A red wine polyphenolic extract reduces the activation phenotype of cultured human liver myofibroblasts

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Abstract

AIM: To test the effect of a standardized red wine polyphenolic extract (RWPE) on the phenotype of human liver myofibroblasts in culture.

METHODS: Human myofibroblasts grown from liver explants were used in this study. Cell proliferation was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. Signaling events were analyzed by western blot with phospho-specific antibodies. Matrix-metalloproteinase activity was measured with gel zymography.

RESULTS: We found that cell proliferation was dose-dependently decreased by up to 90% by RWPE while cell viability was not affected. Exposure to RWPE also greatly decreased the phosphorylation of ERK1/ERK2 and Akt in response to stimulation by the mitogenic factor platelet-derived growth factor BB (PDGF-BB). Finally, RWPE affected extracellular matrix remodeling by decreasing the secretion by myofibroblasts of matrix-metalloproteinase-2 and of tissue inhibitor of matrix-metalloproteinases-1.

CONCLUSION: Altogether, RWPE decreases the activation state of liver myofibroblasts. The identification of the active compounds in RWPE could offer new therapeutic strategies against liver fibrosis.

Key words: Liver fibrosis; Myofibroblasts; Hepatic stellate cells; Wine; Phosphorylation; Proliferation

INTRODUCTION

Liver fibrosis is a serious health problem worldwide. It is a complication of most chronic liver diseases whether due to excessive alcohol consumption, chronic viral hepatitis B or C, non alcoholic steatohepatitis, hemochromatosis or others. The pathophysiology of liver fibrosis has been extensively studied (recently reviewed in[1,2]). Whatever the initial insult, the abundant extracellular matrix (ECM) characteristic of liver fibrosis is synthesized by myofibroblastic cells. Myofibroblasts are mostly absent from the normal liver but at least two types of resident liver cells can be differentiated into myofibroblasts during liver disease: hepatic stellate cells, and portal fibroblasts[3]. Myofibroblastic differentiation is characterized by a high rate of cell proliferation and of ECM synthesis and by cytoskeletal changes, notably expression of alpha smooth muscle actin (ASMA) that confers contractile properties to the cells[4]. In addition, degradation of the normal liver ECM results from an increased secretion of the enzyme matrix metalloproteinase-2 (MMP-2) by myofibroblasts, while the proteolytic degradation of the abnormal ECM is inhibited due to a high level synthesis of a MMP inhibitor, tissue inhibitor of MMP-1 (TIMP-1)[1,2]. In the recent years, a series of natural products were shown to be of potential benefit against liver fibrosis[5,6]. For instance, we and others found that a polyphenolic component of red wine, trans-resveratrol, was able to strongly deactivate liver fibrogenic cells[7,8], while a related molecule, trans-piceid, was uneffective[8]. However, red wine contains many other polyphenolic substances, and red wine polyphenolic extracts (RWPE) showed many interesting biological effects in other settings, notably in the prevention of experimental atherosclerosis[9]. One of the mechanisms postulated in this context is related to the inhibitory effect of RWPE on the proliferation of vascular smooth muscle cells[10]. Since vascular smooth muscle cells share many characteristics with myofibroblasts, we tested the hypothesis that RWPE would affect the phenotypic characteristics of human liver myofibroblasts, especially those related to their pro-fibrogenic activity.

MATERIALS AND METHODS

Materials

Preparation and characterization of the polyphenolic extract (RWPE) from a red French wine (Corbières, A. O. C.) was as described previously[11,12]. One liter of red wine produced 2.9 g of extract, which contained 471 mg/g total phenolic compounds expressed as gallic acid. Phenolic levels in the extract were obtained according to HPLC analysis procedure. In particular, the extract contained 8.6 mg/g catechin, 8.7 mg/g epicatechin, dimers (B1: 6.9 mg/g; B2: 8.0 mg/g; B3: 20.7 mg/g; and B4: 0.7 mg/g), anthocyanins (malvidin-3-glucoside: 11.7 mg/g; peonidin-3-glucoside: 0.66 mg/g; and cyanidin-3-glucoside: 0.06 mg/g), and phenolic acids (gallic acid: 5.0 mg/g; caffeic acid 2.5 mg/g; and caftaric acid: 12.5 mg/g). Stock solutions (1 mg/mL) were prepared in distilled water containing 1% ethanol and further diluted in culture medium. All dilutions were adjusted so as to contain 0.1% ethanol, and medium with 0.1% ethanol was used as a control.

