

The Use of Synthetic Analogues of Arg-Gly-Asp (RGD) and Soluble Receptor of Tumor Necrosis Factor to Prevent Acute and Chronic Experimental Liver Injury

Rafael Bruck,^{a,b} Rami HersHKoviz,^c Ofer Lider,^c Haim Shirin,^b
Hussein Aeed^b and Zamir Halpern^b

^b*Department of Gastroenterology, E. Wolfson Medical Center, Holon and
Sackler School of Medicine, Tel-Aviv University, and*

^c*Department of Cell Biology, Weizmann Institute of Science, Rehovot, Israel*

(Received May 16, 1997; returned for revision June 25, 1997; accepted October 1, 1997)

In chronic viral hepatitis, autoimmune hepatitis, and some chronic cholestatic liver diseases, T-lymphocytes serve as effector cells of the immunostimulatory processes. Cellular interactions of immune cells with extracellular matrix (ECM) components are regulated primarily via the $\beta 1$ subfamily of integrin receptors. The target epitope of several such integrin receptors is the Arg-Gly-Asp (RGD) sequence, a cell adhesion motif shared by several matrix-associated adhesive glycoproteins. We review the use of synthetic nonpeptidic analogues of RGD and of soluble receptor of tumor necrosis factor (TNF)- α in the prevention of immune-mediated, concanavalin A-induced liver damage in mice and of RGD analogues in inhibiting the development of liver cirrhosis in rats. The concanavalin A-induced elevation of serum transaminases and TNF- α , and the infiltration of liver tissue by inflammatory cells, were inhibited by pretreatment of the mice with the synthetic RGD mimetics and soluble TNF receptor. In rats, the progression of thioacetamide-induced liver cirrhosis was markedly inhibited by the coadministration of the RGD mimetic SF-6,5. The compounds described here may be examined therapeutically for pathological conditions in the liver, manifested as necroinflammation, cholestasis and fibrosis.

In several liver diseases, including chronic viral hepatitis, autoimmune chronic active hepatitis, and cholestatic liver diseases such as primary biliary cirrhosis and primary sclerosing cholangitis, T-lymphocytes serve as effector cells of the immunostimulatory processes. The inflammatory cell infiltrate is thought to contribute to liver injury either as a primary event, as in autoimmune hepatitis and primary biliary cirrhosis, or as a secondary response to another process such as chronic viral infection. In response to acute injury, T-cell activation results in production of cytokines such as tumor necrosis factor (TNF)- α^d and IL (interleukin)-2, which maintain and augment the level of the inflammatory process and may induce acute toxicity. The accumulation of mononuclear cells at the site of injury depends in part on their ability to penetrate the sinusoidal endothelial cells and their adhesion to the extracellular matrix in the space of Disse. Both these processes are mediated by integrins, a family of cell surface adhesion receptors. Several matrix and plasma-associated glycoproteins such as fibronectin (FN) are recognized by integrins via the Arg-Gly-Asp (RGD) cell adhesion epitope.

^a To whom all correspondence should be addressed: Rafael Bruck, M.D., Dept. of Gastroenterology, The E. Wolfson Medical Center, Holon 58100, Israel. Tel.: 972-3-5028499; Fax: 972-3-5044634.

^d Abbreviations: TNF, tumor necrosis factor; FN, fibronectin; RGD, Arg-Gly-Asp; RGE, Arg-Gly-Glu; TGF, transforming growth factor; ECM, extracellular matrix; VLA very late activation; Con A, concanavalin A; TAA, thioacetamide, HSC, hepatic stellate cells.

In this paper, we will review the use of synthetic analogues of the extracellular matrix-associated epitope RGD and soluble receptor of TNF in the prevention of immune-mediated hepatitis, and of RGD mimetics in inhibiting hepatic fibrosis in experimental models of acute and chronic liver injury.

THE EXTRACELLULAR MATRIX-ASSOCIATED EPITOPE Arg-Gly-Asp (RGD) IN CELL ADHESION

Cellular interactions of immune cells with ECM components are regulated primarily by a family of transmembrane heterodimeric cell surface adhesion receptors of the integrin family [1-4]. Each integrin is composed of a noncovalently associated α and β subunit. The $\beta 1$ subfamily of integrins, also referred to as VLA (very late activation) receptors, are specific for various glycoprotein ligands of the ECM, such as FN, laminin, collagens, and vitronectin [4, 5]. Although the characterization and localization of integrin recognition sites on ECM glycoproteins has not been completely elucidated, the involvement of the Arg-Gly-Asp- (RGD) epitope in cell adhesion processes has been indicated [2, 6]. The RGD cell adhesion motif is also present in several matrix and plasma-associated glycoproteins such as FN, and is recognized by several integrins, including $\alpha_{IIb}\beta_3$ on platelets, $\alpha_3\beta_1$ (VLA-3), $\alpha_5\beta_1$ (VLA-5), and most of the α_v -containing integrins [3]. Upon cell activation, the RGD-containing sequence is recognized by several integrin receptors that mediate RGD-dependent matrix adhesion of cells. Interactions between immune cells and FN determine the subsequent activation and proliferation of the cells, and the secretion of cytokines such as TNF α , TGF β and fibroblast growth factor [8, 9].

The use of RGD-containing peptides to prevent T cell-mediated inflammatory reactions has been suggested [7]. However, since these short RGD-containing peptides are highly susceptible to proteolysis, their preventive effect required pretreatment of the migrating cells with high concentrations of the RGD peptides, rather than *in situ* administration of the peptides [7]. Therefore, the clinical usage of such compounds to prevent damage due to T-cell-mediated inflammatory reactions *in vivo* is not practical.

