LIVER DISEASE

Overexpression of Bcl-2 by activated human hepatic stellate cells: resistance to apoptosis as a mechanism of progressive hepatic fibrogenesis in humans

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Background and aims: Myofibroblast-like cells, originating from activation of hepatic stellate cells (HSC/MFs), play a key role in liver fibrosis, a potentially reversible process that may rely on induction of HSC/MFs apoptosis. While this possibility has been shown in cultured rat HSC, very limited data are currently available for human HSC/MFs.

Methods: Cultured human HSC/MFs were exposed to several proapoptotic stimuli, including those known to induce apoptosis in rat HSC/MFs, and induction of cell death and related mechanisms were investigated using morphology, molecular biology, and biochemical techniques.

Results: In this study we report that fully activated human HSC/MFs did not undergo spontaneous apoptosis and survived to prolonged serum deprivation, Fas activation, or exposure to nerve growth factor, tumour necrosis factor α (TNF-α), oxidative stress mediators, doxorubicin, and etoposide. Induction of caspase dependent, mitochondria driven apoptosis in HSC/MFs was observed only when protein synthesis or transcription were inhibited. Importantly, the process of HSC activation was accompanied by changes in expression of a set of genes involved in apoptosis control. In particular, activated human HSC/MFs in culture overexpressed Bcl-2. The role of Bcl-2 was crucial as Bcl-2 silenced cells became susceptible to TNF-α induced apoptosis. Finally, Bcl-2 was markedly expressed in HSC/MFs present in liver tissue obtained from patients with hepatitis C virus related cirrhosis.

Conclusions: Human activated HSC/MFs are resistant to most proapoptotic stimuli due to Bcl-2 overexpression and this feature may play a key role in the progression of fibrosis in chronic liver diseases.

Fibrotic progression of chronic liver diseases (CLDs) of different aetiology is accompanied by perpetuation of liver injury (that is, parenchymal necrosis) and/or chronic hepatitis, associated with persisting activation of tissue repair mechanisms and excess deposition of extracellular matrix.[1–5] A key role in fibrogenesis has been attributed to hepatic stellate cells (HSC) that, in CLDs or in culture, undergo a characteristic process of activation in which they (trans)differentiate from a quiescent phenotype (that is, vitamin A storing phenotype) into a highly proliferative, fibrogenic, proinflammatory, and contractile myofibroblast-like phenotype (HSC/MFs).[1–5] Although myofibroblast-like cells in CLDs have also been proposed to originate from portal (myo)fibroblasts[6–8] or even bone marrow derived stem cells,[9] most of the data concerning molecular and cellular mechanisms of liver fibrogenesis derive from studies performed in cultured rat and human HSC/MFs.

An emerging issue in hepatology is that liver fibrosis and even cirrhosis may be potentially reversible.[1 10] Recovery from acute or chronic injury in animal models is characterised by apoptosis of HSC/MFs and, as a consequence, reduction of tissue inhibitor of metalloproteinase (TIMP) levels and progressive degradation of fibrotic matrix.[11–13] Candidate survival factors for HSC/MFs, including transforming growth factor β1, insulin-like growth factor 1, TIMP-1, and type I collagen rich matrix have been proposed,[1 10] together with persistent activation of nuclear factor κB (NFκB), induction of novel Rel-like factors, and prolonged changes in the expression of IkB family proteins.[14] Recent studies indicate that rat HSC/MFs can indeed undergo apoptosis induced by Fas ligand,[15–17] nerve growth factor (NGF),[18] tumour necrosis factor (TNF) related apoptosis inducing ligand,[19] agonists of the peripheral type benzodiazepine receptor,[20] activated Kupffer cells,[21] gliotoxin,[22] or following adenoviral mediated transfer of p53 or retinoblastoma protein.[23] Gliotoxin also significantly decreased the number of activated HSC/MFs in rats treated in vivo with carbon tetrachloride.[22]

Regression of liver fibrosis and, possibly, cirrhosis has also been reported in patients with chronic liver disease (reviewed by Friedman,[1] Iredale,[10], and Desmet and Roskams[24]) once cessation of the causative agent was ensured. Although the possibility of complete regression of cirrhosis is uncertain,[24] specific induction of apoptosis in human HSC/MFs may represent a realistic objective for cell targeted therapy of liver fibrosis. However, information concerning the susceptibility to apoptosis of human HSC/MFs,[25] immortalised HSC/MFs,[26] or human hepatic myofibroblasts[27] is still scarce.

Abbreviations: CLDs, chronic liver diseases; DAPI, 4,6-diamidine-2-phenylindole di-hydrochloride; Δψm, mitochondrial membrane potential; ECL, enhanced chemiluminescence; ERK, extracellular regulated kinase; FasL, Fas ligand; HCV, hepatitis C virus; HSC, hepatic stellate cells; HSC/MFs, activated hepatic stellate cells in myofibroblast-like phenotype; JC-1, J-aggregate forming lipophilic cation; LDH, lactate dehydrogenase; mAb, monoclonal antibody; NGF, nerve growth factor; NFκB, nuclear factor κB; PARP, poly (ADP ribose) polymerase; SFI medium, serum free Iscove's medium; α-SMA, α smooth muscle actin; siRNA, small interfering RNA; TBS, Tris buffered saline; h-TERT, human telomerase catalytic subunit; TIMP, tissue inhibitor of metalloproteinases; TNF-α, tumour necrosis factor α; TrkA, tyrosine kinase A

