Transforming growth factor-β_1 inhibits expression of selenoprotein P in cultured human liver cells

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Abstract The effect of cytokines on the expression of selenoprotein P (SeP) in the human liver cell line HepG2 was investigated. Treatment with interleukin-1β, interferon-γ, and tumor necrosis factor-α had no effect on SeP levels in culture media or on SeP mRNA expression. Conversely, Western analysis revealed a dose-dependent reduction of SeP content in culture medium after treatment with transforming growth factor (TGF)-β_1 with an IC_{50} of 31 pM. Treatment with 100 pM TGF-β_1 for 48 h led to a decrease to 21 ± 9% of controls. RT-PCR analysis of SeP mRNA expression demonstrated an inhibition of SeP transcription to 40 ± 2% of control levels after 24 h. The expression of a luciferase reporter construct under control of the human *SeP* promoter was downregulated by TGF-β_1 treatment in a dose-dependent fashion indicating a transcriptional regulation of the *SeP* gene by TGF-β_1.

Key words: Selenoprotein P; Glutathione peroxidase; Inhibition; Transcriptional regulation; Cytokine; Transforming growth factor-β_1; (HepG2)

1. Introduction

Selenoprotein P (SeP) is a selenocysteine-containing protein that accounts for about 50% of total plasma selenium in humans [1]. It differs from all other mammalian selenoproteins identified so far by its high selenium content. The mRNA of human SeP predicts the occurrence of selenocysteine at 10 positions [2] whereas other selenoproteins contain only one selenocysteine residue per subunit. The physiological function of SeP is not completely understood, but a role as a part of the body's antioxidant defense line seems most likely. In rat and human plasma, several isoforms of SeP were detected [3–5]. Human plasma contains at least two distinct isoforms with different selenium contents and molecular masses of 61 and 51 kDa, respectively [6].

Recently, a role of SeP in human plasma as a protective agent against the oxidation and nitration reactions mediated by peroxynitrite, a potent oxidant generated in vivo, was demonstrated [7].

Additionally, the 5′-flanking region of the human *SeP* gene was shown to contain interferon-γ (IFN-γ) responsive elements and a negative regulation of the *SeP* promoter by pro-inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor-α (TNF-α) was found [8]. In this study, we investigated whether cytokines like IL-1β, IFN-γ, TNF-α, or transforming growth factor (TGF)-β_1 regulate the expression of SeP.

2. Materials and methods

2.1. Materials

Recombinant human IL-1β, IFN-γ, and TNF-α were from Cytogen. Recombinant human TGF-β_1, sodium selenite, ammonium acetate, TRIreagent total RNA isolation kit, phenylmethylsulfonyl fluoride (PMSF), cycloheximide (CHX), and actinomycin D (ActD) were supplied by Sigma (Taufkirchen, Germany). RPMI 1640 medium was from PAA (Linz, Austria), penicillin/streptomycin, basal medium supplement (BMS), fetal calf serum (FCS), sodium hydrogen carbonate, and L-glutamine were purchased from Seromed (Berlin, Germany).

2.2. Cell culture and treatment

The human hepatocarcinoma line HepG2 was cultured in RPMI 1640 medium containing 10% FCS, 1% penicillin/streptomycin, 1 mg/ml L-glutamine, and 0.15% $NaHCO_3$. Cells were maintained under standard conditions at 37°C in 5% CO_2. Forty-eight hours before treatment, cells were cultured in RPMI 1640 medium containing 5% BMS, 1% penicillin/streptomycin, 1 mg/ml L-glutamine, and 0.15% $NaHCO_3$. Cells were then treated as indicated. During treatment, 250 nM sodium selenite was present in media to allow selenoprotein synthesis.