NONPEPTIDIC MIMETICS OF RGD

We recently described the design and synthesis of novel nonpeptidic mimetics of RGD that contain guanidinium and carboxylate groups separated by an atom spacer, thus mimicking the functional groups of RGD [8]. Compound SF-6,5, is a nonpeptidic mimetic of RGD, which has an 11 atom spacer between the guanidinium and carboxylic groups that mimics the atomic spacing between the two groups in the RGD peptide. Compound NS-11 is a conformationally constrained RGD mimetic molecule, that was obtained by substituting a piperidine unit into a spacing chain [9]. The ring substitution in compound NS-11 restricts the conformational freedom of the carboxylate and guanidinium moieties in a constant spatial arrangement (Figure 1). These mimetics specifically inhibited (i) RGD-dependent platelet aggregation, and (ii) binding of T-lymphocytes and tumor cells to immobilized fibronectin and vitronectin. Also, we demonstrated that an RGD mimetic specifically inhibits actively induced, delayed-type hypersensitivity reactions and tumor cell colonization in mice [9, 10], suggesting the involvement of RGD recognition in regulation of immune responses and migration of cells involved in pathological responses. The RGE (Arg-Gly-Glu acid) mimetic, SF-6,6, which has a 12-atom spacing chain between the two ionic functional groups (Figure 1), and therefore lacks integrin specificity, was synthesized to be used as control.

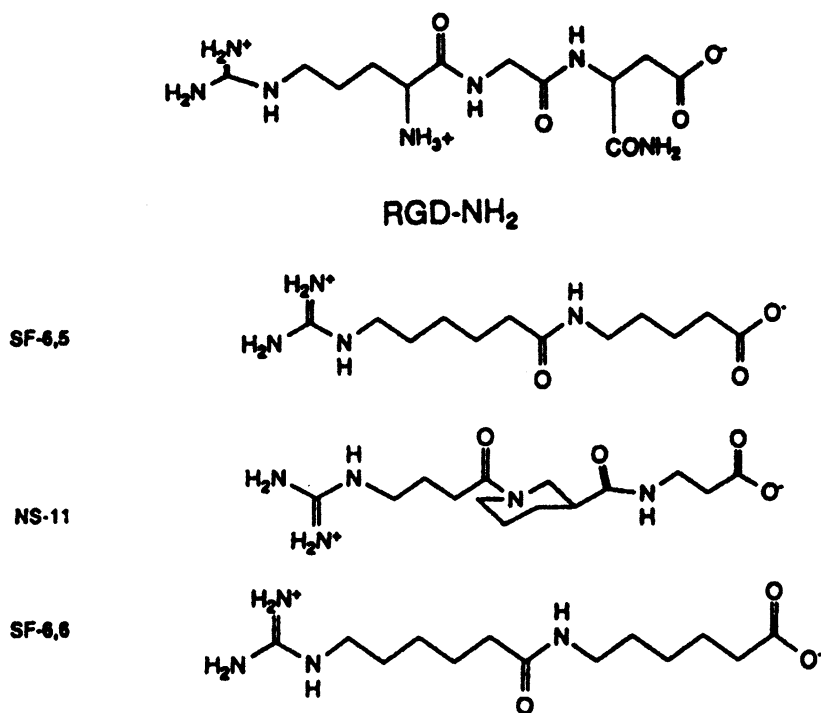


Figure 1. Schematic presentation of the RGD-peptide, and the RGD and RGE mimetics. Adapted from Ref. [15].

EFFECTS OF THE RGD MIMETICS ON T-CELL ADHESION TO FIBRONECTIN AND LAMININ

To compare the ability of the mimetics to inhibit the adhesion of $\beta 1$ integrin-expressing cells, the binding of murine T-cells to immobilized fibronectin and laminin in the presence of the mimetics and RGD and RGE-containing peptides (100 $\mu\text{g/ml}$) was studied. Compounds SF-6,5, NS-11 and the RGD-containing peptide, GRGDSP, inhibited T-cell adhesion to fibronectin (Table 1; Ref. [15]). Compound SF-6,6 did not inhibit T-cells adhesion, which is consistent with the inability of RGE-peptides to interfere with integrin recognition of RGD-containing ECM ligands [5]. The inhibitory effect of the RGD mimetics was not due to a toxic effect since: (i) the T-cells completely recovered their ability to adhere to fibronectin when the compounds were removed by washing and the cells maintained in culture for 24 hr, (ii) neither NS-11 nor SF-6,5 inhibited the binding of T-lymphocytes to laminin (Table 1; Ref 15), and (iii) none of the mimetics inhibited T-cell proliferation in response to concanavalin A (Table 2; Ref. [15]). These findings indicate that the inhibitory actions of SF-6,5 and NS-11 on T-cells adhesion are specific and restricted to immune cell-ECM interactions.