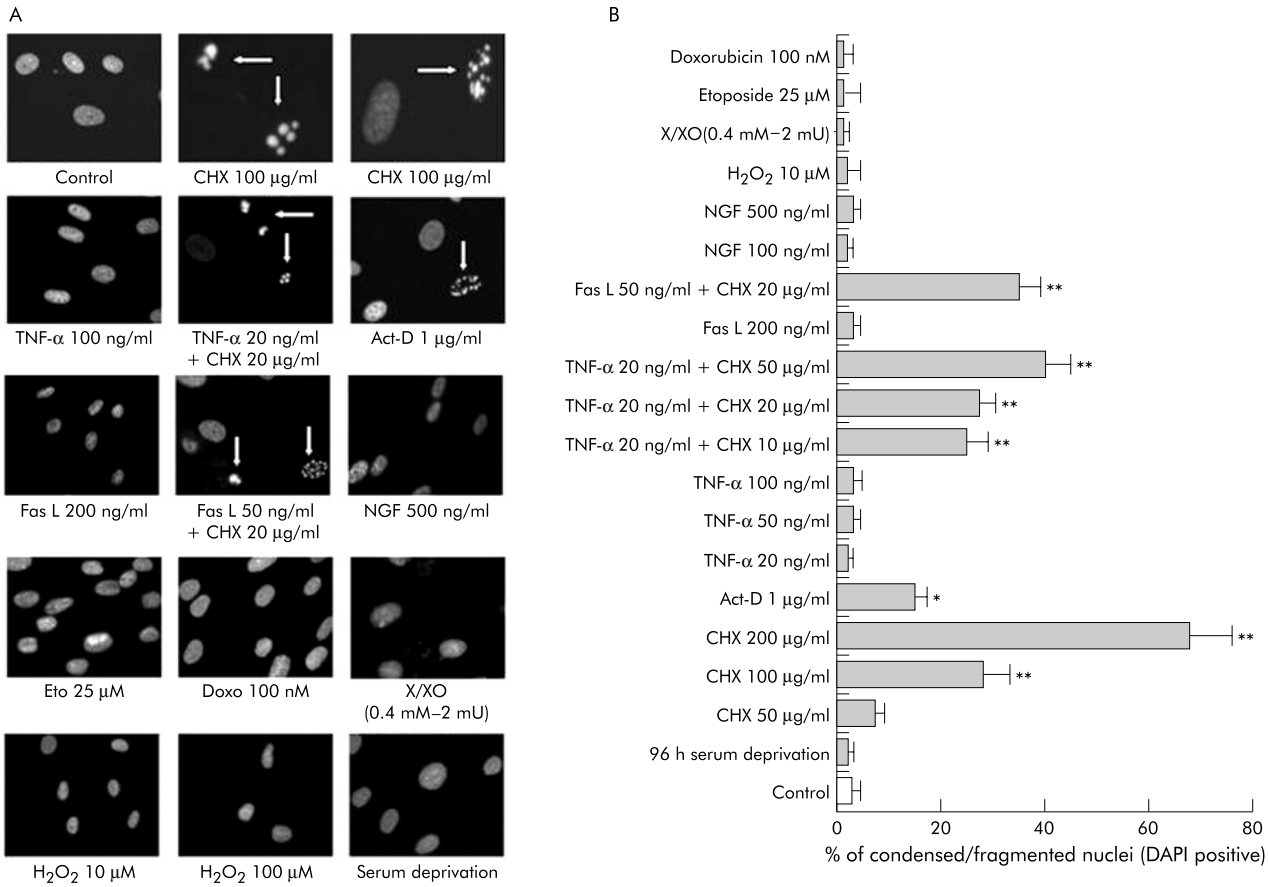

Figure 1 Human activated hepatic stellate cells in myofibroblast-like phenotype (HSC/MFs) survive most proapoptotic stimuli. (A) DAPI fluorescent staining for DNA was performed in human HSC/MFs exposed for 24 hours to different proapoptotic stimuli at the indicated concentrations. Representative images, relative to the highest non-necrotic doses used, are presented in (A) (original magnification ×400). Arrows indicate nuclear changes (that is, DNA fragmentation) that may be immediately indicative of classical apoptosis. (B) Data are expressed as means (SEM) (three experiments for each condition) and summarise evaluation of per cent of condensed/fragmented nuclei for any single tested condition. *$p<0.05$, **$p<0.01$ versus control values. Act-D, actinomycin D; CHX, cycloheximide; DAPI, 4,6-diamidine-2-phenylindole di-hydrochloride; Doxo, doxorubicin; Eto, etoposide; FasL, Fas ligand; H_2O_2, hydrogen peroxide; NGF, nerve growth factor; TNF-α, tumour necrosis factor α; X/XO, hypoxanthine/xanthine oxidase system generating superoxide anion.

In the present study, the susceptibility of activated human HSC/MFs to different proapoptotic stimuli was tested. Activated human HSC/MFs survive most proapoptotic stimuli of potential pathophysiological or therapeutic relevance, possibly due to an activation dependent altered balance between pro- and antiapoptotic molecules, with a major mechanistic role for Bcl-2 overexpression.

METHODS

Materials

Enhanced chemiluminescence (ECL) reagents and nitrocellulose membranes Hybond-C extra were from Amersham Pharmacia Biotech (Cologno Monzese, Milano, Italy), J-aggregate forming lipophilic cation (JC-1) dye from Molecular Probes Europe BV (Leiden, the Netherlands), NGF from Peprotech EC Ltd (London, UK), and Fas ligand (FasL), as activating anti-Fas IgM, from Upstate Biotech. (Lake Placid, New York, USA). Monoclonal and polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, California, USA) except those against Fas, Bcl-2, and Bax (Oncogene, San Diego, California, USA). All the other reagents were from Sigma Chemical Co. (Sigma Aldrich Spa, Milano, Italy).

Cell isolation and culture

The use of human material was approved by the Human Research Review Committee of the University of Florence, where cells were isolated and characterised from surgical wedge sections of human livers not suitable for transplantation, as extensively described elsewhere.[28] Cells obtained from samples of different human livers were cultured in Iscove's medium supplemented with 20% fetal bovine serum. After reaching confluence in the primary culture, serial passages were obtained always applying a 1:3 split ratio. Unless otherwise stated, cell were used between serial passages 4 and 7. At these stages of culture, HSCs show phenotypic features of fully activated HSC/MFs and a profile of cell surface markers identical to that of ''interface'' myofibroblasts described in fibrotic and cirrhotic human livers.[7 8] HSC/MFs were plated in order to obtain the desired subconfluence level (65–70%) and then incubated for 24 hours in serum free Iscove's (SFI) medium in order to obtain cells at the lowest level of spontaneous proliferation before addition of the different stimuli. Mean distribution for cell cycle in cells serum starved for 24 hours and then cultured for an additional 20 hours is approximately 50% in G0/G1 phase, 35% in S phase, and the remaining in G2/M phase.[29] Moreover, 24 hour serum deprived cells continue to increase in cell number in serum free medium for at least an additional 96 hours, at which time the number of cells is found to be doubled.[30] Accordingly, in some experiments, cells were also left in SFI medium for up to 72–96 hours in order to evaluate susceptibility to serum deprivation.

