Fibronectin Increases Survival of Rat Hepatic Stellate Cells - A Novel Profibrogenic Mechanism of Fibronectin

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Key Words

Apoptosis • Fibronectin • Fibrosis • Hepatic stellate cells • Myofibroblasts

Abstract

The aims of this study were to determine whether fibronectin increases survival of hepatic stellate cells (HSCs) in starving conditions, and to identify the signal transduction pathways involved in this effect. Methods: Primary culture of rat HSCs were plated on fibronectin-uncoated or coated culture wells, and grown in the presence of 0.2% or 20% fetal calf serum. Cell apoptosis was measured by an ELISA procedure. Signal transduction pathways were analyzed by inhibiting major intracellular transduction pathways with appropriated inhibitors and by detecting phosphorylated proteins. Results: Fibronectin increased survival of serum deprived HSCs. This effect was abrogated by the presence of the RGD peptide, by silencing FAK expression, and by inhibiting PI3K with LY294002 or wortmannin. Growth of HSCs on fibronectin induced integrin $\alpha 5\beta 1$ expression, tyr^{397} , ser^{473} , and ser^{136} phosphorylation of FAK, Akt, and Bad, respectively, and the binding of phosphorylated Bad to 14-3-3 proteins. Likewise, fibronectin increased

Bcl2/Bax ratio and reduced release of mitochondrial cytochrome *c* into the cytoplasm, formation of apoptosome, and caspase 9 and 3 activity. These effects were avoided by treatment of cells with PI3K inhibitors. Conclusion: Fibronectin increases survival of HSCs via a pathway involving integrin $\alpha 5\beta 1$ receptors, FAK, PI3K, Akt and proteins of Bcl2 family.

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Introduction

Hepatic fibrosis represents a consequence of chronic liver injury of any cause that is involved in the pathophysiology of most clinical complications of end-stage liver diseases. It is characterized by an excessive production and deposition of extracellular matrix (ECM)⁽¹⁾ components, mainly collagen type I and fibronectin (FN). Activated hepatic stellate cells (HSCs) have been shown to play a major role in the production of most ECM proteins. Nevertheless, liver myofibroblasts have been shown to contribute to the accumulation of ECM in the liver [1, 2]. The number and activity of these cells are

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associated with the intensity of the fibrogenic process. During liver injury, the number of HSCs is increased and the production of ECM is also elevated. When liver aggression diminishes, the number of fibrogenic cells decreases and production of new ECM ceases. Mechanisms of clearance of activated HSCs are not completely elucidated. However, apoptosis is likely a potential way by which these cells disappear during resolution of liver injury [3]. Since, stimulation of HSCs apoptosis reduces scarring in animal models of liver fibrosis. [4], understanding of mechanisms involved in the apoptotic death of these cells is essential for the development of antifibrotic therapies.

FN is a constituent of the ECM composed of two similar subunits joined by a pair of disulfide bonds near the carboxyl-terminus. It is folded into a series of globular domains that are binding sites for components of the ECM. Moreover, FN interacts with cell surface receptors, including integrins [5] and non-integrin receptors [6]. These integrin-mediated interactions with ECM components play crucial roles in many fundamental aspects of cell migration, growth, and differentiation [7]. FN is a profibrogenic protein, since it contributes to the organization of the ECM, helps cells attach to it, promotes activation [8] and proliferation of HSCs, induces collagen gene expression [9] and precedes the deposition of other components of the ECM in the process of liver fibrogenesis. Moreover, FN might also contribute to the liver fibrosis by increasing HSCs survival. Although it is known that FN acts as survival factor in a number of cell lines [10, 11], its effect on the survival of HSCs has rarely been explored.

The aims of the present study were to determine the effect of FN on the survival of serum-starved cells, and to identify the signal transduction pathways implicated in this effect.

Materials and Methods

HSC isolation and culture

HSCs were isolated from adult male Sprague-Dawley rats using sequential pronase-collagenase digestion followed by arabinogalactan (Larcol, SIGMA Chemical, Alcobendas, Spain) density gradient centrifugation as described by Rippe et al. [12]. The purity of HSC culture was tested by flow cytometry at day 2. This study showed that most isolated cells were reelin-positive (98.2%) or glial fibrillary acidic protein-positive (93.3%), and only a minority of them were smooth muscle actin-positive (0.9%), or fibulin-2-positive (2.19%) [13]. Isolated HSCs were expanded up to passage 2 in Dulbecco's

modified Eagle's medium (Lonza, Verviers, Belgium) supplemented with 20% fetal calf serum (FCS) and standard antibiotics in 95% air, 5% CO₂ humidified atmosphere at 37°C. At this time, most cells were reelin-positive (99.3%), glial fibrillary acidic protein-positive (96.1%), and smooth muscle actin-positive (87.6%). HSCs were plated in serum on fibronectin-uncoated or coated tissue culture plastic wells (Nunc A/S, Roskilde, Denmark). Coating was done with 3 µg/cm² human plasma FN (SIGMA Chemical, Alcobendas, Spain) in PBS at room temperature for one hour. All animal procedures were performed in accordance with the Spanish Guidelines for the Care and Use of Laboratory Animals.

Apoptosis assay

Apoptosis of floating and attached cells was analyzed using the "Cell Death Detection ELISA" kit (ROCHE Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol.

Western blot analysis

Whole cell protein extracts were prepared from HSCs cultured on plastic or FN until confluence as previously described [10]. The filters containing proteins were then incubated with appropriated polyclonal antibodies [integrin α5, integrin β1, focal adhesion kinase (FAK), tyrosine³⁹⁷ phosphorylated FAK, Akt, serine⁴⁷³ phosphorylated Akt, Bad, serine¹³⁶ phosphorylated Bad, 14-3-3 proteins, cytochrome c, Apaf-1, β-actin]. Signals were detected using the ECL Western Blotting Detection Reagent (Amersham Iberica, Madrid, Spain).

Blue native one-dimensional electrophoresis

Apoptosome complex was isolated by one-dimensional Blue Native-PAGE using a gradient gel of 3-12% acrylamide (NativePAGE 3-12% Bis-Tris Gel, Invitrogen, Carlsbad, CA) as described by Schagger and von Jagow [14] and Triepels [15]. Immunoreactive material was visualized by chemiluminescence. ECL signals were quantified using the IMAGEJ image analysis software [16].

