itself promoted strong JNK1/2 activation and apoptosis, in some analogy to Ro 318220, a characterized JNK1/2 activator. In contrast, activation of p42/p44 mitogen activated protein kinases (ERK1/2) afforded some protection towards NO-evoked apoptosis that was antagonized by the upstream ERK1/2 inhibitor PD 98059.

During our studies with MC we unexpectedly



**Fig. 2.** NO-evoked apoptosis in glomerular MC is attenuated by costimulation with  $O_2^-$ . NO initiated programmed cell death is accompanied by p53 and Bax accumulation, JNK1/2 activation, Bcl-2 down-regulation, and cleavage/activation of caspase-3 followed by typical morphological and biochemical apoptotic parameters such as chromatin condensation and DNA fragmentation. This process can be attenuated by coadministration of  $O_2^-$  which leads to an increase of oxidized glutathione (GSSG) and up-regulation of Bcl-2, thus resulting in cell protection. Under these conditions, no apoptotic alterations are detectable whereas depletion of intracellular glutathione by BSO redirects the NO/ $O_2^-$  protective pathway to MC apoptosis and/or necrosis. For further details see text.

noticed that NO-mediated apoptotic cell death was antagonized by the simultaneous formation of superoxide ( $O_2^-$ ) and vice versa (Brüne et al., 1997; Sandau et al., 1997a,b). Part of the signal transmission of both NO and  $O_2^-$  may stem from their diffusion controlled interaction that results in the formation of peroxynitrite (ONOO<sup>-</sup>) (Pryor and Squadrito, 1995). We addressed the NO/ $O_2^-$ -interaction by exposing cells to NO donors and  $O_2^-$ -generating systems such as DMNQ or the hypoxanthine/xanthine oxidase system, thereby allowing a continuous radical formation. The balanced and simultaneous generation of NO and  $O_2^-$  turned out to be protective for mesangial cells, whereas the unopposed radical generation elicited apoptosis and in higher concentrations necrotic cell death. While apoptosis was accompanied by typical apoptotic features these alterations were attenuated under conditions of NO/ $O_2^-$ -coadministration (Sandau et al., 1997a) although a massive formation of oxidized glutathione became apparent. NO/ $O_2^-$ -coformation not only attenuated the occurrence of late apoptotic parameters such as DNA fragmentation but also suppressed signal components that appeared much earlier as shown for p53 accumulation. Moreover, activation of JNK1/2 was abolished under conditions of NO/ $O_2^-$ -cogeneration. Intriguingly, depletion of reduced glutathione by addition of the  $\gamma$ -glutamylcysteine synthetase inhibitor BSO (L-buthionine-sulfoxamine) reversed NO/ $O_2^-$ -evoked survival to cell destruction and reinstalled JNK1/2 activity (Sandau et al., 1999). In order to achieve protection by NO/ $O_2^-$ -coformation the simultaneous presence of both radicals is of particular importance. If the generation of either NO or  $O_2^-$  is offset, protection is less efficient. We conclude that signaling mechanisms as a consequence of the NO/ $O_2^-$ -interaction redirect apoptotic initiating signals to harmless pathways. The presence of sufficient reduced GSH is indispensable for protection which is substantiated by the fact that a massive oxidative stress signal, i.e. the formation of oxidized glutathione, occurs during protection. However, under these conditions we noticed accumulation of the anti-apoptotic protein Bcl-2, which was upregulated at the mRNA and protein level. Further studies lead to the conclusion that oxidative stress in general elicits Bcl-2 expression as revealed by using different oxidizing agents such as diamide or butyl hydroperoxide. As diamide was non-toxic to MC, we used it as a Bcl-2 activator to protect MC against a subsequent toxic dose of NO. In conclusion, redox changes regulate the Bcl-2 level in rat MC either as a consequence of NO/ $O_2^-$ -coincubation or as a result of oxidation in general. It appears that Bcl-2 can confer cellular protection under conditions of low oxidative stress only, whereas a stronger oxidative signal initiates apoptosis/necrosis despite Bcl-2 expression. Signal strength and the onset of anti-apoptotic Bcl-2 actions relative to the activation of pro-apoptotic signaling pathways may be an important determinant (Sandau and Brüne, 2000).