IMMUNE-MEDIATED, CONCAVALIN (CON) A - INDUCED HEPATITIS

Recently, an animal model of immune-mediated, T-cell-dependent, liver damage was introduced in mice. In this experimental model, liver damage is recognizable by significant elevation of serum aminotransferases 6-8 hr after the intravenous administration of

the lectin concanavalin (Con A) [11]. The induction of Con A hepatitis depends on the interaction between CD4⁺ T-helper cells and macrophages [11], and as recently reported, on the production of cytokines, notably TNF- α , and IFN- γ [12, 13]. When examined by electron microscopy, livers from Con A-treated mice demonstrated severe cellular damage as early as 4-6 hr after Con A administration [11], whereas histologic changes could be demonstrated by light microscopy only 24 hours after the inoculation of Con A [13]. A recent study have suggested that Con A-induced apoptosis of liver cells is dominantly mediated by a perforin-dependent pathway through ICAM-1/LFA-1 interaction and not through the Fas ligand pathway [14]. This mouse model of acute liver damage, might be useful for the investigation of the pathogenesis and the efficacy of experimental treatment modalities in chronic hepatic inflammation and in some cholestatic liver diseases as well, since similar processes take place during an immune response to chronic liver insult.

Table 1. Effects of RGD-peptide and mimetics on T-cells adhesion to fibronectin and laminin.

Inhibitory compound (100 μ g/ml)	Percent adhesion of activated T-cells to:	
	fibronectin	laminin
None	34 \pm 4	38 \pm 5
GRGDSP	17 \pm 2* (50) ^a	36 \pm 4
GRGESP	33 \pm 4	40 \pm 4
NS-11	13 \pm 3* (61)	36 \pm 5
SF-6,5	19 \pm 2* (56)	37 \pm 6
SF-6,6	34 \pm 2	34 \pm 6

Adhesion of activated murine T-cells to ECM-glycoproteins in the presence of various inhibitors (100 μ g/ml).

^a Percent inhibition is indicated. *p < .01: experimental vs. control group. Unless indicated, no significant inhibition was observed. n = 5. Adapted from Ref. [15].

Table 2. Effects of the nonpeptidic mimetics of RGD and RGE on T-cell proliferation.

Addition (100 μ g/ml)	T-cell proliferative response (CPM \times 10 ³ \pm SD)	
	Control	Con A
None	5 \pm 1	172 \pm 10
SF-6,6	3 \pm 2	169 \pm 13
NS-11	2 \pm 1	180 \pm 17
SF-6,5	4 \pm 2	180 \pm 17
GRGDSP	4 \pm 1	170 \pm 11

T-cells were purified from spleens of BALB/c mice, left untreated (control), or activated with Con A, in the presence or absence of the nonpeptidic mimetics of RGD and RGE. One of three experiments, which yielded essentially the same results (SD < 13%). Adapted from Ref. [15].

PREVENTION OF CON A-INDUCED HEPATITIS BY SYNTHETIC RGD ANALOGUES

Inhibition of liver enzymes elevation

BALB/c mice were injected intravenously with 10 mg/kg Con A, and treated daily for 5 days prior to Con A inoculation, with either 500 $\mu\text{g}/\text{mouse}$ of one of the RGD analogues SF-6,5 or NS-11, or the RGE analogue SF-6,6 (as a control group). In a previous study, the RGD analogue SF-6,5 inhibited delayed type hypersensitivity reaction in mice, with the best results obtained when the mice were injected daily for 5 days prior to the experiment [8]. As expected, the control RGE mimetic, SF-6,6, which has a 12-atom spacing chain between the two ionic functional groups, and therefore lacks integrin specificity, did not prevent liver damage induced by Con A inoculation. In contrast, the RGD mimetic SF-6,5 effectively decreased the high serum levels of both liver enzymes tested (Table 3; Ref. [15]). When SF-6,5 was administered at a single i.p. dose of 500 $\mu\text{g}/\text{mouse}$ 1 hour prior to Con A injection or orally (at 500 $\mu\text{g}/\text{mouse}$) for 5 days prior to the induction of hepatitis, the increase in serum levels of liver enzymes was inhibited by only ~50 percent (data not shown). Based on the above data, we concluded that pre-treatment of the animals with the RGD mimetics for 5 days was more effective in the prevention of T-cell-dependent immune-mediated hepatitis in mice.

Table 3. The effects of RGD mimetics and peptides on Con A-induced liver injury in mice.

Inhibitory compound (500 $\mu\text{g}/\text{mouse}$)	AST (IU/l)	ALT (IU/l)
NaCl 0.9%	2422 \pm 311	4105 \pm 473
SF-6,6 (RGE mimetic)	2730 \pm 387	4358 \pm 680
SF-6,5 (RGD mimetic)	197 \pm 43**	167 \pm 30**
NS-11 (RGD mimetic)	190 \pm 32**	192 \pm 35**
GRGDSP (RGD peptide)	2160 \pm 410	3360 \pm 570

Mean \pm SEM; n = 6; **p < .01 compared to Con A alone.

Serum levels of liver enzymes were measured 24 hr after Con A (10 mg/kg, i.v.) administration. Similar results were obtained when liver enzymes were assessed 6 h after the administration of Con A (not shown). The RGE and RGD mimetics were administered i.p. daily for 5 days prior to Con A administration. Adapted from Refs. [15, 16].

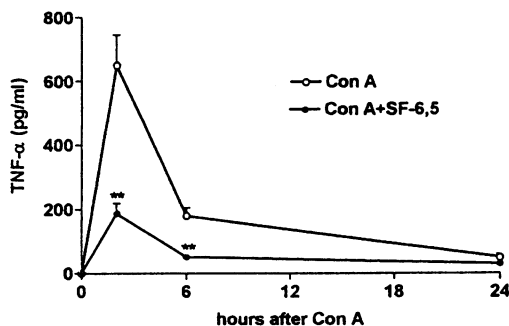


Figure 2. Effect of the synthetic RGD analogue SF-6,5 on serum levels of TNF- α in mice. Serum levels of TNF- α were measured 2-48 hr after the administration of 10 mg/kg Con A. The RGD analogue (500 $\mu\text{g}/\text{mouse}$) was administered daily for 5 days prior to and on the day of Con A inoculation. Serum levels of TNF- α were significantly decreased 2 and 6 hr after Con A injection in the SF-6,5 pretreated mice compared to control mice that received only Con A. Adapted from Ref [16].

