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Augmenter of liver regeneration (ALR) protects human hepatocytes against apoptosis

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ABSTRACT

Augmenter of liver regeneration (ALR) is known to support liver regeneration and to stimulate proliferation of hepatocytes. However, it is not known if ALR exerts anti-apoptotic effects in human hepatocytes and whether this protective effect is cell type specific. This is relevant, because compounds that protect the liver against apoptosis without undesired effects, such as protection of metastatic tumour cells, would be appreciated in several clinical settings. Primary human hepatocytes (phH) and organotypic cancer cell lines were exposed to different concentrations of apoptosis inducers (ethanol, TRAIL, anti-Apo, TGF-β, actinomycin D) and cultured with or without recombinant human ALR (rhALR). Apoptosis was evaluated by the release of cytochrome *c* from mitochondria and by FACS with propidium iodide (PI) staining.

ALR significantly decreased apoptosis induced by ethanol, TRAIL, anti-Apo, TGF-β and actinomycin D. Further, the anti-apoptotic effect of ALR was observed in primary human hepatocytes and in HepG2 cells but not in bronchial (BC1), colonic (SW480), gastric (GC1) and pancreatic (L3.6PL) cell lines.

Therefore, the hepatotrophic growth factor ALR acts in a liver specific manner with regards to both its mitogenic and its anti-apoptotic effect. Unlike the growth factors HGF and EGF, rhALR acts in a liver specific manner. Therefore, ALR is a promising candidate for further evaluation as a possible hepatoprotective factor in clinical settings.

1. Introduction

The liver has a regenerative potential, which permits recovery from functional disorders induced by hepatic injury [1]. During liver regeneration, regenerative factors released from parenchymal and non-parenchymal liver cells stimulate the proliferation of hepatocytes by induction of immediate early genes and stimulating the release of growth factors [2].

Besides well-known growth factors like hepatocyte growth factor (HGF) or epidermal growth factor (EGF), augmenter of liver regeneration (ALR) is another cytokine of vital importance. For both EGF and HGF, mitogenic effects on not only hepatic cells but also trophoblasts and myoblasts, respectively have been shown [3,4]. ALR belongs to a novel group of so called cytozymes as it acts as a growth factor and a sulfhydryl oxidase enzyme, that binds FAD containing a redox-active CxxC disulfide proximal to a flavin ring [5]. ALR is known to support liver regeneration in experimental animals [6,7]. Previous studies have shown that ALR activates the ras/Mek/Erk as well as the PI3K/Akt pathways [8]. The influence of ALR on signaling pathways differs from that of other growth factors, such as EGF. For instance, ALR causes a transient and EGF a permanent increase in ERK phosphorylation [8]. Although an activation of the PI3K/Akt signaling pathway has recently been shown, an anti-apoptotic effect of ALR on human hepatocytes has not been studied yet. This possible new feature of ALR was tested

Abbreviations: rhALR, recombinant human augmenter of liver regeneration; EGF, epidermal growth factor; ERK-1/2, extracellular signal–regulated kinase 1/2; FCS, fetal calf serum; HGF, hepatocyte growth factor; LPS, lipopolysaccharide; MAP kinase, mitogen-activated protein-kinase; MAPKK/MEK, mitogen-activated protein kinase kinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; TRAIL, tumor necrosis factor related apoptosis-inducing ligand; TGF-β, transforming growth factor beta; act D, actinomycin D; NEAA, non essential amino acids; FACS, fluorescence activated cell sorting; phH, primary human hepatocytes.

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by examining ALR's protective effects against various apoptotic inducers. Particularly in the field of cancer treatment, either of primary liver cancer or liver metastases, a therapy that protects the liver against apoptosis would be highly welcome [9].

For HGF, as one of the growth factors influencing the liver metabolism, anti-apoptotic and [10–13] protective effects [14,15] have already been found. However, cytokines or growth factors, such as EGF, HGF or IL-6 are problematic in this context, because they influence a diversity of organ systems [8]. For example, EGF activated ERK and stimulated proliferation not only in liver but also in cell lines of colonic, bronchial, pancreatic and gastric origin [8]. In contrast, ALR induced proliferation only in liver cell systems. However, to our knowledge the cell type specific anti-apoptotic effects of ALR have not yet been studied. Therefore the aim of this study was to investigate the promising characteristics of ALR regarding a possible liver-specific anti-apoptotic effect *in vitro*.