Fetal calf serum (FCS) was from Dutscher (Brumath, France) and human serum from Etablissement Français du Sang (Bordeaux, France). Epidermal growth factor (EGF) was from Peprotech (Tebu, Le Perray en Yvelines, France) and recombinant platelet-derived growth factor BB (PDGF-BB) was from Eurobio (les Ulis, France). Phospho Thr308 AKT-1 and AKT-1 antibodies, phospho-ERK1/ERK2 and total ERK antibodies were from Cell Signaling Technology (Ozyme, Saint Quentin Yvelines, France). ASMA and vimentin antibodies were from Dako (Trappes, France). The TIMP antibody was from Santa Cruz Biotechnologies (Santa Cruz, CA). Beta-actin antibody was from Sigma (Saint Quentin Fallavier, France). IRDye 680 and IRDye 800 conjugated secondary antibodies; Odyssey Blocker and Odyssey infrared imaging system were from LI-COR Biosciences (ScienceTec, Les Ulis, France).

Cell isolation and culture

Human hepatic myofibroblasts were obtained from explants of non-tumor liver resected during partial hepatectomy and characterized as described previously[13,14]. Specifically, the procedure, based on the selective growth advantage of myofibroblasts in the culture conditions used, allowed for 100% pure myofibroblasts population, as shown by positive staining for ASMA and vimentin, and negative staining for CD68 (a Kupffer cell marker), von Willebrand factor (an endothelial cell marker) or cytokeratin (an epithelial cell marker). This procedure is in accordance with INSERM ethical regulation imposed by French legislation. Myofibroblasts were used between the 3rd and the 6th passage, and were grown in DMEM containing 5% FCS, 5% pooled human serum and 5 ng/mL EGF. EGF was removed from the medium at least 3 d before experiments.

Cell proliferation assay

Cells were seeded at a density of 10^4/well in 24-well plates. On the following day, the medium was replaced by DMEM with 5% FCS and RWPE dilutions to be tested. After three or seven days, the medium was removed and the cells

incubated with 1 mg/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] for 2 h at 37℃[15]. The crystals were then dissolved with DMSO and the optical density was recorded at 540 nm. Results were expressed as proliferation index = (B-A)/(C-A) where A is the optical density recorded at d 0, B is the optical density with the test compound and C is the optical density obtained in the control wells.

Measure of cell DNA content

Cells were grown to confluence in 24-well plates, serum-starved for 24 h, then incubated with RWPE. After 24 h, the cells were lysed with NaH_2PO_4/Na_2HPO_4 50 mmol/L, pH 7.4, NaCl 2 mol/L, EDTA 2 mmol/L, and DNA content was measured as described[16].

Western blot

ERK and Akt phosphorylation was measured essentially as described previously[17]. Briefly, cells were grown to confluency and serum-starved for three days. They were then pre-incubated with RWPE in serum-free Waymouth medium for 1 h, and then exposed to 20 ng/mL PDGF-BB for 10 min. At the end of the incubation, cell lysates were prepared in the presence of proteases and phosphatases inhibitors as described[18]. Proteins were measured with a Bio-Rad assay. Equivalent amounts of proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and analyzed by two color Western blotting with antibodies to total-ERK and phospho-ERK, or phospho-Akt-1 and β-actin. Blots were blocked in Odyssey Blocker and incubated simultaneously with both primary antibodies, followed by both IR-labeled secondary antibodies. Signals were detected and quantified using the Odyssey infrared imaging system.

The expression of ASMA and of vimentin was measured in cell extracts from cells exposed for seven days to RWPE using Western blot as described[8].