Table 4. Effects of the RGD and RGE mimetics (*SF-6,5 and *SF-6,6) and soluble receptor of TNF- α on liver histology in Con A-induced liver damage.

	Inflammation (0-3)	Necrosis (0-3)
Con A only	2.2 \pm 0.4	2.3 \pm 0.6
SF-6,6	2.4 \pm 0.5	2.5 \pm 0.5
SF-6,5	0.6 \pm 0.2**	0.4 \pm 0.2**
sTNF-R (100 μ g/mouse)	0.3 \pm 0.1**	0.2 \pm 0.1**

*500 μ g/mouse; Mean \pm SD; n = 5; **p < .01 compared to Con A only.

INHIBITION OF TNF- α RELEASE BY SF-6,5

As reported recently, Con A-induced hepatic injury is mediated by cytokines such as TNF- α , IL-2 and IFN- γ [11-13]. The increase in serum levels of TNF- α in response to Con A, was inhibited by the RGD mimetic SF-6,5 (Figure 2; Ref. [16]). Thus, the prevention of Con A-induced liver injury by the RGD mimetic is also associated with inhibition of TNF α release.

Effects of the RGD mimetic SF-6,5 on liver histology

Livers of control rats, treated with Con A only or with the inactive RGE analogue SF-6,6, demonstrated areas of intralobular necrosis and inflammatory cell infiltrates around the central veins and the portal tracts. Inflammatory infiltrates consisted mainly of mononuclear cells, many of which were positively stained by anti-CD4 by immunohistochemistry. Compatible with previous studies, these results further establish the major role of the CD4⁺ T-cell sub population in the etiology and pathogenesis of Con A-induced hepatitis [11]. As expected, the control RGE mimetic SF-6,6 did not prevent the apparent liver inflammation. In contrast, in mice treated by the RGD analogue SF-6,5 liver damage was minimal: no intralobular necrosis or significant inflammatory infiltration could be demonstrated by light microscopy (Table 4).

Inhibition of liver damage by soluble receptor

Hepatic injury in Con A-induced hepatitis is mediated primarily by TNF- α , and this damage could be prevented by the use of polyclonal TNF- α antiserum [13]. Recombinant soluble TNF receptors (sTNF-R) have been developed and can be used to neutralize TNF- α *in vivo* [5]. Therefore, sTNF-R (p55 recombinant human soluble TNF receptor, Interpharm, Israel), was administered intravenously, 1 hour prior to Con A injection in order to prevent liver damage and to confirm the major role of TNF- γ in Con A-induced hepatitis.

sTNF-R, at a molar ratio of 1:10³ or 1:10⁴ to TNF- α (1 or 10 μ g/mouse respectively, based on TNF- α serum level measured 2 hr after Con A inoculation), had no effect on the release of aminotransferases (Table 5). However, sTNF-R, at a molar ratio of 1:10⁵ to TNF- α (100 μ g/mouse), effectively inhibited the Con A-induced elevation of hepatic enzymes and of TNF- α (Table 5, Figure 3). Thus, *in vivo* administration of sTNF-R, that inhibits the increase of serum TNF levels in response to Con A, appears to decrease the biochemical manifestations of experimentally-induced liver damage.

The histopathologic manifestations of liver damage were also prevented by the administration of the sTNFR at a dose of 100 μ g/mouse (Table 4). Apoptosis of liver cells

Table 5. Effect of sTNF-R on Con A-induced liver injury in mice.

sTNF-R	AST (IU/l)	ALT (IU/l)
None	2422 ± 311	4105 ± 520
(1 µg/mouse)	2670 ± 421	4264 ± 411
(10 µg/mouse)	2111 ± 321	3956 ± 508
(100 µg/mouse)	118 ± 27**	22 ± 5**

Mean ± SD; **p < .001 compared to Con A alone; n = 5; Serum levels of liver enzymes were measured 24 hr after Con A administration (10 mg/kg, i.v.). Adapted from Ref. [16].

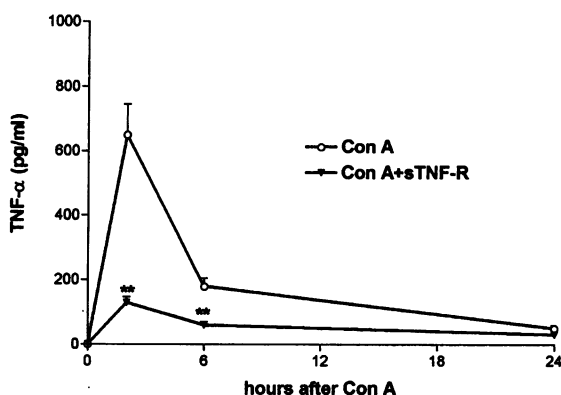


Figure 3. Effect of soluble receptor of TNF on serum levels of TNF-α in mice. Soluble receptor of TNF was administered i.v. at a dose of 100 µg/mouse 1 hr prior to Con A administration. Serum levels of TNF-α were measured 2-24 hr after the administration of 10 mg/kg Con A, and were significantly decreased 2 and 6 hr after Con A injection in the sTNF-R pretreated mice, compared to control mice that received only Con A. Lower doses of sTNF-R (1 and 10 µg/mouse) were not effective in preventing Con A-induced hepatitis. Adapted from Ref. [16].

is an early event in Con A-induced hepatitis, that could be demonstrated histopathologically as early as 3 hr after the inoculation of Con A [12]. In liver sections taken from rats treated with sTNF-R, which were examined 2 and 6 hr after the injection of Con A, apoptosis of liver cells was not detected.