Part of our studies concerning the role of oxidative versus nitrosative stress are in analogy to *in vitro*

experiments performed by Wink and colleagues (Wink et al., 1993, 1997). They observed GSSG formation by incubating NO donors,  $O_2^-$ , and reduced glutathione and concluded NO-evoked nitrosative reactions to be quenched by the resultant oxidative stress. In some analogy NO attenuated  $O_2^-$ -mediated toxicity in chondrocytes (Blanco et al., 1995), stretch-induced programmed myocyte cell death that resulted from  $O_2^-$  formation, or abrogated toxicity of oxidized low-density lipoprotein in endothelial cells (references in: Brüne et al., 1998, 1999). Moreover, it is reported that  $O_2^-$  acts as a self-protecting factor for NO toxicity in neurons (Koshimura et al., 1998). These observations are in line with the protective function of NO during ischemia-reperfusion, peroxide-induced toxicity, lipid-peroxidation, or myocardial injury (Oury et al., 1992; Wink et al., 1993; Rubbo et al., 1994).

As a general concept it appears that in some systems the balanced formation and interaction of physiologically relevant radicals (NO and  $O_2^-$ ) resembles a protective principle thereby eliminating harmful reactions that are operating as a consequence of unopposed radical generation. Analysis of cytokine-stimulated MC may add to these considerations. Cytokine treatment of MC provoked iNOS upregulation without driving cells into apoptosis (Sandau et al., 1997a). In contrast, cytokines in combination with pyrrolidine dithiocarbamate, which blocks endogenous superoxide dismutase, allowed p53 and Bax accumulation as well as DNA fragmentation, suggesting a modulatory role of endogenously produced  $O_2^-$ . In addition, sublethal application of GSNO in addition to cytokines produced MC death, which was antagonized by an iNOS inhibitor, thus implying a role of endogenously generated NO as well. In line with these considerations we noticed that MC expressing iNOS did not display signs of apoptosis and, vice versa, cell showing characteristic features of apoptosis did not stain for iNOS (Nitsch et al., 1997). We conclude that the formation of NO does not necessarily promote cell death, i.e. apoptosis and that the production of  $O_2^-$  can be considered as a fine tuning systems that redirects NO-actions from apoptosis towards cell protection.

## Conclusions

Nitric oxide is a versatile messenger that combines physiological as well as pathophysiological signaling properties. This is exemplified for rat mesangial cells where NO participates in regulating relaxation/contraction besides acting as a cytotoxic molecule. In line with the general, however simplified concept we would attribute physiological signaling to soluble guanylyl cyclase activation and concomitant cGMP formation. Evidently, as seen for alterations in F-actin disassembly, the production of cGMP may provoke signaling which refers to the cAMP-system. Therefore, cyclic nucleotides (cGMP and cAMP) in general have to be considered when MC respond to low level of NO generation. This is in contrast to NO actions in

provoking cell death along an apoptotic pathway. Toxicity is unrelated to cyclic nucleotide formation but is subjected to multiple regulatory components. Toxicity of NO is affected by the existing biological milieu, relative rates of NO formation, its redox state, the combination with oxygen, superoxide, as well as other biomolecules.

It will be mandatory to define the versatility of NO-signaling mechanisms in relation to both, physiological as well as pathophysiological signaling and to explore how NO-responsive targets serve both sensory and regulatory roles in transducing a signal. The switch from destructive to protective pathways and the molecular recognition of this balance will be central to understand NO-actions in MC and to open avenues for therapeutical interventions with the implication to substitute NO or to attenuate its generation for the benefit of the kidney mesangium.

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## **Invited Review**

# **Liver fibrosis, the hepatic stellate cell and tissue inhibitors of metalloproteinases**

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**Summary.** Liver fibrosis occurs as a consequence of net accumulation of matrix proteins (especially collagen types I and III) in response to liver injury. The pathogenesis of liver fibrosis is underpinned by the activation of hepatic stellate cells (HSC) to a myofibroblast like phenotype with a consequent increase in their synthesis of matrix proteins such as interstitial collagens that characterise fibrosis. In addition to this there is increasing evidence that liver fibrosis is a dynamic pathologic process in which altered matrix degradation may also play a major role. Extracellular degradation of matrix proteins is regulated by matrix metalloproteinases (MMPS) – produced by HSC - which in turn are regulated by several mechanisms which include regulation at the level of the gene (transcription and proenzyme synthesis), cleavage of the proenzyme to an active form and specific inhibition of activated forms by tissue inhibitors of metalloproteinases (TIMPS). Insights gained into the molecular regulation of HSC activation will lead to therapeutic approaches in treatment of hepatic fibrosis in the future, and could lead to reduced morbidity and mortality in patients with chronic liver injury

**Key words:** Hepatic Stellate Cell (HSC), Matrix Metalloproteinase (MMP), Tissue Inhibitor of Metalloproteinase types 1 to 4 (TIMPs 1, 2, 3 & 4), Liver fibrosis, Collagenase