The concentration of TIMP-1 in conditioned medium was also measured by Western blot. Confluent cells were incubated for two days with RWPE, the medium was collected, centrifuged and aliquots, normalized for DNA content of the cell layers, were analyzed by Western blot.

Zymography for MMP-2 activity

The detection of MMP-2 in conditioned medium was performed by gelatin zymography[19], essentially as previously described[8,20]. Briefly, cells were grown to confluence in 24-well plates, serum-starved for 24 h, then incubated with RWPE. The medium was collected, centrifuged and aliquots, normalized for DNA content of the cell layers, were analyzed on 8% SDS-PAGE gels containing 1 mg/mL gelatin. Following staining with Coomassie blue, MMP activity is detected as a white zone on a blue background.

Immunofluorescence detection of alpha-smooth muscle actin

Cells were seeded at a density of 10^4/well on glass coverslips in 24-well plates. On the following day, the medium was replaced by DMEM with 5% FCS and RWPE
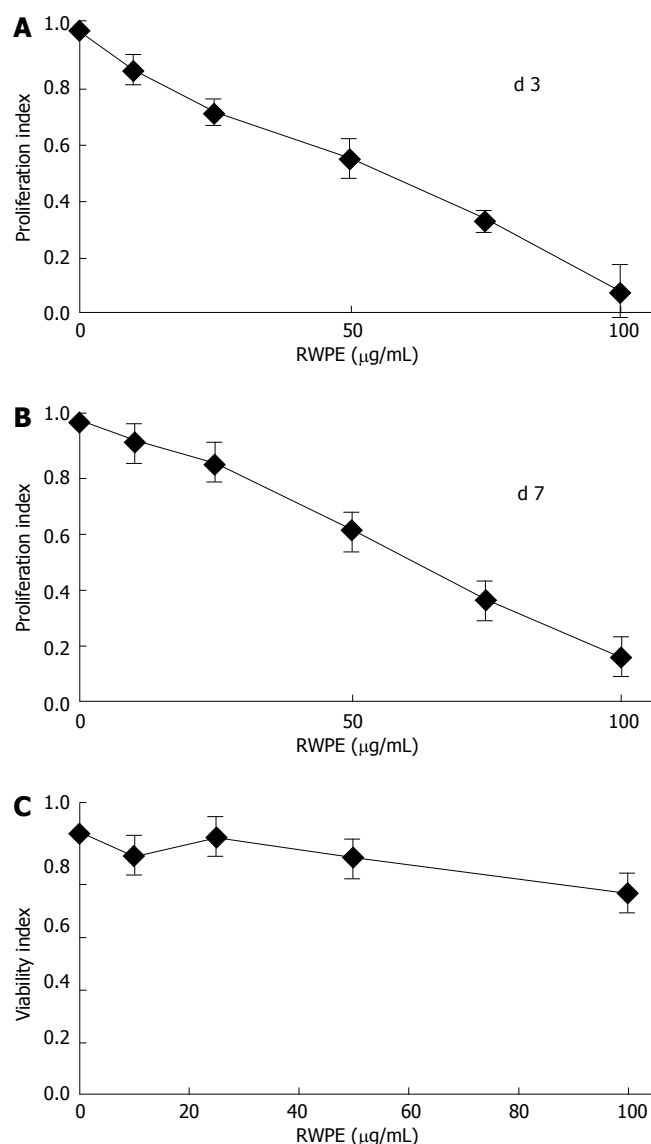