2.3. Enrichment of selenoprotein P from culture media

To 15 ml of culture medium, PMSF was added to give a final concentration of 1 mM. Samples of 10 ml were applied to a 1 ml HiTrap heparin agarose column (Pharmacia). The column was washed with 10 volumes of ice-cold wash buffer (100 mM ammonium acetate, 50 mM Tris-HCl, pH 7.4), and the SeP-containing fraction was eluted with wash buffer containing 2 M ammonium acetate. One milliliter of the eluate was collected for Western analysis.

2.4. Western blotting

Western detection of SeP was performed as described previously using affinity-purified anti-SeP IgG [6].

2.5. RT-PCR

RT-PCR analysis of mRNA expression was performed as described elsewhere [9]. Primer sequences and PCR conditions are given in Table 1.

Transcripts with common PCR conditions (SeP/GAPDH, extracellular/phospholipid hydroperoxide glutathione peroxidase (GPX3/GPX4)) were coamplified.

2.6. Plasmids and cell culture for transfection

The 1800 bp *Bgl*II/*Kpn*I SeP promoter fragment was isolated from the plasmid pBK15 [8] and subcloned into the luciferase reporter gene

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Abbreviations: ActD, actinomycin D; BMS, basal medium supplement; CHX, cycloheximide; FCS, fetal calf serum; GPX3, extracellular glutathione peroxidase; GPX4, phospholipid hydroperoxide glutathione peroxidase; HRP, horseradish peroxidase; IFN, interferon; IL, interleukin; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; RT, reverse transcription/reverse transcriptase; SeP, selenoprotein P; TNF, tumor necrosis factor

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plasmid pGL3basic revealing plasmid BK4GL3. Transfection was performed as described previously [8]. After 24 h, the transfection solution was replaced by DMEM-F12 medium containing 250 nM sodium selenite (controls) and human recombinant TGF-β_1 (0.1–100 pM). After a further incubation period of 16 h, the cells were harvested and luciferase and β-galactosidase activity was determined as described before [8].

2.7. Data analysis

Bands from Western blots and RT-PCR were scanned with an OmniMedia Scanner and quantified with Whole Band Analyzer software (Millipore, Überlingen, Germany). Curve fitting and statistical analysis (Student's *t*-test) were done by GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Effect of pro-inflammatory cytokines on SeP expression

Treatment of HepG2 cells with IL-1β (50 ng/ml), IFN-γ (100 ng/ml), and TNF-α (50 ng/ml) for 2, 8, 16, or 24 h revealed no effect either on SeP secretion or on SeP mRNA expression (data not shown).

3.2. Dose-dependent inhibition of SeP secretion by TGF-β_1

When HepG2 cells were incubated for 48 h with different doses of TGF-β_1, levels of SeP in culture medium were markedly decreased in a dose-dependent fashion as detected by Western analysis (Fig. 1). The half-maximal inhibitory concentration (IC_{50}) was about 30 pM. Treatment with 100 pM TGF-β_1 reduced the SeP concentration to 21 ± 9% (mean ± S.E.M., $n = 3$) of controls.

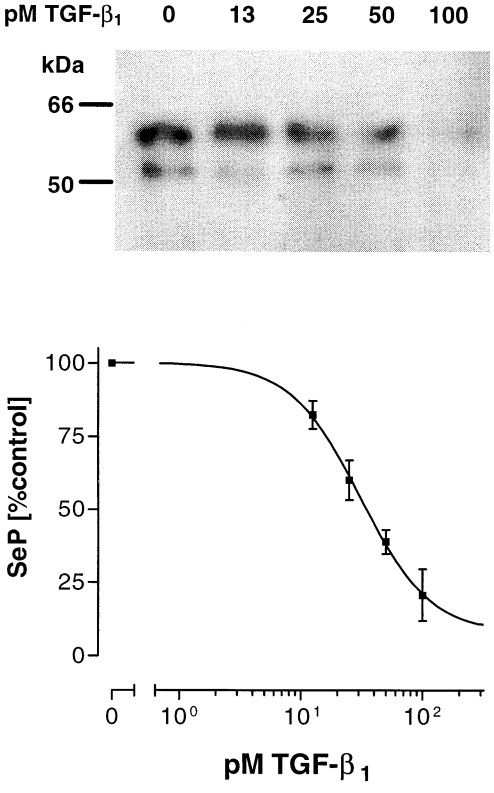

Fig. 1. Influence of TGF-β_1 on secretion of SeP by HepG2 cells. Top: Result of a Western blot. Bottom: Dose-effect relationship for SeP_{61}.

