Epidermal growth factor, but not hepatocyte growth factor, suppresses the apoptosis induced by transforming growth factor-beta in fetal hepatocytes in primary culture

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Abstract We studied whether the TGF-β-induced apoptosis in fetal hepatocyte primary cultures may be modulated by the presence of mitogenic stimuli, such as EGF or HGF. EGF prevented cell death, showing a dose dependence that was identical to that observed for its effect on DNA synthesis stimulation. HGF, in contrast, had no effect, even at high concentrations. EGF blocked apoptosis, since in the presence of this factor cells did not show DNA fragmentation. Moreover, EGF, but not HGF, blocked c-*fos* induction associated with the apoptotic process induced by TGF-β in these cells.

Key words: TGF-β; Apoptosis; Fetal hepatocyte; HGF; EGF

1. Introduction

Apoptosis, or programmed cell death, is essential in many aspects of normal development and is required for maintaning homeostasis [1]. Because apoptosis plays a critical role in deleting cells from tissues, it is not surprising that failure of apoptosis leads to imbalanced cell proliferation and is now recognized as a mechanism of carcinogenesis. Lethal cellular programs that lead to apoptosis may be triggered by a variety of exogenous and environmental stimuli. As in all biological systems, there are activators and inhibitors, growth factors and cytokines clearly playing an important regulatory role. During recent years, increasing evidence supports a role of apoptosis in normal liver biology and as a pathophysiological mechanism of cell death during hepatobiliary disease (for a review, see [2]). However, very little is known about the possible role of apoptosis during the growth and differentiation of fetal liver.

Transforming growth factor-beta (TGF-β) is one of the best known physiological inhibitors of epithelial cell proliferation and recent interest has been focused on its possible role in cell death. It is a member of a large family of structurally related factors that play a critical role during embryogenesis in mammals, frogs and flies [3,4]. We have reported that TGF-β inhibits the epidermal growth factor (EGF) – and/or hepatocyte growth factor (HGF) – induced DNA synthesis in fetal hepatocytes in primary culture [5,6] and modulates proto-oncogene and liver-specific gene expression in these cells [7,8]. However, TGF-β might have other functions in the liver, since higher concentrations of this factor both inhibit proliferation and increase apoptosis in fetal [9] and adult [10] rat hepatocytes and in hepatoma cells [11]. Cell proliferation occurs during stimulation of fetal or regenerating hepatocytes by mitogens such as TGF-α, EGF or HGF [5,6,12]. It has been described that when the mitogenic stimulus is withdrawn in vivo, the excess cells are removed by apoptosis, resulting in a regression of the original hyperplasia [13]. However, it is not yet clear if all growth factors may be survival factors for the liver.

In this work we have studied whether TGF-β-induced apoptosis may be modulated by the presence of mitogenic stimuli, such as EGF or HGF, in fetal hepatocytes in primary culture. The results show that both mitogens behave in a different manner. Whereas EGF blocks apoptosis induced by TGF-β, HGF does not. This is the first evidence of a differential response of fetal hepatocytes to EGF and HGF, which clearly might implicate differential roles for both mitogens during liver development, regeneration and hepatocarcinogenesis.

2. Materials and methods

2.1. Materials

TGF-β was from Austral Biologicals (San Ramon, CA). Collagenase was from Boehringer (Mannheim, Germany). Fetal and neonatal calf serum and culture media were from Imperial Laboratories (Hampshire, UK). Radiochemicals were from ICN (Irvine, CA). Multiprimer DNA labeling system was from Amersham (Buckinghamshire, UK). The nick-translation labeling system was from Gibco BRL (Gaithersburg, USA).

2.2. Isolation and culture of fetal rat hepatocytes

Hepatocytes from 20-day old fetal Wistar rats were isolated and plated in arginine-free M-199 medium as previously described [5,14]. Cells were incubated in 5% CO_2, at 37°C for 4 h, allowing cell attachment to plates. The medium was changed at that time and replaced by one of the same composition except that the 10% fetal calf serum was replaced with 2% newborn bovine serum. After 18–20 h, the medium was again replaced for one of identical composition but in the absence of serum. 2 h later, growth factors were added.

2.3. TGF-β-mediated cytotoxicity assay

After cell incubation in the absence or presence of the different factors, the medium was discarded and the remaining viable adherent cells were stained with crystal violet (0.2% in 2% ethanol) for 20 min, as previously described [15]. Remaining viable cells were calculated as % absorbance with respect to control cells (incubated in the absence of growth factors).

