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Bidirectional effects of Kupffer cells on hepatocyte proliferation in vitro

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The control of rat hepatocyte DNA synthesis in vitro by Kupffer cells and transformed perisinusoidal lipocytes, i.e. myofibroblast-like cells was studied. Conditioned media from Kupffer cells inhibit the replicative (hydroxyurea-sensitive) DNA synthesis dose-dependently in primary cultures of hepatocytes stimulated by epidermal growth factor (EGF). The cytokine responsible for the inhibition was identified as transforming growth factor β (TGFβ). After neutralization of activated TGFβ in these media, DNA synthesis is stimulated in quiescent hepatocytes via transforming growth factor α (TGFα) demonstrated by competitive TGFα/EGF-receptor blocking on hepatocytes. Results similar to those obtained with Kupffer cells were found with conditioned media of myofibroblast-like cells. Northern blot hybridization confirms the expression of both TGFβ and TGFα in Kupffer cells and myofibroblast-like cells, respectively. These findings support the notion that Kupffer cells and myofibroblast-like cells might regulate in both directions liver regeneration depending on the proportion of secreted TGFα and TGFβ and on the activation status of TGFβ, of which a significant fraction is secreted in the latent form.

Cell interactions; Kupffer cell; Hepatocyte; Proliferation; Transforming growth factor α, β

1. INTRODUCTION

There are indications of metabolic cooperation between Kupffer cells and hepatocytes which may be important for the regulation of normal and pathologic liver function [1,2]. As examples, Kupffer cell secretions affect protein synthesis [3], influence protein phosphorylation [4] and depress cytochrome P450 functions in parenchymal liver cells [5]. Kupffer cells have been implicated also in the regulation of hepatocyte proliferation in vitro but contradictory results were communicated [6–8]. Whereas previous reports indicate a reduction of hepatocyte proliferation [6,7], recent studies have shown a heatlabile, not yet clearly identified factor in conditioned media from primary cultures of Kupffer cells isolated from intact and regenerating remnant livers after partial hepatectomy which stimulates DNA synthesis in hepatocytes [8]. We have shown recently that Kupffer cells have the ability to modulate the mitotic activity of cultured perisinusoidal lipocytes, a sinusoidal type of liver cells specialized in the storage and metabolism of retinoids [9] in both ways depending on the activity of transforming growth factors (TGF) type α and β [10]. TGFα [11] and TGFβ [12] have been identified as important activators and inhibitors, respectively, of hepatocyte proliferation and are thought to play central roles in the regulation of liver cell regeneration [13]. In the present study we present evidence that Kupffer cells have the potency to stimulate and inhibit hepatocyte DNA synthesis in vitro determined by the ratio of active TGFβ to TGFα. This finding offers an explanation for the above cited uncertainty concerning the effects of Kupffer cells on the regulation of hepatocellular multiplication.

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Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modification of Eagle's medium; EGF, epidermal growth factor; FCS, fetal calf serum; KC, Kupffer cells; KCcm, Kuppfer cell conditioned medium; MFblC, myofibroblast-like cells; MFblCcM, myofibroblast-like cell conditioned medium; PC, parenchymal cells; PL, perisinusoidal lipocytes; TGF, transforming growth factor

2. MATERIALS AND METHODS

2.1. *Chemicals*

Radiochemicals were purchased from NEN-Du Pont (Dreieich, Germany), ICN (Eschwege, Germany) and from Amersham/Buchler (Braunschweig, Germany). Rat TGFα and rat TGFα-fragment (amino acid residues 34–43) [14] were obtained from Bachem Biochemica (Heidelberg, Germany) and human platelet TGFβ from Paesel (Frankfurt, Germany). Neutralizing antibodies against porcine and human TGFβ were supplied by British Biotechnology Ltd. (Oxford, UK). The 44 base long TGFα oligonucleotide probe was purchased from Dianova (Hamburg, Germany). The TGFβ cDNA probe (pTGFβ33) [15] was kindly provided by Dr P. Kondaiah (NIH, Bethesda, USA).

2.2. *Methods*

2.2.1. Isolation and culture of hepatocytes, perisinusoidal lipocytes and Kupffer cells

All cells were prepared from male Sprague–Dawley rats following previously described procedures [16–18].

Hepatocytes were isolated following the collagenase perfusion method of Seglen [19]. Normal rats were starved overnight before cell isolation. Viability of isolated hepatocytes checked by Trypan blue

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exclusion was greater than 90% and contamination with vimentin/desmin-positive cells was below 0.4%. The cells were seeded with a density of 5×10^4/cm^2 in 6-well plates and cultured in Hanks F-12 medium containing 0.2% BSA, 0.02 u/ml insulin, and 1% penicillin/streptomycin. Medium was changed 2 h and 20 h after seeding. Additions of factors (TGFα,β) and of conditioned media were made at the same times.

