IL-6 and extracellular matrix remodeling

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ABBREVIATIONS

AP1: activator protein 1; CAT: Chloramphenicol acetyl transferase; ECM: Extracellular matrix; HSCs: Hepatic stellate cells; Jak: Janus tyrosine kinases; JNK: c-Jun N-terminal kinase; MMPs: Matrix metalloproteinases; PP2A: Protein phosphatase 2A; TIMPs: Tissue inhibitor MMPs; IL-6: Interleukin-6.

INTRODUCTION

Hepatic fibrosis is a characteristic feature of chronic liver diseases. It is the result from the excessive deposition of *extracellular matrix* (ECM) proteins into the liver in response to a number of liver injuries, including chronic ethanol ingestion, viral hepatitis and iron deposition. Hepatic fibrosis has important physiopathologic consequences on the liver function and structure. First, it may contribute to increase intrahepatic vascular resistances to the portal flow and to originate portal hypertension. Activated hepatic stellate cells may contribute to this effect,

since these cells exhibit contractile capacity and can modulate blood flow at the sinusoidal level (1). Second, perisinusoidal fibrosis represents a barrier to the exchange of nutrients and metabolites between sinusoidal blood and hepatocytes. Furthermore, deposition of ECM in the space of Disse induces the loss of fenestrae of the sinusoidal endothelial cells (2). Third, ECM is a physiological reservoir for cytokines and growth factors in the close cell surrounding. The binding of these factors to ECM component modulates their biological activity and protects them from proteolysis (3).

ECM of the liver is composed of three distinct types of proteins including collagen, noncollagenous glycoproteins, and proteoglycans (Fig. 1). *Collagen type I, III and V* are heterotrimer molecules that form a triple helical structure with a tendency to form supramolecular aggregates (4,5). *Type V*, a pericellular collagen in the normal liver, is also present in increased amount in septa and portal tracts. *Collagen IV* is the major collagen of basement membrane. It is an unusual collagen type in that molecules cross-link at their terminal domains to form a network. This sheet of collagen is physically intermeshed or directly bound to other components of the basement membrane like laminins, nidogen, or perlecan.

In the hepatic fibrosis and cirrhosis, all types of collagens are increased. When hepatic fibrosis is mild, collagen type I and III are equally increased. However, when hepatic fibrosis is severe, the predominant collagen in the connective tissue bands is type I, and the ratio of collagen I to collagen III increased to 4:1 (5). In these conditions, collagen IV is deposited mainly in the space of Disse, transforming sinusoids into capillaries. Although collagen I becomes the predominant ECM constituent in fibrosis and cirrhosis, many other proteins also are part of the fibrous complex. Thus, glycoproteins (fibronectin, laminins, tenascin, undulin, thrombospondin, SPARC) and proteoaminoglycans (perlecan, biglycan, decorin, aggrecan) also are increased in scars and septa.

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Fig. 1.- Composition of the extracellular matrix.
Matriz extracelular.

Although it is likely that each cell population in the liver plays a role in the production of ECM components, *hepatic stellate cells* (HSCs) appear to be the major fibrogenic cell type (6). HSCs are resident nonparenchymal cells located in the subendothelial space between hepatocytes and sinusoidal endothelial cells. In normal liver, HSCs are relatively quiescent with respect to ECM synthesis (7,8). However, during liver injury, they undergo a complex process that transforms quiescent HSC to one activated cell that is proliferative, fibrogenic, and contractile (9,10). HSC activation is associated with changes in the metabolism (increased DNA synthesis, cellular proliferation), the pattern of gene expression [collagen I, III, IV, laminin, fibronectin, MMPs (*Matrix Metalloproteinases*), TIMPs (*Tissue Inhibitor of MMPs*)], and the capacity to synthesize cytokines, growth factors and their receptors.

Extensive fibrosis in experimental liver injury may resolve upon withdrawal of the injurious agent. Thus, fibrosis or cirrhosis induced by biliary obstruction or carbon tetrachloride intoxication in rat is reversible over a period of one to three months (11). Likewise, reversible fibrosis has been shown in mouse and rabbit models of hepatosplenic schistosomiasis (12). Current evidence indicates that regression of liver fibrosis and advanced micronodular cirrhosis is a real phenomenon if the original cause of the liver injury is effectively removed (13-17). However, this process may be incomplete over a prolonged period and results in the development of macronodular cirrhosis. Remodeling of this residual lesion is limited by matrix cross-linking (18). Transglutaminase-mediated cross-linking of collagen provides matrix proteins with resistance to MMP-mediated degradation (19). Thus, these linking may limit matrix degradation even in the presence of active MMPs.

Degradation of the ECM in the hepatic fibrosis is a complex process involving MMPs, specific TIMPs, and enzymes that activate latent MMPs (20). In an experimental model of liver fibrosis, transient MMPs overexpression in the liver effectively attenuates established fibrosis (21).

MMPs constitute a large family of structurally related zinc-dependent endopeptidases capable of degrading a wide variety of ECM components (22-24), and, therefore, they may play a key role in the resolution of liver fibrosis upon withdrawal of the injurious agent. MMP-1 and MMP-13 cleave interstitial collagens (types I, II, III and X). MMP-2 (gelatinase A) and MMP-9 (gelatinase B) degrade denatured interstitial collagen (gelatins), type V and IV collagens, and noncollagen proteins (fibronectin, laminin, elastin). MMP-3 (stromelysin-1), MMP-10 (stromelysin-2) and MMP-7 (matrilysin) degrade a broad range of substrates, including proteoglycans, and collagen and noncollagen proteins. Membrane-type MMPs (MMP-14 to MMP-25) are anchored to the surface of cells through their carboxyl-terminal transmembrane domain. These membrane-type MMPs degrade collagen and noncollagenous proteins, proteoglycans and play an important role in the activation of MMP-2 and MMP-13. In rats and mice, there is only one interstitial MMP, the MMP-13, which shares 86% homology with human MMP-13. MMP-13 is capable of degrading type I, II, III, IV, X, and XIV collagens, tenascin, fibronectin and the aggrecan protein (25).