Figure 1 RWPE decreases myofibroblasts proliferation. Myofibroblasts were grown for either three (**A**) or seven days (**B**) in the presence of the indicated concentrations of RWPE. Results are the mean ± SD of 4 independent experiments conducted in quadruplicate. The effect of RWPE was highly significant using ANOVA ($P < 0.0001$). In control experiments, myofibroblasts were exposed to RWPE for 24 h and the DNA content of the cell layer was measured (**C**). Results are expressed as the percentage of the values in treated cells as compared to cells treated with the solvent alone and are the mean ± SD of 3 independent experiments conducted in quadruplicate. There were no significant differences between conditions.
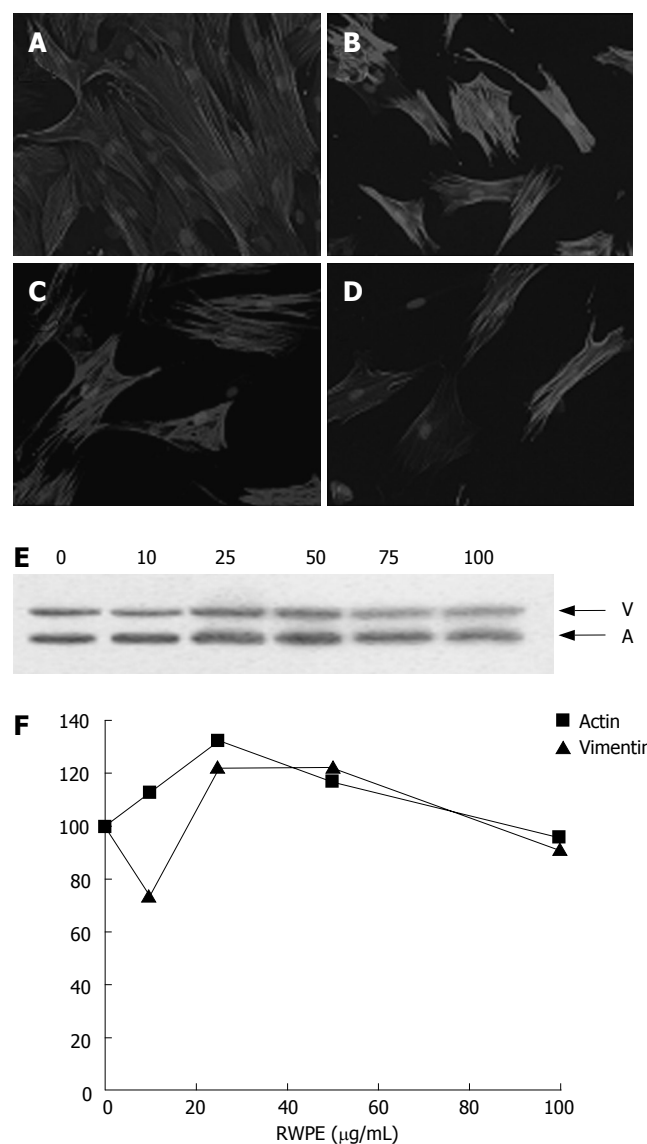
Figure 2 Effect of the RWPE on expression of alpha-smooth muscle actin. **A**: Myofibroblasts were incubated for seven days in the absence of RWPE (A) or with 50 (**B**), 75 (**C**), or 100 (**D**) μg/mL RWPE. Aliquots of cell extracts grown in the same conditions were analyzed by Western blot (**E**) simultaneously for ASMA (A) and vimentin (V). **F**: The signals were quantified as described in Materials and Methods. The graph shows the mean of 2 separate experiments.