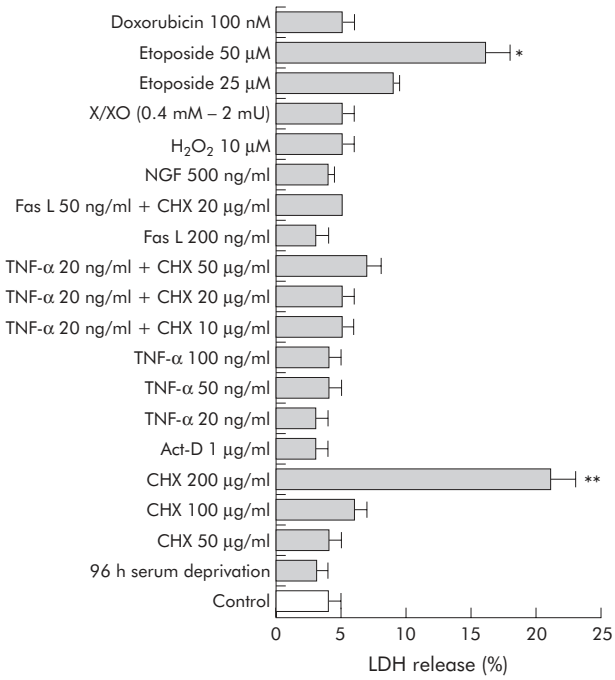

Figure 2 Analysis of necrotic cell death. Analysis of lactate dehydrogenase (LDH) release in culture medium was analysed in human activated hepatic stellate cells in myofibroblast-like phenotype (HSC/MFs), exposed for 24 hours to the indicated conditions. Data are expressed as means (SEM) (three experiments for each condition). *$p<0.05$, **$p<0.01$ versus control values. Act-D, actinomycin D; CHX, cycloheximide; FasL, Fas ligand; H_2O_2, hydrogen peroxide; NGF, nerve growth factor; TNF-α, tumour necrosis factor α; X/XO, hypoxanthine/xanthine oxidase system generating superoxide anion.

In some experiments the following cell preparations were also used: (a) freshly isolated (that is, quiescent and non-cultured) HSC; (b) late passaged, slowly, or non-proliferating flattened/enlarged cells in culture, mostly (usually more than 90%) positive to senescence associated β-galactosidase histochemical staining.[31]

Western blot

Cell lysates obtained by HSC/MFs were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis on 10% or 7.5% acrylamide gels. Blots were incubated with the desired primary antibodies and then incubated with peroxidase conjugated antimouse or antirabbit immunoglobulins in Tris buffered saline (TBS)-Tween containing 2% (wt/vol) non-fat dry milk, as previously described,[25 32 33] and developed with ECL reagents according to the manufacturer's instructions.

Bcl-2 silencing by small RNA interference

The chosen small interfering RNA (siRNA) sequence is specific for human Bcl-2 based on BLAST search (NCBI database) and targets human Bcl-2 mRNA at sequence 5′-GTA CAT CCA TTA TAA GCT G-3′.Sense siRNA sequence was 5′-GUA CAU CCA UUA UAA GCU GdT dT-3′. Antisense siRNA was 3′-d(TT) CAU GUA GGU AAU AUU CGA C-5′. The siRNA and related non-silencing control (negative control) were synthesised by Qiagen-Xeragon (Germantown, Maryland, USA). Transfection of human HSC/MFs was performed using the Amaxa nucleofection technology (Amaxa, Koeln, Germany), as previously described.[34] Bcl-2 protein levels were analysed by western blot 72 hours after transfection.

Evaluation of cell death and apoptosis

Necrotic cell death (release in culture medium of lactate dehydrogenase), DAPI fluorescence staining to detect nuclear DNA, and caspase 3-like activity (fluorometric) were evaluated as reported elsewhere.[25] DNA fragmentation was evaluated using the DNA ladder technique.[35] Mitochondrial membrane potential ($\Delta\psi m$) was monitored using the JC-1 dye technique and analysed in a FACScan (Becton Dickinson, Milan, Italy, equipped with CellQuest software).[36] Plasma membrane expression of Fas was detected by cytofluorimetric analysis, as previously described.[37]

Human tissues and immunohistochemistry

These experiments were conducted on frozen surgical sections of human liver, as described in detail elsewhere.[34 38] Normal human liver tissue was obtained from two surgical liver biopsies from two patients undergoing uncomplicated cholecystectomy. Liver tissue characterised by evident cirrhosis (METAVIR F4) was obtained from four patients with hepatitis C virus (HCV) related liver cirrhosis undergoing orthotopic liver transplantation. The use of this material conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the University of Florence Human Research Review Committee. Serial cryostat sections were dried and then sequentially incubated with either the primary anti-Bcl-2 monoclonal antibody (mAb) or with the primary anti-α-smooth muscle actin (SMA) mAb and, after washing, with an affinity purified rabbit antimouse antibody. At the end of the incubation, sections were washed twice in TBS and then incubated with alkaline antialkaline phosphatase and developed. A non-immune mouse IgG primary antibody was used as a negative control.

Statistical analysis

Data in bar graphs represent means (SEM) and were obtained from average data of at least three independent experiments. Luminograms and morphological images are representative of at least three experiments with similar results. Statistical analysis was performed using the Student's *t* test or ANOVA for analysis of variance when appropriate ($p<0.05$ was considered significant).

RESULTS

Fully activated human HSC/MFs survive most proapoptotic stimuli

Fully activated human HSC/MFs (passages 4–7) survived to serum deprivation for as long as 96 hours or when exposed to TNF-α (20–100 ng/ml), NGF (10–500 ng/ml), FasL (50–200 ng/ml), doxorubicin (10–200 nM), etoposide (1–25 μM), and oxidative stress mediators such as hydrogen peroxide (0.1–100 μM), superoxide anion and, as already reported in detail elsewhere, 4-hydroxynonenal.[25] Lack of induction of apoptosis by these agents was supported by the absence of nuclear condensation/fragmentation (fig 1A, B), poly (ADP ribose) polymerase (PARP) cleavage, or activation of caspase 3-like activity (not shown). In addition, no evidence of significant cell detachment was detected within 48 hours of treatment (72–96 hours for serum deprivation experiments, not shown). Only etoposide, that did not induce apoptosis at any concentration (fig 1A,B), caused dose dependent necrotic cell death, as shown by lactate dehydrogenase (LDH) release (fig 2). Gliotoxin (0.1 and 1.0 μM) represented the only exception being very effective in causing rapid detachment of cells from the culture support and nuclear condensation, in agreement with previous reports.[22 26] However, these changes were neither prevented by the pan-caspase inhibitor zVAD.fmk nor associated with nuclear fragmentation or a significant increase in caspase 3-like

