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# Time-dependent onset of Interferon-α2b-induced apoptosis in isolated hepatocytes from preneoplastic rat livers

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**Abstract**

We have already demonstrated that interferon alfa-2b (IFN-α2b) induces apoptosis in isolated hepatocytes from preneoplastic rat livers via the secretion of transforming growth factor $\beta_1$ (TGF-$\beta_1$), and this process is accompanied by caspase-3 activation. The aim of this study was to further investigate the mechanism of this activation. Isolated hepatocytes from preneoplastic livers induced DNA fragmentation in response to IFN-α2b, which was completely blocked when anti-TGF-$\beta_1$ was added to the culture media. IFN-α2b mediated radical oxygen species (ROS) production that preceded the loss of mitochondrial transmembrane potential ($\Delta\Psi$), release of cytochrome *c*, and activation of caspase-3. Bax levels increased in a time-dependent fashion, and Bcl-$x_L$ was down-regulated in the early hours of IFN-α2b treatment. The delayed translocation of Bid into the mitochondria was in concordance with late caspase-8 activation. In conclusion, endogenous TGF-$\beta_1$ secreted under IFN-α2b stimulus seems to induce cytochrome *c* release through a mechanism related to Bcl-2 family members and loss of mitochondrial $\Delta\Psi$. Bax protein could be responsible of the release of cytochrome *c* during the initial hours of IFN-α2b-induced apoptosis via TGF-$\beta_1$. Activated Bid by caspases could amplificate the mitochondrial events, enhancing the release of cytochrome *c*.


*Keywords:* Preneoplastic rat hepatocyte; TGF-$\beta_1$; Caspase; Bcl-2 family protein; Cytochrome *c*

## 1. Introduction

Interferon alfa (IFN-α) belongs to the IFN family of cytokines that elicit a pleiotropic biological action. They have antiviral, antiproliferative, and immunomodulatory effects. In oncology, IFN-α is used for the treatment of a number of solid tumors and hematological malignancies [1]. However, the benefit derived from IFN-α treatment of hepatocarcinoma remains controversial [2]. In this regard, it has been stated that IFN-α applied in the early stages of tumor evolution could have a very important clinical effect, whereas its activity in advanced stages would be minimal [3]. We have previously shown that IFN-α2b induces apoptosis in isolated hepatocytes from preneoplastic rat livers [4]. The apoptotic effect of IFN-α2b was mediated by the production of TGF-$\beta_1$ from hepatocytes, due to the fact that when anti-TGF-$\beta_1$ was added to the culture media, IFN-α2b-induced apoptosis was completely blocked.

Apoptosis, or programmed cell death, is an essential physiologic process required for normal development and maintenance of liver homeostasis. However, apoptosis is

*Abbreviations:* IFN-α2b, interferon alfa-2b; TGF-$\beta_1$, transforming growth factor $\beta_1$; ROS, radical oxygen species; $\Delta\Psi$, transmembrane potential; DEN, diethylnitrosamine; 2-AAF, 2-acetylaminofluorene; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; TMRM, tetramethylrhodamine methyl ester.

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also involved in pathologic conditions, including liver regression, physical and chemical liver injury, viral hepatitis, and liver carcinogenesis [5]. The aspartate-specific-cysteine-protease (caspase) cascade is now believed to be the main pathway by which cellular death is orchestrated [6]. There are two pathways by which caspase activation is triggered: the extrinsic and the intrinsic. Both pathways converge on the activation of caspase-3. This caspase is ultimately responsible for the majority of the effects [7]. The extrinsic pathway is activated by the engagement of death receptors on the cell surface. Caspase-8 is the key initiator caspase in the death-receptor pathway [8]. The intrinsic pathway or non-receptor-mediated apoptosis is triggered by various extracellular and intracellular stresses, such as growth factor withdrawal, hypoxia, DNA damage, and oncogene induction. Signals that are transduced in response to these stresses result in the permeabilization of the outer mitochondrial membrane, release of cytochrome *c* and other pro-apoptotic molecules, formation of the apoptosome, and caspase activation [9]. Among these processes, only the permeabilization step is regulated, and several members of the Bcl-2 family are involved in this regulation. The anti-apoptotic proteins Bcl-2 and Bcl-$x_L$ can stop the march towards apoptotic death by preventing cytochrome *c* release [10], whereas translocation of pro-apoptotic proteins (Bax and Bid) to the mitochondria can induce the release of cytochrome *c* contained in the intermembrane space. Once cytochrome *c* is released, the downstream cascade of caspase activation is irreversible [11].

The endogenous transforming growth factor $\beta_1$ (TGF-$\beta_1$) is an important physiological mediator for apoptosis in both normal and neoplastic liver [12]. It has been reported that TGF-$\beta_1$ plays an important role as an inducer of apoptosis in a variety of cells, including primary hepatocytes [13], fetal rat hepatocytes [14], and hepatoma cell lines [15–17]. Although the mechanisms by which TGF-$\beta_1$ induces apoptosis in hepatocytes have not been fully elucidated, it is well established that TGF-$\beta_1$ mediates radical oxygen species (ROS) production that contributes to the down-regulation of Bcl-$x_L$, the release of cytochrome *c*, and the activation of at least caspases-2, -3, -8, and -9 [14–19]. However, the involvement of Bcl-2, Bax, and Bcl-$x_L$ proteins in TGF-$\beta_1$-induced apoptosis has not been completely established. TGF-$\beta_1$ decreased the anti-apoptotic protein Bcl-$x_L$ in diverse hepatoma cell lines [16,20] whereas in other hepatoma cells no changes in Bax or Bcl-$x_L$ were observed [17]. On the other hand, overexpression of Bcl-2 blocked induction of apoptosis by TGF-$\beta_1$ in human hepatoma cells [21].