The aim of this study was to show a protection of hepatic cells by ALR administration from apoptosis induced by ethanol, tumor necrosis factor related apoptosis-inducing ligand (TRAIL), anti-Apo, transforming growth factor β (TGF-β) and actinomycin D (Act D) and that this protection occurs in primary human hepatocytes but not in pancreatic, colonic, bronchial and gastric cell lines. This would imply a possible medical usage of ALR regarding protection of liver cells during apoptosis inducing therapies.

2. Materials and methods

2.1. Reagents

Recombinant human ALR (rhALR) was prepared as described previously [16]. Briefly, fractions containing rhALR protein were combined and dialyzed against dialysis buffer (25 mM Hepes, 0.1% Tween 20, and 1 mM EDTA, pH 8.2) at 4 °C, with three buffer changes. Afterwards, rhALR protein was concentrated using a 5 kDa cut-off ultrafree-15 centrifugal filter device (Millipore GmbH, Schwalbach, Germany) [17]. All antibodies used were purchased from Cell Signaling Technology (Beverly, MA, USA). TRAIL and TGF-β were obtained from Sigma–Aldrich (Taufkirchen, Germany). Actinomycin D was purchased from AppliChem (Darmstadt, Germany) and anti-Apo from Alexis (San Diego, CA, USA).

2.2. Isolation of primary human hepatocytes

Remnant liver samples were obtained from patients with informed consent through the Grosshadern Tissue Bank after partial hepatectomy. This tissue bank is regulated according to the guidelines of the non-profit state-controlled HTCR (Human Tissue and Cell Research) foundation following study approval [18] according to the local ethical committee of the Ludwig Maximilians University. Human hepatocytes were isolated using a modified two-step EGTA/collagenase perfusion procedure as described previously [19]. Viability of isolated hepatocytes was determined by trypan blue exclusion. Cell suspensions with viabilities more than 80% were plated and cultured for further experiments.

2.3. Primary hepatocytes culture

Cells were plated on biocoated collagen I 6-Well plates (Becton Dickinson, Heidelberg, Germany) for Western Blots and 12-Well plates for FACS analysis in 1–2 ml of culture media. The medium consisted of Dulbecco's modified Eagle's medium (DMEM, Lonza, Cologne, Germany) with 5% fetal calf serum (FCS, Biochrom, Berlin, Germany), 2 mM L-glutamine (Biochrom, Berlin, Germany) and supplements as follows: 1.7 mU/ml insulin (B. Braun Melsungen AG, Melsungen, Germany), 3.75 ng/ml hydrocortisone (Sigma–Aldrich, Taufkirchen, Germany), 100 μg/ml streptomycin and 100 U/ml penicillin (Lonza, Cologne, Germany) and 1 μg/ml glucagon (Novo Nordisk Pharma GmbH, Mainz, Germany). For starvation media, the mixture of supplements was reduced to: 0.5 U/l insulin, 100 kU/l penicillin/streptomycin and 2 mM L-glutamine. Cells were incubated at 37 °C in a humidified incubator with 5% CO_2. Viability of hepatocytes during the culture period was monitored by cell morphology (light microscopy, image analysis).

2.4. Cell lines and culture conditions

The bronchial (BC1) and gastric (GC1) cell lines were kindly provided by Dr. N. van den Engel, Ludwig Maximilians University, Munich [20]. Human hepatic cell lines (HepG2, Chang) were cultured in RPMI medium (Lonza, Cologne, Germany) supplemented with 10% FCS, 4 mM L-glutamine, 100 U/ml penicillin/streptomycin. Bronchial (BC1), colonic (SW480), and gastric (GC1) cell lines were cultured in RPMI medium supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non essential amino acids (NEAA) and 50 μg/ml gentamycin. Pancreatic (L3.6PL) carcinoma cell lines were cultured in DMEM with 1 g/ml glucose, 4 mM L-glutamine, 1 mM sodium pyruvate, 12% FCS, 2x MEM vitamin mixture, 0.2 mM MEM NEAA (Lonza, Cologne, Germany), 120 U/ml penicillin and 20 μg/ml streptomycin. All cell lines were cultivated in 5% CO_2 at 37 °C.