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Abbreviations: TGF-β, transforming growth factor-beta; EGF, epidermal growth factor; HGF, hepatocyte growth factor; HNF-4, hepatocyte nuclear factor-4.

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2.4. DNA synthesis assay

After incubation of the cells for 48 h in the absence or presence of the mitogen, DNA synthesis was evaluated by [^{3}H]thymidine incorporation into trichloroacetic acid-precipitable material, as we have previously described [5].

2.5. Analysis of nuclear DNA content by flow cytometry

The ploidy determination of hepatocyte nuclei was estimated by flow cytometry DNA analysis as previously described [5]. Cells were detached from dishes by addition of 0.25% trypsin-0.02% EDTA and the DNA content per nucleus was evaluated in a FACSCAN flow cytometer (Becton-Dickinson, San Jose, CA), after using the Cycle test DNA reagent kit (Becton- Dickinson) to stain nuclei with propidium iodide. For the computer analysis, only signals from single nuclei were considered (10 000 nuclei/assay).

2.6. Analysis of DNA fragmentation

A modified version of the method of Lyons et al. [16] was used to assess the fragmentation of hepatocyte DNA. Rat fetal hepatocytes were washed twice with PBS, then scraped and pelleted at 4°C. Cells were resuspended in 500 μl of buffer containing 10 mM EDTA, 0.25% Triton X-100, 2.5 mM Tris-HCl, pH 8.0 and stored at 4°C for 15 min. Intact nuclei were pelleted and eliminated by centrifugation at 500×*g* for 10 min and the supernatant centrifuged at 25 000×*g* at 4°C for 30 min. DNA in the supernatant was precipitated at −80°C after addition of 2 vols. of ethanol (70% final concentration), pelleted by microcentrifugation at 4°C for 15 min, dried, resuspended in 200 μl 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE buffer) and incubated at 37°C for 30 min with 0.1 mg/ml RNAse A and for 2–3 h with 0.25 mg/ml proteinase K. DNA was purified by phenol-chloroform extraction and precipitated at −70°C after adding (1/10 vol. of) 3 M sodium acetate, pH 5.3 and (2 vols. of) ethanol. Precipitated DNA was dissolved in TE buffer containing 30% glycerol, 1 μg/ml ethidium bromide and electrophoresed in a 1.5% agarose gel. The gel was visualized and photographed under transmitted UV light with a Polaroid camera.

2.7. RNA Isolation and Northern blot analysis

Total RNA was isolated as described by Chomczynski and Sacchi [17]. For each assay, RNA was extracted from the pooled cells of two 92 mm diameter dishes, denatured in 50% formamide, 2.2 M formaldehyde, 20 mM MOPS, pH 7.0, 6% glycerol at 65°C for 15 min, separated by size on gels containing 0.9% agarose and 0.66 M formaldehyde and blotted on GeneScreen membranes (NEN Research Products, Dupont, Boston MA). Hybridizations were performed as previously described [7]. α-Fetoprotein [18], albumin [19] and 18S ribosomal [20] cDNAs were labeled with [α-^{32}P]dCTP by nick-translation, whereas v-*fos* [21], H-*ras*, [22] and HNF-4 [23] were labeled by random priming reaction. Serial hybridizations with the different probes were performed succesively.

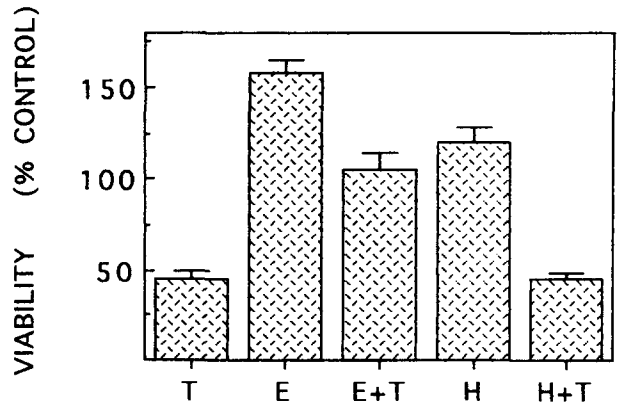

Fig. 1. Effect of epidermal growth factor or hepatocyte growth factor on cell death induced by TGF-β in rat fetal hepatocytes in primary culture. Cells were cultured in the absence or presence of 1 ng/ml TGF-β (T), 20 ng/ml EGF (E), 10 ng/ml HGF (H), or the combination of EGF and TGF-β (E+T), or HGF and TGF-β (H+T) for 24 h. After this time cells were stained with crystal violet and the viability was quantified as described in section 2. Results are expressed as % of control and are means ± S.E.M. from three independent experiments of triplicate dishes.