In the current study, we wished to further investigate the mechanism of caspase activation during IFN-α2b-induced apoptosis via TGF-$\beta_1$. For this purpose, experiments were performed to characterize the time-dependent effects of IFN-α2b via TGF-$\beta_1$ on hepatocytes from preneoplastic livers.

## 2. Materials and methods

### 2.1. Chemicals

Diethylnitrosamine (DEN), 2-acetylaminofluorene (2-AAF), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), and collagenase were obtained from Sigma Chemical Co. IFN-α2b was kindly provided by BioSidus Laboratory (Buenos Aires, Argentina). Anti-caspase-3 (L-18), anti-caspase-8 (H-134), anti-cytochrome *c*, anti-Bax, anti-Bcl-$x_L$, anti-Bid, and secondary antibodies were obtained from Santa Cruz Biotechnology. The fluorogenic substrates for caspase-3 *N*-acetyl-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) and for caspase-8 *N*-acetyl-Ile-Glu-Thr-Asp-AMC (Ac-IETD-AMC) were purchased from Alexis Co. Fluorescent probe tetramethylrhodamine methyl ester (TMRM) was from Fluka. All other chemicals were of the highest grade commercially available.

### 2.2. Animals and treatment

Adult male Wistar rats (330–380 g) were maintained in a room at constant temperature with a 12 h light–dark cycle, with free access to food and water. The experimental protocol was performed according to the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health, publication no. 25–28, revised 1996). The animals were subjected to a two-phase model of rat hepatocarcinogenesis, for details see Ref. [22]. The initiation stage was performed by the administration of two i.p. necrogenic doses of DEN (150 mg/kg body weight) 2 weeks apart. One week after the last injection of DEN, the rats received 20 mg/kg body weight of 2-AAF by gavage for four consecutive days per week during 3 weeks.

### 2.3. Cell isolation and culture

Hepatocytes were isolated by collagenase perfusion and mechanical disruption as described previously [23]. A hepatocyte-enriched population from the entire liver containing both preneoplastic hepatocytes plus hepatocytes from the surrounding non-preneoplastic liver tissue was obtained by centrifugation twice at 40 g [24]. To remove residual adherent cells from the hepatocytes, the suspension was incubated twice for 20 min in plastic dishes and the hepatocytes were transferred on culture plates.

Cells were cultured at 37 °C in a humidified atmosphere of 5% $CO_2$ in RPMI medium with L-glutamine supplemented with 10% FCS, penicillin (100 U/mL), and streptomycin (100 μg/mL). Two hours later, the medium was changed to fresh medium supplemented with 0.2% FCS, and cells were treated with $10^5$ U/mL IFN-α2b or left untreated. This dose of IFN-α2b induced *in vitro* a similar increment of hepatocytes apoptosis than the one observed *in vivo*, when rats with preneoplasia received IFN-α2b at therapeutic doses [22]. Cells were cultured at different times: 1, 4, 7, 20, and 24 h.

The viability of all cell suspensions used, checked by Trypan blue exclusion, was between 85% and 95%.

### 2.4. TGF-$\beta_1$ assay

Hepatocyte-conditioned media were collected at the different times and TGF-$\beta_1$ was quantitated using an ELISA test (R&D Systems).

### 2.5. Analysis of apoptosis by DNA fragmentation

Hepatocytes cultured at different times were scrapped off and centrifuged, lysed with 5 mmol/L Tris/HCl (pH 8.0), 20 mmol/L EDTA, 0.5% Triton X-100, and stored at 4 °C for 15 min. Intact nuclei were eliminated by centrifugation and the supernatant was centrifuged at 15,000*g* for 30 min at 4 °C. DNA in the supernatant was purified as described previously [25]. Cytosolic DNA was subjected to 10% PAGE. The gel was incubated for 30 min with 0.5 μg/mL ethidium bromide and visualized under transmitted ultraviolet light by using the BioChemi System.

### 2.6. Analysis of apoptosis by annexin V staining

Apoptosis was investigated using the FITC-Annexin V Apoptosis Kit (BD Biosciences). Cultured hepatocytes were stained according to the manufacturer's suggestions. Then cells were washed, fixed, and viewed under fluorescence microscope. Apoptotic cells were counted by examining >1000 cells/dish and expressed per 100 hepatocytes.

### 2.7. Measurement of intracellular ROS

Formation of ROS was determined as previously described [26,27], with slight modifications. Cells cultured at different times were loaded with 5 μM DCFH-DA for 20 min at 37 °C with 5% $CO_2$. Fluorescence was measured on a Shimadzu Spectrofluorophotometer RF-5301 PC (excitation wavelength 488 nm, emission wavelength 525 nm).