2.5. Cell treatment

After 16–20 h attachment of primary human hepatocytes or plating of cell lines, medium was changed to starvation medium and media with 1% FCS, respectively for the next 12–19 h [21]. Primary human hepatocytes and cell lines were incubated with 750 ng/ml rhALR for 4 h. Subsequently, apoptosis was induced in the presence or absence of rhALR by 24 h incubation with 100 mM ethanol, 20 ng/ml TRAIL, 100 ng/ml anti-Apo, 10 ng/ml TGF-β or 10 μg/ml act D.

2.6. Western blot analysis

The cells were washed with PBS and lysed in cell lysis buffer (New England Biolabs GmbH, Frankfurt, Germany) containing 20 mM Tris–HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin. Cell lysates were sonicated briefly, centrifuged at 14,000*g* (10 min/4 °C) and supernatants (10–20 μg protein) were subjected to electrophoresis through a 4–15% polyacrylamide gel. Proteins were then transferred to PVDF membranes. After electro-transfer, the blots were blocked for 1 h at room temperature in blocking buffer containing 20 mM Tris, 137 mM NaCl, 0.1% Tween 20 and 5% milk (pH 7.6). The blots were then incubated with primary antibodies (New England Biolabs GmbH, Frankfurt, Germany) with 1:1000 dilutions (cytochrome *c*) in blocking buffer overnight. Following several washes in buffer containing 20 mN Tris–HCl, 137 mM NaCl and 0.1% Tween 20 (pH 7.6), the blots were incubated in 1:2000 dilution of anti-rabbit IgG HRP-linked as secondary antibody (New England Biolabs GmbH, Frankfurt, Germany) diluted in blocking buffer 1 h at room temperature. Following several washes in buffer, the immunoreactive proteins were visualized and quantified by densitometric analysis using ImageJ software (Wayne Rasband, National Institutes of Health, USA). For all western blots, GAPDH was used as a reference gene.

2.7. Isolation of mitochondrial and cytosolic fraction

For analyzing the compartmentalization of cytochrome *c* protein, subcellular fractions such as mitochondria and cytosol were

isolated using a mitochondrial isolation kit for cultured cells (Pierce, Rockford, USA). The mitochondrial pellets were lysed with 2% CHAPS in Tris buffered saline. The cytosolic fraction was desalted using Slide-A-Lyzer® Dialysis Cassettes 7KD (Fischer Scientific GmbH, Schwerte, Germany) according to manufacturer's instructions.

2.8. FACS analysis

All cell lines and primary cells were plated on 12-well plates for FACS analysis. Both primary human hepatocytes and hepatic cell lines were stained with propidium iodide after treatment to measure the percentage of apoptotic nuclei in hypotonic buffer [22]. After incubation with propidium iodide overnight at 4 °C, stained cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences, NJ, USA).

2.9. Statistical analysis

Statistical analysis was performed using two-tailed Student's *t*-test. *P* levels <0.05 were considered as significant.

3. Results

3.1. ALR decreases cytochrome c release from mitochondria

As an early sign of apoptosis, administration of ethanol resulted in an increase in cytochrome *c* release into the cytosol in primary human hepatocytes (Fig. 1). When treated with ethanol, cells cultured in the presence of rhALR exhibited a clear decrease in cytosolic cytochrome *c* compared to the cells incubated with ethanol alone (Fig. 1). The influence of ALR on the release of cytochrome *c* from mitochondria into the cytosol suggests that the anti-apoptotic effect of ALR is at least partially mediated via the intrinsic pathway.

3.2. ALR protects hepatocytes against apoptosis induction by different stress signals

To analyze the protective effect of ALR against apoptosis, different hepatic cells were treated with apoptosis inducing agents. Apoptosis caused by different mechanisms, such as ligand-receptor signaling via CD 95 (TRAIL, anti-Apo), transmembrane serine/threonine kinase receptors (TGF-β) and inhibition of RNA-synthesis (act D), was measured using FACS analysis with propidium iodide staining (Fig. 2). Co-incubation with rhALR significantly decreased TRAIL induced apoptosis in primary human hepatocytes (Fig. 2A). Similarly, the hepatic cell line HepG2 showed a significant increase in apoptosis after incubation with 100 ng/ml anti-Apo, whereas co-incubation with rhALR reduced apoptosis significantly (Fig. 2B). A similar anti-apoptotic effect by rhALR was obtained for TGF-β induced apoptosis in chang cells (Fig. 2C). Also actinomycin D induced apoptosis was antagonized by rhALR in HepG2 cells (Fig. 2D).