MMPs are secreted as inactive proenzymes; therefore they need to be subsequently activated by several mechanisms including enzymatic cleavage (serine protease plasmin, stromelysin-1, membrane-type MMPs) (20,26). Activated MMPs produce a limited number of cleavages within the triple helix of the three α -chain of interstitial collagen molecules, e.g. in type I collagen, cleavage occurs between Gly⁷⁷⁵ and Ile⁷⁷⁶ in the α_1 chain and the corresponding Gly/Leu in the α_2 chain, to yield typical three-fourths length amino-terminal and one-fourth length carboxyl-terminal fragments. This initial single site cleavage of interstitial collagens results in their partial unfolding, which then renders them susceptible to degradation to small fragments by gelatinases. The activity of MMPs is regulated by a complex mechanism at the level of gene expression, proenzyme activation, or binding of proenzyme or active enzyme to TIMPs.

TIMPs are low molecular weight glycoproteins capable of irreversibly binding to proenzyme or active form of MMPs with a 1:1 stoichiometry and inactivating a wide variety of MMPs degrading enzymes. Because of the one-to-one interactions, small changes in levels of TIMPs can lead to significant changes in the biological activity of MMPs. Excess production of TIMPs relative to MMPs could contribute to the progression of liver fibrosis, while the enhancement of matrix breakdown may be achieved by either reducing the synthesis of TIMPs or increasing the expression of MMPs. All members of this

family are able to inhibit all of the MMPs. However, some TIMPs have specific relationship with individual MMPs (27,28). TIMPs are multifunctional molecules that not only inhibit MMPs but also stimulate proliferation of various cell types and can protect cells from apoptosis (29,30).

The severity of hepatic fibrosis is the result of the degree of collagen synthesis and the fate of the collagen deposited into the ECM of the liver, which, in turn, depend on the balance between the production and the activation of MMPs and the levels of TIMPs. In chronic liver disease in human and in animal models of liver fibrosis, expression of MMP-1/MMP-13 does not change significantly, as hepatic fibrosis develops and progresses, but there is a striking increase in expression of TIMP-1 and TIMP-2 (31). In homogenates prepared from fibrotic livers, the level of TIMP-1 protein increases approximately five-fold in comparison with normal liver. Regression of hepatic fibrosis is characterized by degradation of ECM and restoration of normal liver histology. Following cessation of injury, there is a five-fold increase in collagenase activity in the liver, which is associated with a progressive decrease in the level of expression of TIMP-1 and TIMP-2, but no change in the level of expression of MMP-13. Studies in cultured HSCs have explored the molecular basis of the up-regulation of TIMP-1 during hepatic fibrogenesis. These studies have identified a key region in the TIMP-1 promoter that is critical to gene expression (32). However, up to now, it has not been identified the proteins interacting with this region. Many cell types, particularly HSCs, Kupffer cells and inflammatory cells, produce several MMPs in culture (20,26).

Very few studies have examined the effect of cytokines on ECM degradation in HSCs. However, it is well known that MMP production can be up-regulated by several hormones (PTH, cortisol), growth factors (platelet derived growth factor, basic fibroblast growth factor), cytokines (TNF α , IL-1 α , IL-6, IL-10, IFN γ , oncostatin M, macrophage migration inhibitory factor) and tumor promoters (phorbol esters). On the other hand, MMP-13 is down-regulated by transforming growth factor (TGF β), insulin-like growth factor (IGF) (20,26,33-40). Likewise, several cytokines (TNF α , IL1 β , IL-6, oncostatin M, IFN α) and growth factors (TGF β) up-regulate (41-43) or inhibit (44-47) TIMPs expression by cultured HSCs.

Interleukin-6 (IL-6) is a multifunctional glycoprotein produced by activated monocytes, macrophages, endothelial cells, and hepatic stellate cells that mediates a wide variety of functions in different cells, including fibroblasts, hepatocytes, and hepatic stellate cells (48). IL-6 promotes cell proliferation and differentiation and regulates specific gene expression (49), particularly the expression of the acute phase proteins in liver cells (48,50). Acute and chronic liver diseases, particularly alcoholic liver diseases, are similar to the acute phase response in some respects. Thus, patients with alcoholic

hepatitis show fever, muscle wasting, neutrophilia, and increased production of C-reactive protein, α_1 -antitrypsin and amyloid A (50,51). High levels of IL-6 have been detected in the sera of patients with alcoholic liver cirrhosis (52-54) and some authors have shown a correlation between circulating concentrations of IL-6 and serum concentrations of C-reactive protein (55,56), an IL-6-induced protein. Thus, IL-6 seems to be one of the most important factors regulating inflammatory response in the liver.

We have shown that IL-6 increases MMP-13 protein levels and the steady-state levels of MMP-13 mRNA in a dose- and time-dependent fashion (Fig. 2) and that this effect is produced acting within the gene promoter (57). This effect is evident after 6 hours of treatment but it is particularly significant after 24 hours. This prolonged incubation time required by IL-6 to activate MMP-13 gene expression suggests that it may be required the synthesis

of a new protein. The IL-6-induced increase in MMP-13 mRNA levels can be blocked by inhibiting the protein synthesis with cycloheximide (Fig. 2C), which supports this requirement.

This enhanced MMP-13 gene expression is associated with a marked increase in the immunoreactive MMP-13 protein (Fig. 2D). These results agree with those reported by Franchimont et al. (58) and Kusano et al. (59), who also demonstrated that IL-6, in the presence of its soluble receptor, increased MMP-13 mRNA levels, immunoreactive MMP-13 and its biological activity. It is likely that IL-6 has an effect on the extracellular metabolism of MMP-13, since the effect of this cytokine on MMP-13 secreted into the culture medium is much higher than that induced on the steady-state level of MMP-13 mRNA. Although some authors have shown that IL-6 significantly enhanced TIMP-1 production and TIMP-1 mRNA expression in human fibroblasts and other cell lines (60-64), in our experience, IL-6 also increases immunoreactive TIMP-1, but to a lesser extent (Fig. 2D). In fact, TIMP-1 and MMP-13 promoters contain common regulatory elements, i.e. an AP1 binding site (TRE) and PEA-3 (65). Therefore, factors that up-regulate MMP-13 gene expression may also stimulate TIMP-1 expression.