Perisinusoidal lipocytes were seeded with a density of 3.8×10^6 in 75 cm^2 tissue culture bottles and maintained as monolayers in DMEM containing 10% (v/v) FCS. Medium changes were made every second day beginning 16 h after seeding. After reaching confluency about 6 days after seeding, primary cultured cells were subcultured by trypsinization are reseeding. Cells were passaged for a 2nd time after a further incubation period of 14 days. The purity of freshly isolated cells used in this study was at least 90%, cell viability checked by Trypan blue exclusion was more than 80% and the yield ranged from 2×10^7 to 5×10^7 cells/liver. With the first medium change most of the contaminating cells were removed and the monolayers were essentially free of impurities. PL were identified by their typical light microscopic appearance, transmission electron microscopy [20], positive immunochemical staining for vimentin, desmin, α-smooth-muscle-actin and vitamin A-specific autofluorescence [20].

Kupffer cells were seeded with a density of 0.4×10^6 cells/cm^2 in 75 cm^2 bottles and incubated for 2 h in 15 ml DMEM containing 10% FCS. After 2 h the medium was changed to 0.2% BSA instead of FCS. The viability was greater than 90%. Identification and assessment of the purity of the Kupffer cells were performed by light- and electron microscopy, staining for endogenous peroxidase, by phagocytosis of latex beads (for 60 min at 37°C) and by demonstration of positive immunofluorescent staining for vimentin and negative staining for desmin [16]. According to these criteria the purity of seeded Kupffer cells was greater than 95%.

2.2.2. Preparation of conditioned medium

Medium was harvested after a 24 h incubation period in absence of fetal calf serum. The medium was centrifuged (450 × g, 7 min, 4°C) and stored at −80°C. Half of the conditioned medium was acidified (pH 2.0) with HCl for 30 min, after which time the medium was neutralized. Native and transiently acidified media were dialyzed in tubings with a molecular weight cut off 3500 against 100 vol Hanks F-12 medium at 4°C.

2.2.3. [^{3}H]Thymidine incorporation

The incorporation of [^{3}H]thymidine (1 μCi/ml medium) into the DNA of hepatocytes was measured over a 24 h incubation period beginning 20 h after seeding following a previously described protocol [20].

2.2.4. Competitive radioligand assay

TGFβ and TGFα in Kupffer cell conditioned media were quantitated using competitive radioligand binding assays according to previously described methods [21,22]. Monolayers of secondary cultured PL (myofibroblast-like cells) were washed with binding buffer [128 mmol/l NaCl, 5 mmol/l KCl, 1.2 mmol/l $CaCl_2$, 1.2 mmol/l $MgSO_4$, 50 mmol/l HEPES, pH 7.5, with 5 mg/ml BSA) and incubated for 30 min in binding buffer at 37°C. After equilibration for 10 min at 22°C appropriate concentrations of [^{125}I]TGFβ or [^{125}I]EGF, unlabeled peptide (TGFβ, TGFα) and KC conditioned media, dialyzed against binding buffer, respectively, were added. Incubations proceeded for 3 h and were terminated by aspiration of the binding buffer and washing the monolayers with ice-cold binding buffer. Bound radioactivity was liberated with 1% (v/v) Triton X-100 in isotonic saline solution, pH 7.4 and measured in a gamma counter.

2.2.5. Northern and dot blotting

Total RNA from KC was isolated as described by Chomczynski and Sacchi [23]. To examine, whether TGFβ and TGFα are expressed in KC, total RNA was separated by gel electrophoresis and blotted on Hybond N membranes. The measurement of ribosomal S6-protein gene expression was used as internal standard [24]. The 0.8 kb probe for TGFβ was obtained by *SacI-PvuI* digestion of the pTGFβ33 plasmid, subsequent separation by gel electrophoresis followed by elution of the 0.8 kb fragment, which was labeled with [^{32}P]dCTP. The TGFα oligonucleotide probe was 3'-labeled with [^{32}P]ddATP. Hybridization was carried out as described by Maniatis et al. [25]. The blots were exposed for 4–14 days at −80°C to Hyperfilm MP using an intensifying screen.

2.2.6. General techniques

DNA content of the cell cultures was measured fluorometrically using calf thymus DNA as a standard [26].