### 2.8. Preparation of mitochondria-enriched fractions and cytosolic extracts

Hepatocytes were scrapped off the plates, and centrifuged, washed, and resuspended in 300 mmol/L sucrose with protease inhibitors. Cells were disrupted by sonication and incubated at 4 °C for 15 min and lysates were centrifuged at 1000*g*. Mitochondrial and cytosolic fractions were then produced as described by de Duve et al. [28]. Proteins were quantified according to Lowry et al. [29]. Enrichment of each cytosolic and mitochondrial fractions was determined by the measurement of specific activities of succinate cytochrome *c* reductase (mitochondrial marker) and lactate dehydrogenase (cytosolic marker) [30]. The specific activity of lactate dehydrogenase in cytosol was 9 ± 2-fold higher than in the mitochondrial fraction, whereas the specific activity of succinate cytochrome *c* reductase in mitochondria was 17 ± 2-fold higher than in cytosol.

### 2.9. Analysis of caspases-3 and -8 activities

Caspases-3 and -8 activities were evaluated by measuring the proteolytic cleavage of their fluorogenic substrates Ac-DEVD-AMC and Ac-IETD-AMC, respectively, according to the manufacturer's suggestions. Briefly, cytosolic extract (50 μg of protein) was added into reaction buffer containing 20 mmol/L Hepes (pH 7.5), 10% glycerol, 2 mmol/L DTT, and 200 μmol/L caspase substrate. The reaction mixture was incubated at 37 °C for 1 h. After that time, enzymatic activity was measured in a luminescence spectrometer (Aminco-Bowman Series 2) (excitation wavelength 380 nm, emission wavelength 460 nm).

### 2.10. Western blot analysis

Procaspases-3 and -8 were detected in cytosolic extracts (20 μg per lane), cytochrome *c* was analyzed in mitochondrial and cytosolic fractions (5 μg protein/condition). Bax, Bcl-$x_L$, and Bid were explored in mitochondria-enriched fractions (30 μg protein for Bax and Bcl-$x_L$, and 15 μg for Bid). Samples were resolved by SDS–PAGE and electroblotted onto polyvinyl difluoride membranes. Immunoblots were blocked and incubated overnight at 4 °C with the primary antibody (1:500). Finally, they were incubated with peroxidase-conjugated secondary antibody (1:5000) and bands were detected by ECL detection system (Amersham Pharmacia Biotech). The immunoreactive bands were quantified using the Gel-Pro Analyzer (Media Cybernetics) software. In all Western blotting analysis, the uniformity of protein loading was checked using Ponceau S.

### 2.11. Analysis of mitochondrial transmembrane potential

To examine mitochondrial $\Delta\Psi$, cultured hepatocytes were loaded with TMRM, a membrane-permeable cationic fluorophore that accumulates in mitochondria in proportion to their $\Delta\Psi$ [31]. $\Delta\Psi$ was determined by using a ratio fluorescence approach [32].

### 2.12. Statistical analysis

The results were expressed as means ± SE. Significance in differences was tested by one-way ANOVA, followed by Tukey test. Differences were considered significant when the $p$ value was $< 0.05$.

## 3. Results

### 3.1. Secretion of TGF-$\beta_1$ to the culture media

Cultured hepatocytes from preneoplastic rat livers did not show increased levels of secreted TGF-$\beta_1$ when they

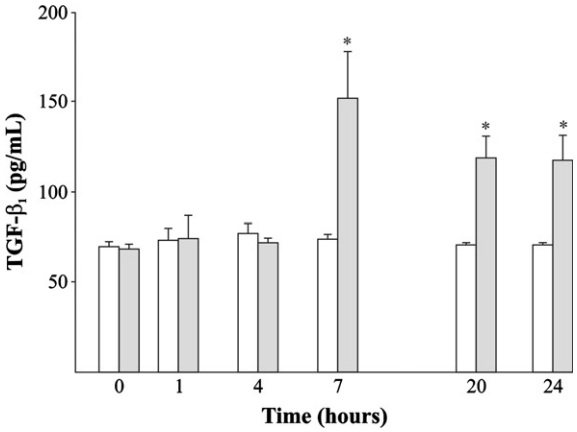


Fig. 1. Release of TGF-$\beta_1$ (pg/mL) by cultured hepatocytes without (white bars) and with (gray bars) IFN-α2b addition. Data are expressed as means ± SE. $^*p < 0.05$ vs. unstimulated hepatocytes from each corresponding studied time.

were stimulated with IFN-α2b at 1 and 4 h of culture. However, IFN-α2b presence in the culture media induced a twofold increase of TGF-$\beta_1$ secretion at 7 h of culture. TGF-$\beta_1$ levels continued significantly increased at 20 and 24 h of culture (Fig. 1).

### 3.2. DNA fragmentation analysis

According to other authors [25], only cytosolic, fragmented DNA was isolated, due to the fact that in total DNA isolation experiments it was difficult to observe the typical oligosomal ladder.

A representative time dependence study of the cell death process is shown in Fig. 2a. Cultured hepatocytes did not show DNA ladder after 1 and 4 h of IFN-α2b stimulus. A very small amount of low molecular weight DNA fragmentation was first observed at 7 h of incubation with IFN-α2b, and continuously increased for the next studied hours, reaching a maximum at 24 h of culture. In contrast, fragmented DNA was not observed in cultured cells that were left untreated at all studied times (data not shown).