Fig. 2.- (A) Dose-response effect of IL-6 on MMP-13 mRNA. Confluent Rat-1 fibroblasts were incubated for 24 hours in the absence or presence of increasing concentration of IL-6. (B) Time-response effect of 20 ng/mL IL-6 on MMP-13 mRNA. (C) MMP-13 mRNA in cells incubated for 24 hours in control medium (Ctr) or in medium containing 20 ng/mL IL-6 in the presence or absence of 0.1 mM cycloheximide (CHX). MMP-13 mRNA was measured by Northern blot analysis. The autoradiograms were quantitated by scanning laser densitometry. Bars represent the level of MMP-13 mRNA in each sample after normalization to the level of 18S RNA. Values are given as fold over the value in control cells. (D) Medium harvested from cells untreated and treated with 10, 20, or 40 ng/ml was analyzed by Western blot with antibody against human MMP-13. Likewise, samples of medium harvested from cells untreated or treated with 20 or 40 ng/ml IL-6 were concentrated, and 30 μ g protein from each sample were analyzed by Western blot with specific antibody against rat TIMP-1. There is a dose-dependent increase in the amount of immunoreactive MMP-13 and TIMP-1. (A) Efecto de la dosis de IL-6 sobre el ARNm de la MMP-13. *Fibroblastos Rat-1* fueron incubados durante 24 horas en ausencia o presencia de concentraciones crecientes de IL-6. (B) Efecto del tiempo de exposición a 20 ng/mL de IL-6 sobre el ARNm de la MMP-13. (C) ARNm de la MMP-13 en células incubadas durante 24 horas en medio control o en medio que contenía 20 ng/mL IL-6 en presencia o ausencia de 0,1 mM de cicloheximida (CHX). El ARNm de la MMP-13 fue determinado por Northern blot. Las autoradiografías fueron cuantificadas mediante densitometría de láser. Las barras representan los niveles de ARNm de la MMP-13 en cada muestra tras su normalización con los niveles de ARN 18S. Los valores son expresados como veces sobre el valor en las células controles. (D) Se analizaron las proteínas del medio de cultivo de células tratadas y no tratadas con 10, 20 o 40 ng/mL mediante Western blot utilizando para ello anticuerpos frente a la MMP-13 humana. Igualmente, se concentraron muestras de medio de cultivo de células tratadas con 20 o 40 ng/mL de IL-6. Treinta microgramos de estas proteínas fueron analizadas mediante Western blot empleando anticuerpos frente al TIMP-1 de rata. Se produce un aumento dependiente de la dosis en la cantidad de MMP-13 y de TIMP-1 inmunorreactivas.

Rat MMP-13 promoter displays a general organization similar to that of other members of the MMP family (66,67), particularly to human (68) and rabbit MMP-1 genes (69). All share a common 10-exon organization (67,68,70) and contain a typical TATA box in addition to TRE and polyomavirus enhancer activator 3 (PEA-3) consensus site in their promoter region (67,71-74), suggesting a common regulatory mechanism of gene transcription. Rat MMP-13 gene also contains several consensus transcription factor recognition sequences such as C/EBP, Cbfa1, p53, AP-2, and AP1 (75) (Fig. 3). In cells transfected with plasmids whose activity is driven by portions of different length of the MMP-13 gene promot-

er, we found that sequences upstream of the base pair -79 are dispensable in IL-6-mediated stimulation of MMP-13 gene expression. These experiments also show that integrity of the TRE site appears to be essential for the IL-6 induced response (Fig. 4). The TRE site has been implicated in the expression of many of the MMP genes (76,77), including MMP-13 (77). In cells transfected with a luciferase construct containing two copies of the TRE upstream of a minimal promoter, we were able to confirm this stimulatory effect of IL-6 on the TRE site (Fig. 5). Very little information exists about the effect of IL-6 on the activation of genes with a TRE. However, while Dafada et al. (78) found that IL-6 had no effect on the ex-

Fig. 3.- Schematic representation of the rat MMP-13 gene promoter. (+) Sign indicates the transcription start site.

Representación esquemática del promotor genético de la MMP-13 de rata. El signo (+) indica el lugar de inicio de la transcripción genética.

		CAT activity (Fold over control)		
5'	3'			
P(-2000)MMP-13 CAT		1	2.5	3.3
P(-1000)MMP-13 CAT		1	2.3	2.9
P(-500)MMP-13 CAT		1	1.9	3.0
P(-284)MMP-13 CAT		1	2.2	3.0
P(-76)MMP-13 CAT		1	1.8	2.7
P(-76)MMP-13 CAT mutated		1	0.9	1.0
		0	20	40
		IL-6 (ng/mL)		

Fig. 4.- Rat 1 fibroblasts were transiently transfected with a series of MMP-13-CAT constructs obtained by removing progressively more 5'-flanking sequences of the MMP-13 promoter and exposed to 0, 20, or 40 ng/mL IL-6 for 24 hours. Cell lysates were assayed for chloramphenicol acetyl transferase (CAT) activity. CAT activity is expressed as fold over control activity. Deletion of promoter sequences upstream of base pair -76 relative to the transcription start site does not abrogate the effect of IL-6 on MMP-13 gene expression. This effect disappears in cells transfected with a construct in which the AP1 binding site has been mutated (X).

Fibroblastos Rat 1 fueron transfectados con una serie de plásmidos MMP-13-CAT obtenidos eliminando progresivamente secuencias de mayor tamaño del extremo 5' del promotor MMP-13. Estas células fueron expuestas a 0, 20 ó 40 ng/mL de IL-6 durante 24 horas. La actividad CAT fue determinada en los lisados de esas células. La actividad cloranfenicol acetil transferasa (CAT) la expresamos en forma de veces la actividad existente en las células controles. La eliminación de las secuencias proximales al par de bases -76 en relación con el inicio de la transcripción no elimina el efecto de la IL-6 sobre la expresión del gen de la MMP-13. Este efecto desaparece cuando la secuencia consenso AP1 es mutada (X).

Fig. 5.- Cells were transfected with the p2xTRE-Luc construct and cultured in the absence or presence of 20 ng/ml IL-6. Luciferase activity was measured after 6, 12 or 24 hours of treatment with IL-6. Values are given as fold over the activity in control cells. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ as compared with control cells.