INHIBITION OF HEPATIC FIBROSIS BY THE NON-PEPTIDIC MIMETIC OF RGD, SF-6,5

A chronic insult induces a prolonged inflammatory process that may lead to the development of liver fibrosis. The process of fibrosis appears to result from complex interactions between extracellular matrix macromolecules, hepatic stellate cells, cytokines and growth factors. Secreted soluble cytokines, probably link the inflammatory and reparative phase of liver cirrhosis, by activating hepatic stellate cells (HSC) cells. Upon activation, HSC proliferate and initiate the fibrogenic process by depositing matrix components, such as collagen, glycoproteins (e.g., laminin) and proteoglycans in the space of Disse, and as the chronic liver insult persists it eventually progresses to nodule formation and cirrhosis [17]. Transforming growth factor (TGF) β contributes to the fibrotic process by modulating matrix formation by inducing the synthesis of fibronectin, laminin, collagen I, proteoglycans and tissue inhibitors of metalloproteinases [18, 19]. TGFβ also enhances β1 integrin

expression in a manner that increases cellular adhesion to the matrix proteins [20]. Hence, the management of hepatic fibrosis should involve, in addition to suppressing or eliminating the causative agents of chronic hepatic inflammation, a specific action on the ECM.

RGD ANALOGUES AND THE INHIBITION OF LIVER FIBROSIS IN RATS

Administration of thioacetamide and the RGD and RGE analogues

Liver fibrosis was induced by the administration of thioacetamide (TAA) 0.03 percent (Sigma Chemical Co., St. Louis, MO) in the drinking water for 12 weeks. The synthesized SF-6,5 and SF-6,6 were administered i.p. 5 days a week. Rats were treated as follows: One group received TAA and i.p. injections of 0.9 percent NaCl 5 days a week for 12 weeks (cirrhotic controls for the RGD-treated groups). Other groups received TAA orally and either i.p. SF-6,5 (100 µg/day), SF-6,5 (500 µg/day), SF-6,6 (500 µg/day) the RGD-containing peptide (GRGDSPK), (Sigma Chemical Co., St. Louis, MO) 500 µg/day for 5 days a week. The control groups consisted of one that received tap water without TAA for 12 weeks (normal controls) and a group of rats that received only SF-6,5 for 12 weeks to look for adverse effects.

Liver histopathology

Liver sections were processed for light microscopy including staining the sections with hematoxylin and eosin, and Masson trichrome. The degree of inflammation and fibrosis was expressed as the mean of 10 different fields in each slide that had been classified on a scale of 0-3 according to Muller et al. [21].

The administration of the RGD-containing peptide (GRGDSPK) or the RGE analogue SF-6,6 for 12 weeks did not prevent the development of liver cirrhosis (Table 6) in TAA-treated rats. The livers of rats that received TAA and a low dose (100 µg/day) of the RGD analogue for 12 weeks showed cirrhotic lesions similar to the group treated with TAA alone for 12 weeks (Table 6; Ref 22). Thus, a low dose of SF-6,5 was not effective in preventing liver cirrhosis. In contrast, the livers of rats that received TAA and a higher dose (500 µg/day) of SF-6,5 for 12 weeks showed slight portal and peri-portal inflammation with mild bridging fibrosis, but no cirrhotic nodules or passive fibrotic septa [22].

Table 6. The effects of nonpeptidic mimetics of RGD and RGE on liver histopathology of TAA*-treated rats.

Compound used for treatment	Analogue (µg/day) (12 w)	Inflammation (0-3)***	Nodule formation (0-3)***	Fibrotic septa (0-3)***
None	0	0	0	0
TAA	0	1	3	3
TAA+SF-6,5	100	2	2-3	2-3
TAA+SF-6,5	500	1-2	1	1-2
TAA+SF-6,6	500	1	3	3
TAA+GRGDSP	500	1-2	3	3

* 0.03% in drinking water for 12 weeks, unless otherwise indicated.

For 5 days a week. n = 6 in each group.*No change, 0; slight changes, 1; stronger changes, 2; and 3; intense changes. Adapted from Ref. [22].

Quantitative analysis of liver fibrosis

Hepatic fibrosis was quantitated by computerized imaging morphometry with a computerized video-imaging system (Biological Detection System, Pittsburgh, PA), as previously described [23]. The mean integrated optical density values of the histologic slides of the TAA-treated group were significantly higher than those of the TAA plus SF-6,5 (500 $\mu\text{g}/\text{day}$) and the control groups (Figure 4A). This quantitative morphometric method confirmed the results of the histopathologic scoring.

Spleen weights

An indirect measure of portal hypertension was obtained by measuring the weights of the rats spleens when treatment was ended. After 12 weeks, the mean spleen weight of rats receiving TAA daily was about 80 percent higher than those receiving normal tap water and injections of 0.9 percent NaCl (Figure 4B). The mean spleen weight of rats that received SF-6,5 (500 $\mu\text{g}/\text{day}$) in addition to TAA for 12 weeks was only about 30 percent higher than that of controls.

Side effects of SF-6,5

A control group consisting of 5 rats received daily only the RGD analogue for 12 weeks. Upon the end of treatment, no mortality or major adverse effects such as infections or bleeding were observed in the treated rats and blood chemistry and liver histology in this group appeared normal.