3.3. ALR exerts a liver-specific anti-apoptotic effect

Since ALR shows a protective effect on hepatic cells against apoptosis induced by ethanol, TRAIL, anti-Apo, TGF-β and act D, it was of interest to analyze whether this effect is hepatocyte specific or can be observed in different cell types. For this purpose, cultivated primary human hepatocytes, the human hepatoma cell line HepG2, as well as bronchial (BC1), colonic (SW480), gastric (GC1) and pancreatic (L3.6PL) cell lines were tested. Interestingly, rhALR ameliorated TRAIL induced apoptosis only in human hepatocytes and in HepG2 cells (Fig. 3). In contrast, apoptosis was not significantly reduced in BC1, SW480, GC1 and L3.6PL cells. Therefore, ALR shows a protective effect on liver cells but not on cells of bronchial, colon, gastric and pancreatic origin. This feature may offer the possibility of a selective liver protection, which could be helpful in several clinical settings.

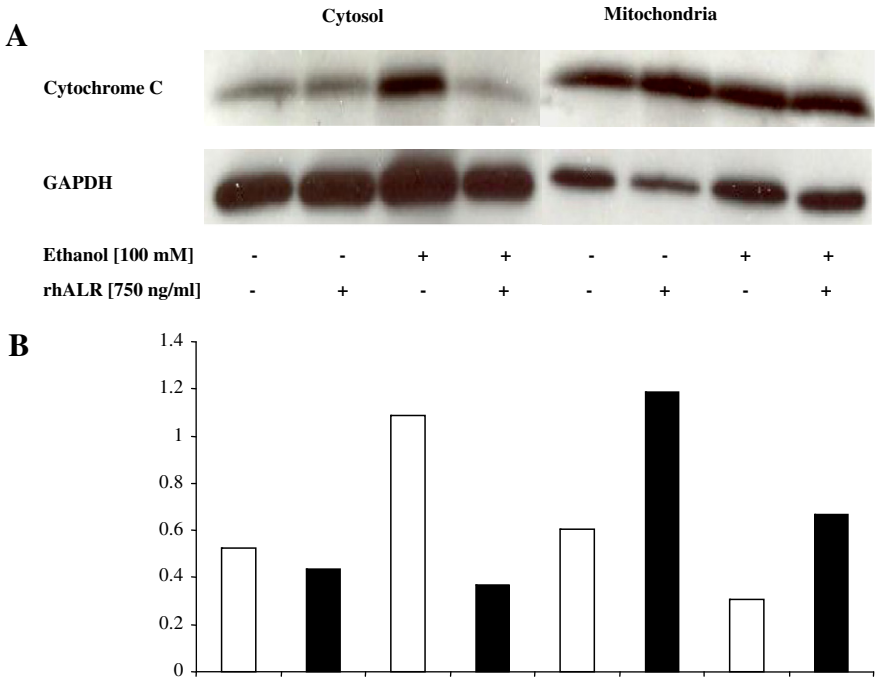

Fig. 1. Western blot analysis showing subcellular cytochrome *c* compartmentalization of in primary human hepatocytes after treatment with or without ethanol (100 mM) in the presence or absence of rhALR (750 ng/ml). Cells were incubated with recombinant human ALR (rhALR) 4 h before treatment with ethanol and ALR was kept in the culture media for the duration of the treatment (24 h). (A) Representative western blot, (B) quantitative analysis of the western blot.