Las células fueron transfectadas con el plásmido p2xTRE-Luc y cultivadas en ausencia o presencia de 20 ng/mL de IL-6. Se midió la actividad luciferasa tras 6, 12 o 24 horas de tratamiento con IL-6. Los valores son expresados en forma de veces por encima de la actividad en las células controles. *: $p < 0,05$; **: $p < 0,01$; ***: $p < 0,001$ comparados con las células controles.

pression of pTRE-CAT in transient transfected cells, suggesting that AP1 is not induced by IL-6 treatment, Melamed et al. (79) showed that IL-6 induced the formation of a TRE-binding complex, which was abrogated by anti-Jun specific antibodies.

Upregulation of gene expression is usually induced by the binding of transcription factors to specific cis-acting elements in gene promoters. Our study suggested that IL-6 upregulates MMP-13 gene expression via transcription factor AP1 and through the cis-acting element TRE. Thus, gel DNA retardation experiments show that IL-6 promotes a dose- and time-dependent binding of nuclear proteins to TRE site (Fig. 6). Nuclear protein-DNA complexes induced by the IL-6 treatment contain phosphorylated c-Jun as shown by the incubation of nuclear extract with a phosphorylated c-Jun-specific antibody prior to the gel retardation assay, which lead to the formation of two supershifted complexes. These results support the role played by TRE and AP1 in mediating the effect of IL-6 on rat MMP-13 gene expression.

The AP1 transcription factor is organized into Jun-Jun, Jun-Fos, or Jun-ATF dimmers, and the presence of Jun family members in these dimmers enables AP1 to bind cis-acting elements of gene promoters. These proteins form a variety of homo- and heterodimers that bind to a common DNA recognition site, the TRE (58,80,81). As figures 7 shows, culture of fibroblasts with IL-6 induces c-Jun, JunB and c-Fos mRNA (Fig. 7A), c-jun and c-fos promoters (Figs. 7B and 7C) and c-Jun and c-Fos proteins (Figs. 8A and 8B). Other authors have also shown

Fig. 6.- (A) DNA binding was analyzed by gel retardation assay. A double stranded radiolabeled AP1 oligonucleotide was incubated with nuclear proteins extracted from control cells or cells treated with 20 ng/mL IL-6 for 1 to 6 hours. (B) Nuclear extracts from cells treated for 24 hours with increasing concentrations of IL-6 were incubated with radiolabeled AP1 probe. Bars represent the intensity of the AP1-DNA complexes in each experimental condition.

(A) La fijación al ADN a las proteínas nucleares fue analizado mediante la prueba del retardo del ADN en gel. Un oligonucleótido de doble cadena marcado con las secuencias consenso de AP1 fue incubado con un extracto de proteínas nucleares obtenidas de células controles o células tratadas con 20 ng/mL de IL-6 durante 1 a 6 horas. (B) Extractos nucleares de células tratadas durante 24 horas con concentraciones crecientes de IL-6 fueron incubados con una sonda marcada de AP1. Las barras representan la intensidad de los complejos AP1-ADN en cada condición experimental.

that IL-6 stimulated *junB* gene expression in a variety of cells (82-86) and Cressman et al. (87) have shown that the expression of *junB* is markedly reduced in the liver of IL-6-deficient mice.

Transcriptional activity of AP1 depends not only on the quantity of AP1 components and their ability to bind DNA but also on the degree of phosphorylation of these proteins (80,88). Phosphorylation of c-Jun in its activation domain at serine 63 and 73 prolongs its half-life and potentiates the ability of c-Jun to activate transcription as either a homodimer or as a heterodimer with c-Fos (80). Western blot using a specific monoclonal antibody for serine 63 phosphorylated c-Jun demonstrates that IL-6 induces an increase in this form of c-Jun, which is particularly marked after 12 hours of treatment (Fig. 8C). Lütticken et al. (84) also showed that IL-6 triggers a delayed phosphorylation of STAT3 at serine residues. A va-

Fig. 7.- (A) Fibroblasts were cultured with 0-30 ng/mL IL-6 for 24 hours and total RNA was isolated and analyzed by Northern blotting. The blot was hybridized with 32 P-labeled probes specific for c-Jun, JunB and c-Fos. Autoradiograms were quantitated by scanning laser densitometry. Bars represent mRNA levels expressed as fold over levels in control cells. (B and C) Fibroblasts were transfected with the reporter plasmids pJun-Luc or pFos-Luc. Transfected cells were incubated with 0 or 20 ng/mL IL-6 for 3 to 24 hours. After the indicated time, luciferase activity was measured in cell homogenates. Fold represents luciferase activity in the presence of IL-6 divided by that obtained in the absence of IL-6. RLU are arbitrary luminescence units. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

(A) Los fibroblastos fueron cultivados con 0 a 30 ng/mL de IL-6 durante 24 horas. Se aisló y analizó el ARN total mediante Northern blot. Los blots fueron hibridados con sondas específicas para c-Jun, JunB y c-Fos marcadas con 32 P. Las autorradiografías fueron cuantificadas mediante densitometría láser. Las barras representan los niveles de ARNm expresados en forma de veces por encima del nivel en las células control. (B y C) Los fibroblastos fueron transfectados con el plásmido pJun-Luciferasa (B) o con el pFos-Luciferasa (C). Una vez transfectadas, las células fueron incubadas durante 3 a 24 horas con 0 a 20 ng/mL de IL-6. Tras el tiempo indicado, la actividad luciferasa fue determinada en los homogenados celulares. Las barras representan la actividad luciferasa en las células tratadas con IL-6 dividida por la actividad en ausencia de IL-6. RLU son unidades arbitrarias de luminiscencia. *: $p < 0,05$; **: $p < 0,01$; ***: $p < 0,001$.

riety of protein kinases, including JNK (c-Jun N-terminal kinase), pp42, pp54, pp44-, and p38-mitogen-activated protein kinases (89), p34cdc2, protein kinase C, casein kinase II, efficiently phosphorylates c-Jun (90). JNK,

Fig. 8.- Whole cell protein extracts from cells incubated in the absence or presence of increasing concentrations of IL-6 (A) for 1 to 8 hours (B) were separated by SDS-polyacrylamide gel electrophoresis and transferred to membrane for immunoblot analysis. Immunoblots were probed with either specific anti-c-Jun or anti-c-Fos antibodies and detected by enhanced chemiluminescence. In C, immunoblots were probed with specific anti-phosphoserine-63 c-Jun antibody. Bars represent fold protein content in IL-6 treated cells divided by the protein content in untreated cells.