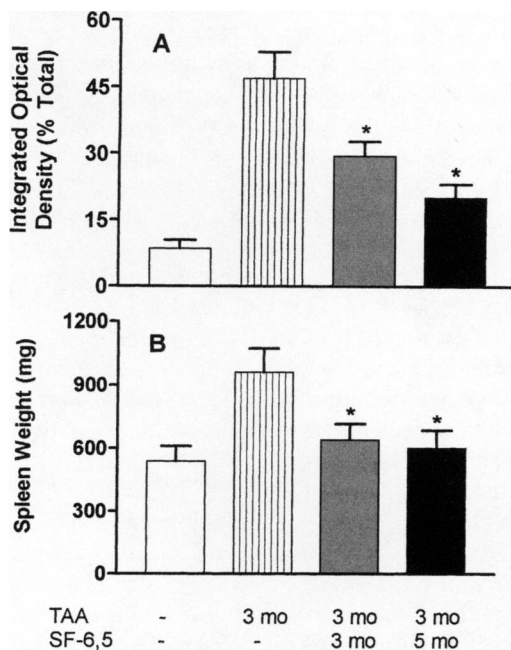


Figure 4. A) Quantitative determination of the fibrosis in the liver biopsies by computerized imaging morphometry. Integrated optical density depicted as the Mean \pm SD; * $p < .01$ compared with TAA group. B) Effects of administration of TAA (0.03 percent) and the RGD analogue SF-6,5 (500 $\mu\text{g}/\text{day}$, 5 days/wk) on the spleen weights of rats. Mean \pm SD; * $p < .01$ compared with the group that received only TAA. Note the further decrease in fibrosis and spleen weights when the treatment with the RGD analogue SF-6,5 was continued for another 2 months after the discontinuation of TAA. Adapted from Ref. [22].

DISCUSSION

Liver inflammation in Con A-treated mice, characterized by areas of necrosis and infiltration of liver tissue by mononuclear cells, predominantly CD4⁺ T-lymphocytes, could be prevented by synthetic analogues of the RGD sequence and by sTNF-R. Moreover, the elevation of serum TNF- α , was also inhibited by pretreatment of mice with either the RGD mimetic or sTNF-R, suggesting that either neutralization of serum TNF- α , or inhibition of the interaction between T-lymphocytes and the ECM effectively prevent the cytokine response, and might be one of the mechanisms by which these compounds protect against Con A hepatitis. The inhibition of Con A hepatitis by sTNF-R is consistent with previous studies that suggested a pivotal role for TNF- α , in the induction of Con A-induced liver injury in mice [11-13], and confirm that neutralization of endogenous TNF- α , by sTNF-R, can prevent liver injury in this model.

The synthetic RGD analogue SF-6,5 was also effective in alleviating hepatic fibrosis induced by the hepatotoxin TAA. In this rat model, liver cirrhosis results from the inhibition of respiratory metabolism and enzymatic activity within the nuclei of liver cells by reactive oxygen species rather than by a direct stimulation of immune cells (although immune cells are involved in the induction of cirrhosis by TAA) [24].

During the process of hepatic fibrogenesis, HSC, which in chronically injured liver are activated by cytokines such as platelet derived growth factor [25], TGF β 1 [26] and other factors, acquire a myofibroblast-like phenotype, are the major source of matrix components in both normal and fibrotic livers [27]. Normal liver subendothelial space is known to lack a true basement membrane, although the presence of ECM glycoproteins typical of basement membrane such as collagens, laminin and fibronectin have been described [28]. Cell-matrix interaction may be important in HSC activation [29]. Recent studies have shown that the interactions of epithelial cells with ECM components such as fibronectin via integrins play an important role in wound healing [30], fibrosis [31] and hepatic regeneration. One of the earliest detectable changes in the ECM of the insulted liver is an increase in total FN [32]. In human tenon's capsule fibroblasts adhesion to FN is an RGD-dependent process [33]. A recent study in HSC in culture have demonstrated that α_5 and β_1 integrin subunits (fibronectin receptor) are present in both quiescent and activated HSC, and the increased expression of the α_5 and β_1 subunits during HSC activation suggests that the fibronectin receptor that binds the RGD sequence may modulate the differentiation and activation of HSC in response to changes in the ECM [34]. Hence, compounds that interfere with the interactions between $\alpha_5\beta_1$ integrin receptors and the RGD sequence were expected to inhibit fibrogenesis in the liver.

Prevention of RGD-dependent cell-ECM interactions by SF-6,5, may alleviate the chronic inflammatory response that initiate hepatic fibrogenesis. Inhibition of the interactions between inflammatory cells and the ECM by RGD mimetics was proposed, since the RGD-dependent interaction of resting T-cells and macrophages with ECM components results in secretion of TNF- α and fibroblast growth factor. Moreover, when ECM is physically damaged, cytokine secretion is enhanced [35]. As shown in mice, the administration of SF-6,5 effectively inhibited the release of TNF- α in response to Con A administration. During inflammation, proteases released by lymphocytes can degrade normal ECM components, and in particular FN, to biologically active peptides that are chemoattractants for fibroblasts [36]. During fibrogenesis, these chemoattracted myofibroblasts complete the reparative phase by depositing ECM constituents. Therefore, inhibition of myofibroblast accumulation or activation may alleviate fibrogenesis [34].