3. RESULTS

3.1. *Effect of Kupffer cell conditioned medium on the proliferation of EGF-stimulated hepatocytes*

Conditioned medium from Kupffer cells caused a dose-dependent inhibition of [^{3}H]thymidine incorporation into the DNA of hepatocytes stimulated in culture by addition of 25 ng/ml EGF (Fig. 1). Medium, which had been transiently acidified before addition to hepatocyte cultures, was constantly more potent than native (untreated) conditioned medium. The inhibitory activity of KC-conditioned medium could be reduced and even completely abolished by preincubation of the medium with increasing concentrations of neutralizing polyclonal antibodies against TGFβ (Fig. 2).

3.2. *Effects of Kupffer cell conditioned medium on the proliferation of quiescent hepatocytes*

Native and transiently acidified Kupffer cell media had no effect on the [^{3}H]thymidine incorporation into the DNA of hepatocytes cultured in Hanks F-12 medium in the absence of growth factors. When preincubated with 20 μg/ml neutralizing anti-TGFβ antibodies a dose-dependent, hydroxyurea-sensitive growth stimulatory effect of KCcM was observed (Fig. 3). By competitive inhibition of the binding of TGFα to its receptor on hepatocytes induced by addition of the 10 amino acid long TGFα(aa 34–43)-

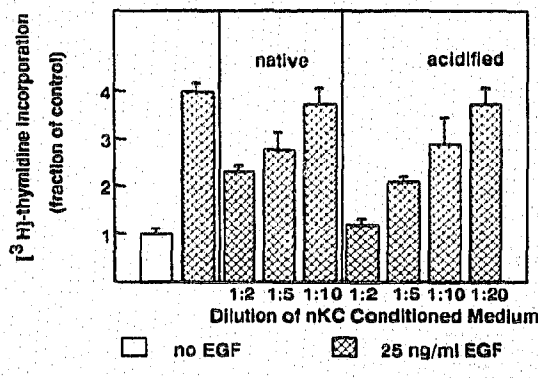

Fig. 1. Inhibition of EGF stimulated DNA synthesis in hepatocytes by native and transiently acidified conditioned medium from cultured Kupffer cells (nKC). Epidermal growth factor (EGF) was added 2 h and 20 h after plating simultaneously with conditioned medium. Control represents DNA synthesis of parenchymal cells in the absence of EGF. Mean values ± SD of 4 experiments are shown.

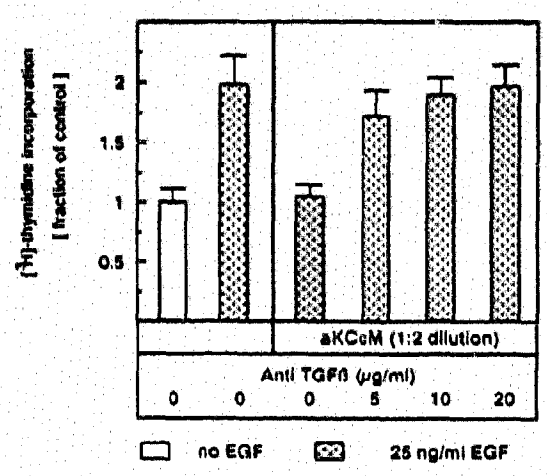

Fig. 2. Effect of anti-TGFβ antibodies on the inhibitory action of conditioned medium from Kupffer cell cultures on hepatocellular [^{3}H]thymidine incorporation. Transiently acidified KC medium (aKCeM) at a 1:2 dilution was incubated for 2 h with increasing amounts of neutralizing anti-TGFβ prior to the addition to hepatocytes stimulated by EGF. Mean values ± SD of 4 experiments are shown.

polypeptide [14], the proliferation stimulatory effect of TGFβ-blocked KCeM was reduced and even completely abolished at a peptide concentration of 12 pmol/ml (Fig. 4).

3.3. *Concentrations of TGFα and TGFβ in Kupffer cell conditioned media and Northern blot hybridizations*

The TGFβ concentration in the medium of Kupffer cells was calculated from data obtained with a competitive radioligand binding assay. It was found to be