Extractos de proteínas de células incubadas en presencia o ausencia de concentraciones crecientes de IL-6 (A) durante 1 a 8 horas (B) fueron separadas por electroforesis en un gel de poliácridamida-SDS y transferidas a una membrana para análisis por inmunoblot. Los inmunoblots fueron expuestos a anticuerpos específicos frente a anti-c-Jun o anti-c-Fos y las proteínas fueron detectadas por quimioluminiscencia. En C, los inmunoblots fueron revelados con anticuerpos específicos anti-c-Jun fosforilado en serina-63. Las barras representan el contenido de esas proteínas en las células tratadas con IL-6 dividido por su contenido en las células control.

also known as stress-activated protein kinase (SAPK), is the most efficient member of the mitogen-activated protein kinase (MAPK) family involved in c-Jun phosphorylation on serines 63 and 73 of c-Jun and in potentiating its

transactivation function (91,92). Moreover, JNK is a critical MAPK pathway for IL-1 induced collagenase gene expression in a variety of cell types (93). However, our study indicates that IL-6 does not induce the phosphorylation of c-Jun by stimulating JNK activity. As figure 9A shows incubation of cells with 10 and 20 ng/ml IL-6 for 15 minutes, but not for 5 minutes, decreased JNK activity to 84 and 68%, respectively, of that of control cells.

Fig. 9.- (A) IL-6 decreases JNK activity in Rat-1 fibroblasts. Recombinant GST-c-Jun was incubated with 25 μ g of whole cell protein extracts from cells treated without or with 10 or 20 ng/mL for 5 and 15 minutes. JNK-mediated phosphorylation of GST-c-Jun is assessed by the incorporation of [γ - 32 P]ATP. *: $p < 0.01$. (B) Effects of IL-6 on JNK phosphorylation. Twenty-five micrograms of protein extracts from cells incubated in the presence of 20 ng/mL IL-6 for 0 to 60 minutes were separated by 8% SDS-polyacrylamide gel electrophoresis and transferred to membrane for immunoblot analysis. Immunoblot was then probed with specific anti-JNK as control (■) for the subsequent blotting with the anti-phosphorylated JNK antibody (▨).

(A) La IL-6 disminuye la actividad JNK en fibroblastos Rat-1. GST-c-Jun recombinante fue incubado con 25 μ g de extractos de proteínas de células tratadas con o sin 10 ó 20 ng/mL durante 5 a 15 minutos. La fosforilación de GST-c-Jun mediada por la JNK se valora por la incorporación de [γ - 32 P]ATP. *: $p < 0,01$. (B) Efectos de la IL-6 sobre la fosforilación de JNK. Veinticinco microgramos de extractos de proteínas de células incubadas en presencia de 20 ng/mL durante 0 a 60 minutos fueron separados por electroforesis en gel de 8% SDS-poliacrilamida y transferidas a una membrana para análisis por inmunoblot. Los inmunoblots obtenidos fueron revelados con anticuerpos anti-JNK específicos como control (■) para posteriormente ser revelados con anticuerpos anti-JNK fosforilado (▨).

Moreover, Western blots using specific polyclonal antibodies against JNK reveal one single band located at 46 kDa, the density of which does not change after incubation with IL-6. Likewise, this treatment does not modify phosphorylated JNK, as reveal Western blots using specific monoclonal IgG antibody raised against phosphorylated JNK (Fig. 9B). As dual phosphorylation of serine/threonine amino residues by the MAP kinase kinase (MKK4 and MKK7) is necessary for JNK activation (94), these results support the conclusion that this kinase is not involved in the IL-6-induced increase in phosphorylated c-Jun and MMP-13 gene expression. These results concurs with those reported by a number of authors showing that IL-6 has no detectable effects on JNK (95,96) or even inhibits the activation of this MAP kinase (97,98).

As mentioned above, in addition to JNK, a variety of protein kinases, including ERK, p38 kinase, casein kinase II, and PKC (99) can efficiently phosphorylate c-Jun (90,100). Moreover, in some cell lines, ERK pathway seems to play a role in mediating some effects of IL-6 (98). However, blocking these kinases with specific inhibitors does not abrogate the stimulatory effect of IL-6 on c-Jun phosphorylation (Fig. 10A), AP1 binding to DNA (Fig. 10B) or MMP-13 gene expression (Fig. 10C). In a recent study (101), we blocked ERK with PD98059, p38 MAP kinase with SB203580, protein kinase C (PKC) with H7 and GF 109203X, protein kinase A with H8 and H89, calmoduline-dependent protein kinase with calmidazolium, and phosphatidylinositol-3-kinase with wortmannin. Taken together, these results indicate that none of these protein kinases is likely required for the effect of IL-6 on MMP-13 gene expression.

Phosphorylation state of c-Jun is a dynamic process controlled by both serine/threonine protein kinases and protein phosphatases 2A (PP2A) (102). Therefore, the increase in phosphorylated c-Jun may result not only by an enhanced kinase activity but also by a reduced PP2A activity. In fact, a number of studies have clearly demonstrated that inhibition of protein phosphatases 2A results in an induction of collagenase, JunB, and c-Fos mRNA and a potent activation of AP1, through serine/threonine phosphorylation (103-106). There are four classes of serine/threonine-specific protein phosphatases. These include PP1, PP2A, PP2B (calcineurin), and PP2C. PP2A and PP1 are widely distributed in the cytoplasm of mammalian cells and have been reported to be involved in signalling pathways, modifying the activity of a variety of protein kinases (107). Measurement of the PP2A activity in fibroblasts treated with increasing concentrations of IL-6 shows that this cytokine decreased this activity in a dose dependent fashion (Fig. 11A). Likewise, incubation of cells with 20 ng/ml IL-6 for 15 minutes to 24 hours resulted in a decrease in serine/threonine phosphatase activity, which was particularly marked between three and six hours. At 12 and 24 hours, this activity remained under the control activity (Fig. 11B).