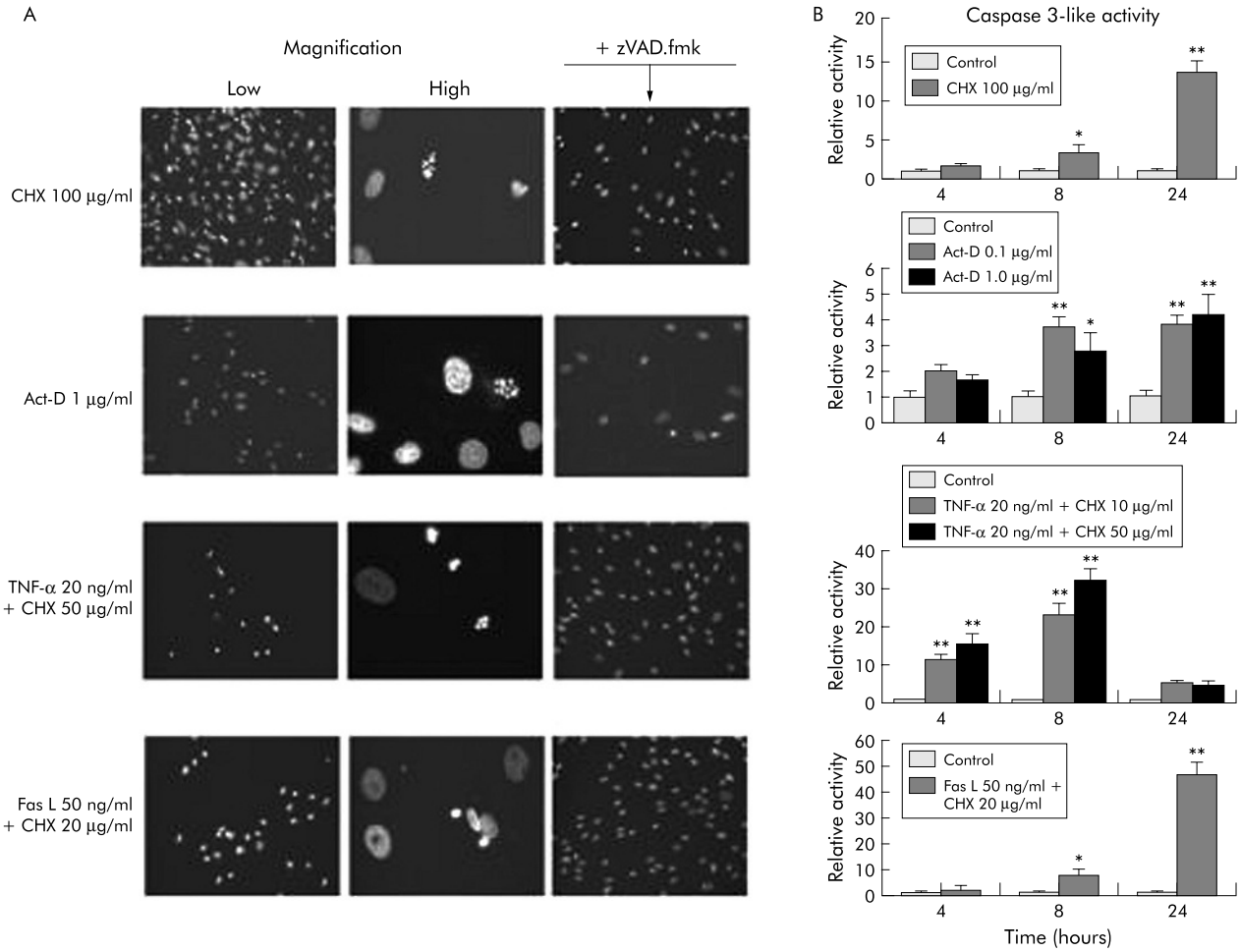

Figure 3 Induction of apoptosis in human activated hepatic stellate cells in myofibroblast-like phenotype (HSC/MFs) requires inhibition of protein synthesis or of transcription. (A) Apoptosis induction was analysed in human HSC/MFs exposed to the indicated concentrations of cycloheximide (CHX), actinomycin D (Act-D), tumour necrosis factor α (TNF-α)/CHX, and Fas ligand (FasL)/CHX. Evidence of nuclear condensation/fragmentation (DAPI staining, 24 hours), as shown in representative images (original magnification: low ×100, high ×400) in the absence or presence of the pan-caspase inhibitor zVAD.fmk. (B) Time dependent fluorometric analysis of caspase 3-like activity. Data are expressed as means (SEM) (three experiments for each condition). *p<0.05, **p<0.01 versus control values.

activity (data not shown), suggesting that gliotoxin may induce a form of caspase independent, apoptosis-like programmed cell death.[39]

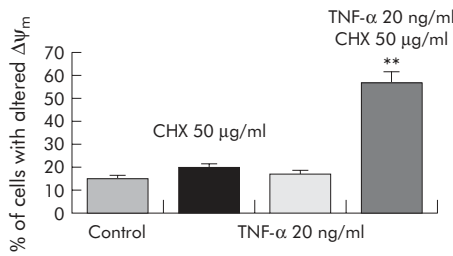

Figure 4 Cycloheximide (CHX) and tumour necrosis factor α (TNF-α)/CHX induce apoptosis in human activated hepatic stellate cells in myofibroblast-like phenotype (HSC/MFs) by involving mitochondria. Cytofluorimetric analysis of changes in mitochondrial membrane potential (Δψm) in untreated (control) activated human HSC/MFs (HSC34, passage 7) or in the same cells exposed for six hours to either effective proapoptotic stimulus (TNF-α/CHX, used at the indicated concentrations) or to non-significantly effective stimuli represented by TNF-α alone (20 ng/ml) or CHX alone (50 µg/ml). Data are expressed as means (SEM) (three experiments for each condition). **p<0.01 versus control values.

Lack of apoptosis induction could not be ascribed to the absence of the cognate receptors for some of the agents used or to an incomplete apoptotic machinery because human HSC/MFs indeed express Fas (CD-95), pro-caspase 8, and pro-caspase 9 (data not shown) as well as TNF-α receptors[7] and both p75 and tyrosine kinase A receptors for NGF.[40]

Our results clearly indicate that caspase dependent apoptosis is inducible in human HSC/MFs only by actinomycin D (0.1–1.0 µg/ml) or relatively high dose of cycloheximide (100 µg/ml), as well as by TNF-α or FasL when used in combination with lower concentrations of cycloheximide (that is, not proapoptotic per se). This was shown by the presence of nuclear condensation/fragmentation (fig 1A,B; 3A), absence of significant LDH release at the time of maximal apoptosis (fig 2), significant effect of pan-caspase inhibitor zVAD.fmk in preventing cell death (fig 3A), and activation of caspase 3-like activity (fig 3B).