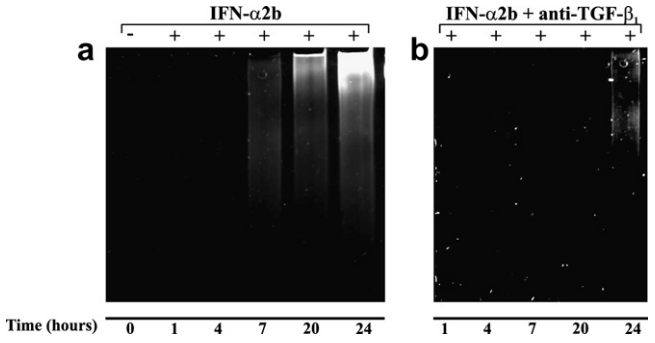


Fig. 2. Effect of IFN-α2b on DNA fragmentation. Hepatocytes from preneoplastic livers were incubated for 1, 4, 7, 20, and 24 h without (not shown) and with IFN-α2b (a), and also with IFN-α2b plus anti-TGF-$\beta_1$ (b). Cells were lysed and subjected to cytoplasmic DNA extraction. Purified low molecular weight DNA was electrophoresed on 10% polyacrylamide gel and visualized by ultraviolet fluorescence by staining with ethidium bromide. A representative experiment is shown.

Table 1
Effect of IFN-α2b on hepatocytes apoptosis determined by annexin V staining

| Hours | Percentage of apoptotic hepatocytes | | |
|---|---|---|---|
| | Without IFN-α2b | With IFN-α2b | With IFN-α2b plus anti-TGF-$\beta_1$ |
| 1 | 8.4 ± 0.9 | 9.1 ± 0.5 | 8.2 ± 1.3 |
| 4 | 8.9 ± 0.6 | 8.9 ± 0.7 | 8.8 ± 0.7 |
| 7 | 9.3 ± 0.2 | 8.8 ± 0.5 | 9.8 ± 0.2 |
| 20 | 9.3 ± 0.3 | 14.3 ± 1.4* | 10.7 ± 1.9 |
| 24 | 9.1 ± 0.4 | 21.2 ± 0.1* | 9.1 ± 0.3 |

Results are expressed as means ± SE from three independent experiments with duplicate dishes for each point.
* $p < 0.05$ vs. unstimulated cells from each period of time.

In addition, experiments were performed with IFN-α2b plus anti-TGF-$\beta_1$ to demonstrate whether the DNA fragmentation observed in IFN-α2b-treated cells is mediated by the production of TGF-$\beta_1$ from hepatocytes. When anti-TGF-$\beta_1$ was added to the culture media, no DNA cleavage was detected (Fig. 2b).

### 3.3. Annexin V staining analysis

Table 1 shows the percentage of apoptotic hepatocytes by annexin V staining at the different hours of culture. The percentage of apoptotic cells in IFN-α2b-treated cultures significantly increased over unstimulated cultures at 20 and 24 h. When anti-TGF-$\beta_1$ was added to the culture media, no increase in the percentage of apoptotic hepatocytes could be detected.

### 3.4. ROS production analysis

It has been described that TGF-$\beta_1$-induced apoptosis in rat fetal hepatocytes is mediated by ROS production [25]. In this work, we examined whether the generation of ROS is involved in the IFN-α2b-induced apoptotic cell death of hepatocytes from rat preneoplastic livers. For this purpose, we used DCFH-DA, an oxidation-sensitive fluorescent probe [27]. Cells were incubated at different times (0.5, 1, 1.5, 2, 3, 4, 7, 20, and 24 h) with or without IFN-α2b. Hepatocytes stimulated with this cytokine showed a maximum increase in ROS production at 1 h of culture (Fig. 3). After this time, ROS decreased, returning to control values. The presence of anti-TGF-$\beta_1$ did not affect the peak of ROS observed at 1 h of culture.

### 3.5. Caspases-3 and -8 activation analysis

In this work, time-course experiments to analyse the activities of caspases-3 and -8, and the expressions of procaspases-3 and -8 following IFN-α2b treatment were performed. These procaspases are converted into active subunits during the course of activation [16]. Thus, decreases in densities of procaspase-3 or -8 bands indicate activation of the respective caspase.

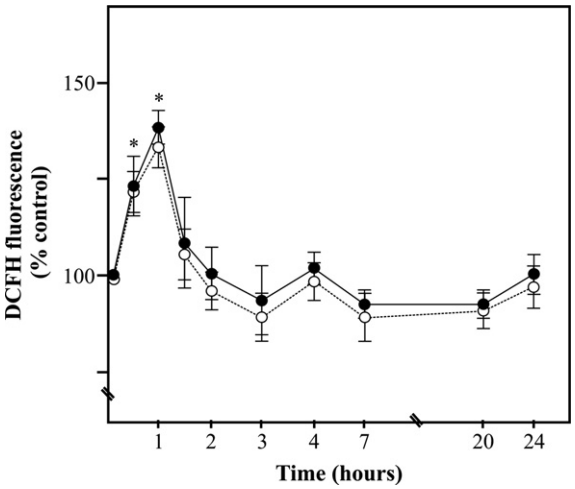


Fig. 3. IFN-α2b induction of intracellular reactive oxygen species content. Hepatocytes from preneoplastic livers were cultured for different times (0.5, 1, 1.5, 3, 4, 7, 20, and 24 h) in the absence or presence of IFN-α2b. The production of ROS was analyzed as described in Section 2. Results from IFN-α2b- (black circles) and IFN-α2b plus anti-TGF-$\beta_1$- (white circles) treated hepatocytes are expressed as percentages of control (untreated) cells and are means ± SE from at least four independent experiments. $^{*}p < 0.05$ vs. untreated cells.