### **Introduction: Liver Fibrosis**

Fibrosis is the liver's wound healing response to a variety of chronic insults including autoimmune damage (primary biliary cirrhosis, chronic active hepatitis), infection (particularly hepatitis B and C), parasitic infestation (schistosomiasis) and toxic damage (principally alcohol) (Friedman, 1997). The high

prevalence of these illnesses make liver fibrosis a worthy cause of study: 250x10<sup>6</sup> people worldwide are infected with Hepatitis B infection alone. To date, progressive fibrosis and cirrhosis have been viewed as a static irreversible response to chronic injury. Evidence is accumulating that liver fibrosis can now be considered as a dynamic and potentially reversible process in which changes in matrix degradation occur in addition to matrix synthesis: moreover it mirrors parenchymal wound healing in other tissues.

### **Pathogenesis**

Hepatic fibrosis is characterised by an accumulation of extracellular matrix (ECM) in response to chronic liver injury and is usually distinguished from cirrhosis (advanced fibrosis) which is considered irreversible and in which thick bands of matrix fully encircle the parenchyma forming abnormal nodules. By definition cirrhosis results in the disruption of the normal liver architecture. However this definition is potentially misleading; fibrosis and cirrhosis should be considered part of a disease process continuum in which cirrhosis represents the most advanced stage. Similarities of the wound healing response in liver, kidney, lung and arteries have increased our knowledge of how tissues respond to ongoing injury. Central to the processes in all of these tissues is the recruitment of inflammatory cells, and the activation of mesenchymal myofibroblast like cells (Stellate cells in liver) onto which cell signals converge. The activated myofibroblast cells proliferate and secrete interstitial or fibrillar collagens (collagen I and III). Ultimately the activated myofibroblast-like stellate cells express cytokines in an autocrine manner. Central too, is the release of matrix degrading proteases, matrix metalloproteinases (MMPs) and their regulation by the specific inhibitors - the so called tissue inhibitors of metalloproteinases (TIMPs) and other protease inhibitors. Experimental liver injury and human cirrhosis are characterised by an increased content of extracellular matrix (ECM) constituents predominantly the interstitial or fibrillar collagen: collagen types 1 and III (Rojkind et al., 1979; Siebold et al., 1988). Indeed quantitative as

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well as qualitative changes in other matrix components occur including sulphated proteoglycans (Arenson et al., 1988), and matrix glycoproteins including laminin (Maher et al., 1988), cellular fibronectin (Martinez-Hernandez, 1985) and tenascin (Ramadori et al., 1991). The changes in matrix composition are similar whatever the liver insult: this underscores the importance of understanding the central regulatory elements of the fibrotic process which in turn may identify novel therapeutic strategies that may intervene or reverse the fibrosis response.

### The hepatic stellate cell

Central to the changes observed in matrix production (fibrogenesis) and degradation (fibrolysis) is the Hepatic Stellate Cell (HSC) which has now been identified as the major source of matrix in chronic liver injury (Friedman, 1997; Kawada, 1997). The HSC is a mesenchymal cell, which lies in the Space of Disse between the specialised hepatic sinusoidal epithelium and the palisades of hepatocytes (Wake, 1980). In normal liver HSC are distinguished by prominent intracellular lipid droplets that contain vitamin A stored as retinyl esters (Hendriks et al., 1988). In health HSC are the principal storage site for retinoids and the endogenous ultraviolet fluorescence of vitamin A which in these cells provides a convenient cellular marker for these cells and imparts a buoyancy which assists in their purification (Friedman et al., 1985).

HSCs lie in close proximity to a matrix consisting

primarily of type IV collagen, laminin and heparan sulphate proteoglycans (Burt et al., 1990). In chronic injury the HSCs become activated - undergoing a phenotypic change to a myofibroblast type cell that expresses a smooth muscle actin (Rockey et al., 1992), proliferate and have been shown to secrete matrix proteins: specifically collagen I and III (Milani et al., 1989, 1990a,b). The primary location of fibrotic change exists according to the site of injury (eg perivenular in CCl<sub>4</sub> intoxication and periductular following bile duct ligation in rats) but with progressive injury will spread to become panlobular. HSC can be demonstrated to undergo a transitional cell stage before becoming more fully activated in the early stages of fibrotic models (McGee and Patrick, 1972; Minato et al., 1983; Senoo et al., 1984; Maher et al., 1988; Takahara et al., 1988; Maher and McGuire, 1990). Studies of HSC biology have been enhanced by the observation that when primary HSC cultures are plated onto uncoated plastic they demonstrate the phenotypic changes which mirror activation. This observation has greatly facilitated the study of HSC activation and the culture model of activation is widely used and accepted in studies of hepatic fibrogenesis.

