Transforming growth factor-β1 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)-β1 with an IC50 of 31 pM. Treatment with 100 pM TGF-β1 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-β1, treatment in a dose-dependent fashion indicating a transcriptional regulation of the SeP gene by TGF-β1.

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Key words: Selenoprotein P; Glutathione peroxidase; Inhibition; Transcriptional regulation; Cytokine; Transforming growth factor-β1; (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)-β1 regulate the expression of SeP.

2. Materials and methods

2.1. Materials

Recombinant human IL-1β, IFN-γ, and TNF-α were from Cyto- gen. Recombinant human TGF-β1, 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, and fetal calf serum (FCS) were from PAA GmbH, penicillin/streptomycin, from PAA (Linz, Austria), penicillin/streptomycin, 1 mg/ml l-glutamine, and 0.15% NaHCO3. RPMI 1640 medium was from PAA (Linz, Austria), penicillin/streptomycin, 1 mg/ml l-glutamine, and 0.15% NaHCO3. Cells were then treated as indicated. During treatment, 250 nM sodium selenite was present in media to allow selenoprotein synthesis.

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% NaHCO3. Cells were maintained under standard conditions at 37°C in 5% CO2. 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% NaHCO3. 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 (SePGAPDH, extracellular/ phospholipid hydrolase glutathione peroxidase (GPX3/ GPX4) were coamplified.

2.6. Plasmids and cell culture for transfection

The 1800 bp BgII/KpnI SeP promoter fragment was isolated from the plasmid pBK15 [8] and subcloned into the luciferase reporter gene

Abbreviations: ActD, actinomycin D; BMS, basal medium supplement; CHX, cycloheximide; FCS, fetal calf serum; GPX3, extracellular glutathione peroxidase; GPX4, phospholipid hydrolase 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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PII: S 0 0 1 4 - 5 7 3 9 ( 9 9 ) 0 1 2 9 8 - 3

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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-β1 (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-β1

When HepG2 cells were incubated for 48 h with different doses of TGF-β1, 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 (IC50) was about 30 pM. Treatment with 100 pM TGF-β1 reduced the SeP concentration to 21 ± 9% (mean ± S.E.M., n = 3) of controls.

3.3. Effect of TGF-β1 on SeP, GPX3, and GPX4 expression

HepG2 cells were incubated with 100 pM TGF-β1 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-β1 had no effect on GPX3 and GPX4 mRNA expression in HepG2 cells.

3.4. Effect of protein synthesis inhibitor CHX on TGF-β1-mediated mRNA decrease

To characterize the mechanism of TGF-β1 action on SeP expression, cells were coinubated with 35 μM CHX and 100 pM TGF-β1 for 16 h. The presence of CHX neutralizes the inhibitory effect of TGF-β1 on SeP mRNA expression (data not shown).

3.5. Influence of TGF-β1 on SeP mRNA stability

To examine whether TGF-β1 influences SeP mRNA stability, experiments with ActD, an inhibitor of mRNA synthesis, were performed. Cells were first treated simultaneously with 100 pM TGF-β1 and 5 μg/ml ActD for 6 and 16 h. The inhibitory effect of TGF-β1 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-β1 treated and control cells in presence of ActD (Fig. 3, bottom).

When cells were treated for 3 h with 100 pM TGF-β1 prior to addition of ActD, the inhibitory effect of TGF-β1 was
restored (Fig. 3, top, lanes 6–11). These results indicate that enhanced mRNA degradation is unlikely to be responsible for the effect of TGF-β1 on SeP expression.

3.6. TGF-β1 modulates SeP promoter activity

To further characterize the transcriptional effect of TGF-β1 on SeP expression, reporter gene assays under control of the human SeP promoter were conducted. As shown in Fig. 4, TGF-β1 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-β1 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-β1 thus indicating a specific inhibition of SeP promoter activity by TGF-β1 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-β1 exerts an inhibitory effect on SeP secretion as well as on SeP mRNA abundance via negative regulation of the SeP promoter. TGF-β1 produces a variety of effects in a number of tissues and cell lines. TGF-β1-deficient mice show multifocal inflammatory responses and tissue necrosis leading to fatal organ failure which suggests a central role of TGF-β1 in the regulation of inflammatory reactions [11,12]. During inflammation, TGF-β1 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-β1 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5' → 3')</th>
<th>Annealing temperature (°C)</th>
<th>Number of cycles</th>
<th>Ref.</th>
</tr>
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<tbody>
<tr>
<td>SeP</td>
<td>FP: CAT CAG CAC CTT GGC AGC AGT, RP: CAA CTG GCA CTG GCT TCT GTG</td>
<td>61</td>
<td>18</td>
<td>[2]</td>
</tr>
<tr>
<td></td>
<td>FP: TGA AGG TCG GAG TCA ACG GAT TGG GT</td>
<td>61</td>
<td>18</td>
<td>[25]</td>
</tr>
<tr>
<td>GAPDH</td>
<td>FP: CAG TGG GGC CAT GAG GTC CAC CAC</td>
<td>61</td>
<td>18</td>
<td>[25]</td>
</tr>
<tr>
<td>GPX3</td>
<td>FP: TAC ATC TGA CCG CCT CCT CTG, RP: CAC ACA CAC AAT CAC GCA TAC</td>
<td>60</td>
<td>23</td>
<td>[26]</td>
</tr>
<tr>
<td>GPX4</td>
<td>FP: CGC TGC TCT GTG GGG CTC TGG, RP: ACG CTG GAT TTT CCG GTC TGC</td>
<td>60</td>
<td>23</td>
<td>[27]</td>
</tr>
</tbody>
</table>

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-β1 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-β1 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-β1 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.

References