Real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells using the Tri-reagent (Sigma-Aldrich, Steinheim, Germany) according to the manufacturer's instructions. cDNA was generated from 1 µg sample RNA using 1st Strand cDNA Synthesis Kit for RT-PCR (Roche Applied Science, Indianapolis, IN) and the sense/antisense primers described in table 1: The amplification conditions were: denaturation 95°C, 30s; annealing 54°C, 30s; extension 72°C, 45s. The amplification products were size-resolved by electrophoresis.

Real-time quantitative polymerase chain reaction

PCR was performed on a Light Cycle 1.0 (Roche Applied Science) in 20 µl with 50 ng cDNA, 0.5 µM primers, 2 µl FastStart DNA Master SYBR Green I (Roche Applied Science, Mannheim, Germany). Data from the real-time, quantitative PCR were analyzed following the 2^{-ΔΔC_T} method as described by Livak et al. [17]. Sequence of primers used in these experiments are shown in table 1. The amplification conditions were an initial 30

Table 1. Primers used in real-time quantitative PCR and siRNA employed for silencing FAK mRNA.

Primers used in real-time quantitative PCR		
Rat GAPDH	Sense:	5'-ACC ACA GTC CAT GCC ATC AC-3';
	Antisense:	5'-TCC ACC ACC CTG TTG CTG TA-3'
Rat FAK	Sense:	5'-CCT TCC GAT TCA GAA GAT-3';
	Antisense:	5'-AGA CTC TGC TGT GGT TCT G-3'.
Rat Bcl-2,	Sense:	5'-GAC GCG AAG TGC TAT TG-3';
	Antisense:	5'-GTA GCG ACG AGA GAA GT-3';
Rat Bax,	Sense:	5'-CGG CGA ATT GGA GAT G-3';
	Antisense:	5'-CAC CCT GGT CTT GGA T-3'.
siRNA used for silencing mRNA		
FAK	Sense :	5'-AGA AAU AGC UGA UCA AGU ATT-3'
	Antisense:	3'-TTU CUU UAU CGA CUA GUU CAU-5.
Integrin $\alpha 5$	Sense:	5'-CAC UGU UCC UCA UCU UCA ATT-3'
	Antisense:	3'-UUG AAG AUG AGG AAC AGU GTT-5'

s at 48°C, 10 min at 95°C, 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min. [18]. The correct size of the amplified products was checked by electrophoresis.

RNA interference

The sequences of siRNA for focal adhesion kinase (FAK) (BioNova Cientifica S.L. Madrid, Spain) are shown in table 1. The non-specific control RNA was also purchased from BioNova and was used as negative control. For the transfection experiments, cells were seeded in 6-well plates. At 60% confluence, cells were transfected with siRNA at a final concentration of 10 nM using LipofectAMINE 2000 (Invitrogen Corp. Carlsbad, CA), according to the manufacturer's protocol. Cells were grown on plastic or fibronectin plates and incubated at 37°C in 5% CO₂ for 72 hours before the western blot analysis. Forty-eight hours after transfection, RNA was collected and RT-PCR was done for the FAK and GAPDH transcripts.

Activity of caspases 3 and 9

The activity of these two caspases was measured using colorimetric assay kits (Caspase-3/CPP32 and Caspase-9/Mch6 Colorimetric assay Kits. MBL, Woburn, MA) according to the manufacturer's protocol. These assays are based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labelled substrate LEHD-pNA or DEVD-pNA in the case of caspase-9 or caspase-3, respectively.

Isolation of mitochondrial and cytosolic cytochrome c

Cells were homogenized at 4°C in 150 μ l Tris/sucrose buffer (0.2 mM EDTA), 0.25 M sucrose, 10 mM Tris/HCl, pH 7.6) and centrifuged at 800 g for 15 minutes. The pellet was discarded and mitochondria were obtained after centrifugation of the supernatant at 10000 g for 15 minutes. After this centrifugation, the supernatant contained the cytoplasmic fraction and the pellet the mitochondrial fraction. To wash the mitochondria, the pellet was resuspended in 50 μ l isolation buffer (0.5 μ g/ml leupeptin, 0.5 μ g pepstatin, 1 mM PMSF, 250 mM sucrose, 1 mM EGTA, 1 mM EDTA, 10 mM Tris/HCl, pH 7.5), and kept on ice for 30 min. Mitochondrial pellet was centrifuged at 16000 g for 5 min at 4°C and the supernatant (mitochondrial fraction) was collected and stored at -80°C.

Statistical analysis

All results are expressed as mean $\pm$ SD unless otherwise mentioned. Data were analyzed using the nonparametric Mann-Whitney test, A *p* value <0.05 was considered statistically significant.

Results

Fibronectin decreases apoptosis induced by serum deprivation

To determine whether serum withdrawal induces apoptosis in rat HSCs, we measured apoptotic death in cells grown in the presence of either 0.2% or 20% FCS. Figure 1A shows that HSCs were sensitive to serum depletion in a time-dependent manner. After 72 hours of serum withdrawal, apoptosis increased 2.03 $\pm$ 0.21-fold as compared with control cells grown with FCS (*p*<0.01). To assess whether FN prevents apoptosis induced by serum deprivation, HSCs were plated on FN-coated dishes and grown in starving conditions for 24 to 92 hours. At 72 hours and in the presence of serum, the apoptotic death decreased by 18.11 $\pm$ 3.5% when cells were cultured on FN. In the absence of serum, the growth of cells on FN decreased the apoptotic rate by 59.30 $\pm$ 3.83% (*p*<0.01) (Fig. 1A). This protective effect intensified as FN concentration increased from 1 to 3 μ g/cm². However, higher concentrations of FN were not associated with a greater protection against apoptosis (Fig. 1B).