Figs. 4a and b show that caspase-3 like and caspase-8 activities significantly increased at 24 h of culture under IFN-α2b stimulus (40% and 85%, respectively, compared with untreated cells). Before this time, no increase in caspases-3 and -8 activities could be detected. According to these results, procaspase-3 and procaspase-8 levels significantly decreased after 24 h of IFN-α2b treatment (Figs. 4c and d). Besides, immunoblotting studies showed that procaspase-3 levels started to diminish at 20 h of culture in presence of IFN-α2b, whereas procaspase-8 levels started to decrease at 7 h of stimulation with IFN-α2b, although these decreases were not statistically significant. On the other hand, no changes in procaspase-3 or procaspase-8 protein levels were observed in unstimulated cultured hepatocytes from preneoplastic livers (Figs. 4c and d).

These data showed that caspases-3 and -8 were processed and activated during IFN-α2b-induced apoptosis via TGF-$\beta_1$. However, there was no clear evidence if one caspase is being activated before the other.

### 3.6. Release of cytochrome c and mitochondrial transmembrane potential analysis

We studied the intracellular distribution of cytochrome *c* in IFN-α2b-stimulated hepatocytes from preneoplastic livers. As early as 4 h after treating with IFN-α2b, there was a significant increase in the cytochrome *c* content of the cytosol and its levels increased over time, reaching a maximum at 20 h. At the same time, cytochrome *c* content decreased in the mitochondria. These results showed that the release of the cytochrome *c* was an early cellular response to TGF-$\beta_1$ (Figs. 5a and b).

The disruption of mitochondrial transmembrane potential ($\Delta\Psi$) has been defined as an early irreversible stage of apoptosis, preceding caspase-3 activation [10]. In agreement with results previously reported for adult and fetal rat hepatocytes [33,34], in this work we presented evidence that the loss of mitochondrial $\Delta\Psi$ was coincident with the

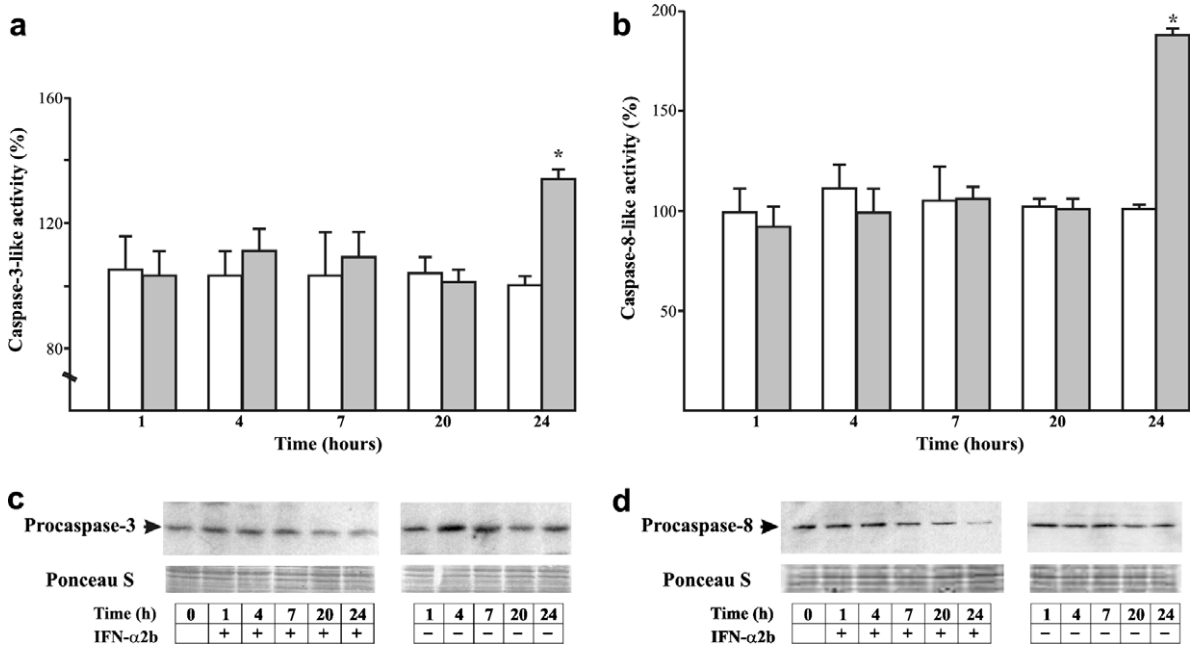


Fig. 4. Time course analysis of caspase-3 and caspase-8 activation during IFN-α2b-induced apoptosis in hepatocytes from rat preneoplastic livers. Time course analysis of (a) caspase-3 and (b) caspase-8 activities. Cells were incubated without (white bars) or with (gray bars) IFN-α2b for different periods of time (1, 4, 7, 20, and 24 h), and caspases-3 and -8 activities were assayed. Results are expressed as percent values as means ± SE from three independent experiments with duplicate dishes for each point. $^{*}p < 0.05$ vs. unstimulated cells from each period of time. Time course analysis of (c) procaspase-3 and (d) procaspase-8 levels. Hepatocytes were untreated (−) or treated (+) with IFN-α2b and procaspases-3 and -8 expressions were studied by Western blot.