Fig. 10.- (A) IL-6-induced phosphorylation of c-Jun is not abrogated by the blockade of protein kinases. Twenty five micrograms of whole cell protein extracts from control cells (Ctr) or cells treated with 20 ng/ml IL-6 for 12 hours in the presence or absence of protein kinase inhibitors were separated as described in figure 8. Protein kinase C, ERK and p38 MAP kinase were inhibited with 20 μ M H7 (H7), 25 μ M PD98051 (PD), or 1 μ M SB203580 (SB). Immunoblot was probed with specific anti-phosphorylated c-Jun antibody. (B) Blockade of protein kinases does not inhibit the effect of IL-6 on AP1 binding activity. We analyzed DNA binding activity by gel retardation assay. A double-stranded radiolabeled AP1 oligonucleotide was incubated with nuclear proteins extracted from control cells (Ctr) or cells treated with 20 ng/ml IL-6 (IL), in the absence or presence of 20 μ M H7 (H7), 25 μ M PD98051 (PD), or 1 μ M SB203580 (SB). Bars represent the intensity of the DNA binding in the different experimental conditions. (C) Steady-state levels of MMP-13 mRNA in fibroblasts treated with 20 ng/ml IL-6 in the absence or presence of a number of protein kinase inhibitors. Confluent Rat-1 fibroblasts were incubated for 24 hours in control medium (Ctr) or medium containing 20 ng/ml IL-6 in the presence or absence of protein kinase inhibitors [25 μ M PD98051 (PD), 1 μ M SB203580 (SB), 20 μ M H7, 10 nM GF 109203X (GF), 20 μ M H8 and 50 nM H89, 10 μ M calmidazolium (CM), and 0.1 μ M wortmannin (Wo)]. Protein kinase inhibitors were added to the cells 45 minutes before IL-6. mRNA was analyzed as described in figure 2 and autoradiograms were quantitated by scanning laser densitometry.

(A) La fosforilación de c-Jun provocada por la IL-6 no es evitada por el bloqueo de las quinasas de proteínas. Veinticinco microgramos de extracto proteico de células control (Ctr) o de células tratadas con 20 ng/ml

de IL-6 durante 12 horas en presencia o ausencia de inhibidores de quinasas de proteínas fueron separadas como se describe en la figura 8. Las quinasas de proteínas PKC, ERK y p38 MAP quinasa fueron inhibidas con 20 μ M H7 (H7), 25 μ M PD98051 (PD) o 1 μ M SB203580 (SB). Los inmunoblots fueron revelados con anticuerpos específicos anti-c-Jun fosforilado. (B) El bloqueo de las quinasas de proteínas no inhibe los efectos de la IL-6 sobre la actividad fijadora del AP1. Analizamos la actividad fijadora del AP1 al ADN mediante el procedimiento de retraso en gel. Un oligonucleótido de AP1 marcado con 32 P fue incubado con extractos nucleares de proteínas obtenidas de células controles (Ctr) o de células tratadas con 20 ng/ml de IL-6 (IL) en ausencia o presencia de 20 μ M H7 (H7), 25 μ M PD98051 (PD) o 1 μ M SB203580 (SB). Las barras representan la intensidad de la unión de AP1 al ADN en las diferentes condiciones experimentales. (C) Niveles de ARNm de la MMP-13 en fibroblastos tratados con 20 ng/ml de IL-6 en ausencia o presencia de diversos inhibidores de quinasas de proteínas. Fibroblastos Rat-1 confluentes fueron incubados durante 24 horas en medio control (Ctr) o medio de cultivo con 20 ng/ml de IL-6 en presencia o ausencia de inhibidores de quinasas de proteínas [25 μ M PD98051 (PD), 1 μ M SB203580 (SB), 20 μ M H7, 10 nM GF 109203X (GF), 20 μ M H8, 50 nM H89, 10 μ M calmidazolium (CM) y 0,1 μ M wortmannina (Wo)]. Los inhibidores de quinasas fueron añadidos a las células 45 minutos antes de la IL-6. El ARNm fue analizado como se describe en la figura 2 y las autorradiografías fueron cuantificadas mediante densitometría láser.

The key role of PP2A in the IL-6-induced MMP-13 gene expression is also supported by experiments in which Rat-1 fibroblasts are pretreated for 20 minutes with 25 nM okadaic acid, a potent inhibitor of protein phosphatase 1 and PP2A (108). In this experimental condition, MMP-13-mRNA levels increase markedly and the addition of 20 ng/ml IL-6 for 24 hours does not modify this effect (Fig. 12). In contrast, as expected, inhibition of tyrosine phosphatases with 0.1 mM pervanadate does not change the stimulatory effects of IL-6 on MMP-13 gene expression. Furthermore, okadaic acid mimics the effects of IL-6 on MMP-13 gene expression. Many other authors have shown these effects of the okadaic acid on MMP gene expression, as well as on c-Jun phosphorylation and AP1 binding activity (105,109,110). I_2^{PP2A} , another potent inhibitor of PP2A, have produced similar effects (106). This decrease in PP2A activity is not due to a reduced amount of PP2A protein in fibroblasts exposed to IL-6. Thus, measurement of these levels in cell extracts from fibroblasts treated with 20 ng/ml IL-6 for three to sixteen hours demonstrates that this cytokine did not induce any change in intracellular PP2A levels.

Core structure of PP2A consists of complexes of three subunits (102,111,112). A 36 kDa catalytic C subunit (PP2AC) is complexed with a structural A subunit of 65 kDa (PP2A-A). This dimer is associated with a third, variable regulatory B subunit, that likely influences substrate specificity or cellular localization (102). The phosphorylation on tyrosine-307 at the C-terminus of the catalytic subunit of PP2A regulates phosphatases activity (113,114). It is well known that this phosphorylation is

Fig. 11.- Effects of IL-6 on PP2A activity in cultured fibroblasts. (A) Dose-response curve. Rat-1 fibroblasts were incubated without and with 10 to 80 ng/ml IL-6 for 3 hours. Phosphatase activity was assessed by the free phosphates released from the substrate (serine/threonine phosphopeptide) when incubated with cell lysates for 30 minutes. (B) Time-response curve. Cells were incubated with 20 ng/ml IL-6 for 15 minutes to 24 hours. *: $p < 0.01$, as compared with the activity in control cells.