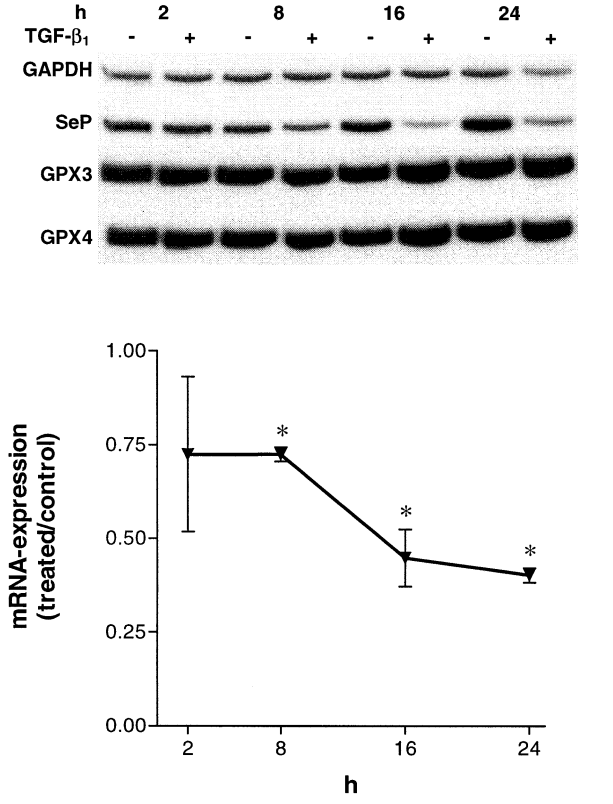

Fig. 2. Time course of SeP mRNA expression in HepG2 cells after treatment with 100 pM TGF-β_1. Top: Result of a typical RT-PCR. Bottom: Band intensities representing SeP mRNA expression normalized to the respective untreated controls are given ($n = 3$, mean ± S.E.M.). Significant ($P < 0.05$) differences to controls are indicated by an asterisk.

3.3. Effect of TGF-β_1 on SeP, GPX3, and GPX4 expression

HepG2 cells were incubated with 100 pM TGF-β_1 for 2, 8, 16, or 24 h. After 24 h, a maximum reduction of SeP mRNA levels to 40 ± 2% ($n = 3$) of the respective control was observed (Fig. 2). TGF-β_1 had no effect on GPX3 and GPX4 mRNA expression in HepG2 cells.

3.4. Effect of protein synthesis inhibitor CHX on TGF-β_1-mediated mRNA decrease

To characterize the mechanism of TGF-β_1 action on SeP expression, cells were coincubated with 35 μM CHX and 100 pM TGF-β_1 for 16 h. The presence of CHX neutralizes the inhibitory effect of TGF-β_1 on SeP mRNA expression (data not shown).

3.5. Influence of TGF-β_1 on SeP mRNA stability

To examine whether TGF-β_1 influences SeP mRNA stability, experiments with ActD, an inhibitor of mRNA synthesis, were performed. Cells were first treated simultaneously with 100 pM TGF-β_1 and 5 μg/ml ActD for 6 and 16 h. The inhibitory effect of TGF-β_1 was counteracted by this treatment (Fig. 3, top, lanes 1–5), indicating that mRNA synthesis is required for negative regulation of SeP mRNA. Densitometric analysis of band intensities revealed no difference in SeP-mRNA abundance between TGF-β_1 treated and control cells in presence of ActD (Fig. 3, bottom).