It has been reported that RGD-containing peptides can dramatically signal the secretion of proteases such as collagenase and stromelysin from cultured synovial fibroblasts [37]. Thus, enhancement of collagen degradation due to increased production or activity

of matrix metalloproteinases may also play a role in the inhibition of hepatic fibrosis by RGD analogues, and this possibility should be tested in future studies.

Thus, nonpeptidic mimetics of cell adhesion motifs can pharmacologically affect pathological processes involved in hepatic fibrosis. Although the mechanism(s) by which the RGD analogue induced the antifibrotic effect was not elucidated in our studies, the processes that may be affected by these mimetics during chronic liver injury include immune cell migration, cytokine release, activation and adhesion of activated HSC to ECM or enhancement of collagen degradation due to increased production or activity of matrix metalloproteinases.

Thus, nonpeptidic analogues of the versatile ECM adhesion epitopes may be effective in preventing chronic hepatic inflammation and fibrosis, and should be considered therapeutically to inhibit pathological conditions in the liver. In preliminary studies, no apparent side effects attributable to SF-6,5 were observed in rats that received only the RGD mimetic for 12 weeks. However, further efficacy and toxicity studies are indicated before considering these compounds therapeutically for patients with liver diseases.

ACKNOWLEDGEMENT: Supported by a grant from the Chief Scientist, Ministry of Health, Israel

REFERENCES

1. Springer, T.A. Adhesion receptors of the immune system. *Nature* 346:425-434, 1990.
2. Hynes, R.O. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69:11-25, 1992.
3. Ruoslahti, E. Integrins. *J. Clin. Invest.* 87:1-5, 1991.
4. Ruoslahti, E. Fibronectin and its receptors. *Ann. Rev. Biochem.* 57:375-393, 1988.
5. D'Souza, S.E., Ginsberg, M.H., and Plow, E.F. Arginyl-Glycyl-Aspartic acid (RGD): a cell adhesion motif. *TIBS* 16:246-50, 1991.
6. Shimizu, Y., van Seventer, G.A., Horgan, K.J., and Shaw, S. Regulated expression and binding of three VLA (β 1) integrin receptors on T-cells. *Nature* 345:250-253, 1990.
7. Ferguson, T.A., Mizutani, H., and Kupper, T.S. Two integrin-binding peptides abrogate T-cell-mediated immune responses in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 88:8072-8076, 1991.
8. Greenspoon, N., Hershkoviz, R., Alon, R., Varon, D., Shemkman, B., Marx, G., et al. Selective inhibition of integrin-mediated platelet and T-cell function by novel organic surrogates of the Arg-Gly-Asp cell adhesion motif. *Biochem.* 32:1001-1008, 1993.
9. Hershkoviz, R., Greenspoon, N., Mekori, Y.A., Hadari, R., Alon, R., and Lider, O. Inhibition of CD4⁺ T lymphocyte binding to fibronectin and immune-cell accumulation in inflammatory sites by novel peptidic mimetics of the Arg-Gly-Asp cell adhesion motif. *Clin. Exp. Immunol.* 95:270-276, 1994.
10. Hardan, I., Hershkoviz, R., Greenspoon, N., Weiss, L., Alon, R., Cahalon, L., et al. Inhibition of metastatic cell colonization in murine lungs and tumor-induced morbidity by nonpeptidic mimetics of the Arg-Gly-Asp motif. *Int. J. Cancer* 55:1-6, 1993.
11. Tiegs, G., Hentchel, J., and Wendel, A. A T-cell-dependent experimental liver injury in mice inducible by Concanavalin A. *J. Clin. Invest.* 90:196-203, 1992.
12. Gantner, F., Leist, M., Lohse, A.W., Germann, P.G., and Tiegs, G. Concanavalin A-induced T-cell-mediated hepatic injury in mice: the role of tumor necrosis factor. *Hepatology* 21:190-198, 1995.
13. Mizuhara, H., O'Neill, E., Seki, N., Ogawa, T., Kusunoki, C., Otsuka, K., et al. T-cell activation-associated hepatic injury: mediation by tumor necrosis factors and protection by interleukin 6. *J. Exp. Med.* 179:1529-1537, 1994.
14. Watanabe, Y., Morita, M., and Akaike, T. Concanavalin A induces perforin-mediated but not Fas-mediated hepatic injury. *Hepatology* 24:702-710, 1996.
15. Hershkoviz, R., Lider, O., Bruck, R., Aeed, H., Greenspoon, N., and Halpern, Z. Treatment of immune cell-mediated liver damage by nonpeptidic mimetics of the extracellular matrix-associated Arg-Gly-Asp epitope. *J. Hepatology* 22:158-164, 1995.
16. Bruck, R., Shirin, H., Hershkoviz, R., Lider, O., Kenet, G., Aeed, H., Matas, Z., Zaidel, L., and Halpern, Z. Analysis of Arg-Gly-Asp mimetics and soluble receptor of tumor necrosis factor as therapeutic modalities for concanavalin A induced hepatitis in mice. *Gut* 40:133-138, 1997.