Overexpression of Bcl-2 in human HSC/MFs: a crucial ''in vitro'' and ''in vivo'' antiapoptotic feature of activated cells

To investigate molecular mechanisms underlying the resistance to apoptosis of human HSC/MFs, cytofluorimetric analysis was performed using the fluorescent tracer JC-1, a dye sensitive to mitochondrial transmembrane potential

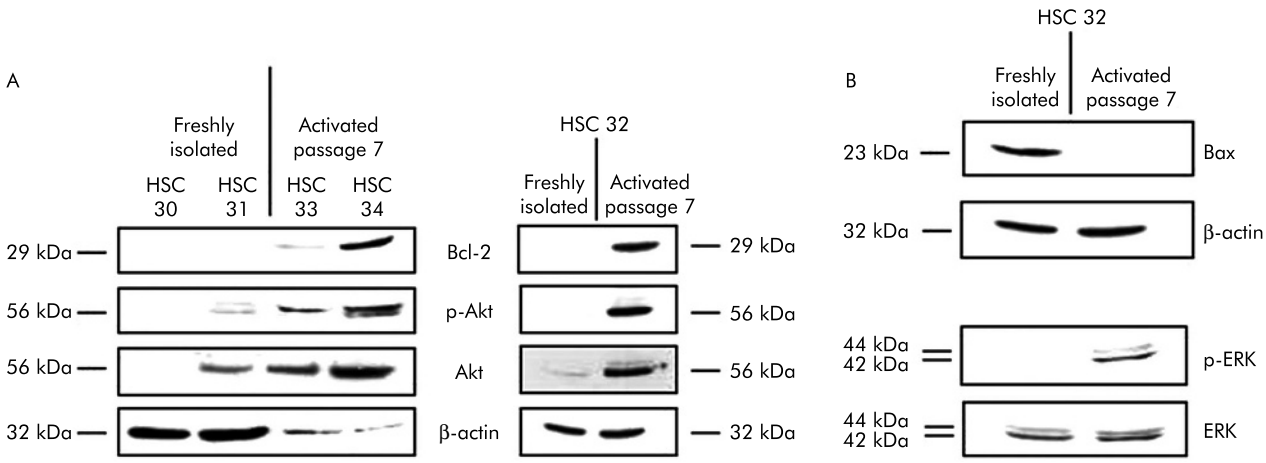

Figure 5 Activation dependent changes in levels of pro- and antiapoptotic proteins. (A) Comparative analysis of proteins involved in the control of apoptosis in human activated hepatic stellate cells in myofibroblast-like phenotype (HSC/MFs) (passage 7) and freshly isolated HSC. In the first series of experiments, western blot analysis for Bcl-2, phospho-Akt, and c-Akt was performed on lysates obtained from three different lines of activated HSC/MFs and from three lines of freshly isolated HSC. In the left part of (A), loading of samples (as shown in any image by protein levels of β-actin) was deliberately higher for samples from freshly isolated cells. In order to unequivocally determine whether bcl-2 bands were really absent from freshly isolated cells, Bcl-2 blots were also exposed for longer time. Data obtained from the same line (HSC 32) with equal loading are shown on the right panel of (A). (B) In the second series of experiments, additional parameters were investigated by western blot analysis performed on lysates obtained from the same line HSC 32. Images are representative of at least three experiments for any parameter.

($\Delta\psi m$). These experiments indicate that TNF/CHX induced apoptosis occurred with loss of $\Delta\psi m$ in a significant number of cells (fig 4). As a consequence, we compared different proteins or signalling pathway elements involved in the regulation of $\Delta\psi m$ in three different preparations of fully activated HSC/MFs and in freshly isolated (quiescent) non-cultured human HSC (fig 5A, B). While freshly isolated HSC showed high levels of the proapoptotic molecule Bax and undetectable expression of Bcl-2, activated HSC/MFs had a complete reversal in the Bcl-2/Bax ratio, as indicated by high levels of Bcl-2 and disappearance of Bax. In agreement with these findings, levels of phospho-extracellular regulated kinase (ERK) and phospho-Akt, which correlate with their activation status, were remarkably higher in activated cells. Interestingly, re-blotting the same membranes for the non-phosphorylated form of Akt indicated that activated cells constitutively express higher total levels of this protein (fig 5A).

In order to assess whether or not overexpression of Bcl-2 is present under in vivo conditions characterised by long term activation of HSC/MFs, immunohistochemistry for either Bcl-2 or α-SMA (a marker of activated myofibroblast-like cells in chronic liver diseases) was performed on serial cryostat sections of human HCV related cirrhosis (METAVIR score F4). Expression of Bcl-2 in normal liver, similar to that described previously in the literature,[41] was confined to some epithelial cell lining bile ductules and small interlobular bile ducts as well as some portal lymphocytes but not hepatocytes or other non-parenchymal cells (data not shown). In human cirrhotic liver with extensive fibrosis and cirrhosis (fig 6A, B), hepatocytes were still negative and Bcl-2 positivity, apart from mononuclear (that is, lymphocytes, macrophages) cells or some biliary epithelial cells, was clearly detectable in sinusoidal/perisinusoidal cells characterised by the typical elongated myofibroblast-like shape, with Bcl-2 staining distributed uniformly in the cytoplasm, closely corresponding to α-SMA positive staining, detectable in cells in corresponding serial sections. These aspects were particularly concentrated in areas located at the interface between fibrotic septa and the parenchyma of cirrhotic nodules, whereas they were scarce or absent in the centre of cirrhotic nodules. In addition, these findings were consistently present in similar areas of each section and were comparable in the four different human liver specimens analysed.

Bcl-2 silencing results in increased sensitivity of human HSC/MFs to TNF-α induced apoptosis

To provide a more mechanistic link between changes in the levels of proteins controlling apoptosis and susceptibility to cell death, we first analysed the behaviour of late passaged/senescent cells (usually more than 90% positive to senescence associated β-galactosidase staining, see fig 7A). These late passaged/senescent cells showed (fig 7B) a dramatic decrease in Bcl-2 levels and, to a less extent, phospho-Akt levels. Moreover, these late passaged/senescent cells became sensitive to TNF-α alone which induced a mitochondrial dependent cell death accompanied by loss of $\Delta\psi m$ (fig 7C). Remarkably, these changes were observed in cells derived from the same human liver as that used for the experiment shown in previous figures (HSC 34, compare figs 4 and 5 with figs 7B and 7C).