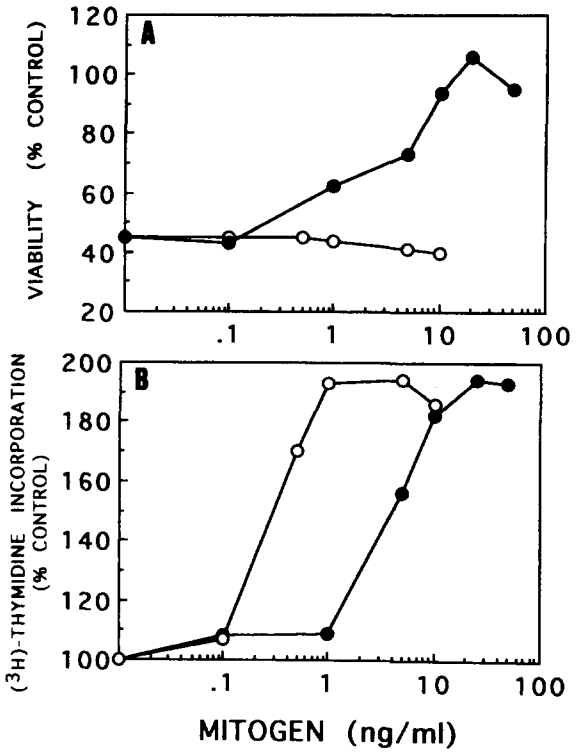

Fig. 2. (A) Dose-response analysis of the effect of EGF or HGF on the apoptosis induced by TGF-β in fetal hepatocytes. After culture of cells for 24 h in the presence of 1 ng/ml TGF-β and in the absence or presence of different concentrations of EGF (●) or HGF (○), cell viability was calculated as described in Fig. 1. (B) Dose-response analysis of the mitogenic effect of EGF or HGF. After culture of cells for 48 h in the absence or in the presence of different concentrations of EGF (●) or HGF (○), DNA synthesis was calculated by [^{3}H]thymidine incorporation as described in section 2. In both (A) and (B), results are expressed as % control (untreated) cells and are a representative experiment of at least three.

3. Results and discussion

3.1. Effect of growth factors on cell death induced by TGF-β in fetal hepatocytes

We have previously reported that TGF-β regulates death of fetal hepatocytes in primary culture [8]. These cells die in the presence of the factor in a dose-dependent manner, presenting clear evidence of the involvement of an apoptotic process [9]. However, nothing is known about the possible protective role of growth factors on TGF-β-induced apoptosis in hepatocytes. For this reason, as a first approach we investigated whether or not known mitogenic factors for these cells could prevent TGF-β-induced cell death. When hepatocytes extracted from 20-day-old fetuses were incubated for 24 h in the presence of 1 ng/ml TGF-β, 50% of the cells died. A similar result was observed when TGF-β was combined with 10 ng/ml HGF. However, in the presence of 20 ng/ml EGF, TGF-β was ineffective, the percentage of viable cells being maintained as in control cells (Fig. 1).

A dose-dependence study of the EGF-protective effect on cell death induced by TGF-β showed that low concentrations of the factor did not have any effect. Concentrations of 1–20