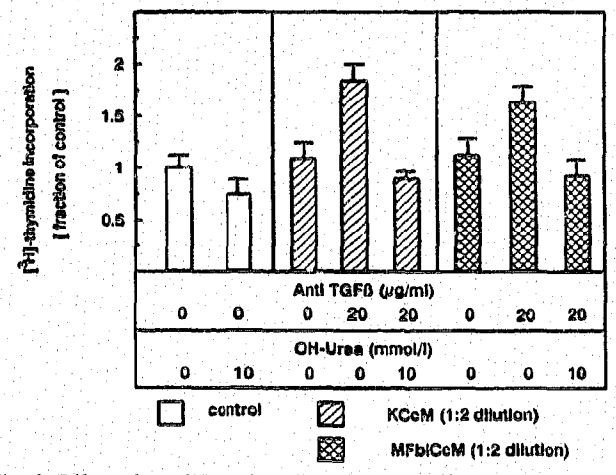

Fig. 3. Effect of conditioned medium from Kupffer cells (KCeM) and transformed PL (myofibroblast- like cells (MFblCeM) on unstimulated hepatocellular DNA synthesis in absence and presence of hydroxyurea (10 mM). Conditioned media and OH-urea were added 2 h and 20 h after plating. In one portion of the conditioned medium TGFβ was neutralized by preincubation at 37°C for 2 h with 20 µg/ml anti-TGFβ before addition to hepatocyte cultures. Mean values ± SD of 4 experiments are shown.

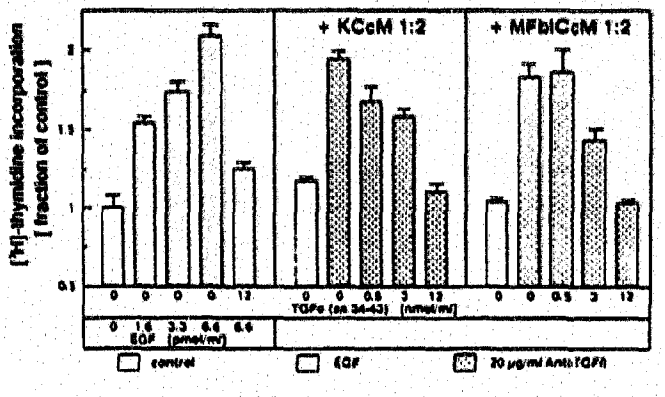

Fig. 4. Effect of TGFα/EGF-receptor blocking on the stimulation of hepatocellular DNA synthesis induced by TGFβ-blocked Kupffer cell conditioned medium (KCeM) and myofibroblast-like cell conditioned medium (MFblCeM). Conditioned medium was pretreated for 2 h with anti-TGFβ and added 2 h and 20 h after plating simultaneously with increasing amounts of competing TGFα peptide (amino acid residues 34-43). Mean values ± SD of 4 experiments are shown.

2.0 ± 0.8 ng/ml, produced by 10^6 Kupffer cells per 24 h (Fig. 5). A single Kupffer cell derived from normal liver produced 0.08 fmol TGFβ/24 h. For determining TGFα concentrations in KCeM, competition with [^{125}I]EGF binding was achieved using rat TGFα. The production rate of TGFα was 20 ± 3 ng/10^6 KC/24 h (Fig. 5). The conditioned medium contained 4.0 ± 0.6 pmol/ml TGFα.

Hybridization of total RNA extracted from 24 h cultured KC with ^{32}P-labeled TGFβ_1 and TGFα probes, respectively resulted each in a single band with a size of 2.6 kb for TGFβ_1 mRNA and about 4.7 kb for TGFα mRNA (Fig. 6).

3.4. *Effects of conditioned medium from transformed perisinusoidal lipocytes (myofibroblast-like cells) on hepatocyte proliferation*

Previously we have shown, that conditioned medium from myofibroblast-like cells, i.e. the transformed

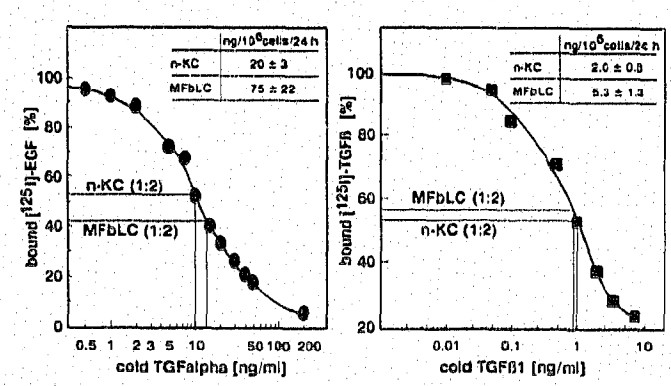

Fig. 5. Binding of [^{125}I]TGFβ and [^{125}I]TGFα to myofibroblast-like cells in the presence of increasing concentrations of unlabeled TGFβ and TGFα, respectively. Competive inhibition of binding by conditioned media from Kupffer cells and myofibroblast-like cells are indicated. Mean values of duplicate experiments are shown.