Finally, to unequivocally investigate in fully activated human HSC/MFs the possible mechanistic role of Bcl-2 overexpression in conferring resistance to apoptosis, Bcl-2 expression was knocked down by RNA interference strategy. Specifically designed siRNA effectively reduced Bcl-2 protein levels (fig 8A) and sensitised Bcl-2 silenced HSC/MFs, but not control cells (that is, the same cells treated with a non-silencing siRNA), to TNFα-induced apoptosis, as indicated by altered morphology, nuclear condensation and fragmentation, and detachment of approximately 15–20% of cells from culture dishes (figs 8B, 9, 10).

DISCUSSION

The present study represents the first comprehensive attempt to analyse the susceptibility of activated human HSC/MFs in their myofibroblast-like phenotype to undergo caspase dependent apoptosis. Human HSC/MFs can survive a number of well established proapoptotic stimuli or culture conditions, some of which have been reported previously to induce caspase dependent apoptosis in cultured rat HSC/MFs. Indeed, the available experimental evidence indicates that cultured rat HSC/MFs can undergo spontaneous apoptosis, a phenomenon typically seen in parallel with HSC activation.[15]

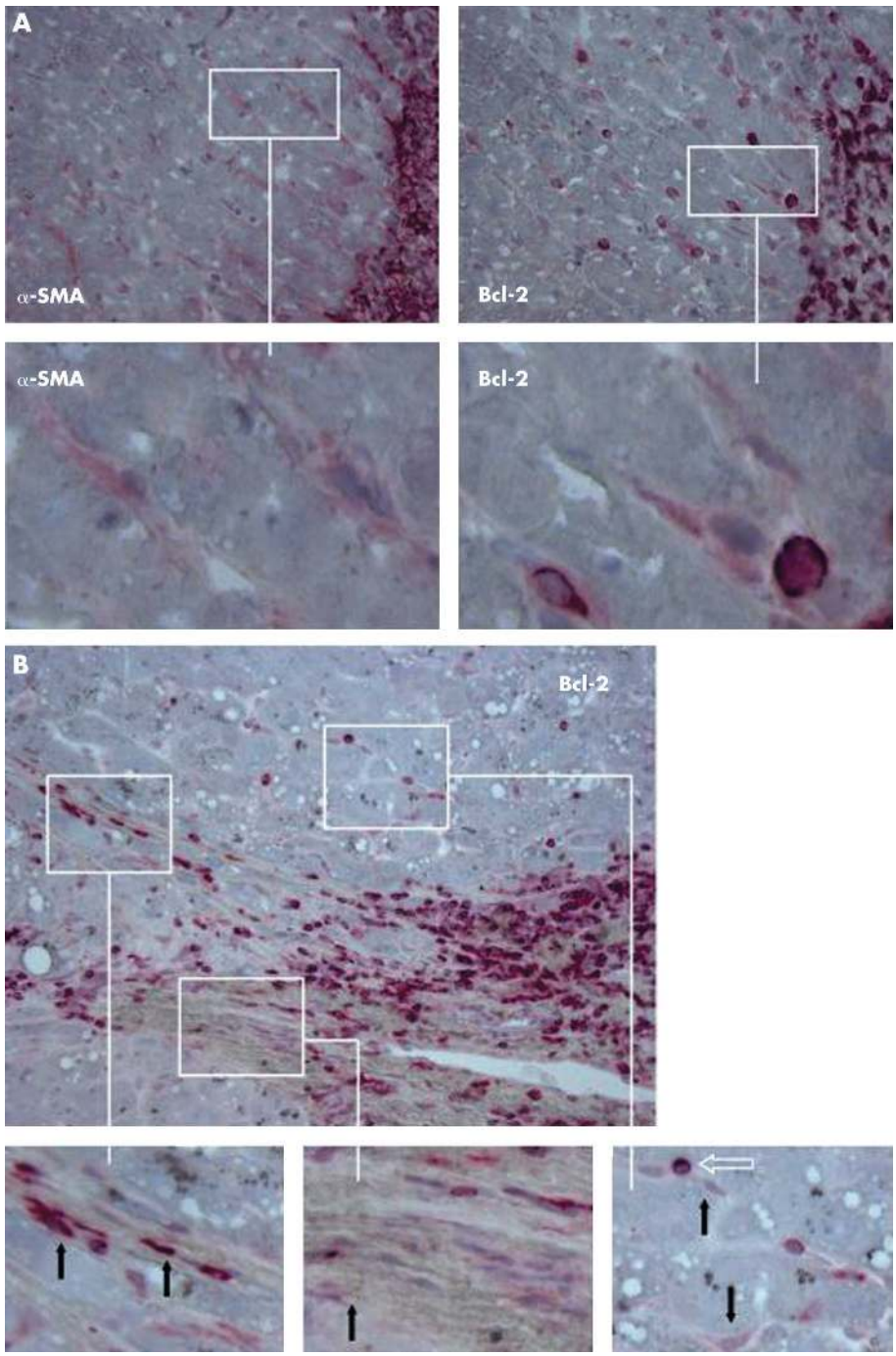

Figure 6 Distribution and localisation of Bcl-2 in cirrhotic human liver tissue. Immunohistochemical staining (alkaline antialkaline phosphatase) on serial sections of cirrhotic human liver tissue (hepatitis C virus related) with anti-α-smooth muscle actin (α-SMA) monoclonal antibody (mAb) (A) or with anti-Bcl-2 mAb (A, right images, and B). Nuclear counterstaining with haematoxylin. Insets represent digital magnification of selected fields of acquired images (original magnification ×200). The solid arrows in (B) insets indicate Bcl-2 positive myofibroblast-like cells whereas the open arrow indicates a mononuclear cell positive for Bcl-2.

Moreover, the process of activation in rat HSC apparently leads to enhanced sensitivity to the proapoptotic action of FasL.[15 16] However, the sensitivity of rat HSC/MFs to FasL alone was reported in only one study[15] whereas in others induction of apoptosis by FasL required the use of sensitisers (cycloheximide or actinomycin D).[16 17] Human HSC/MFs do not undergo significant spontaneous apoptosis, survive for as long as 72–96 hours to serum deprivation and to FasL concentrations 4–5 times higher than those necessary to induce apoptosis in Jurkat T cells (data not shown). Moreover, inhibition of protein synthesis by cycloheximide or of transcription by actinomycin D is an absolute requirement for induction of caspase dependent cell death in human cells.