### Evidence for HSC as the main source of matrix in liver injury

In injury, HSC express collagen types I, III, IV (Takahara et al., 1988; Nakatsukasa et al., 1990) and laminin (Milani et al., 1990b) but little or no messenger RNA is localised to parenchymal cells (see Table 1). HSCs are therefore now considered to be the major source of fibrotic matrix in liver injury (Maher and McGuire 1990; Pietrangelo et al., 1992).

Activation consists of early (initiation) and late (perpetuation) phases (Friedman, 1997). Early activation may be provoked by rapid deposition of cellular fibronectin and release of soluble stimuli by Kupffer cells (hepatic macrophages) (Friedman and Arthur, 1989). The late phases of activation are characterised by proliferation (Wong et al., 1994), fibrogenesis, contractility, release of cytokines (Maher et al., 1988) and certain MMPs. The most effective stimulus for proliferation described to date is platelet derived growth factor (PDGF) - a cytokine which also plays a role in smooth muscle cell proliferation during atherosclerosis. The expression of PDGF receptors is a feature of HSC activation (Wong et al., 1994). Other factors that may be important include epidermal growth factor (Marra et al., 1996), fibroblast growth factor (Pinzani et al., 1989), endothelin (Housset et al., 1994), insulin-like growth factor (Pinzani et al., 1990), thrombin (Marra et al., 1995) and transforming growth factor alpha (TGF $\alpha$ ).

In fibrogenesis, alongside the increased ECM accumulation through HSC proliferation, matrix production per cell is also increased. The most effective stimulus to collagen-1 synthesis yet described is transforming growth factor beta 1 (TGF  $\beta_1$ ). TGF  $\beta_1$  can

**Table 1.** Products and components of hepatic stellate cells.

1 <i>Vitamin A related compounds</i>	
Retinoids	
Nuclear retinoid receptors	
2 <i>Cytoskeletal markers</i>	
desmin, alpha-smooth muscle actin	
3 <i>Extracellular Matrix</i>	
Collagens	Types I, III, IV, V, VI, XIV
Proteoglycans	eg Heparan sulphate
Glycoproteins	eg laminin, tenascin
4 <i>Proteases and Inhibitors</i>	
Matrix proteases:	eg Interstitial Collagenase/MMP-1, Gelatinase A/MMP-2 Stromelysin/MMP 3 Membrane-type metalloproteinase 1(MT-1 or MT-MMP)/MMP 14
Protease Inhibitors:	eg TIMPs 1-4 and PA-1
5 <i>Cytokines, growth factors and inflammatory mediators</i>	
Prostaglandins	
Acute Phase components	
Mitogens	
Vasoactive Mediators	eg Endothelin 1 (ET-1), nitric oxide (NO)
Fibrogenic compounds	eg TGF-beta 1-3
6 <i>Receptors</i>	
Cytokine receptors	
7 <i>Signaling molecules and transcription factors</i>	
Signaling components	eg MAP kinase
Transcription factors	eg SP-1, NFkB, c-myb

be derived from HSC (autocrine) (Bachem et al., 1992; Houglum et al., 1994) and from Kupffer cells (paracrine), (Friedman and Arthur, 1989). TGF  $\beta_1$  binding to HSC increases with HSC activation (Houglum et al., 1994), and activation of latent TGF  $\beta_1$  itself may also contribute to a net increase in its activity. Activation of HSC may also be associated with an increase in contractile activity, a quality common to myofibroblast-like cells in other tissues. HSC contractility is regulated by endothelin (Housset et al., 1994), nitric oxide (Rockey and Chung, 1995) and potentially by eicosanoids and modifiers of cyclic AMP (Pinzani et al., 1990).

HSC may regulate the inflammatory response to liver injury by releasing cytokines chemotactic for leukocytes. Colony stimulating factor (Pinzani et al., 1992) and monocyte chemotactic peptide (Marra et al., 1993), both secreted by HSC, are upregulated during HSC activation. These findings may contribute to the marked infiltration of mono-nuclear leukocytes which accompanies most forms of experimental and human liver injury. Most recently HSC have been demonstrated to release IL-10, a cytokine with modulatory effects on macrophages suggesting that rather than being a passive recipient of fibrogenic signals the HSC may influence the inflammatory response by down regulating Kupffer cell activity (Thompson et al., 1998a,b).

### Matrix degradation in liver fibrosis

The resorption of extracellular matrix is a normal event in the remodelling seen in a variety of tissues eg. in embryogenesis, trophoblastic implantation, angiogenesis, tissue morphogenesis and growth. In pathological processes the accelerated breakdown occurring in for example arthritides, periodontal disease,

tumour invasion and metastases may in part be due to a breakdown in the tight control of degradative processes (Murphy et al., 1991).