As many cell-to-FN relationships are mediated by the interaction of cell surface integrins with the Arg-Gly-Asp (RGD) motif in the FN molecule, we plated HSCs on FN-coated and -uncoated cell culture dishes in the presence of 1.38 mM of either RGD or Arg-Gly-Glu (RGE) peptides (Sigma-Aldrich. Saint Louis, MI). Twenty four hours after plating, HSCs were exposed to these

Fig. 1. Fibronectin increases rat HCS survival. A. Serum deprivation increases apoptosis of HSCs, but fibronectin prevents the death of these cells. Primary culture of HSCs was grown on FN-coated (■) or uncoated (□) culture plastic wells in the presence or absence of serum. After 24, 48, 72, and 92 hours, apoptosis was measured as described under “Methods”. S+, presence of serum; S-, absence of serum. (*, $p < 0.001$ as compared with apoptosis at 24 hours; (a), $p < 0.001$ as compared with cells grown without fibronectin. Representative experiment that was repeated three times by quadruplicate. B. Dose response effect of FN and type I collagen on apoptosis induced by serum deprivation. Rat hepatic stellate cells were grown in culture plastic wells coated with 1 to 10 $\mu\text{g}/\text{cm}^2$ FN in the presence (FCS+) or absence of serum. After 72 hours, apoptosis rate was measured as described under “Methods”. Representative experiment that was repeated twice by triplicate. Data are represented as fold the apoptosis rate in cells cultured with serum for 72 hours. NS, not significant; *, $p < 0.05$; **, $p < 0.001$ as compared with apoptosis in cells grown in the presence of serum.

peptides and maintained in starving conditions (0.2% FCS) for another 72 hours. The apoptotic rate was measured after this time. In the presence of FN, apoptosis induced by serum deprivation decreased by $47.1 \pm 6.4\%$ ($p < 0.01$). This protective effect was suppressed when HSCs were grown on FN-coated dishes in the presence of the soluble RGD peptide, but persisted in cells incubated in the presence of the control RGE peptide (Fig. 2A). Since $\alpha 5 \beta 1$ integrin are cell surface receptors for FN, we wanted to know whether these integrins are expressed in HSCs and whether this expression is affected by FN. As figure 2B shows, both integrins were expressed by HSCs and this expression increased markedly in cells cultured on FN-coated plates. These cell receptors mediate survival induced by FN, as silencing integrin $\alpha 5$ expression abrogated protection caused by FN (Fig. 2C).

Because FAK is a tyrosine kinase that is involved in some integrin signaling, we silenced FAK expression by transient transfection of HSCs with a specific siRNA for FAK and examined its impact on cell survival. As figure 3A clearly shows, silencing FAK expression abrogated completely the effect of FN on HSC survival. Our study also shows that cell adhesion to FN induced FAK phosphorylation at Tyr³⁹⁷, which was particularly marked between 30 and 120 minutes (Fig. 3B). On the contrary, the total FAK protein levels did not change significantly in this time. Phosphorylation of FAK was avoided by silencing integrin $\alpha 5$ expression (Fig. 2C) or by the presence of soluble RGD peptide (Fig 3D). These results indicate that FAK activation is implicated in the survival signal triggered by the interaction of HSCs with FN.

Fibronectin-induced survival of HSCs is mediated by phosphatidylinositol 3-Kinase (PI3K)

Because FAK can activate other downstream pathways, including JNK, NF κ B, ERK, and PI3K [19], we treated serum starved HSCs grown on FN-coated or uncoated culture dishes with 20 μM SP600125, 200 μM gliotoxin, 50 μM PD98059, 12.5 μM LY294002 or 100 nM wortmannin (Calbiochem-Merck, Darmstadt, Germany), specific inhibitors of these kinases. Apoptotic rate was measured 48 hours later. As figure 4A shows, the cell death induced by the serum deprivation was reduced to the $40.8 \pm 6.20\%$ of the basal level when grown on FN-coated dishes. Inhibition of JNK, NF κ B, or ERK with 20 μM SP600125, 200 μM gliotoxin or 50 μM PD98059, respectively, increased significantly the apoptosis rate induced by starvation. However, the protective effect of FN persisted when JNK, NF κ B or ERK was blocked with the above mentioned inhibitors (Fig. 4A). Although apoptosis rate decreased significantly in these inhibited HSCs cultured on FN, it remained elevated as compared with apoptosis of control cells cultured on plastic in absence of serum. Likewise, pretreatment of cells with 12.5 μM LY294002 or 100 nM wortmannin, two inhibitors of PI3K, intensified significantly apoptosis induced by serum deprivation, but these treatments abrogated the protective effect of FN. To confirm that PI3K signaling pathway is activated by FN, we measured the Ser⁴⁷³ phosphorylation of PKB/Akt, a downstream effector of PI3K, by immunoblotting using specific antibody. Figure 4B shows that FN induced ser⁴⁷³ phosphorylation of Akt reaching its peak

Fig. 2. Tripeptide RGD and integrin $\alpha 5\beta 1$ are involved in the transmission of the survival signal initiated by fibronectin. **A.** Effects of RGD and RGE peptides on fibronectin-induced survival of HSCs. Cells were cultured on FN-uncoated (FN-) or FN-coated culture wells (FN+). Twenty four hours later, 1.38 mM of either RGD or RGE peptide was added to the cells. After another 24 hours, cells were maintained in starving conditions (0.2% FCS) for 72 hours. The apoptotic rate was measured after this time. Results are presented as means $\pm$ SD of a representative experiment that was performed in triplicate. NS, not significant; *, $p < 0.01$ as compared with control cells [FN (-), RGD (-), RGE (-)]. **B.** Fibronectin induces the expression of integrin $\alpha 5$ and $\beta 1$ in cultured HSCs. Cells were cultured on FN-coated (FN) or uncoated (control) culture plates. After 72 hours of culture, proteins were extracted and analyzed by Western blot. Blots are representative of an experiment that was repeated three times. **C.** Silencing $\alpha 5$ integrin abrogates the survival effect of fibronectin. To downregulate integrin $\alpha 5$ expression in HSCs, cells were transfected with either 10 nM non-specific control RNA (siCtr) or 10 nM siRNA $\alpha 5$ oligonucleotide against the synthesis of $\alpha 5$ integrin (siRNA- $\alpha 5$). After 24 hours, cells were grown in starving conditions on FN-coated or uncoated wells. Seventy-two hours later, apoptosis was measured. RNA was collected and RT-PCR was done for integrin $\alpha 5$ (integ. $\alpha 5$ cDNA) and GADPH (GADPH cDNA) transcripts. Likewise, proteins from transfected cells were collected and analyzed by Western blot using anti-integrin $\alpha 5$ and anti- β -actin antibody. Results are presented as means $\pm$ SD of a representative experiment that was performed three times in triplicate. NS₁, not significant as compared with control cells (siCtr, serum +, FN-); *, $p < 0.001$ as compared with control. NS₂, not significant as compared with cells grown on FN-uncoated wells.

level at 30 min and that this effect was abolished by pretreatment of cells with 12.5 μ M LY294002.