dilutions to be tested. After three days, cells were fixed with methanol at -20℃, then incubated sequentially with an anti-ASMA monoclonal antibody and a Texas Red-conjugated secondary antibody. The slides were examined with a Zeiss Axioplan fluorescence microscope.

RESULTS

Effect of the RWPE on cell proliferation

The RWPE dose-dependently inhibited the proliferation of myofibroblasts down to 7.8% ± 4.5% of control at d 3 and 15.8 ± 8.0 at d 7 (Figure 1A and B). The concentration that reduced growth by 50% was 50 μg/mL. A toxic effect of RWPE on cells could be ruled out because no morphological signs of toxicity nor cell detachment were observed (Figure 2); furthermore, when confluent cells were exposed to a dose range of RWPE, there was no decrease in DNA content of the cell layers even at the highest concentrations (Figure 1C).

Effect of the RWPE on expression of alpha-smooth muscle actin

Expression of the cytoskeletal protein ASMA is hallmark of activated liver fibrogenic cells. We found that long term (up to seven days) exposure of liver myofibroblasts to RWPE did not affect ASMA expression. This was shown both by immunofluorescence and by Western blot (Figure 2). In addition, the expression of another cytoskeletal protein, vimentin, which expression is independent of fibrogenic cell activation, was also unaffected by RWPE treatment (Figure 2C).
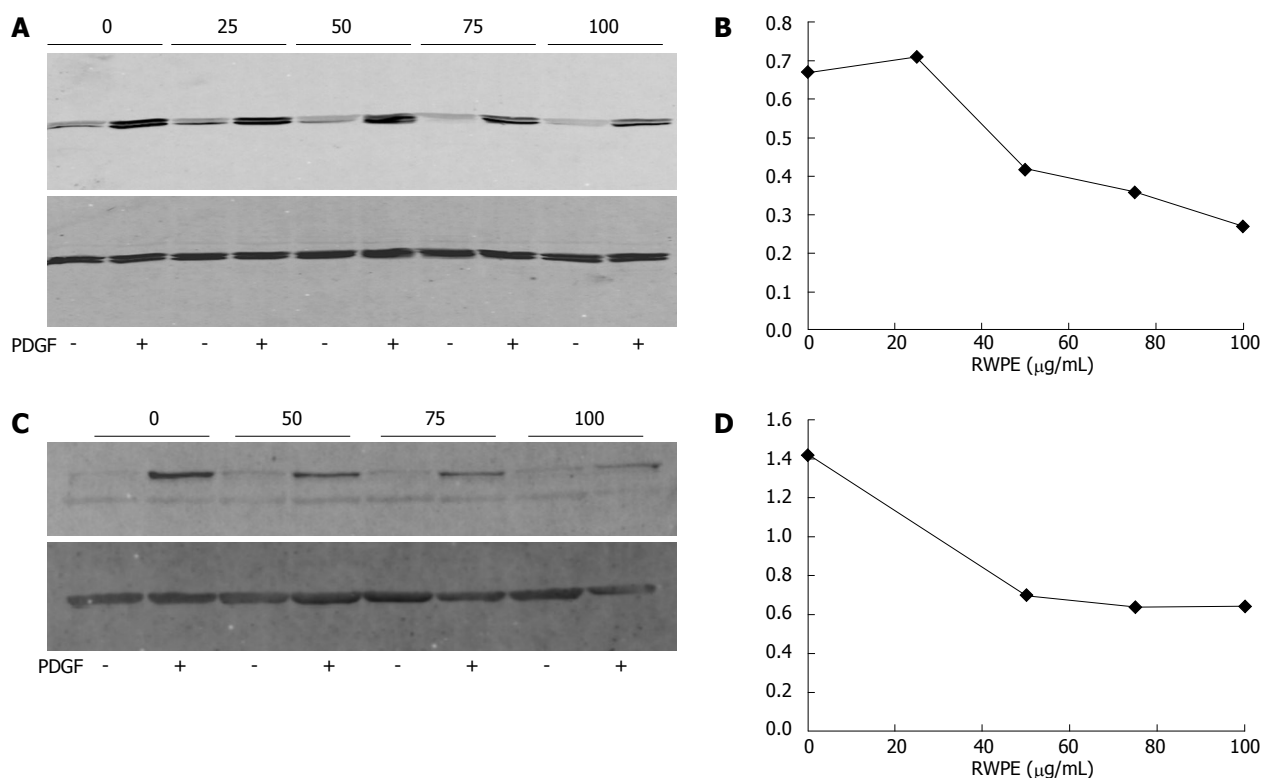
Figure 3 Effect of the RWPE on the phosphorylation of MAPK and Akt. **A**: Myofibroblasts were pre-incubated for 1 h with the indicated concentrations of RWPE (in μg/mL) or solvent, then exposed for 10 min to 20 ng/mL PDGF-BB or buffer. Identical amounts of cell extracts were analyzed by Western blot with antibodies to phospho-ERK1/ERK2 (top panel) and to total ERKs (bottom panel). The picture is representative of 3 experiments; **B**: Quantitative analysis of the experiment shown in (**A**). The activation index refers to the ratio between the levels of phospho-ERK to those of total ERK; **C**: Same as in A except that the blot was labelled with an anti-phospho-Akt antibody (top panel) and an antibody to β-actin (bottom panel); **D**: Quantitative analysis of the experiment shown in (**C**). The activation index refers to the ratio between the levels of phospho-Akt to those of β-actin.