When cells were treated for 3 h with 100 pM TGF-β_1 prior to addition of ActD, the inhibitory effect of TGF-β_1 was

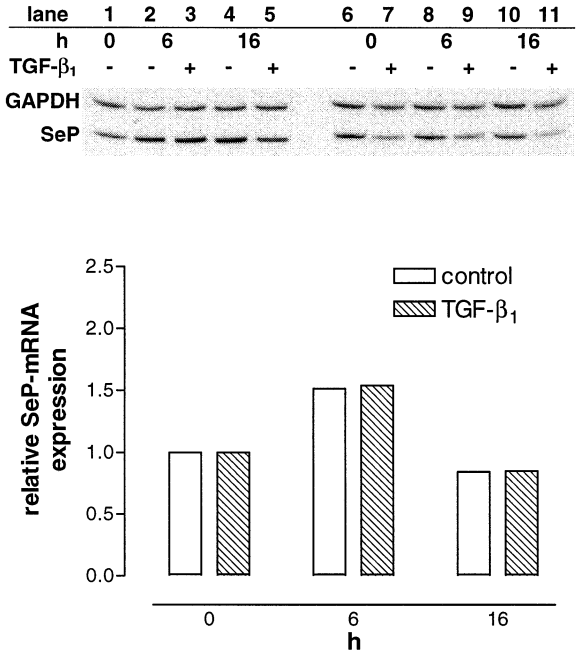

Fig. 3. Effect of ActD on the TGF-β_1-mediated inhibition of SeP mRNA expression. Top: Cells were treated simultaneously with TGF-β_1 and ActD (lanes 1–5) or preincubated with TGF-β_1 for 3 h before addition of ActD (lanes 6–11). Bottom: Band intensities from lanes 1–5 were normalized to GAPDH expression and shown as relative expression to the untreated cells (0 h). Means of two independent experiments are given.

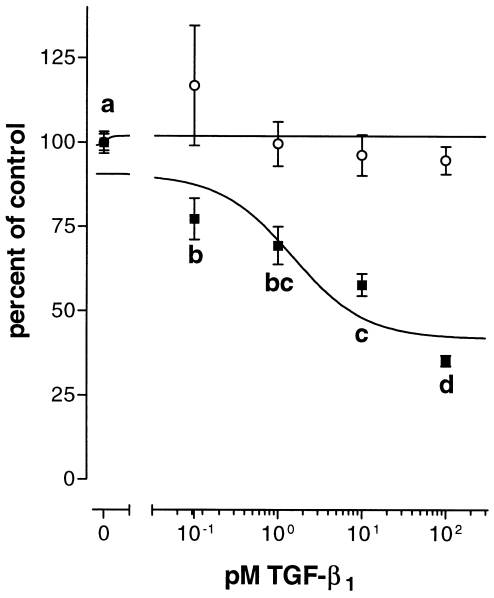

Fig. 4. SeP promoter activity in HepG2 cells after TGF-β_1 treatment. Cells were transfected with pGL3basic (open circles) or BK4GL3 (closed squares). Values normalized for β-galactosidase activity represent means ± S.E.M. in percent of controls, of three to four independent experiments, each run in duplicate or triplicate. Values not sharing a common letter are significantly different from each other ($P < 0.01$, data shown for BK4GL3 only).

restored (Fig. 3, top, lanes 6–11). These results indicate that enhanced mRNA degradation is unlikely to be responsible for the effect of TGF-β_1 on SeP expression.