Since Akt can phosphorylate and activate Bad (*Bcl-2-antagonist of cell death*), a pro-apoptotic protein, we examined whether the contact of HSCs with FN induces Bad phosphorylation at serine¹³⁶. Therefore, cells were serum-starved for 72 hours, detached and held in suspension (control cells) or plated on tissue culture plates coated with FN for 15 to 60 minutes at 37°C. Bad phosphorylation was analysed by Western blot using specific antibody against Bad or phosphorylated Bad at Ser¹³⁶. As figure 4C shows, adhesion of HSCs to FN induced ser¹³⁶ phosphorylation of Bad mainly at 30 minutes. This phosphorylation was abrogated when PI3K activity was inhibited with 12.5 μ M LY294002. To examine whether phosphorylated Bad was sequestered in the cytosol bound to the 14-3-3 proteins [20, 21], we

immunoprecipitated cell proteins with antibody against 14-3-3 proteins and Western blotted precipitated proteins with anti-ser¹³⁶ phosphorylated Bad. This experiment demonstrated that growth of HSCs on FN for 30 minutes resulted in an increased binding of phosphorylated Bad to 14-3-3 proteins (Fig. 4D). This binding was also abrogated by pretreatment of cells with LY294002.

In addition to Bad, other proteins involved in the apoptotic process are Bcl-2 (*B-cell lymphoma 2*) and Bax (*Bcl-2 associated X protein*), among others. While Bcl-2 is an anti-apoptotic protein, Bax is a pro-apoptotic factor. The ratio of Bcl-2 to Bax is considered to determine whether a cell lives or dies by apoptosis [22]. In this study, we measured the effects of FN on the Bcl-2 and Bax gene by quantitative RT-PCR. As figure 5A illustrates, growth of HSCs on FN determined a marked increase in the ratio Bcl-2/Bax which was mainly

Fig. 3. Phosphorylated FAK mediates the effect of fibronectin on cell survival. **A.** Downregulation of FAK abrogates the protection of HSCs by fibronectin. To downregulate FAK expression in HSCs, cells were transfected with either 10 nM non-specific control RNA (siCtr) or 10 nM siRNA oligonucleotide against the synthesis of FAK (siRNA-FAK). After 24 hours, cells were grown in starving conditions on FN-coated or uncoated wells. Seventy-two hours later, apoptosis was also measured. Likewise, proteins from transfected cells were collected and analyzed by Western blot using anti-FAK and anti- β -actin antibody. RNA was also collected and RT-PCR was done for FAK (FAK cDNA) and GAPDH (GAPDH cDNA) transcripts. NS₁, not significant; *, $p < 0.001$; as compared with control (siCtr, serum +, FN-). NS₂, not significant as compared with cells grown on FN-coated wells. Representative experiment that was repeated three times in triplicate. **B.** Adhesion of HSCs to fibronectin results in the 397 tyrosine phosphorylation of FAK. Cells were grown in starving conditions for 72 hours. After this time, cells were trypsinized and seeded on FN-coated culture wells (FN) for 0 to 120 minutes. Total FAK and phosphorylated FAK (pFAK) were measured in these cells by Western blot. Blots are representative of three separate experiments. **C.** Downregulation of $\alpha 5$ integrin synthesis blocks FAK tyrosine phosphorylation. Cells were transfected with either 10 nM non-specific control RNA (siCtr) or 10 nM siRNA oligonucleotide against the synthesis of $\alpha 5$ integrin (siRNA- $\alpha 5$). After 72 hours serum deprivation, cells were trypsinized and seeded on FN-coated culture wells (FN) for 0 to 120 minutes. Total FAK and phosphorylated FAK (pFAK) were measured in these cells by Western blot. Blots are representative of three separate experiments. **D.** RGD tripeptide blocks FAK phosphorylation induced by fibronectin. Cells were cultured on FN-coated culture wells (FN). After 72 hours serum deprivation (0.2% FCS), cells were trypsinized and seeded on FN-coated culture wells (FN) for 0 to 120 minutes in the presence of 1.38 mM of either RGE or RGD tripeptide. Total FAK and phosphorylated FAK (pFAK) were measured in these cells by Western blot. Blots are representative of two separate experiments.

due to an increase in the Bcl-2 gene expression (Fig. 5B). Bax gene expression decreased only slightly in HSCs growth on FN.

Fibronectin reduces release of mitochondrial cytochrome c, formation of apoptosome and activation of caspase 9 and 3

As cytochrome *c* mediates apoptosis after its release from the mitochondrial intermembrane space into the cytoplasm, we measured cytochrome *c* in the mitochondria and cytosol after culturing HSCs on FN for 3 to 72 hours. We found that mitochondrial cytochrome *c* decreased and cytosolic cytochrome *c* increased in serum deprived HSCs cultured on plastic dishes (Fig. 6A). However, these changes did not occur in cells cultured

on FN. In these cells, mitochondrial cytochrome *c* increased and cytosolic cytochrome *c* decreased during serum deprivation. Since cytosolic cytochrome *c* binds Apaf-1 (*apoptotic protease activating factor-1*) and contributes to form the apoptosome, we isolated apoptosome complex by blue native electrophoresis and identified Apaf-1 and cytochrome *c* by Western blot using appropriate antibody. Figure 6B illustrates that formation of the apoptosome complex was decreased in HSCs grown on FN-coated plates, but this reduction did not occur in cells pretreated with 12.5 μ M LY294002. Finally, activity of caspase 9 and 3 decreased markedly in cells grown on FN. However, this effect was avoided in cells pretreated with 12.5 μ M LY294002 (Fig. 6C) indicating that PI3K participates in the