Survival to TNF-α alone is in principle not surprising and may be the consequence of persistent status of activation of NFκB serving as a survival signal in activated HSC/MFs.[14] In this respect, human HSC/MFs behave similarly to several non-transformed or neoplastic cell types that undergo TNF-α dependent apoptosis only in the presence of cycloheximide or actinomycin D.[16 17 39 42] However, persistent activation of NFκB may not be the main explanation for resistance to apoptosis induction in human cells. Indeed, although blockade of NFκB using an adenovirus expressing an IkB dominant negative protein is known to sensitise rat HSC/MFs to TNF-α induced apoptosis,[43] the same procedure has been shown to be ineffective in human cells.[44] Accordingly, in a series of preliminary experiments, we were unable to

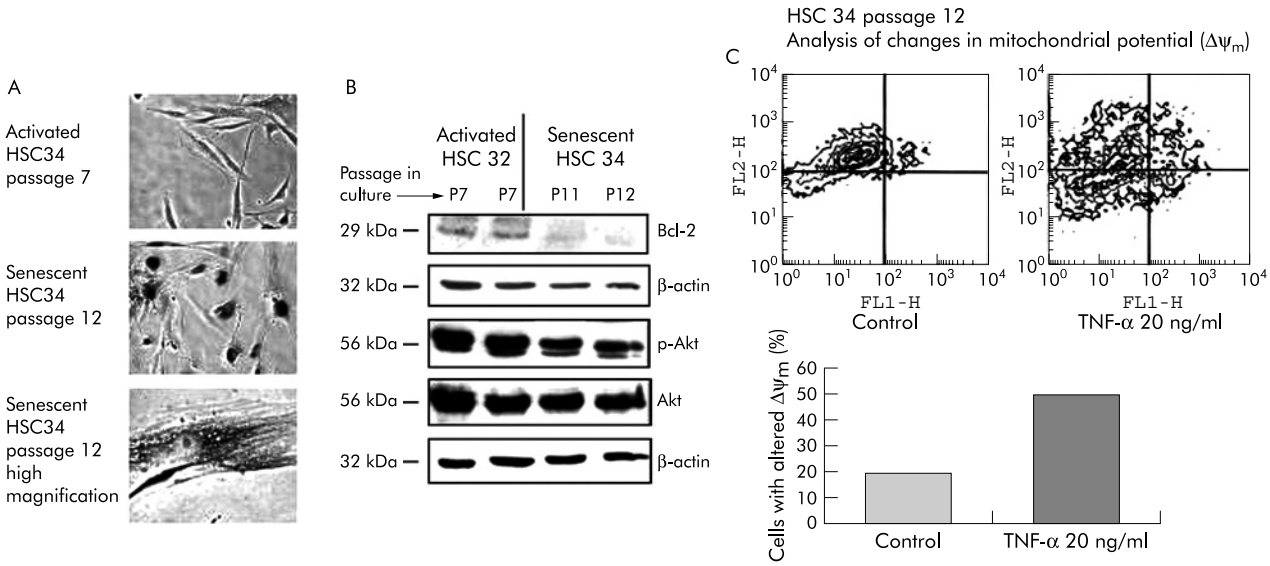

Figure 7 Senescence dependent downregulation of survival attitude in human activated hepatic stellate cells in myofibroblast-like phenotype (HSC/MFs). Senescent human HSC/MFs (positive for senescence associated β-galactosidase staining (A); original magnification ×200) change their pattern of expression for critical proteins involved in the control of apoptosis, as shown by western blot analysis (B), and become sensitive to mitochondrial induced apoptosis by tumour necrosis factor α (TNF-α) alone, as shown by cytofluorimetric analysis of mitochondrial membrane potential (Δψm) (C, original images plus evaluation of cells with altered potential in bar graph). For western blot analysis, equal loading of samples was monitored by analysing levels of β-actin. Data and images are representative of three different experiments.

sensitise human HSC/MFs to TNF-α by pretreating cells with a number of pharmacological inhibitors of NFκB (data not shown).

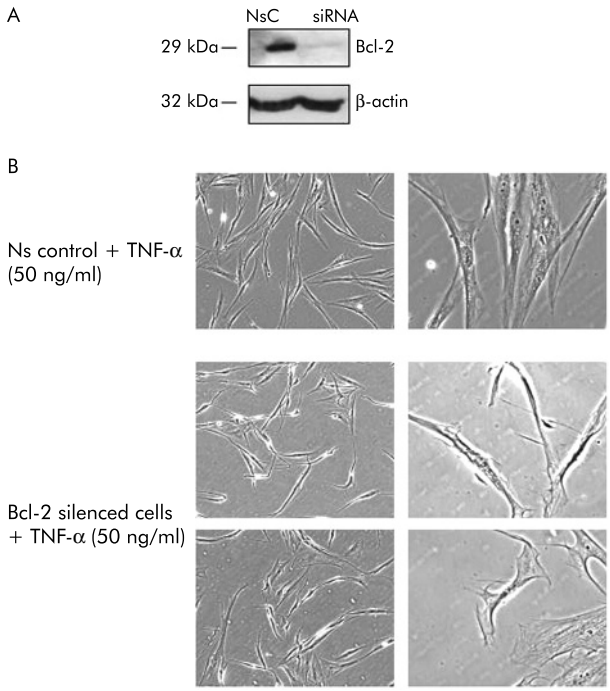

Figure 8 Bcl-2 silencing by RNA interference strategy turns off survival attitude in human activated hepatic stellate cells in myofibroblast-like phenotype (HSC/MFs). Bcl-2 silenced cells, but not cells transfected with a non-silencing control small interfering RNA (siRNA) (NsC), become sensitive to apoptosis induced by tumour necrosis factor α (TNF-α) alone. (A) Western blot analysis of Bcl-2 protein levels 72 hours after transfection, with equal loading of samples monitored by analysing levels of β-actin. (B) Phase contrast analysis (original magnification ×100 and ×400). Data and images are representative of three different experiments.