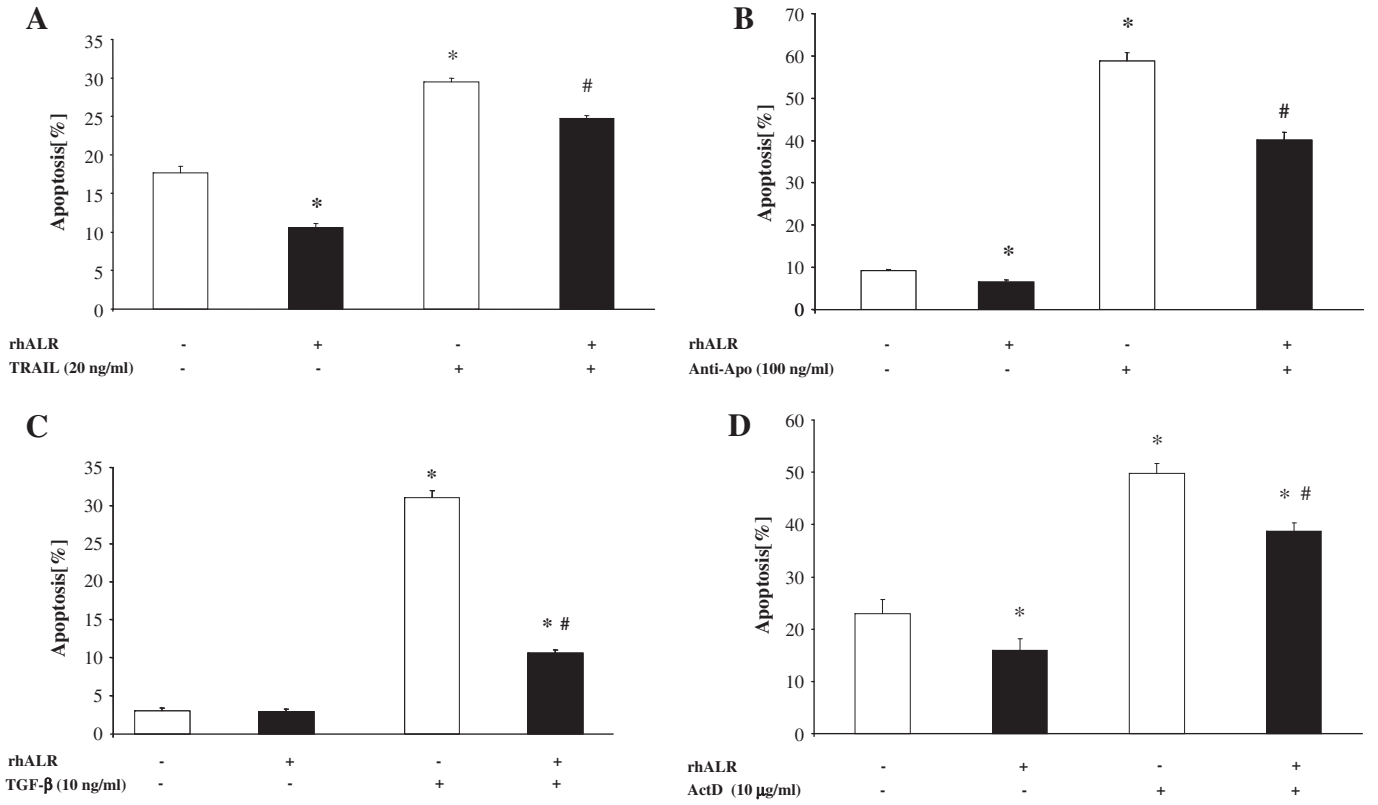

Fig. 2. Apoptosis (%) induced in (A) primary human hepatocytes by TRAIL (20 ng/ml), (B) HepG2 cells by anti-Apo (100 ng/ml), (C) Chang cells by TGF-β (10 ng/ml) and (D) HepG2 cells by actinomycin D (10 µg/ml). The treatments shown are with or without apoptosis inducers in the presence or absence of rhALR (750 ng/ml). $^{*}p < 0.05$ vs. untreated cells using students *t*-test. $^{\#}p < 0.05$ vs. cells treated with apoptosis inducer using students *t*-test.