Fig. 4. Fibronectin induces Bad phosphorylation through PI3K. A. Inhibition of PI3K with LY294002 (LY) or wortmannin (W) abrogates FN-induced survival of HSCs. Cells were cultured in starving conditions (0.2% FCS) on FN-coated (FN+) or uncoated (FN-) culture wells for 24 hours. After this time, cells were treated with 20 μ M SP600125 (SP), 200 μ M gliotoxin (GL), 50 μ M PD98059 (PD), 12.5 μ M LY294002 (LY) or 100 nM wortmannin (W) for 45 minutes. Then, cells were trypsinized and seeded on FN-coated culture wells (FN) for 48 hours in the presence of kinase inhibitors. Apoptotic index was determined 48 hours later. Results are presented as means $\pm$ SD of a representative experiment that was performed five times in triplicate. NS, not significant; *, $p < 0.01$. (a), $p < 0.01$; (b), $p < 0.001$ as compared with apoptosis of cells cultured on FN-uncoated plated in starving conditions and in the absence of inhibitors [FN (-), Inhibitor (-)] B. Fibronectin induces ser⁴⁷³ phosphorylation of Akt in cultured HSCs. Cells were grown in starving conditions for 24 hours. After this time, cells were trypsinized and seeded on FN-uncoated (Ctr) or FN-coated culture wells (FN) for 5 to 60 minutes in the absence or presence of 12.5 μ M LY294002. Total Akt (Akt) and ser⁴⁷³ phosphorylated Akt (pAkt⁴⁷³) were measured in protein extracts from these cells by Western blot using specific antibody. Blots are representative of three separate experiments. C. Growth of HSCs on fibronectin-coated dishes leads to the ser¹³⁶ phosphorylation of Bad. Cells were grown in starving conditions for 24 hours. After this time, cells were trypsinized and seeded on FN-uncoated (Ctr) or FN-coated culture wells (FN) for 15 to 60 minutes without or with pre-treatment of cells with 12 μ M LY294002 for 45 minutes. Total Bad (Bad) and ser¹³⁶ phosphorylated Bad (pBad¹³⁶) were measured in protein extracts from these cells by Western blot using specific antibody. Blots are representative of three separate experiments. D. Growth of HSCs on fibronectin-coated dishes results in the physical interaction of ser¹³⁶ phosphorylated Bad with 14-3-3 proteins. Cells were grown in starving conditions for 24 hours. After this time, cells were trypsinized and seeded on FN-coated culture wells (FN) for 30 minutes with or without pre-treatment of cells with 12 μ M LY294002 for 45 minutes. Cell proteins were immunoprecipitated with anti-14-3-3 antibody. Ser¹³⁶ phosphorylated Bad (p-Bad¹³⁶) and 14-3-3 proteins were detected in the precipitated proteins by Western blot using specific antibody against pBad¹³⁶ or 14-3-3 proteins. As control, samples were also precipitated with rabbit unspecific IgG antibody and analyzed by Western blot using polyclonal anti-14-3-3 antibody. Blots are representative of three separate experiments.

mediation of the FN-induced inhibition of both caspases.

Discussion

FN is considered a profibrogenic protein, since it has been shown that FN deposits early in various fibrotic diseases and seems to function as a template for deposition of other extracellular matrix proteins, such as collagen and laminin, in the injured area [23, 24]. Moreover, a number of studies have documented that FN influence HSC adhesion, proliferation, migration, and differentia-

tion [8], induces collagen gene expression [9], wound repair, and tissue regeneration [25]. In addition of all these profibrogenic effects, in this study, we show that FN also contributes to the fibrogenic process by providing survival signals for serum depleted HSCs.

Cells survival depends on multiple factors, including soluble growth factors present in serum and its interactions with ECM proteins, particularly, with FN [26, 27]. Our study shows that serum deprivation for 72 hours increased apoptosis two-fold in HSCs cultured on FN-uncoated plates. However, the amount of cells that underwent apoptosis decreased to the control level when

Fig. 5. Fibronectin increases anti-apoptotic Bcl2, Bcl_{XL} expression and Bcl2/Bax ratio. A and B. Hepatic stellate cells were grown on FN-coated or -uncoated culture plastic wells in starving conditions. After 24, 48, and 72 hours, Bcl2 and Bax gene expression were measured by quantitative RT-PCR using endogenous GAPDH as reference. FN (-), FN-uncoated wells; FN (+) FN-coated wells. *, $p < 0.01$.

grown on FN-coated plates (Fig. 1A). These results confirm other studies showing that ECM functions as cell survival factor for many cell types [19, 26, 28, 29]. The present study also demonstrates that survival signals induced by FN were suppressed when HSCs were cultured in the presence of the soluble RGD peptide, but not when cells were cultured in the presence of the control RGE peptide (Fig. 2A). This result concurs with those showing that the soluble RGD peptide blocks the adhesion of cells to FN and trigger their apoptosis [30, 31]. The RGD tripeptide has been identified as a functional domain involved in the adhesion of FN to cell surface receptors, mainly to $\alpha 5 \beta 1$ integrin [32]. This integrin is well expressed by activated HSCs [33] and our study

Fig. 6. Fibronectin reduces release of mitochondrial cytochrome c, formation of apoptosome and activation of procaspase 9 and 3. A. Primary culture of HSCs was grown on FN-coated or -uncoated culture wells in the presence (serum) or absence of serum. After 3, 24, and 72 hours of serum deprivation cytochrome c was measured in mitochondrial preparation (Cyt. c Mitochondrial) and in cytosol (Cyt. c Cytosolic) as described under "Methods". FN (-), FN-uncoated wells; FN (+), FN-coated wells. B. Hepatic stellate cells were grown on FN-coated (FN) or uncoated culture (Ctrl) wells in starving conditions and in the absence or presence of 12.5 μ M LY294002. After 72 hours, high molecular weight protein complexes were isolated by blue native polyacrylamide gel electrophoresis as described under "Methods". Apaf-1, cytochrome c, and β -actin were identified by Western blot using specific antibody. Blot representative of one experiment that was repeated five times. C. Hepatic stellate cells were grown in starving conditions on FN-coated (FN) or uncoated (Ctrl) culture plastic wells in absence or presence of 12.5 μ M LY294002 (LY) or 100 nM wortmannin (W) for 45 minutes. After 72 hours activity of caspase 9 or 3 was measured as described under "Methods". *, $p < 0.01$ as compared with control.

demonstrates that its expression increases in HSCs cultured on FN-coated dishes (Fig. 2B). In other cell lines, it

has been shown that the binding of FN to $\alpha 5 \beta 1$ integrin activates $\alpha 5$ gene transcription through phosphorylation of Sp1 by ERK [34]. Silencing gene expression of $\alpha 5$ integrin resulted in a loss of the protective effect of FN against apoptosis (Fig. 2C) indicating that survival signal of FN is transmitted through integrin receptors.