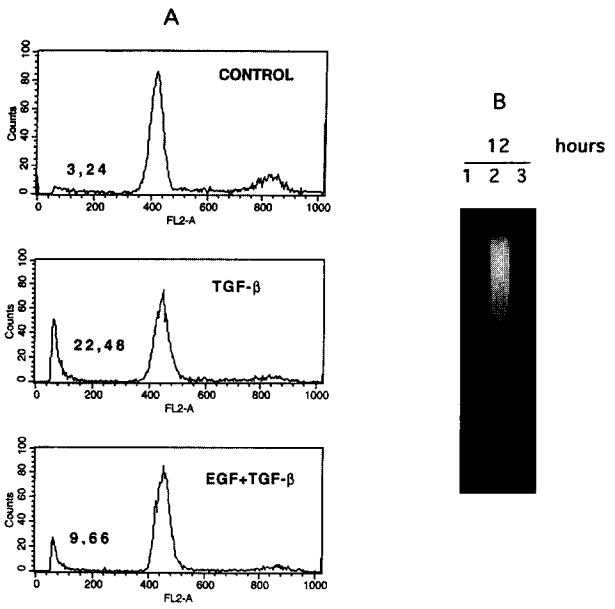

Fig. 3. Effect of EGF on TGF-β-induced DNA fragmentation. (A) Nuclear DNA content of untreated cells, or cells that were treated for 12 h with either 1 ng/ml TGF-β alone or TGF-β in combination with 20 ng/ml EGF was analysed by propidium iodide staining in a flow cytometer. The DNA profiles of a representative experiment (of four) are shown (B). After 12 h incubation of fetal hepatocytes in the absence (1) or presence of TGF-β (2) or TGF-β and EGF (3) cells were lysed and low molecular weight DNA isolated, subjected to gel electrophoresis on a 1.5% agarose gel and visualized by UV fluorescence after staining with ethidium bromide. A representative experiment of three is shown.

ng/ml showed a progressive protective effect that became maximal at 10–20 ng/ml, where cell death was totally abolished (Fig. 2A). This dose dependence was identical to that observed for the DNA synthesis stimulated by EGF in these cells (Fig. 2B). In contrast, HGF did not have any effect even at concentrations higher than those necessary for obtaining the maximal response in terms of cell proliferation (Fig. 2).

In our previous studies on apoptosis elicited by TGF-β we had seen that TGF-β at apoptotic doses induced changes in cell morphology. Hepatocytes responded to TGF-β with an initial loss of cell contacts and migration, followed by degeneration and detachment [9]. HGF did not have any influence on such morphological effects, while cells treated with TGF-β in combination with EGF were perfectly attached to dishes, showing intact morphology (results not shown).

3.2. *EGF prevents DNA fragmentation induced by TGF-β in fetal hepatocytes in primary culture*

In order to assess whether EGF was really blocking the apoptotic process elicited by TGF-β, we first analyzed the nuclear DNA content by flow cytometry, staining nuclei with propidium iodide. Cells that had been incubated for 12 h in the presence of TGF-β showed a significant percentage (about 20–25%) of nuclei with a DNA content lower than 2C, in contrast to 2–3% in control (untreated) cells. In contrast, in the presence of TGF-β and EGF the percentage of nuclei with a DNA content lower than 2C disminished to 8–10%, an important decrease with respect to TGF-β alone (a representative experiment is shown in Fig. 3A).

In an alternative approach, we analyzed if EGF was also able to prevent DNA fragmentation induced by TGF-β. Low molecular weight DNA was extracted from parallel dishes that had been incubated in the absence or presence of TGF-β alone or in combination with EGF, and examined by electrophoresis. Cells that were treated with TGF-β for 12 h showed the typical DNA ladder. In contrast, DNA fragmentation in cells treated with EGF and TGF-β was not observed (Fig. 3B).

All these results suggest that EGF prevents the apoptosis induced by TGF-β in fetal hepatocytes, presenting a dose dependence that is similar to that observed for its mitogenic activity. EGF and TGF-α also inhibit the onset of apoptotic DNA cleavage found in other cell models, such as cultured rat ovarian granulosa cells [24] or mammary epithelial cells [25].

3.3. *EGF, but not HGF, suppresses TGF-β induction of c-fos mRNA levels in fetal hepatocytes in primary culture*

We had observed that apoptotic concentrations of TGF-β induced c-*fos* expression in fetal hepatocytes, that was maximal 24–48 h after adding the factor [9]. It therefore seemed of interest to study whether EGF also prevented the induction of c-*fos* expression. For this purpose, total RNA extracted from