3.6. TGF-β_1 modulates SeP promoter activity

To further characterize the transcriptional effect of TGF-β_1 on SeP expression, reporter gene assays under control of the human SeP promoter were conducted. As shown in Fig. 4, TGF-β_1 treatment of HepG2 cells that were transfected with the SeP promoter construct BK4GL3 resulted in a marked dose-dependent decrease of luciferase activity. Treatment with 100 pM TGF-β_1 for 8 h reduced normalized SeP-promoter activity to 49.4 ± 3.2% of controls (mean ± S.E.M., $n = 10$–12, data not shown), 16 h treatment reduced normalized SeP promoter activity to 35.2 ± 1.6% (mean ± S.E.M., $n = 10$–12). In contrast, background luciferase activity of the pGL3 basic plasmid was not influenced by TGF-β_1 thus indicating a specific inhibition of SeP promoter activity by TGF-β_1 treatment.

4. Discussion

Up to now, SeP secretion by human liver cells was known to be regulated by selenium supply only [10]. In the present study, we show that TGF-β_1 exerts an inhibitory effect on SeP secretion as well as on SeP mRNA abundance via negative regulation of the SeP promoter.

TGF-β_1 produces a variety of effects in a number of tissues and cell lines. TGF-β_1-deficient mice show multifocal inflammatory responses and tissue necrosis leading to fatal organ failure which suggests a central role of TGF-β_1 in the regulation of inflammatory reactions [11,12]. During inflammation, TGF-β_1 is responsible for the induction of extracellular matrix proteins [13] and for inhibition of lymphocyte proliferation and function at femtomolar concentrations [14]. In HepG2 cells, regulation of acute-phase proteins by TGF-β_1 has been demonstrated [15].

In rats, SeP levels have been correlated with protection against free-radical-induced liver necrosis [16]. In human plasma, SeP is effective at preventing both oxidation and nitration reactions mediated by peroxynitrite, a reactive oxygen species

Table 1
Primer sequences and PCR conditions

Gene	Sequence (5′→3′)	Annealing temperature (°C)	Number of cycles	Ref.
SeP	FP: CAT CAG CAC CTT GGC AGC AGT RP: CAA CTG GCA CTG GCT TCT GTG	61	18	[2]
GAPDH	FP: TGA AGG TCG GAG TCA ACG GAT TTG GT RP: CAT GTG GGC CAT GAG GTC CAC CAC	61	18	[25]
GPX3	FP: TAC ATC TGA CCG CCT CTT CTG RP: CAC ACA CAC AAT CAC GCA TAC	60	23	[26]
GPX4	FP: CGC TGC TCT GTG GGG CTC TGG RP: ACG CTG GAT TTT CGG GTC TGC	60	23	[27]

FP: forward primer; RP: reverse primer.

endogenously formed by endothelial cells, activated macrophages and other cells involved in inflammatory processes [7]. SeP has been shown to bind to cellular membranes [17] and associates with endothelial cells in rat tissues [18]. Hence, SeP might act as an endothelial protection factor by inactivating peroxynitrite generated under pathological conditions, e.g. acute lung injury, sepsis, and atherosclerosis [19–21]. Because endothelial cells as well as macrophages and neutrophils do not express SeP (V. Mostert, unpublished observation), there is a necessity for SeP to be secreted in an endocrine fashion by other cell types, e.g. hepatocytes, in which SeP expression is high [22].

The inhibitory effect of TGF-β_1 on the expression of the *SeP* gene is probably not an immediate one since de novo synthesis of proteins as well as of mRNA is necessary for downregulation of SeP expression. Consequently, TGF-β_1 seems to induce the transcription and synthesis of one or several factors which inhibit SeP transcription. The only transcriptional partners for transduction of TGF-β signals identified so far are the Smad proteins [23]. The motif CAGACA which has been shown to act as a Smad binding element in the JunB promoter is present at position −1797 in the SeP promoter [8]. A likely mechanism of Smad proteins inhibiting the transcription of the *SeP* gene would be an obstruction of a binding site for a basal transcription factor.

Our data represent the first observation of SeP regulation by an endogenous mediator. TGF-β_1 often counteracts the induction of target genes by inflammatory cytokines [24]. Further experiments in cell and animal models will further elucidate a possible induction of SeP during inflammation.

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