FN-integrin activates a number of protein tyrosine kinases including FAK. Upon activation by integrin, FAK undergoes autophosphorylation of Tyr³⁹⁷ as well as its association with other intracellular signaling molecules [35, 36]. Phosphorylated FAK has been implicated in many cellular processes, including cell survival [37]. However, in addition to stimulating FAK, integrin receptors can also activate the phosphatidylinositol 3-kinase (PI3K) directly [38]. Our study shows that growth of HSCs on FN-coated plates was followed by a striking increase in the amount of tyr³⁹⁷ phosphorylated FAK (Fig. 3B). Silencing $\alpha 5$ integrin expression or culture of HSCs in the presence of the soluble RGD peptide prevent FAK activation by FN (Figures 3C and 3D). Furthermore, this study also supports that FAK plays a functional role in the signaling pathway triggered by FN, since the percentage of apoptotic cells increased markedly after silencing FAK expression with an appropriated siRNA (Fig. 3A). Although integrin receptors may activate other antiapoptotic pathways [39], the role played by FAK in the protection of cells against apoptosis has been reported by other authors in a number of cell lines, but not in HSCs. Thus, microinjection of antibody to FAK induced apoptosis in fibroblasts. Likewise, introduction of a dominant negative FAK domain [40] or blocking FAK with antisense oligonucleotides resulted in a marked increase in the percentage of apoptotic cells [41]. On the contrary, it has been shown that FAK overexpressing cells are resistant against various apoptotic stimuli [42].

The present study shows that the antiapoptotic effect of FN on HSCs can be reversed by the pharmacological inhibition of PI3K by LY294002 or wortmannin (Fig. 4A). Inhibition of ERK, JNK, or NF κ B increased apoptotic rate following serum starvation (Fig. 4A), which indicated that these kinases and transcription factor are involved in the protection of HSCs against apoptosis caused by serum deprivation. However, in spite of being blocked these pathways, the survival effect of FN persisted (Fig. 4A). Only inhibition of PI3K abrogated the protection exerted by FN. These results indicate that PI3K and its downstream derivatives, but not ERK, JNK or NF κ B, may play a role in the mediation of the survival signal arising from FN adhesion. PI3K is a heterodimeric lipid kinase that has been shown to play a major role in the integrin-

stimulated signaling pathways and in the prevention of apoptosis. It is comprised of a 110 kDa catalytic subunit and an 85 kDa regulatory subunit. The association of phosphorylated FAK with p85 leads to the activation of PI3K, and subsequently of other kinases including Akt [43]. The involvement of the PI3K/Akt pathway in the protection of cells against apoptosis has been demonstrated in other cell lines, but not in HSCs [39, 44]. Akt is activated in response to a number of extracellular stimuli, including integrin adhesion to matrix [45], but, for full activation, Akt requires its phosphorylation at thr³⁰⁸ and ser⁴⁷³ by 3-phosphoinositide-dependent protein kinases 1 and 2 [43]. In the present study, we show that adhesion of HSCs to FN resulted, not only in the prevention of apoptosis, but also in the induction of Akt Ser⁴⁷³ phosphorylation. Pretreatment of cells with LY294002 abrogated this phosphorylation and induced apoptotic death (Fig. 3B). Phosphorylated Akt in turn phosphorylates many downstream substrates [43, 46], including several factors implicated in the apoptosis process, such as Bad.

Mitochondria play a central role in the apoptotic machinery, as they release a number of apoptogenic proteins into the cytoplasm, including cytochrome *c* [47, 48], in response to the increase in pro-apoptotic proteins in the mitochondrial membrane. While Bcl-2 and Bcl-X_L prevent mitochondrial permeabilization and block the release of cytochrome *c*, Bad, Bax and Bak, among others, cause membrane permeabilization, the release of cytochrome *c*, and apoptosis [49]. In death conditions, like serum deprivation, mitochondrial membrane permeability increases and cytochrome *c* translocates from the mitochondrial intermembrane space into the cytosol. Our study shows that growth of HSCs on FN-coated dishes increased markedly anti-apoptotic Bcl-2 gene expression and slightly decreased the pro-apoptotic Bax gene expression. Consequently, the ratio Bcl-2/Bax was strikingly increased in these cells (Fig. 5A). These results concur with those previously reported by others. Thus, a $\alpha 5 \beta 1$ integrin/FAK-dependent increased in Bcl-2 expression has also been found in other cell lines [50]. These changes in the pattern of Bcl-2 proteins in the mitochondrial membrane are likely involved in the survival of HSCs induced by FN. It has been hypothesized that the ratio Bcl-2 or Bcl-X_L to Bax or Bak determines whether cell lives or dies [22]. Bad is localized in the mitochondrial membrane bound to the pro-survival proteins Bcl-2 and Bcl-X_L, where it promotes cytochrome *c* release. When phosphorylated at ser¹³⁶, Bad is sequestered in the cytoplasm by interacting with the 14-3-3 proteins, thereby pre-

venting Bad interaction with Bcl-2/Bcl-X_L on the mitochondrial membrane [20, 21], and reducing apoptosis [51]. Our study shows that adhesion of HSCs to FN induced ser¹³⁶ phosphorylation of Bad by PI3K/Akt pathway and its binding to 14-3-3 proteins (Fig. 4C and 4D). Inhibition of this route with LY294002 abrogated Bad phosphorylation at ser¹³⁶, its physical interaction with 14-3-3 proteins, and the antiapoptotic effect of FN.

Our study also demonstrates that FN avoids the release of cytochrome *c* into the cytoplasm (Fig. 6A). This effect might be ascribed to the decrease in the proapoptotic proteins and to the increase in the anti-apoptotic proteins in the mitochondrial membrane (Bcl-2 and Bcl-X_L). Once released from the mitochondria, cytochrome *c* combines with Apaf-1, ATP/dATP, and caspase 9 [52] to form a complex called apoptosome, which leads to the activation of caspase 9 and afterward of the executioner caspase 3 and 7 [53]. The recruitment of procaspase 9 into the apoptosome leads to its dimerization and activation. Our study shows that activity of caspase 9 and 3 decreased in starving HSCs grown on FN (Fig. 6C). These reduced enzymatic activities are coupled with a marked decrease in the formation of apoptosome (Fig. 6B). Moreover, this effect of FN on caspase 9 activity is dependent on the activity of PI3K, since inhibition of this kinase abrogated FN-induced caspase 9 and 3 inactivation. Although the present study addresses only the integrin-FAK-PI3K-mitochondria pathway, parallel, unpublished studies have shown that other factors, such as survivin, IAP, among others, may also be implicated in the survival signal triggered by adhesion of FN to HSCs.