In fibrotic liver injury there is evidence to suggest that: 1) Degradation of the normal basement membrane-like liver matrix (in the Space of Disse) occurs. This may disturb hepatocyte function and promote deposition of a fibrillar liver matrix. 2) In progressive fibrosis there is a failure to degrade excess fibrillar collagens (Arthur, 1994a). The major class of enzyme expressed by mesenchymal cells to mediate this matrix remodelling are the Matrix Metalloproteinases (MMPs) (see Table 2).

The MMPs are a family of zinc and calcium dependent endopeptidases secreted by connective tissue cells that have activity against the major constituents of matrix including fibrillar (interstitial and banded) and non-fibrillar collagens. MMPs are important in ECM turnover (Murphy et al., 1991), and there is strong evidence for their expression by HSC (Emonard et al., 1990; Herbst et al., 1991; Milani et al., 1992) and Kupffer cells (Winwood et al., 1995). The MMPs can be grouped according their enzymatic substrate (see Table 2). The first group are the collagenases: these MMPs are central to the process of remodelling/repair of fibrotic tissue as they cleave the helix of native fibrillar collagens I, II and III to render the collagen susceptible to degradation by other MMPs to which they were previously resistant (Goldberg et al., 1986). Thus activation of Interstitial Collagenase (MMP-1) or an alternative analogue is crucial to the process of remodelling of fibrotic matrix - the expression of Interstitial Collagenase would be necessary to initiate degradation of the most abundant fibrotic collagen (types I and III) (Friedman, 1997). A recent report that Gelatinase A (MMP-2) may have degradative activity against collagen I (Franklin, 1995) would, if confirmed, have implications for the process of matrix remodelling

**Table 2.** The matrix metalloproteinases family (MMPs).

NAME	NUMBER	SUBSTRATE
<i>The Collagenases</i>		
Interstitial Collagenase	MMP-1	Collagen I, II, III, VII, VIII, X, gelatins, aggrecans, tenascin
Neutrophil Collagenase	MMP-8	Collagen I, II, III, VII, X, aggrecan
Collagenase 3	MMP-13	Collagen I, II, III, VII, X, aggrecans, gelatins
<i>The Gelatinases</i>		
Gelatinase A	MMP-2	Gelatin, collagen types IV, V, VII, X (may also have Interstitial Collagenase activity against collagen 1), fibronectin, elastin, laminin, aggrecan, vitronectin
Gelatinase B	MMP-9	Gelatin, collagen IV, V, VII, X, XI, vitronectin, elastin, aggrecan
<i>The Stromelysins</i>		
Stromelysin 1	MMP-3	Aggrecan, link protein, fibronectin, laminin, elastin, transin, gelatins
Stromelysin 2	MMP-10	Gelatins I, III, IV, V; collagens III, IV, V, VIII, IX, activates procollagenase, fibronectin, laminin, elastin, aggrecan
Stromelysin 3	MMP-11	N-terminal domain cleaves casein
Matrilysin	MMP-7	Gelatins, elastin, aggrecans, fibronectin, link protein, activates procollagenase, vitronectin, tenascin C, entactin, laminin
<i>Membrane-type MMPs</i>		
MT-1	MMP-14	Activates progelatinase A and possibly other MMPs, collagen I, II, III; dermatan sulphate, laminin B chain, fibronectin, gelatin, vitronectin

- in the liver and other organ/tissue systems. This second group of MMPs, the Gelatinases, have activity against denatured collagen (gelatin), some collagens, elastin and laminin. The gelatinases are significant for their important role in degrading normal type IV collagen in the basement membrane in the Space of Disse during the early stages of the fibrotic process (Murphy et al., 1991). The third group, the Stromelysins, have activity against a variety of collagen: II, IV, IX, X, XI, denatured collagen (or gelatin), laminin and fibronectin. Stromelysins are also important in that they activate procollagenase (Murphy et al., 1991; Matrisian, 1992). A fourth group, the membrane type MMPs (MT-MMP), comprises of three members and serve to activate gelatinase and by virtue of a unique structure localize this activity to the cell surface (Sato et al., 1994; Takino et al., 1995; Will and Hinzmann 1995).