Efectos de la IL-6 sobre la actividad PP2A en cultivos de fibroblastos. (A) Curva de respuesta según la dosis. Fibroblastos Rat-1 fueron incubados sin y con 10 a 80 ng/mL de IL-6 durante 3 horas. La actividad fosfatasa fue determinada por los fosfatos libres desprendidos por el sustrato (fosfopéptido serina/treonina) cuando fue incubado con lisados celulares durante 30 minutos. (B) Curva de respuesta según el tiempo. Las células fueron incubadas con 20 ng/mL de IL-6 durante 15 minutos a 24 horas. *: $p < 0,01$, comparado con la actividad en las células controles.

Fig. 12.- Okadaic acid reproduces the effect of IL-6 on MMP-13 mRNA. Confluent cells were incubated for 24 hours in control medium (Ctr) or medium containing 20 ng/ml IL-6. Serine/threonine phosphatases were

inhibited with 25 nM okadaic acid (OA) and tyrosine phosphatase with 0.1 mM pervanadate (VA) added 20 minutes before IL-6. MMP-13 mRNA was analyzed by Northern blotting.

El ácido okadaico reproduce los efectos de la IL-6 sobre el ARNm de la MMP-13. Células confluentes fueron incubadas durante 24 horas en medio de control (Ctr) o en medio que contiene 20 ng/mL de IL-6. Las fosfatasas serina/treonina fueron inhibidas con 25 nM ácido okadaico (OA) y las fosfatasas de tirosinas con 0,1 mM de pervanadato (VA) añadidos a las células 20 minutos antes de la IL-6. El ARNm de la MMP-13 fue analizado mediante Northern blot.

associated with a 90% loss in activity, and thereby with a sustained effect of protein kinases (113,115), including c-Jun phosphorylation. On the contrary, dephosphorylation of PP2A_c reactivates this enzyme (102). Considering this mechanism of regulation of the PP2A activity, we

Fig. 13.- (A) Western blot analysis. Cells were incubated in the absence or presence of 20 ng/ml of IL-6 for 5 to 30 minutes. Cell lysates were subjected to Western immunoblot analysis with anti-tyrosine 307 phosphorylated PP2A catalytic subunit antibody. *: $p < 0.01$; **: $p < 0.001$. (B) Immunoprecipitation with anti-PP2A structural subunit antibody. Cells were treated with 20 ng/ml IL-6 for 5 to 60 minutes. Cell lysates were immunoprecipitated with anti-PP2A structural subunit antibody and Western blotted either with anti-PP2A structural subunit antibody (■) or with anti-phosphotyrosine antibody (▨). Bars represent phosphorylated PP2A catalytic subunit or PP2A structural subunit.

(A) Análisis por Western blot. Las células fueron incubadas en ausencia o presencia de 20 ng/ml de IL-6 durante 5 a 30 minutos. Los lisados celulares fueron sometidos a análisis por Western blot utilizando anticuerpos frente a la unidad catalítica de la PP2A fosforilada en la tirosina 307. *: $p < 0,01$; **: $p < 0,001$. (B) Inmunoprecipitación de las proteínas celulares con anticuerpos frente a la subunidad estructural de la PP2A. Las células fueron tratadas con 20 ng/ml de IL-6 durante 5 a 60 minutos. Los lisados celulares fueron inmunoprecipitados con anticuerpos frente a la subunidad estructural de la PP2A y posteriormente revelados con anticuerpos bien frente a la misma subunidad de la PP2A (■) o bien frente a la anti-fosfotirosina (▨). Las barras representan la subunidad fosforilada de la PP2A o la subunidad estructural PP2A.

could hypothesize that binding of IL-6 to specific receptors on cell surface might inactivate PP2A by increasing tyrosine phosphorylation on its catalytic subunit which, in turn, would prolong activation of AP1. Similar sequence of events has been shown following addition of insulin to skeletal muscle cells (115,116). Moreover, inhibition of PP2A with I_2^{PP2A} increased both the concentration and DNA binding of c-Jun and the transcriptional ac-

tivity of AP1 (106). Likewise, PP2A inhibitors induce threonine phosphorylation of STAT3, other cytoplasmic transcription factor that upon activation translocate into the nucleus where they activate target genes (117,118).

Western blot analysis using specific polyclonal anti-tyrosine 307 phosphorylated PP2A_c antibody shows a marked increase in the tyrosine phosphorylation of PP2A_c (Fig. 13A). On the other hand, while immunoprecipitation of PP2A using specific antibody against the structural subunit of PP2A demonstrates that IL-6 did not affect to the total amount of this subunit, immunodetection of tyrosine phosphorylated proteins with antiphosphotyrosine antibody indicates that treatment of cells with 20 ng/mL IL-6 for 0 to 60 minutes induces a time-dependent increase in the PP2A_c tyrosine phosphorylation (Fig. 13B). This result concurs with those reported by Choi et al. (119), who also found that IL-6 induced PP2A tyrosine phosphorylation.

IL-6 initiates its actions by binding to a specific receptor complex on the cell membrane. This complex is composed of two subunits: an 80-kDa binding protein and a 130-kDa transmembrane signal transducing component (gp130) (48). Other members of the IL-6 cytokine superfamily (IL-11, oncostatin M, leukemia inhibitory factor, ciliary neurotrophic factor, cardiotropin-1, neutrophin-1/B.ccl stimulating factor-3) (120-122) share the same gp130 receptor subunit. Activation of IL-6 signal transduction involved gp130-homodimerization and tyrosine phosphorylation of the cytoplasmic tail of gp130 by gp130-associated Janus tyrosine kinases (JAKs). These kinases are recruited to the plasma membrane and activated upon cell activation by cytokines and growth factors (123). All JAKs contain at least one copy of a peptide sequence, which is part of a consensus for PP2A binding (124,125). This phosphatase has been shown to form stable complexes with various protein kinases, including Jak2 (115,126,127). Thus, it is conceivable that the IL-6-induced PP2A_c phosphorylation and PP2A inactivation might be mediated by Jak2. Thus, as figure 14 shows, pre-treatment of cells for 60 minutes with 5 μ M AG490, a specific inhibitor of Jak2 (128), abrogates completely the IL-6-induced increase in MMP-13 mRNA, binding of nuclear proteins to AP1 consensus sequence, c-Jun and PP2A_c phosphorylation, and PP2A activity. As far as we are aware, no further information is available on the role of Jak kinases and PP2A in mediating the effect of IL-6 on MMP-13 gene expression. However, oncostatin M, another member of IL-6 superfamily cytokine (122), also induces MMP gene expression in astrocytes, fibroblasts and osteoblasts (37,130), and this effect is associated with Jak1 and Jak2 tyrosine phosphorylation.