In conclusion, the present study shows that FN may contribute to the fibrogenic process by increasing survival of serum-deprived HSCs and that this effect is triggered by the binding of integrin $\alpha 5\beta 1$ cell surface receptors to the RGD tripeptides in the FN. After binding, FAK becomes phosphorylated at tyr³⁹⁷, which in turn activates PI3K and Akt. Following Bad phosphorylation and increased Bcl-2 and Bcl-X_L in mitochondrial membrane, cytochrome *c* release decreases and, consequently, the activity of caspase 9 and 3 declines.

Abbreviations

Apaf-1 (apoptotic protease activating factor-1); Bcl-2 (B-cell lymphoma 2); Bad (Bcl-2-antagonist of cell death); Bax (Bcl-2 associated X protein); ECM (extracellular matrix); FAK (focal adhesion kinase); FCS (fetal calf serum); FN (fibronectin); HSCs (hepatic stellate cells); PBS (phosphate-buffered saline); PI3K (phosphatidylinositol 3-Kinase); RGD (Arg-Gly-Asp); RGE (Arg-Gly-Glu); RT-PCR (real-time polymerase chain reaction).

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References

- 1 Friedman SL: Mechanisms of Hepatic Fibrogenesis. *Gastroenterology* 2008;134:1655-1669.
- 2 Urtasun R, Nieto N: Hepatic stellate cells and oxidative stress. *Rev Esp Enferm Dig* 2007;99:223-230.
- 3 Iredale JP, Benyon RC, Pickering J, McCullen M, Northrop M, Pawley S, Hovell C, Arthur MJ: Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. *J Clin Invest* 1998;102:538-549.
- 4 Douglass A, Wallace K, Parr R, Park J, Durward E, Broadbent I, Barelle C, Porter AJ, Wright MC: Antibody-targeted myofibroblast apoptosis reduces fibrosis during sustained liver injury. *J Hepatol* 2008;49:88-98.

- 5 Clark RA, An JQ, Greiling D, Khan A, Schwarzbauer JE: Fibroblast migration on fibronectin requires three distinct functional domains. *J Invest Dermatol* 2003;121:695-705.
- 6 Shenkman B, Brill G, Solpov A, Vitkovsky Y, Kuznik B, Koltakov A, Kotev-Emeth S, Savion N, Bank I: CD4+ lymphocytes require platelets for adhesion to immobilized fibronectin in flow: role of beta(1) (CD29)-, beta(2) (CD18)-related integrins and non-integrin receptors. *Cell Immunol* 2006;242:52-59.
- 7 Madsen CD, Sidenius N: The interaction between urokinase receptor and vitronectin in cell adhesion and signalling. *Eur J Cell Biol* 2008;87:617-629.
- 8 Milliano MT, Luxon BA: Initial signaling of the fibronectin receptor (alpha5beta1 integrin) in hepatic stellate cells is independent of tyrosine phosphorylation. *J Hepatol* 2003;39:32-37.
- 9 Dodig M, Ogunwale B, Dasarathy S, Li M, Wang B, McCullough A.J: Differences in regulation of type I collagen synthesis in primary and passaged hepatic stellate cell cultures: the role of alpha5beta1-integrin. *Am J Physiol Gastrointest Liver Physiol* 2007;293:G154-164.
- 10 Edderkaoui M, Hong P, Lee JK, Pandol SJ, Gukovskaya AS: Insulin-like growth factor-I receptor mediates the prosurvival effect of fibronectin. *J Biol Chem* 2007;282:26646-26655.
- 11 Kao CL, Lin HT, Chen YW, Chen YC, Tsai FT, Chang YL, Chiou SH, Sheu DC, Ku HH: Fibronectin suppresses lipopolysaccharide-induced liver damage and promotes the cytoprotection abilities of hepatocyte-like cells derived from human bone marrow mesenchymal stem cells. *Transplant Proc* 2007;39:3444-3445.
- 12 Rippe RA, Almounajed G, Brenner DA: Sp1 binding activity increases in activated Ito cells. *Hepatology* 1995;22:241-251.
- 13 Díaz-Sanjuan T, García-Ruiz I, Rodríguez-Juan C, Muñoz-Yagüe T, Solís-Muñoz P, Solís-Herruzo JA: Interferon α increases metalloproteinase-13 gene expression through a polyomavirus enhancer activator 3-dependent pathway in hepatic stellate cells. *J Hepatol* 2009;50:128-139.
- 14 Schagger H, von Jagow G: Blue native electrophoresis for isolation of membrane protein complexes in enzymatic active form. *Analytical Biochem* 1991;199:223-231.
- 15 Triepels RH, Hanson BJ, van den Heuvel LP, Sundell L, Marusich MF, Smeitink JA, Capaldi RA: Human complex I defects can be resolved by monoclonal antibody analysis into distinct subunit assembly patterns. *J Biol Chem* 2001;276:8892-8897.
- 16 Abramoff MD, Magelhaes PJ, Ram SJ: Image Processing with Image. *J Biophot Internat* 2004;11:36-42.
- 17 Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta CT$ method. *Methods* 2001;25:402-408.
- 18 Bustin SA: Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assay. *J Mol Endocrinol* 2000;25:169-193.
- 19 Almeida EAC, Illic D, Han Q, Hauck CR, Jin F, Kawakatsu H, Schlaepfer DD, Damsky CH: Matrix survival signalling: from fibronectin via focal adhesion kinase to c-jun NH2-terminal kinase. *J Cell Biol* 2000;149:741-754.
- 20 Masters SC, Yang H, Datta SR, Greenberg ME, Fu H: 14-3-3 inhibits Bad-induced cell death through interaction with serine-136. *Mol. Pharmacol.* 2001;60:1325-1331.
- 21 Subramanian RR, Masters SC, Zhang H, Fu H: Functional conservation of 14-3-3-isoforms in inhibiting Bad-induced apoptosis. *Exp Cell Res* 2001;271:142-151.
- 22 Lassus P, Opitz-Araya X, Lazebnik Y: Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science* 2002;297:1352-1354.
- 23 Brown LF, Dvorak AM, Dvorak HF: Leaky vessels, fibrin deposition, and fibrosis: a sequence of events common to solid tumors and to many other types of disease. *Am Rev Respir Dis* 1989;140:1104-1107.
- 24 Kishimoto C, Kitazawa M, Takada H: Interstitial fibrin-fibronectin deposition with T cell infiltrates precedes fibrosis in murine viral myocarditis. *Int J Exp Path* 1989;79:417-423.
- 25 Crawford DC, Chobanian AV, Brecher P: Angiotensin II induces fibronectin expression associated with cardiac fibrosis in the rat. *Circ Res* 1994;74:727-739.
- 26 Xing H, Cao Y, Weng D, Tao W, Song X, Wang W, Meng L, Xu G, Zhou J, Wang S, Ma D: Fibronectin-mediated activation of Akt2 protects human ovarian and breast cancer cells from docetaxel-induced apoptosis via inhibition of the p38 pathway. *Apoptosis* 2008;13:213-223.
- 27 Navarro-Alvarez N, Rivas-Carrillo JD, Soto-Gutierrez A, Yuasa T, Okitsu T, Noguchi H, Matsumoto S, Takei J, Tanaka N, Kobayashi N: Reestablishment of microenvironment is necessary to maintain in vitro and in vivo human islet function. *Cell Transplant* 2008;17:111-119.
- 28 He Y, Chen J, Ren J, Wang G, Cai G: Type I collagen inhibits hydroxyl radical-induced apoptosis. *J Biochem* 2002;132:373-379.
- 29 Priya S, Sudhakaran PR: Cell survival, activation and apoptosis of hepatic stellate cells: modulation by extracellular matrix proteins. *Hepatol Res* 2008;38:1221-1232.
- 30 Iwamoto H, Sakai H, Tada S, Nakamura M, Nawata H: Induction of apoptosis in rat hepatic stellate cells by disruption of integrin-mediated cell adhesion. *J Lab Clin Med* 1999;134:83-89.
- 31 McGill G, Shimamura A, Bates RC, Savage RE, Fisher DE: Loss of matrix adhesion triggers rapid transformation-selective apoptosis in fibroblasts. *J Cell Biol* 1997;138:901-911.
- 32 Sodek J, Ganss B, McKee MD: Osteopontin. *Crit Rev Oral Biol Med* 2000;11:279-303.
- 33 Iwamoto H, Sakai H, Nawata H: Inhibition of integrin signalling with arg-gly-asp motifs in rat hepatic stellate cells. *J Hepatol* 1998;29:752-759.
- 34 Gingras ME, Larouche K, Larouche N, Leclerc S, Salesse C, Guérin SL: Regulation of the integrin subunit alpha5 gene promoter by the transcription factors Sp1/Sp3 is influenced by the cell density in rabbit corneal epithelial cells. *Invest. Ophthalmol Vis Sci* 2003;44:3742-3755.
- 35 Basson MD: An intracellular signal pathway that regulates cancer cell adhesion in response to extracellular forces. *Cancer Res* 2008;68:2-4.
- 36 Reyes CD, Petrie TA, García AJ: Mixed extracellular matrix ligands synergistically modulate integrin adhesion and signaling. *J Cell Physiol* 2008;217:450-458.
- 37 Chatzizacharias NA, Kouraklis GP, Theocharis SE: Disruption of FAK signaling: a side mechanism in cytotoxicity. *Toxicology* 2008;245:1-10.
- 38 Lee JW, Juliano RL: The alpha5beta1 integrin selectively enhances epidermal growth factor signaling to the phosphatidylinositol-3-kinase/Akt pathway in intestinal epithelial cells. *Biochim Biophys Acta* 2002;1542:23-31.
- 39 Lee JW, Juliano RL: $\alpha 5 \beta 1$ integrin protects intestinal epithelial cells from apoptosis through a phosphatidylinositol 3-kinase and protein kinase B-dependent pathway. *Mol Biol Cell* 2000;11:1973-1987.
- 40 Heidkamp MC, Bayer AL, Kalina JA, Eble DM, Samarel AM: GFP-FRNC disrupts focal adhesions and induces anoikis in neonatal rat ventricular myocytes. *Circ Res* 2002;90:1282-1289.
- 41 Zhang Y, Lu H, Dazin P, Kapila Y: Squamous cell carcinoma cell aggregates escape suspension-induced, p53-mediated anoikis. *J Biol Chem* 2004;279:48342-48349.