### Regulation of MMP expression and activity

The extracellular activity of MMPs is regulated at various stages: 1) by transcriptional activation at the level of the gene; 2) by activation of the latent proenzyme when the propiece is cleaved; and 3) by extracellular inhibition by the specific Tissue Inhibitors of Metalloproteinases (TIMPs) or more general protease inhibition such as  $\alpha$ -2 macroglobulin. A series of growth factors including IL-1, TNF- $\alpha$ , PDGF,  $\beta$ -FGF, and EGF regulate MMPs at the level of the gene (Matrisian, 1990; Murphy et al., 1990, 1991; Murphy and Hembray 1992). An important cytokine in the context of liver fibrosis is TGF- $\beta$ <sub>1</sub> which differentially regulates certain MMPs and is expressed by Kupffer cells (Matsuoka and Tsukamoto, 1990) and activated HSCs (Bachem et al., 1992). Activated HSCs express TGF- $\beta$ <sub>1</sub> receptors (De Bleser et al., 1995), TGF- $\beta$ <sub>1</sub> mRNA and also secrete this cytokine in an autocrine manner. In fibroblasts TGF- $\beta$ <sub>1</sub> down regulates interstitial collagenase (MMP-1): important in the degradation of collagen I (Edwards et al., 1987), whilst upregulating gelatinase A (MMP-2) (Overall et al., 1989) TIMP-1 and collagen I (Matsuoka and Tsukamoto, 1990; Wahl et al., 1993). Hence TGF- $\beta$ <sub>1</sub> is a

profibrogenic cytokine and is likely to play an important role in the fibrotic process.

A second level of control resides where the secreted form of all MMPs (pro-MMP) is converted to the active MMP by cleavage of the propiece (Bachem et al., 1989; Friedman et al., 1989; McDonald, 1989). This process may be mediated by plasmin (Matrisian, 1992; Murphy et al., 1992). Active stromelysin cleaves activated interstitial collagenase (MMP-1) (Murphy et al., 1987; Matrisian, 1990) whilst the fourth group of MMPs: MT-MMP activates gelatinase A (MMP-2) at the cell membrane.

### The TIMPs

Tissue Inhibitors of Metalloproteinases (TIMPs) function at two levels: certain TIMPs stabilise specific pro-MMP species and all TIMPs inhibit all active MMP enzymes: thus acting as an important regulatory brake on metalloproteinase activity (see Table 3) (Docherty et al., 1985; Boone et al., 1990). Four TIMPs have been identified to date (TIMP 1, 2, 3 and 4) - each is a separate gene product (Pavloff et al., 1992; Denhardt et al., 1993; Apte et al., 1994; Wu and Moses, 1998) (see Table 3). Structurally, there are similar features running through the TIMP family. TIMP 1 and 2 share 40% amino acid homology and both have three looped structures stabilised by six disulphide bonds (Boone et al., 1990; Murphy and Docherty, 1992; Denhardt et al., 1993). TIMPs bind to MMPs in a stoichiometric manner which is irreversible under normal physiological conditions. The MMP is rendered inactive when the TIMP binds to its active site (Cawston et al., 1983; Bo and Denhardt, 1992; Murphy and Docherty, 1992). Binding is non-covalent so under certain *in vitro* conditions TIMPs can be separated from the MMP species with the former retaining activity against MMPs (Murphy et al., 1989). TIMPs 1 and 2 inhibit the active form of all MMPs (Denhardt et al., 1993) and TIMPs 3 and 4 probably have the same spectrum of activity. Two functional domains exist on the TIMP molecule: the N terminal is vital for activity against MMPs - truncation

**Table 3.** Properties of tissue inhibitors of metalloproteinases (TIMPs).

	TIMP-1	TIMP-2	TIMP-3	TIMP-4
MMP inhibition	All	All	All?	All?
Mature protein size (kDa)	28.5	21	21	23
Glycosylation	Yes	No	Yes	No
Localization	Diffusible	Diffusible	ECM bound	Unknown
Expression	Inducible	Constitutive Possibly Inducible in Hepatic Stellate Cells	Inducible	Unknown
Major tissue sites	Liver, Bone, Ovary	Liver, Lung, brain ovaries, testis, heart, placenta	Kidney, brain, lung, heart, ovary	Kidney, colon, placenta, testis, brain, heart, ovary, skeletal muscle
Binding to pro-MMP	MMP-9	MMP-2	Unknown	Unknown
Binding to all active MMPs	Yes	Yes	Probably	Probably

ECM: extracellular matrix

of the TIMP molecule which spares the N-terminal allows continued inhibitory activity against MMPs. The C terminal facilitates interaction with the prometalloproteinases (Howard and Banda, 1991; Howard et al., 1991; Ward et al., 1991; Fridman et al., 1992). TIMPs 1 and 2 bind to pro-Gelatinase species (Gelatinase B/MMP 9, Gelatinase A/MMP 2 respectively) thus preventing activation (De Clerck et al., 1991; Goldberg et al., 1992). TIMPs are regulated at the level of transcription by cytokines and growth factors that also govern MMP expression and that are also important both in HSC activation and HSC synthetic function. For example TGF- $\beta_1$  upregulates TIMP-1 and gelatinase A whilst down regulating TIMP-2, interstitial collagenase (MMP-1) and stromelysin (Edwards et al., 1987; Overall et al., 1989, 1991). TNF-alpha upregulates TIMP-1 and interstitial collagenase (MMP -1) (Marshall et al., 1993).