Effect of the RWPE on the phosphorylation of MAPK and Akt

In order to delineate the mitogenic pathways affected by RWPE, myofibroblasts were briefly exposed to PDGF-BB. PDGF-BB is major mediator of liver fibrogenic cell activation, as it stimulates notably their proliferation[13,21] and migration[22,23], and is abundant in serum. We then examined the effect of RWPE on signalization pathways elicited by PDGF-BB. As expected, treatment with PDGF-BB induced a major increase in the phosphorylation of ERK1/ERK2 and of Akt. Exposure to RWPE greatly decreased the effect of PDGF-BB on the phosphorylation of both ERK1/ERK2 and Akt (Figure 3).

Effect of the RWPE on MMP-2 and TIMP-1 expression

A high level expression of the matrix remodeling enzyme MMP-2[24], and of the inhibitor of extracellular matrix degradation, TIMP-1[25], is characteristic of activated liver fibrogenic cells. Gelatin zymography showed a gelatinolytic band migrating at an apparent molecular weight of 72 kDa characteristic of MMP-2. The RWPE strikingly and dose-dependently decreased the secretion of MMP-2 (Figure 4A). It also greatly decreased TIMP-1 secretion, as assessed by Western blot (Figure 4B).

DISCUSSION

We show here that a standardized RWPE has striking effects on the phenotype of human liver myofibroblasts.

This is shown by a decreased proliferation rate together with decreased secretion of MMP-2 and of TIMP-1. These effects are not the consequence of a direct toxicity of the extract on cells as shown by morphological examination and DNA content measurement. We investigated further the mechanism of the decreased proliferation rate and found that RWPE treatment largely abolished the phosphorylation of ERK1/ERK2 and of Akt induced by the mitogenic factor PDGF-BB. Previously, Iijima *et al* showed that a RWPE decreased Akt but not ERK activation in vascular smooth muscle cells stimulated with PDGF[26]. The differences may be due to the fact that different RWPEs were used, or to a true cell specificity. For instance, despite the fact that myofibroblasts and smooth muscle cells are related cells, we found that they had a differential response to trans-resveratrol[8]. Some of the effects of the RWPE are reminiscent of those of trans-resveratrol, raising the possibility that RWPE effects may be due to this compound. However, resveratrol is present only at low concentration in wine (reviewed in[27]) and is unlikely to explain the effects of RWPE. In addition, whereas it does indeed decrease myofibroblasts proliferation, MMP-2 secretion[8] and Akt activation[28], it does not affect ERK activation in response to PDGF[28]. Furthermore, contrary to RWPE, it does decrease ASMA expression[8].