- 42 Sonoda Y, Matsumoto Y, Funakoshi M, Yamamoto D, Hanks SK, Kasahara T: Anti-apoptotic role of focal adhesion kinase (FAK). Induction of inhibitor-of-apoptosis proteins and apoptosis suppression by the overexpression of FAK in a human leukemic cell line, HL-60. *J Biol Chem* 2000;273:16309-16315.
- 43 Cantley LC: The phosphoinositide 3-kinase pathway. *Science* 2002;296:1655-1657.
- 44 Hatano E, Brenner DA: Akt protects mouse hepatocytes from TNF- α - and Fas-mediated apoptosis through NF κ B activation. *Am J Physiol Gastrointest Liver Physiol* 2002;282:G1357-1368.
- 45 Kotha J, Longhurst C, Appling W, Jennings LK: Tetraspanin CD9 regulates beta 1 integrin activation and enhances cell motility to fibronectin via a PI-3 kinase-dependent pathway. *Exp Cell Res* 2008;314:1811-1812.
- 46 Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB: Exploiting the PI3K/Akt pathways for cancer drug discovery. *Nature Rev* 2005;4:988-1004.
- 47 Ashkenazi A, Herbst RS: To kill a tumor cell: the potential of proapoptotic receptor agonists. *J Clin Invest* 2008;118:1979-1990.
- 48 Solís-Herruzo JA, García-Ruiz I, Pérez-Carreras M, Muñoz-Yagüe MT: Non-alcoholic fatty liver disease. From insulin resistance to mitochondrial dysfunction. *Rev Esp Enferm Dig* 2006;98:844-874.
- 49 Daniel PT, Schultze-Osthoff, K., Guner, D: Guardians of cell death: the Bcl-2 family proteins. *Assays Biochem* 2003;39:73-88.
- 50 Lee BH, Ruoslahti E: Alpha5beta1 integrin stimulates Bcl-2 expression and cell survival through Akt, focal adhesion kinase, and Ca²⁺/calmodulin-dependent protein kinase IV. *J Cel Biochem* 2005;95:1214-1223.
- 51 Wang XT, Pei DS., Xu J, Guan QH, Sun YF, Liu XM, Zhang GY: Opposing effects of Bad phosphorylation at two distinct sites by Akt1 and JNK1/2 on ischemic brain injury. *Cell Signal* 2007;19:1844-1856.
- 52 Green DR: Apoptotic pathways: ten minutes to dead. *Cell* 2005;121:671-674.
- 53 Wang X: The expanding role of mitochondria in apoptosis. *Genes Dev* 2001;15:2922-2933.