Studies mapping the gene promoters of TIMPs and MMPs show some common regulatory motifs that differ in individual TIMPs/MMPs in terms of their frequency and position in relation to the transcription start site. For example the murine TIMP-1 and interstitial collagenase (MMP-1) promoters both have AP-1 and PEA-3 binding sites but in different configurations (Edwards et al., 1992; Schorpp et al., 1995). An AP-1 site is noted in the promoters of stromelysin and gelatinase B but not in gelatinase A (MMP-2). The human TIMP-2 gene on chromosome 17 is flanked by 5' AP-1 and AP-2 consensus sequences and several SP-1 sites in association with a TATA box (De Clerck et al., 1994). The AP-1 consensus site in the TIMP 2 promoter is further upstream from the transcription start site than that found in the TIMP-1 promoter and is not associated with a PEA-3 motif. As in TIMP 1 and 2, the TIMP 3 promoter has multiple SP-1 sites which confer a high basal expression in growing cells (Wick et al., 1995). The spatial distribution and differing frequencies of transcription binding sites in the promoters of TIMPs 1 and 2 may explain in part the differential expression observed in response to cytokines such as TGF  $\beta_1$  and TNF- $\alpha$ . The promoters also provide a mechanism whereby TIMPs can be coregulated and independently regulated to inhibit MMP activity in a wide variety of physiological (eg growth and development) and pathological processes (eg arthritides and liver fibrosis). Moreover, from what has been discussed it can be proposed that through relatively small changes in the ratio of TIMP: MMP concentrations alterations in matrix degradation can be effected and regulated.

There are large regions of the TIMP promoter regions still undescribed. Recently for example a novel transcription factor binding site "Upstream TIMP element 1" has been described along with its associated binding proteins (Trim et al., 2000).

#### **Interaction of TIMPs with MMPs in the fibrotic process and evidence for MMP inhibition during liver fibrosis**

Stellate cells express a variety of MMPs: both

human and rat HSCs express gelatinase A (MMP-2) and rat HSC express Stromelysin (MMP-3) (Arthur et al., 1989, 1992; Vyas et al., 1995). Gelatinase A expression is upregulated with HSC activation whilst Stromelysin is transiently expressed with HSC activation over a period of 72 hours. Both MMPs can be immunolocalized to HSC and MMP activity can be detected in cell culture supernatants. Both MMPs are also expressed in acute liver injury in perisinusoidal cells (Herbst et al., 1991; Iredale et al., 1993). Collagenase can be localised to the HSC cytoplasm during activation by culture on uncoated plastic (Arthur, 1994b).

Messenger RNA (mRNA) for collagenase cannot be detected in activated rat HSC but is detected in freshly isolated cells (Iredale et al., 1996). However TNF- $\alpha$  and IL-1 can induce interstitial collagenase expression in activated human HSCs (Emonard et al., 1990; Iredale et al., 1995). Cultured HSC also release interstitial collagenase in response to polyunsaturated lecithin (Li et al., 1992). HSCs therefore possess the ability to remodel matrix during activation and specifically to mediate remodelling of interstitial collagens by expressing interstitial collagenase (MMP-1). Other cells may play a role in matrix degradation such as Kupffer cells which express Gelatinase B (Winwood et al., 1995) and sinusoidal endothelial cells which express stromelysin (Herbst et al., 1991).