Thus, we can conclude that, at least "in vitro", IL-6 is an antifibrogenic cytokine that might contribute to remodelling connective tissue. IL-6 increases MMP-13 gene expression acting at transcriptional level via AP1. IL-6 increases *c-jun* and *c-fos* gene expression, enhances

Fig. 14.- Inhibition of Jak2 abrogates the effects of IL-6 on MMP-13 mRNA (A), AP1 binding activity (B), c-Jun serine phosphorylation (C), and PP2A activity (D). (Panel A) Cells were incubated for 24 hours in control medium (Ctrl) or medium containing 20 ng/mL IL-6, 20 ng/mL IL-6 plus 5 μ M AG490 (AG), 5 μ M AG490, 20 ng/mL IL-6 plus 135 μ g/mL genistein (Gen), 135 μ g/mL genistein. AG490 and genistein were added to the cells 60 minutes before IL-6. MMP-13 mRNA was analyzed by Northern blotting. Bars represent steady-state MMP-13 mRNA levels in cells cultured in these conditions. (Panel B) Cells were incubated for 12 hours without or with 20 ng/mL IL-6 in the absence or presence of 5 μ M AG490 (AG), or 135 μ g/mL genistein (GN). DNA binding was analyzed by gel retardation assay. Ctrl, control cells. (Panel C) Cells were treated for 30 minutes with 20 ng/mL IL-6 in the absence

or presence of 5 μ M AG490, 100 μ M of JAK3 Inhibitor I (J31) added 60 minutes before IL-6. Twenty-five micrograms of protein extracts from cells treated as indicated were subjected to Western immunoblot analysis with anti-tyrosine 307 phosphorylated PP2A catalytic subunit antibody (p-PP2A_c). (Panel D) Cells were incubated for three hours in the same experimental conditions as in panel A. PP2A activity in cell lysates was assessed as described in figure 11. Ctrl, Control cells.

La inhibición de JAK2 anula los efectos de la IL-6 sobre el ARNm de la MMP-13 (A), la actividad de la AP1 (B), la fosforilación de serina en el c-Jun (C) y la actividad de la PP2A (D). (Panel A) Las células fueron incubadas durante 24 horas en medio de control (Ctrl) o en medio que contenía 20 ng/mL de IL-6, 20 ng/mL de IL-6 más 5 μ M AG490 (AG), 5 μ M AG490, 20 ng/mL IL-6 más 135 μ g/mL genisteína (Gen), 135 μ g/mL genisteína. El AG490 y la genisteína fueron añadidos a las células 60 minutos antes del IL-6. El ARNm MMP-134 fue analizado mediante Northern blot. Las barras representan los niveles de ARNm MMP-13 en las células cultivadas en las condiciones mencionadas. (Panel B) Las células fueron cultivadas durante 12 horas con o sin 20 ng/mL de IL-6 en ausencia o presencia de 5 μ M AG490 (AG) o 135 μ g/mL genisteína (GN). La unión al ADN fue analizada mediante la prueba del retardo en gel. Ctrl, células controles. (Panel C) Las células fueron tratadas durante 30 minutos con 20 ng/mL en ausencia o presencia de 5 mM AG490, 100 μ M del Inhibidor de JAK3 (J31) añadidos a las células 60 minutos antes de la IL-6. Veinticinco microgramos de extracto proteico de células tratadas como se ha indicado fueron sometidos a análisis por Western blot con anticuerpos frente a la subunidad catalítica de la PP2A fosforilada en la tirosina. (Panel D) El bloqueo de JAK2 impide la fosforilación de la subunidad catalítica de PP2A y la pérdida de la actividad PP2A. Las células fueron incubadas durante tres horas en las mismas condiciones experimentales que se mencionan en panel A. La actividad PP2A en los lisados celulares fue determinada como se describe en la figura 11. Ctrl, células controles.

phosphorylated c-Jun, and promotes the binding of AP1 to the MMP-13 promoter. The increase in phosphorylated c-Jun is not due to increased protein kinase activity, including Jun N-terminal kinase, but to a decreased serine/threonine phosphatases activity. IL-6, after binding to a specific receptor on the cell membrane, activates tyrosine kinase JAK2, which phosphorylates the catalytic subunit of protein phosphatases 2A (PP2A) at the tyrosine 307 leading to inhibition of its activity and prolongs c-Jun phosphorylation (Fig. 15).

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Fig. 15.- After IL-6 binding to specific receptor (gp80) on the cell membrane (1), tyrosine kinase Jak2 phosphorylates and activates the transmembrane signal transducing component of the IL-6 receptor (gp130) (2). Afterward, Jak2 binds and phosphorylates tyrosine 307 in the catalytic subunit of PP2A (3), which is associated with a loss of its phosphatase activity (4). Because of this low serine/threonine phosphatase activity, c-Jun remains phosphorylated for a longer period of time and contributes to maintain activation of the MMPs gene expression (5) and MMPs synthesis (6). This enhanced MMP proteins and MMP activity might result in the degradation of extracellular matrix, the hepatic stellate cell death by apoptosis, and in the fibrosis remodelling (7).

Tras la unión de la IL-6 a los receptores específicos (gp80) situados en la membrana plasmática de las células (1), la quinasa de proteínas JAK2 fosforila y activa al componente transmembranoso de transmisión de señal de la IL-6 (gp130) (2). Tras ello, JAK2 se une y fosforila a la tirosina 307 de la unidad catalítica de la PP2A (3), lo cual se encuentra asociado con una pérdida de su actividad catalítica (4). Debido a esta baja actividad fosfatasa sobre serinas/treoninas, el c-Jun permanece fosforilado durante más tiempo y contribuye a mantener la activación de la expresión genética de la MMP-13 (5) y la síntesis de la MMP-13 (6). Este aumento de las MMPs y de la actividad de la MMP puede determinar la degradación de la matriz extracelular, la muerte por apoptosis de las células estrelladas del hígado y el remodelamiento de la fibrosis (7).

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