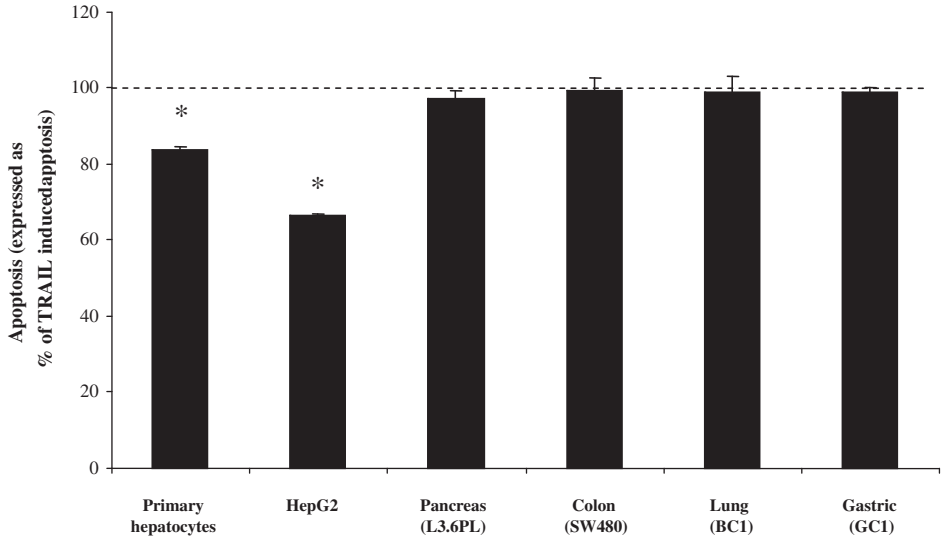

Fig. 3. Apoptosis (%) induced by TRAIL (20 ng/ml) in the presence of recombinant human ALR (750 ng/ml) in cells (primary human hepatocytes, HepG2 cells, pancreatic cells, colonic cells, bronchial cells and gastric cells) expressed as percent of apoptosis caused by TRAIL treatment alone. Cells treated were incubated with rhALR 4 h before treatment with the apoptosis inducer and ALR was kept in the culture media for the duration of the treatment (24 h). $^{*}p < 0.05$ vs. cells treated with TRAIL using students *t*-test.

4. Discussion

Therapeutic options stimulating anti-apoptotic mechanisms in hepatocytes would be highly welcome in several clinical settings, e.g. non-alcoholic steatohepatitis (NASH), alcohol mediated hepatitis and cholestatic liver diseases, to retard fibrotic progression and potentially prevent cirrhosis [23]. As hepatocyte cell death promotes hepatic fibrosis, therapeutic hepatoprotective strategies focusing on protection of hepatocytes and prevention of hepatocytic apoptosis are important. However, systemic administration of cytokines or non-specific growth factors may be problematic because of undesired effects on other cell types. Examples like EGF and HGF that have anti-apoptotic effects on hepatocytes also show a stimulation of proliferation in numerous other cell types [3,4].

Recently, ALR has been described as a hepatocyte specific mitogen [8]. However, its influence on apoptosis in human hepatocytes and its cell-type specificity concerning anti-apoptotic effects is still unknown. In the present study we show that ALR clearly decreases ethanol-induced cytochrome *c* release from mitochondria of human hepatocytes. Similarly, apoptosis induced by TRAIL, a proapoptotic factor in human hepatocytes [24], was antagonized by ALR. Also the agonist antibody anti-Apo, which recognizes the epitope on the extracellular domain of CD 95 [25], the proapoptotic cytokine TGF-β [26] and actinomycin D caused apoptosis in human hepatocytes which could be ameliorated by ALR. In contrast to human hepatocytes, no anti-apoptotic effect of ALR was observed in bronchial (BC1), colonic (SW480), gastric (GC1) and pancreatic (L3.6PL) cell lines. This suggests that similar to the already observed hepatocyte specific mitogenic effect of ALR, the anti-apoptotic effect of ALR may also be hepatocyte specific.

As a possible limitation regarding liver specificity, it should be considered that a protective effect of ALR against hydrogen peroxide was found in human neuroblastoma cells [27] and in rats protecting kidneys from ischemia/reperfusion injury [28]. In contrast the effect of hydrogen peroxide has not been tested in hepatocytes *in vitro* yet as well as the effect of ALR administration with ischemia/reperfusion injury in the liver needs to be further investigated. Positive effects of ALR on hepatic liver diseases and hepatic failure or survival after antisense oligonucleotide transfection was shown already [29–31] but to our knowledge no anti-apoptotic effect of ALR has been reported on primary human cells. Our observation that ALR protects human hepatoma cells corresponds to the results obtained by Cao et al. regarding radiation-induced oxidative stress [32]. Therefore, ALR besides having anti-apoptotic effects in primary hepatocytes seems to also protect tumour cells. That is why research on possible clinical applications of ALR should be limited to treatment in the presence of secondary metastasis in the liver or non-tumour diseases.

In conclusion, it is shown that ALR protects only cells of hepatic lineage against apoptosis and not pancreatic, colonic, bronchial or gastric cells. These are promising findings for further evaluation of ALR as a possible hepatoprotective cytokine in clinical settings.

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