The RWPE effects observed in the present study are potentially of benefit against liver fibrosis, if they held true in the *in vivo* situation. This seems obvious for the reduced cell proliferation that will decrease the number of ECM-
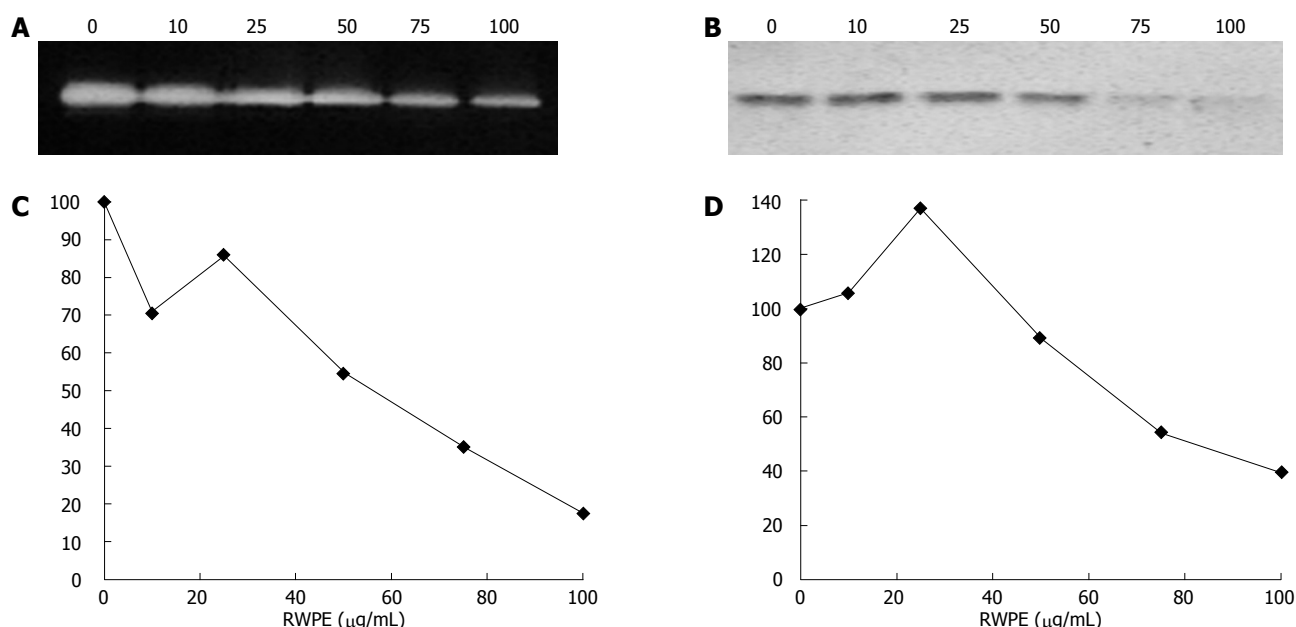
Figure 4 Effect of the RWPE on MMP-2 and TIMP-1 expression. **A**: Myofibroblasts were incubated for 24 h with the indicated concentrations of RWPE (in μg/mL). Aliquots of conditioned medium normalized for the DNA content of the cell monolayers were analyzed on gelatin-containing gels (**A**). The white bands on the dark background indicate gelatinolytic activity. Other aliquots were analyzed by Western blot with an antibody against TIMP-1 (**B**). Another experiment gave similar results; **C**: Quantitative analysis of the experiments. MMP-2: Mean of duplicate samples; TIMP-1: Mean of 2 independent experiments.

producing cells. Notably, the drastic effect on TIMP-1 secretion may have a major implication since TIMP-1 overexpression is considered one of the main determinants of liver fibrosis through the inhibition of ECM-degrading enzymes activity[29,30].

Thus, although excessive wine consumption is one of the major causes of chronic liver diseases, wine itself may unexpectedly contain potent anti-fibrotic compounds. The identification of the active compounds in RWPE could offer new therapeutic strategies against liver fibrosis.

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COMMENTS

Background

Liver fibrosis is a worldwide problem, as it complicates all chronic liver diseases. There is no established treatment for liver fibrosis.

Research frontiers

A series of natural products have shown beneficial effects on liver fibrosis in cell culture and animal models. In addition, red wine polyphenols were shown to reduce the proliferation of vascular smooth muscle cells, a cell type closely related to liver fibrogenic cells.

Innovations and breakthroughs

We found that a standardized red wine polyphenolic extract greatly decreased the proliferation of human liver fibrogenic cells. It also reduced their synthesis of matrix-metalloproteinase-2 and of the tissue inhibitor of matrix metalloproteinase-1, thus suggesting that it could affect the cell ability to remodel the extracellular matrix.

Applications

The identification of the active compound(s) in the extract could lead to *in vivo* testing of its anti-fibrotic activity in liver disease.

Terminology

Liver fibrosis: a common complication of most chronic liver diseases, where an excess of extracellular matrix components are deposited in the liver.

Peer review

This is a very nice communication and provides mechanistic data, is well-written, and ends with a thorough discussion. The results of this study are novel and potentially lay the ground work for the understanding of the effect of wine/wine extracts on human hepatic fibrosis.

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