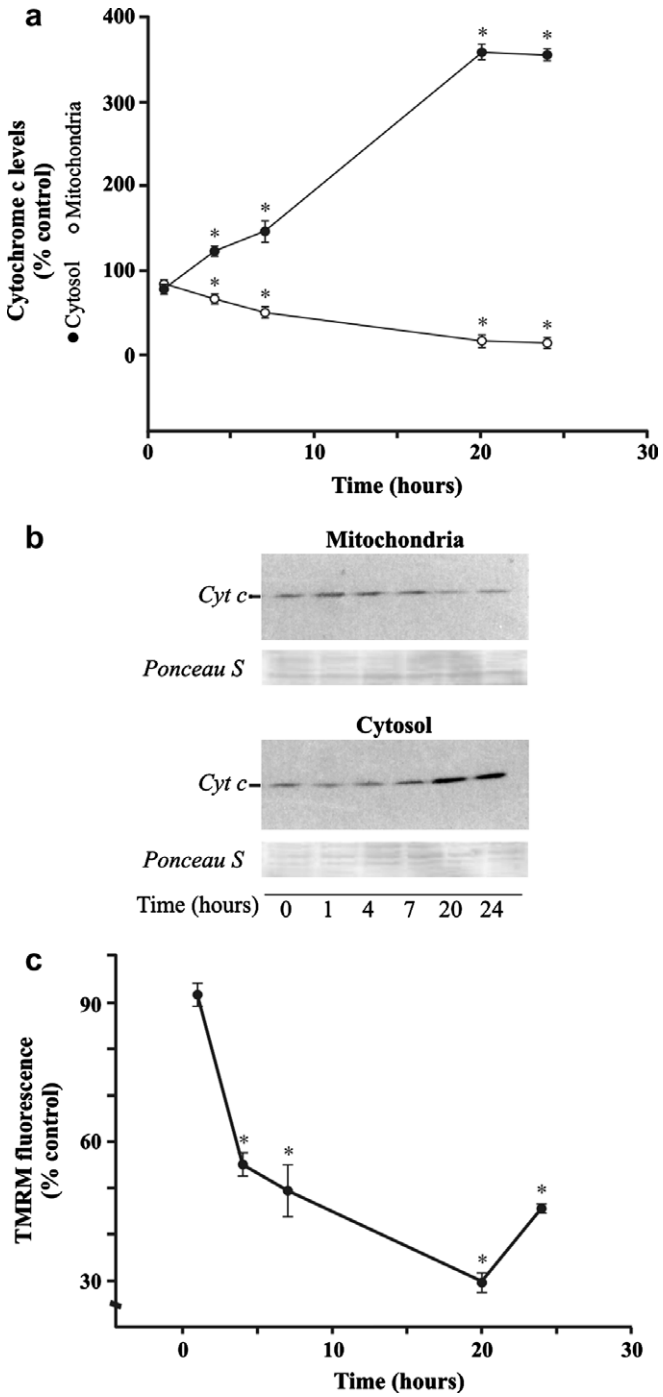


Fig. 5. Time course analysis of the release of cytochrome *c* from mitochondria to cytosol during IFN-α2b-induced apoptosis in hepatocytes from preneoplastic livers. Mitochondria were separated from cytosol and cytochrome *c* content was analyzed by Western blot as described in Section 2. (a) Cytochrome *c* levels in cytosolic and mitochondrial fractions were expressed as percentages of control (untreated) cells, as means ± SE of four independent experiments. $^{*}p < 0.05$ vs. control cells. (b) A representative experiment of a Western blot is shown. (c) Time course analysis of mitochondrial transmembrane potential changes. Cells were incubated without or with IFN-α2b during 1, 4, 7, 20, and 24 h, and TMRM fluorescence was measured. Results are expressed as percentages of control (untreated) cells as means ± SE from three independent experiments. $^{*}p < 0.05$ vs. control cells.

release of cytochrome *c* from mitochondria to cytosol (Fig. 5c).