#### **TIMP expression in progressive fibrosis**

To address the concept that TIMP expression may promote fibrosis by reducing collagenase activity in progressive liver fibrosis, the gene expression of TIMPs 1 and 2 and MMPs have been studied in HSC activation both in tissue culture and in vivo. When HSC are cultured in uncoated tissue culture plastic, a process which recapitulates many of the features of activation including  $\alpha$  Smooth Muscle Actin ( $\alpha$  SMA) and ProCollagen 1 (PC-1) expression, there is an increase in the transcription of TIMP-1 mRNA in activated cells compared to quiescent (freshly isolated) cells (Iredale et al., 1992). TIMP-1 can be immunolocalized to HSC and also detected extracellularly in HSC cell culture supernatants by ELISA. When HSC conditioned media is subjected to gelatin sepharose chromatography, TIMP-1 bound to Gelatinase A (MMP-2) is separated: removal of TIMP-1 is associated with a twenty-fold increase in gelatinase activity. Returning TIMP-1 to the media results in re-inhibition of Gelatinase A and a reduction in its activity (Iredale et al., 1992). Both TIMP 1 and 2 are found in HSC conditioned media and TIMP 2 mRNA is observed in northern analysis of activated HSC total RNA (Iredale et al., 1992; Benyon et al., 1996). It is of interest that HSC activation appears to be associated with an upregulation of TIMP-2 expression: a feature not observed in other cell lines studied to date. HSC may not be the sole source of TIMPs in the liver: TIMPs 1 and 2 are detected in Hep G2 hepatoma cell lines (Kordula et al., 1992; Roeb et al., 1993; Benyon et al., 1996). In these studies TIMP 1 expression was found to increase

in the presence of IL-6, an acute phase cytokine, suggesting that TIMP 1 may be released by hepatocytes in acute liver injury.

A number of other studies have indicated that a reduction in matrix remodelling activity occurs as fibrosis progresses manifested by a fall in interstitial collagenase (MMP-1) activity (essential in the degradation of collagens I and III). This is noted in models of alcoholic liver injury in humans and primates and carbon tetrachloride injury in rats (Okazaki and Maruyama, 1974; Maruyama et al., 1981, 1982; Perez-Tamayo et al., 1987). This suggests that inhibition of matrix degradation is a feature of fibrogenesis. Further evidence to support this concept is provided by models of recovery from hepatic fibrosis. During carbon tetrachloride induced liver injury in the rat progressive fibrosis can be documented and becomes established after 4 weeks of treatment. At this stage prompt withdrawal of the toxin results in resolution of the fibrotic changes over a period of 28 days (Iredale et al., 1998). Analysis of the TIMP/Collagenase relationship in this model indicates that during progressive fibrosis interstitial collagenase continues to be expressed but its activity falls with a concurrent upregulation in TIMP 1 and 2 expression (Mallat et al., 1995). In the recovery phase expression of TIMPs 1 and 2 decreases with a concurrent rise in collagenase activity accompanied by histological evidence of matrix remodelling (Iredale et al., 1998). The expression of Collagenase mRNA remains relatively constant.

In a model of fibrotic liver from murine schistosomiasis expression of interstitial collagenase, detected immunologically, remains relatively constant (Takahashi et al., 1980; Takahashi and Simpsen 1981; Truden and Boros, 1988) whilst collagenase activity decreases emphasizing the importance of expression of collagenase inhibitors during fibrogenesis. Further evidence for the important roles of TIMPs in fibrogenesis come from the analysis of serum in patients with hepatic inflammation and established cirrhosis reveals an increase in TIMP 1 levels by ELISA (Murawaki et al., 1993, 1994; Muzzillo et al., 1993). In addition, when TIMPs 1 and 2, interstitial collagenase and Gelatinase A mRNA expression in fibrotic liver compared to normal were studied by Ribonuclease Protection Analysis (Iredale et al., 1995; Benyon et al., 1996), TIMP 1 and 2 transcripts were increased in fibrotic liver, as are Gelatinase A transcripts (Benyon et al., 1996). In contrast Interstitial collagenase transcripts were only marginally increased in primary sclerosing cholangitis and primary biliary cirrhosis (Benyon et al., 1996). TIMP 1 was also immunolocalized to perisinusoidal cells in fibrotic liver in 75% of biopsies positive for interstitial collagenase suggesting that coexpression of TIMP 1 with interstitial collagenase occurred (Benyon et al., 1996). This data provides powerful evidence that progressive fibrosis is associated with changes in the pattern of matrix degradation in addition to matrix synthesis. Moreover, current evidence

suggests that such changes may be mediated by the powerful MMP inhibitors the TIMPs.

## Conclusion

In summary, the Hepatic Stellate Cell has been examined in the context of progressive fibrotic liver injury. There is considerable evidence that HSC are the principle effector cells for matrix remodeling in liver injury and that during HSC activation TIMPs 1 and 2 mRNA are expressed. Understanding the dynamic changes in TIMP expression in HSC activation will enhance our knowledge of fibrogenesis and may lead to the development of novel therapeutic strategies based on promoting matrix degradation. It is anticipated that further insights into the relative roles of the TIMPs and MMPs will become possible as experimental tools such as the development of TIMP gene knockout mice become available to facilitate definitive mechanistic studies.

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