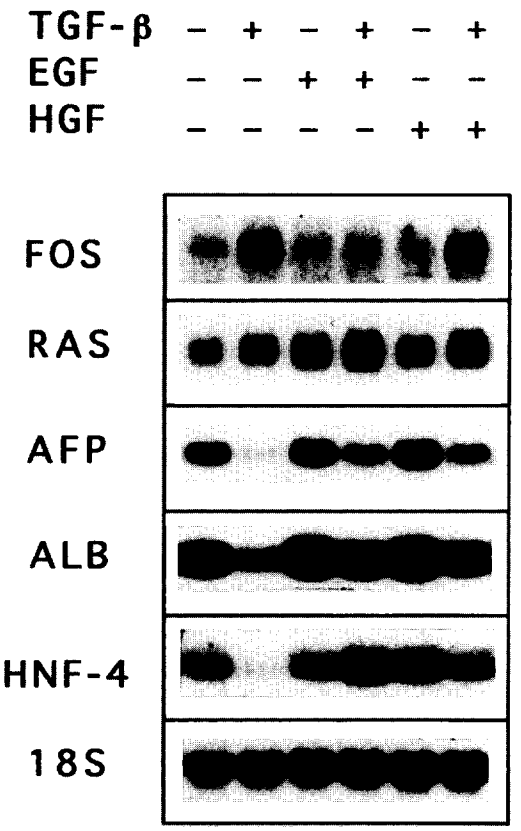

Fig. 4. Role of EGF and HGF in the changes in gene expression induced by TGF-β in fetal hepatocytes in primary culture. Cells were cultured in the absence or presence of 1 ng/ml TGF-β, 20 ng/ml EGF, 10 ng/ml HGF, or the combination of TGF-β and EGF or TGF-β and HGF. After 24 h of treatment, total RNA was isolated and 10 μg were subjected to Northern blot analysis using the cDNA probes specific for c-*fos*, H-*ras*, α-fetoprotein (AFP), albumin (ALB) and HNF-4 mRNAs. 18S Ribosomal probe was used for RNA normalization. A representative experiment of three is shown.

cells incubated for 24 h in the presence of the different factors was subjected to Northern blot analysis. Cells under treatment with TGF-β alone showed induced levels of c-*fos* mRNA (Fig. 4). In agreement with the results observed previously in the viability analysis, cells that were treated with HGF and TGF-β presented similar c-*fos* mRNA levels. In contrast, the presence of EGF completely prevented c-*fos* induction. This difference in c-*fos* expression between EGF- and HGF- treated cells was specific, since the mRNA levels of other proto-oncogenes, such as H-*ras* (Fig. 4), c-*myc* and c-*met* (results not shown) were similar in cells that were treated with either EGF+TGF-β or HGF+TGF-β. Elevated expression of c-*fos* has previously been related to programmed cell death [26]. The time course analysis of c-*fos* mRNA levels showed that in TGF-β-treated fetal hepatocytes its gene expression increased just when cells stopped dying [9]. So, we speculated that c-*fos* might be a survival gene involved in the hepatocyte response to TGF-β. Regardless of its role in the apoptotic process, the results presented here clearly show that EGF prevents the TGF-β-induction of this gene.

The analysis of the mRNA levels of liver differentiation-related genes, such as α-fetoprotein (AFP), albumin (ALB) or transcription factor HNF-4, showed that TGF-β-treated cells clearly lost their expression. However, when EGF or HGF were present, the expression of these liver-specific genes was maintained (Fig. 4). We have recently described that TGF-β, at low concentrations, acts synergistically with EGF and/or HGF maintaining the differentiation state of the fetal hepatocytes [8]. The present results show that this effect can also be observed at higher concentrations of TGF-β (Fig. 4), regardless of whether or not apoptosis was observed.

HGF and EGF are both motogenic and mitogenic for hepatocytes in vitro. However, the spatial and temporal differences exhibited between the two growth factors in terms of signal transduction [27,28], as well as their additive effects on hepatocytes in vitro [6,12] strongly suggest that they probably accomplish their effects via redundant as well as divergent signal transduction mechanisms. The differences between their role in the TGF-β-induced apoptosis presented in this paper may have clear implications during liver development and regeneration or hepatocarcinogenesis. Apoptosis is essential in many aspects of normal development and is required for maintaining homeostasis. Thus, too much or too little cell death may have negative consequences. Hepatic TGF-β gene expression is developmentally regulated. A high level of expression of TGF-β mRNA is found in the liver during mid-gestation; it decreases with increasing fetal maturity and decreases further in the postnatal period [29]. Quiescent liver has very low TGF-β mRNA levels, but normal regenerating, neoplastic proliferating and carcinogen-treated liver all have increased levels of TGF-β gene transcripts [30]. Thus, it has been proposed that TGF-β may be the principal counter-regulator of hepatocyte proliferation during liver regeneration [29]. In accordance with the results shown in this paper, EGF (or TGF-α) might play an important role during early liver development, not only acting as an important mitogen, but also allowing cells to overcome apoptosis and differentiate. HGF, in contrast, might play a more important role in the regenerating liver, where apoptosis elicited by TGF-β might be necessary to counteract the high proliferative rate. Under these conditions, HGF, in addition to its role as an important mitogen in the first steps during liver regeneration, could have an additional role to play later on, i.e. allowing TGF-β to eliminate cells and acting synergistically with this factor in differentiating those cells that survive to the programmed cell death.

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