17. Horn, T., Junge, J., and Christofferson, P. Early alcoholic liver injury: activation of lipocytes in acinar zone 3 and correlation to degree of collagen formation in the Disse space. *J. Hepatol.* 3:333-340, 1986.
18. Czaja, M.J., Weiner, F.R., Flanders, K.C., Giambrone, M.A., Wind, R., Biempical, L., and Zern, M.A. In vitro and in vivo association of transforming growth factor β_1 with hepatic fibrogenesis. *J. Cell Biol.* 108:2477-2482, 1989.
19. Milani, S., Schuppan, D., Herbst, H., and Surrenti, C. Expression of transforming growth factor β_1 in normal and fibrotic human liver. In: A.M. Gressner, G. Ramadori, eds. *Molecular and Cell Biology of Liver Fibrogenesis*. Dordrecht: Kluwer Academic Publishers, 1992. pp. 254-261.
20. Kagami, S., Border, W.A., Ruoslahti, E., and Noble, N.A. Coordinated expression of β_1 integrins and transforming growth factor- β -induced matrix proteins in glomerulonephritis. *Lab. Invest.* 69:68-76, 1993.
21. Schiff, E.R. Hepatic fibrosis - new therapeutic approaches. *N. Engl. J. Med.* 324:987-988, 1991.
21. Muller, A., Machnik, F., Zimmermann, T., and Schubert, H. Thioacetamide-induced cirrhosis-like lesions in rats - usefulness and reliability of this animal model. *Exp. Pathol.* 34:229-236, 1988.
22. Bruck, R., Hershkoviz, R., Lider, O., Aeed, H., Zaidel, L., Matas, Z., Barg, J., and Halpern, Z. Inhibition of experimentally-induced liver cirrhosis in rats by a nonpeptidic mimetic of the extracellular matrix-associated Arg-Gly-Asp epitope. *J. Hepatol.* 24:731-738, 1996.
23. Chevallier, M., Guerret, S., Chossegros, P., Gerard, F., and Grimaud, J.A. A histological semi-quantitative scoring system for evaluation of hepatic fibrosis in needle biopsy specimens: comparison with morphometric studies. *Hepatol.* 20:349-355, 1994.
24. Nuber, R., Teutsch, H.F., and Sasse, D. Metabolic zonation in thioacetamide-induced liver cirrhosis. *Histochemistry* 69:277-288, 1980.
25. Pinzani, M., Gesualdo, L., Sabbah, G.M., and Abboud, H.E. Effects of platelet derived growth factor and other polypeptide mitogens on DNA synthesis and growth of cultured rat liver fat storing cells. *J. Clin. Invest.* 84:1786-1793, 1989.
26. Casini, A., Pinzani, M., Milani, S., Grappone, C., Galli, G., Jezequel, A.M., Schuppan, D., Rotella, C.M., and Surrenti, C. Regulation of extracellular matrix synthesis by transforming growth factor β , in human fat-storing cells. *Gastroenterol.* 105:245-253, 1993.
27. Clement, B., Loreal, O., Rescan, P.Y., Levavasseur, F., Diakonova, M., Rissel, M., L'Helgoualc'h, A., and Guillouzo, A. Cellular origin of the hepatic extracellular matrix. In: Gressner, A.M., Ramadori, G., eds. *Molecular and Cell Biology of Liver Fibrogenesis*. Dordrecht: Kluwer Academic Publishers, 1992. pp. 85-98.
28. Ried, M.L., Florino, A.S., Sigal, S.H., Brill, S., and Holst, P.A. Extracellular matrix gradients in the space of Disse: relevance to liver biology. *Hepatol.* 15:1198-1203, 1992.
29. Friedman, S.L., Roll, F.J., Boyles, J., Aronson, D.M. and Bissel, D.M. Maintenance of differentiated phenotype of cultured rat hepatic lipocytes by basement membrane matrix. *J. Biol. Chem.* 264:10756-10762, 1989.
30. Brown, L.F., Dubin, J., Lavigne, L., Logan, B., Dvork, H.F., and Vandewater, L. Macrophages and fibroblasts express embryonic fibronectins during cutaneous wound healing. *Am. J. Pathol.* 142:793-801, 1993.
31. Barnes, J.L., Hastings, R.R., and De La Garza, M.A. Sequential expression of cellular fibronectin by platelets, macrophages, and mesangial cells proliferative glomerulonephritis. *Am. J. Pathol.* 145:585-597, 1994.
32. Martinez-Hernandez, A. The hepatic extracellular matrix. II. Electron immunohistochemical studies in rats with CCl_4 induced cirrhosis. *Lab. Invest.* 53:166-186, 1985.
33. Hershkoviz, R., Melamed, S., Greenspoon, N., and Lider, O. Nonpeptidic analogues of the Arg-Gly-Asp (RGD) sequence specifically inhibit the adhesion of human tenon's capsule fibroblasts to fibronectin. *Invest. Ophthalmol. Vis. Sci.* 35:2585-2591, 1994.
34. McMorro, B.C., Milliano, M.T., O'Neill, R., and Luxon, B.A. Expression of α_5 and β_1 integrin subunits during activation of hepatic stellate cells. *Gastroenterol.* 112:A1331, 1997.
35. Hershkoviz, R., Gilat, D., Miron, S., Mekori, Y.A., Aderka, D., Wallach, D., Voldavsky, I., Cohen, I.R., and Lider, O. Extracellular matrix induces tumour necrosis factor- secretion by an interaction between resting rat CD4^+ T-cells and macrophages. *Immunol.* 78:50-57, 1993.
36. Hershkoviz, R., Alon, R., Gilat, D., and Lider, O. Activated T-lymphocytes and macrophages secrete fibronectin which strongly support cell adhesion. *Cell. Immunol.* 141:352-361, 1992.
37. Salo, T., Turpeenniemi-Hujansen, T., and Tryggvason, K. Tumor-promoting phorbol esters and cell proliferation stimulate secretion of basement membrane (Type IV) collagen-degrading metalloproteinases by human fibroblasts. *J. Biol. Chem.* 260:8526-8531, 1985.