### 3.7. Analysis of the Bcl-2 family proteins expression

The Bcl-2 family of proteins is related to the mitochondrial changes during apoptosis. Bax and Bcl-$x_L$ involvement in TGF-$\beta_1$-induced programmed cell death is controversial [16,17,20]. So, we analyzed Bax and Bcl-$x_L$ mitochondrial content in hepatocytes from preneoplastic livers. Results showed that the levels of Bax increased in mitochondria of hepatocytes after 4 h of IFN-α2b treatment (30% compared with untreated cells) and augmented in a time-dependent manner, reaching a maximum at 20 h of culture (95% of increase with respect to unstimulated cells) (Fig. 6a). It is well known that IFN-α induces apoptosis in different tumor cell lines through the activation of pro-apoptotic Bax [35], so we have included Bax activation studies in cultured cells treated with IFN-α2b and anti-TGF-$\beta_1$ in order to discard a possible induction of Bax by IFN-α2b itself. The results indicated that the presence of anti-TGF-$\beta_1$ in the culture media of IFN-α2b-stimulated hepatocytes completely blocked the increase of mitochondrial Bax protein levels at the studied times (data not

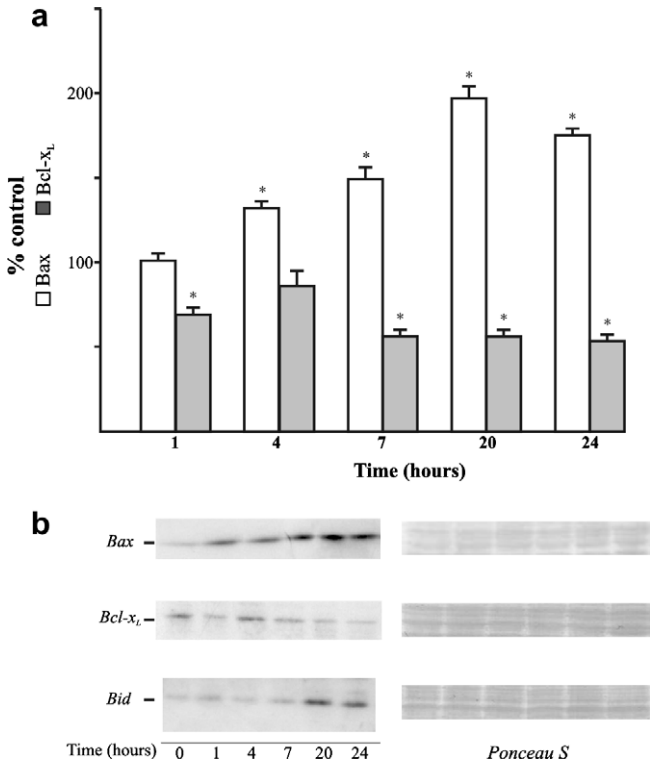


Fig. 6. Role of Bcl-2 family proteins in IFN-α2b-induced apoptosis of hepatocytes from preneoplastic livers. (a) Kinetic study of Bax and Bcl-$x_L$ mitochondrial levels, analyzed by Western blot. Densitometric analyses were performed in at least four independent experiments. Data are expressed as percentages of control (untreated) cells and are means ± SE. $^{*}p < 0.05$ vs. control cells. (b) A representative experiment of a Western blot analysis of Bax, Bcl-$x_L$, and Bid protein levels in mitochondria fractions is shown.

shown). So, Bax up-regulation in IFN-α2b-treated cells is mediated by the TGF-$\beta_1$ secreted from hepatocytes.

On the other hand, Bcl-$x_L$ mitochondrial content was decreased in IFN-α2b-stimulated cells already at 1 h of treatment (30% compared with untreated cells), and reached a diminution of 50% at 7 h (Fig. 6a). This down-regulation of Bcl-$x_L$ in the early hours of apoptosis, although no significant during the time-course study, is in agreement with previous reports [14,16]. Moreover, Bax/Bcl-$x_L$ expression ratio was calculated from the densitometric analysis of Western blots; the ratio clearly increased during the studied times: $1.5 \pm 0.1$, $1.6 \pm 0.2$, $2.7 \pm 0.3$, $3.5 \pm 0.4$, and $3.3 \pm 0.3$, at 1, 4, 7, 20, and 24 h, respectively.

We also examined the mitochondrial levels of pro-apoptotic protein Bid. Caspase-8-mediated cleavage of Bid greatly increases its pro-death activity and results in translocation to mitochondria, where promotes cytochrome *c* release [35]. Fig. 6b shows that truncated Bid increased in mitochondria after 20 h of culture. As expected, this delayed translocation of Bid protein was in concordance with a late caspase-8 activation.

## 4. Discussion

Dysregulation of apoptosis has been implicated in numerous pathological conditions, including cancer, autoimmune disorders, viral infections, and others [5,7]. Efforts aimed at treating these diseases by manipulating cell suicide would seem to have great potential, although they are at a relatively early stage.

In a previous work [4], we showed that IFN-α2b-induced production of TGF-$\beta_1$ by hepatocytes from preneoplastic livers is involved in the apoptotic elimination of preneoplastic foci. *In vitro* studies revealed enhanced apoptosis of IFN-α2b-treated hepatocytes at 24 h of culture, and this effect was mediated by hepatocytic TGF-$\beta_1$. Therefore, we investigated the mechanisms of IFN-α2b-induced apoptosis via TGF-$\beta_1$ in isolated hepatocytes from preneoplastic rat livers by exploring some critical factors involved in the apoptotic pathway.

The present work was made on a mixed population of hepatocytes (preneoplastic plus surrounding cells). Although we were unable to separate both populations, the study of the apoptotic phenomenon induced by IFN-α2b in the whole preneoplastic liver is of great interest, due to the fact that patients with liver diseases treated with this cytokine, such as chronic hepatitis B or C, may already have small, clinically undetectable preneoplastic foci during therapy.