The novel finding of the present study is that the process of activation in human cells is associated with a persistent change in expression of genes and signalling pathways involved in the control of apoptosis, as indicated by comparison of freshly isolated (quiescent) HSC, activated HSC/MFs, and senescent cells. In particular, our data demonstrated a shift towards a higher Bcl-2/Bax ratio together with the process of activation. It is well known that Bcl-2 acts as an antiapoptotic protein by mainly regulating mitochondrial pathways leading to apoptosis.[39] Indeed, in human HSC/MFs, the stimuli able to induce caspase dependent apoptosis, such as cycloheximide or TNF/cycloheximide, were also those able to triggers mitochondrial pathways, as shown by cytofluorimetric analysis of Δψm. Remarkably, both late passaged/senescent cells that spontaneously loose Bcl-2 and Bcl-2 silenced HSC/MFs by RNA interference strategy became sensitive to TNF-α alone. The relevance of Bcl-2 silencing is in agreement with a study that compared the behaviour of senescent human HSC/MFs versus immortalised HSC/MFs expressing the human telomerase catalytic subunit (h-TERT); senescent cells, expressing negligible levels of Bcl-2, underwent spontaneous apoptosis whereas h-TERT cells, expressing higher levels of Bcl-2 and Bcl-XL, showed a very low level of spontaneous apoptosis.[45]

Survival of human HSC/MFs to NGF highlights another apparent difference between rat and human cells. Rat HSC/MFs have been shown to express the low affinity NGF receptor p75 and to undergo apoptosis when exposed to 100 ng/ml NGF; expression of p75 was also reported in human cells in the same study.[18] However, human HSC/MFs survive concentrations of NGF as high as 500 ng/ml with no signs of nuclear condensation, detachment, caspase activation, or PARP cleavage, possibly because human cells, as already reported in abstract form,[40] (full paper submitted) also express the high affinity NGF receptor Trk-A that is functionally active (binding of Trk-A elicited downstream Ras/Erk signalling). Therefore, in human HSC/MFs, the apoptotic inducing effects of p75 may be counterbalanced by Trk-A expression, resulting in the inability of NGF to

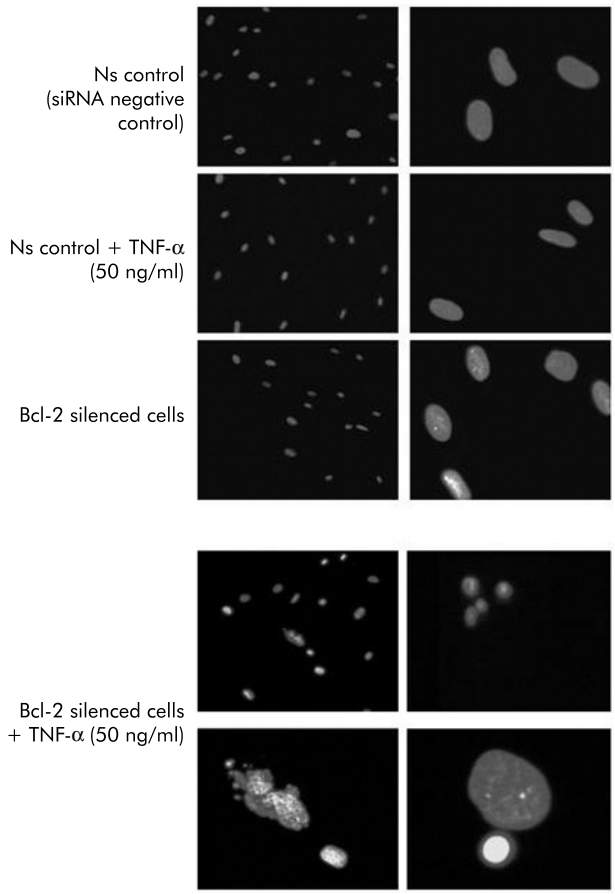

Figure 9 Bcl-2 silencing by RNA interference strategy turns off survival attitude in human activated hepatic stellate cells in myofibroblast-like phenotype (HSC/MFs). Bcl-2 silenced cells, but not cells transfected with a non-silencing control small interfering RNA (siRNA) (Ns control), become sensitive to apoptosis induced by tumour necrosis factor α (TNF-α) alone. DAPI fluorescent staining (original magnification ×100 and ×400) of transfected HSC/MFs exposed or not to TNF-α for 24 hours. Data and images are representative of three different experiments.

mediate apoptosis, as shown for other cells.[46 47] However, the in vivo relevance of these specific data should be interpreted with caution as liver expression of Trk-A in fibrotic or cirrhotic liver is still unclear; evident Trk-A mRNA expression in isolated rat HSC but staining of only vessels walls or inconclusive and weak lobular staining for Trk-A in human liver tissue.[48]

Our data have potential implications for understanding the dynamics of liver fibrosis in humans. Detailed phenotypic characterisation of the cells used in this study demonstrate that the antigen repertoire is similar to that of ''interface'' myofibroblasts that are particularly active in mediating fibrogenesis during chronic active hepatitis.[7 8] Moreover, immunohistochemical data provided in this study clearly indicate that myofibroblast-like cells at the interface between septa and parenchyma significantly overexpress Bcl-2, confirming the possible ''in vivo'' relevance of ''in vitro'' data. Considering the limitations of an in vitro study, our data suggest that molecular mechanisms underlying fibrosis reversal in rodent models may not be extrapolated to humans. Moreover, marked resistance to apoptosis of human HSC/MFs may also suggest that the observed reversal of fibrosis in the human liver may not be entirely dependent on HSC/MFs apoptosis, and explain the longer time frame necessary to observe a significant reduction in the amount of extracellular matrix.[24]

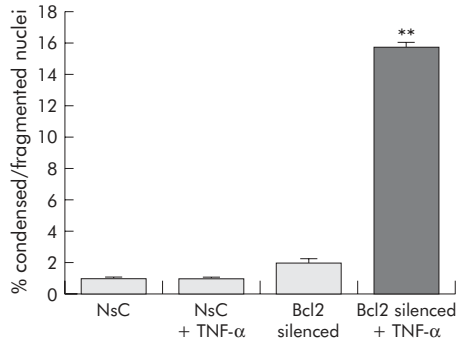

Figure 10 Bcl-2 silencing by RNA interference strategy turns off survival attitude in human activated hepatic stellate cells in myofibroblast-like phenotype (HSC/MFs). Bcl-2 silenced cells, but not cells transfected with a non-silencing control small interfering RNA (siRNA) (NsC), become sensitive to apoptosis induced by tumour necrosis factor α (TNF-α) alone. Data expressed as means (SEM) (three experiments for each condition) are relative to the per cent of condensed/fragmented nuclei for the indicated conditions. **$p<0.01$ versus control values.

In conclusion, the present study indicates that highly proliferative, profibrogenic, and proinflammatory human HSC/MFs are resistant to most proapoptotic stimuli, and inhibition of protein synthesis or transcription is a prerequisite for induction of apoptosis. We suggest that an altered balance between pro- and antiapoptotic molecules, with a major mechanistic role for Bcl-2 overexpression in activated myofibroblast-like cells, represents a key activation related mechanism favouring survival to several different proapoptotic stimuli and, ultimately, limiting fibrosis reversal in human CLDs.

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