Fig. 6. Northern blot hybridization of total RNA from cultured Kupffer cells for TGFβ and TGFα. 20 μg RNA from 24 h cultured Kupffer cells was separated by agarose gel electrophoresis under denaturing conditions, blotted on a hybond N membrane and hybridized with [^{32}P]-labeled probes for TGFβ and TGFα, respectively. The positions of the 28S and 18S RNA are indicated.

counterpart of perisinusoidal lipocytes inhibits via TGFβ the EGF and TGFα stimulated proliferation of hepatocytes in culture [27]. In the following experiments we tested the presence of growth stimulatory acitivity in these conditioned media, if TGFβ was neutralized. The results show that MFblCcM stimulated dose dependently [^{3}H]thymidine incorporation into the DNA of hepatocytes, if TGFβ in the medium was blocked by anti-TGFβ antibodies (Fig. 3). Similar to KCcM the proliferation stimulatory effect was abolished by competition with TGFα receptor binding using the TGFα receptor blocking TGFα-(aa 34-43)-peptide (Fig. 4). The concentration of TGFα/β was found to be 24 ± 7 ng/ml and 1.8 ± 0.4 ng/ml, respectively in the conditioned media added to hepatocyte cultures. The production rates of the factors by MFblC are given in Fig. 5.

4. DISCUSSION

It has been shown previously that Kupffer cells modulate hepatocyte proliferation but conflicting results on the stimulatory and inhibitory actions were reported [6-8]. Furthermore, the nature of the involved mediators was not specified. Kupffer cells produce a large variety of cytokines and cell growth modulation factors [28,29], of which the cooperative action in conditioned media on the regulation of hepatocyte DNA synthesis has not been clearly defined. In the present study we identified in Kupffer cell media TGFα and TGFβ as the most important mediators influencing the hepatocellular proliferation. Conditioned media derived from KC were shown to inhibit the EGF/TGFα stimulated DNA synthesis in hepatocytes and this effect was more pronounced using transiently acidified KCcM, in which latent TGFβ is activated (for review see [30]. These results suggest that the growth inhibitory action is due to active TGFβ, an assumption which is supported by the finding that the growth inhibiting activity can be neutralized by anti-TGFβ antibodies. Hybridization of total mRNA from cultured KC with a cDNA probe for TGFβ$_1$ showed the expression of mRNA encoding for TGFβ. The previously reported finding that KCcM will stimulate hepatocyte proliferation [8] could not be confirmed in our study. Only after neutralization of TGFβ the hydroxyurea-sensitive fraction of DNA synthesis, i.e. the replicative DNA synthesis [31] of quiescent hepatocytes could be stimulated. This effect was abolished by addition of a TGFα specific decapeptide (amino acid residues 34-43), which competes with EGF and TGFα for the receptor but does not affect itself cell proliferation [14]. These data indicate that EGF or TGFα in KCcM might be stimulatory mediator(s) for hepatocyte proliferation. In fact, we measured by radioligand binding assays biologically significant amounts of TGFα/EGF in KCcM. The effect of KCcM, in which TGFβ is blocked, was very similar to that of EGF and TGFα (results not shown) which are known to induce proliferation of hepatocytes in culture [11,32,33]. These results together with the hybridization experiments showing TGFα expression in Kupffer cells let us conclude that TGFα is the effector in KCcM, which stimulates hepatocyte DNA synthesis under culture conditions reported here. Our data suggest that the conflicting results communicated so far on the effects of KC on hepatocyte proliferation might be explained by changing proportions of TGFβ and TGFα in the secretions of Kupffer cells and by a modulation of the activation of latent TGFβ.

Results similar to those with KC were obtained with myofibroblast-like cell conditioned medium showing that transformed perisinusoidal lipocytes produce TGFα and TGFβ [34] which modulate hepatocyte proliferation in vitro.

The above mentioned data and the observation that TGFα and TGFβ are sequentially expressed in regenerating liver [13,35,36] support strongly the assumption that both TGFs play a central role in the up and down regulation of liver regeneration [37]. Cellular sources in the liver of TGFα are hepatocytes [35] and nonparenchymal cells as shown here, but TGFβ seems to be produced only by nonparenchymal cells [12,27, 34,38,39]. The intimate contacts between perisinusoidal lipocytes, Kupffer cells, and hepatocytes in situ [40] and the observation that in regenerating liver TGFα and TGFβ are expressed in nonparenchymal cells in the perisinusoidal area [34-36] suggest that Kupffer cells and activated perisinusoidal lipocytes might be prominent effector cells in a paracrine control of hepatocyte multiplication in situ.

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