The induction of apoptosis by IFN-α2b was determined by annexin V staining. As previously reported [4], the percentage of apoptotic hepatocytes in IFN-α2b-treated cultures increased twofold over unstimulated cultures at 24 h. The same increase was also observed at 20 h of IFN-α2b stimulus. In accordance, the analysis of DNA fragmentation showed a maximal increase of cytosolic DNA at 24 h of culture. The addition of anti-TGF-$\beta_1$ blocked hepatocytes apoptosis, with no increase in DNA fragmentation and percentages of apoptotic hepatocytes, similar to those in unstimulated cultures.

Reactive oxygen intermediates, such as hydrogen peroxides or oxygen radicals, are integral control elements in the cell's decision to enter apoptosis. IFN-α2b-mediated apoptosis via TGF-$\beta_1$ in hepatocytes from preneoplastic livers was preceded by an enhancement of ROS production. An early ROS production induced by TGF-$\beta_1$ has been previously described in fetal rat hepatocytes [14,34]. However, in the present study the maximum increase in ROS at 1 h after IFN-α2b treatment was not coincident with the highest concentration of TGF-$\beta_1$ in conditioned media detected after 7 h of treatment. Moreover, the peak of ROS at 1 h was not affected by the presence of anti-TGF-$\beta_1$ in the culture media. In consequence, the increase of ROS might be mediated by IFN-α2b but not by TGF-$\beta_1$. It has been shown that IFN-γ induces ROS generation via upregulation of NADPH oxidase, a ROS-producing system [37]. The same mechanism has been described to be activated by IFN-α in human neutrophils [38]. So, it is possible that in our model IFN-α2b could be raising ROS levels by NADPH oxidase induction. Moreover, further work with scavengers of reactive species is in process to provide direct evidence that the generation of ROS is involved in the IFN-α2b-induced apoptosis.

In the current study, we observed that caspases-3 and -8 were activated in IFN-α2b-treated hepatocytes from preneoplastic livers. Previous studies have indicated that activation of caspase-8 precedes the activation of caspase-3 in TGF-$\beta_1$-induced apoptosis in hepatoma cells [16]. However, we were unable to define an ordered and sequential processing of individual caspases. On the other hand, Scaffidi et al. [39] previously reported that in some types of cells such as liver cells there is a minimal amount of receptor-activated caspase-8 and probably insufficient to induce downstream effector caspase cleavage. This low caspase-8 activity is thought not to be usually detected but to be enough to cleave Bid. Truncated Bid then translocates to the mitochondria where initiates mitochondria-dependent apoptotic signals including cytochrome *c* release and efficient activation of both caspases-8 and -3. In view of the fact that procaspase-8 levels started to decrease at 7 h of IFN-α2b treatment, we studied Bid protein mitochondrial levels. The delayed translocation of Bid to the mitochondria (after 20 h of culture, see Fig. 6b) allowed us to ascertain that the mechanism postulated by Scaffidi et al. [39] is unlikely to contribute significantly to the apoptotic effect of IFN-α2b on hepatocytes from preneoplastic livers.

In the present study, Bax increased in a time-dependent fashion, and Bcl-$x_L$ was down-regulated in the early hours of IFN-α2b treatment. Therefore, the relative ratio increased during the studied times, clearly favoring the apoptotic process. It has been reported that TGF-$\beta_1$ can down-regulate Bcl-$x_L$ protein levels in some hepatoma cells [16,20] and fetal rat hepatocytes [18] without affecting Bax

expression. Therefore, to our knowledge, this is the first study where TGF-$\beta_1$ induced mitochondrial Bax overexpression in hepatic cells.

We have also shown that cytochrome *c* is early released into the cytoplasm in the time course of TGF-$\beta_1$-induced apoptosis (Fig. 5). This suggested that an apoptosome-like-caspase-activation mechanism could be involved. The exact mechanism by which cytochrome *c* is released from the mitochondria during the apoptosis has not been elucidated. Current ideas suggest that Bcl-2 or Bcl-$x_L$ prevent the release of cytochrome *c* and, therefore, suppress apoptosis, whereas Bax and cleaved Bid promotes cytochrome *c* release and apoptotic cell death [9,40]. In our study, the release of cytochrome *c* into the cytosol was coincident with the increase of Bax protein levels in mitochondria. These data are in good agreement with previous observations that Bax protein can directly induce the release of cytochrome *c*, without apparent requirement for caspases [41]. On the other hand, it has been reported that Bid is a much more potent cytochrome *c*-releasing factor than Bax [36]. In accordance with this, the maximal levels of cytochrome *c* found in cytosol at 20 h were corresponded with augmented levels of Bid protein in mitochondria.

In conclusion, IFN-α2b treatment of hepatocytes from preneoplastic rat livers produced an increase in ROS and TGF-$\beta_1$ secretion. This growth factor seems to induce cytochrome *c* release through a mechanism related to Bcl-2 family members. Bax protein could be responsible of the release of cytochrome *c* during the initial hours of IFN-α2b-induced apoptosis via TGF-$\beta_1$, whereas at later times (after 20 h of culture) activation of Bid by caspases could amplificate the mitochondrial events, enhancing the release of cytochrome *c*.

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