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PI-3 kinase pathway can mediate the effect of TGF-β1 in inducing the expression of *SHARP-2* in LLC-PK1 cells*

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Abstract: We aim to investigate the effect of transforming growth factor (TGF)-β1 on the expression of enhancer of split- and hairy-related protein-2 (SHARP-2) messenger RNA (mRNA) and its signaling pathway. In this study, several cell lines including LLC-PK1 (a porcine kidney tubular epithelial cell line), MDCK (Madin-Darby canine kidney) and CTLL-2 (cytotoxic T-lymphocyte line) were treated with recombinant human TGF-β1, and a series of experiments were carried out, involving Northern blot analysis of total RNA from these cells. Further, several specific chemical inhibitors were applied before TGF-β1 treatment to probe the signaling pathway. The results showed that TGF-β1 can significantly up-regulate SHARP-2 mRNA expression in the LLC-PK1 cell line. The peak level of induction was found 2 h after TGF-β1 stimulation. While one phosphoinositide 3-kinases (PI-3) kinase inhibitor, LY294002, completely blocked the effect of TGF-β1 on SHARP-2 mRNA expression in LLC-PK1 cells at a low concentration, other inhibitors, including PD98059, staurosporine, AG490, wortmannin, okadaic acid and rapamycin, had no effect. The effect of LY294002 was dose-dependent. We conclude that, in LLC-PK1 cells at least, TGF-β1 can effectively induce the SHARP-2 mRNA expression and that the PI-3 kinase pathway can mediate this effect.

Key words: Transforming growth factor (TGF)-β1, Split- and hairy-related protein-2 (SHARP-2), Phosphoinositide 3-kinases (PI-3) kinase pathway, LY294002

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INTRODUCTION

We have cloned the rat enhancer of split- and hairy-related protein 2 (*SHARP-2*) (Hirano *et al*., 2004), a member of the basic/helix-loop-helix (bHLH) protein family (Rossner *et al*., 1997). Mouse and human orthologs have been designated stimulation of retinoic acid 13 (*Stra13*) and differentiated embryo chondrocytes 1 (*DEC1*), respectively (Boudjelal *et al*., 1997; Shen *et al*., 1997). The functions of SHARP-2/DEC1/Stra13 have not yet been fully elucidated; however, defective T-cell activation and the genesis of autoimmune disorders have been reported in aging *Stra13*-deficient mice (Sun *et al*., 2001). Also DEC1 and a closely related protein DEC2 are expressed in the suprachiasmatic nucleus in a circadian fashion and are regulators of the mammalian molecular clock (Sun *et al*., 2001; Honma *et al*., 2002).

It has been reported that the production of SHARP-2 messenger RNA (mRNA) is induced by nerve growth factor, retinoic acid, cyclic adenosine monophosphate (cAMP), serum starvation, and hypoxia in various cells and tissues (Sun and Taneja, 2000; Ivanova *et al*., 2001). We recently found that

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the expression of SHARP-2 mRNA in the rat liver is rapidly increased by insulin (Yamada *et al*., 2003). Zawel *et al*.(2002) reported that DEC1 is a novel down stream target of the transforming growth factor (TGF)-β1. It is unclear whether TGF-β1 can also induce the SHARP-2 mRNA expression and, if so, which pathway mediates the signaling from TGF-β1 to induce SHARP-2 mRNA expression. Here we report our preliminary findings of our work in this area.

MATERIALS AND METHODS

Total RNA was prepared from LLC-PK1 (a porcine kidney tubular epithelial cell line), MDCK (Madin-Darby canine kidney, a canine kidney cell strain) and CTLL-2 (cytotoxic T-lymphocyte line) cells (Riken Cell Bank, Tsukuba, Japan) using TRIzol reagent (Invitrogen, USA). Total RNA (10 μg/lane) was subjected to 0.8% (w/v) denaturing agarose gel electrophoresis and then transferred to a Biodyne membrane (PALL, ICN Biomedicals, Inc., Glen Cove, NY). The filter was pre-hybridized in ExpressHyb hybridization solution at 68 °C for 30 min and then hybridized with a ^{32}P-labeled probe (Amersham Biosciences, USA) and 20 μg/ml heat-denatured herring testis DNA (Takara, Japan) for 1 h. After washing at 50 °C in 0.1× 0.15 mol/L NaCl/0.015 mol/L sodium acetate (SSC) and 0.1% (w/v) sodium dodecyl sulfate (SDS) (Sigma, USA), the filter was exposed to a FUJIX imaging plate (Kanagawa, Japan). Hybridization signals were detected and quantified using a FUJIX BAS-2000 image analyzing system.

The preparation of the Northern blot analysis probes for SHARP-2 was as described previously (Hirano *et al*., 2004; Kawata *et al*., 2004; Yamada *et al*., 2003). For cDNA cloning of mouse 36B4, a ribosomal protein, reverse transcription-polymerase chain reaction was carried out using oligonucleotides 5'-ATGCCCAGGGAAGACAGGGCGACC-3' and 5'-TTAGTCGAAGAGACCGAATCCCATA-3' as primers and mouse liver cDNA as a template (Shou *et al*., 2003; 2004). The product was sub-cloned into the pGEM-T Easy to give pGEM-T Easy 36B4. A 954-bp *Eco*RI fragment was used as a probe. Probe DNAs were labeled with [α-^{32}P]dCTP (Amersham Biosciences) using the BcaBest DNA labeling kit (Takara Biomedicals, Kyoto, Japan).

RESULTS

TGF-β1 can induce significant expression of SHARP-2 mRNA in LLC-PK1 cells

We investigated whether TGF-β1 could induce the expression of SHARP-2 mRNA in three kinds of cell lines: LLC-PK1, an epithelial-like cell line originating from pig kidney tubule epithelial cells; MDCK, the corresponding cell line from dog; and CTLL-2, a suspension cell line. As expected, the level of SHARP-2 mRNA following TGF-β1 stimulation at a final concentration of 2 ng/ml varied greatly among the three cell lines. While a significant induction effect was observed in LLC-PK1 cells, the effects in the other two cell lines were only marginal (Fig.1). Thus, we chose the LLC-PK1 cell line for further studies.

Time-course and dose-effect studies

We first studied the time-course of the effect of TGF-β1 stimulation on the expression of SHARP-2 mRNA in LLC-PK1 cells. The induction effect began after only 1 h, peaked at 2 h, and then decreased (Fig.2a). We also investigated the dose-effect of stimulation using various final concentration of TGF-β1. We found no clear dose-effect. Although we used a final concentrations of 2 ng/ml TGF-β1 for stimulation in the main study, we found that a lower (0.4 ng/ml) or higher (5.0 ng/ml) concentration gave almost the same induction effect (Fig.2b).

PI-3 kinase pathway can mediate the signaling from TGF-β1 to induce SHARP-2 mRNA expression in LLC-PK1 cells

The main purpose of this study was to investigate the signaling pathway by which TGF-β1 induces the expression of SHARP-2 mRNA. First we investigated whether specific chemical inhibitors could inhibit the effect of TGF-β1 on the expression of SHARP-2 mRNA. A PI-3 kinase pathway specific inhibitor LY294002 (Sigma, USA) almost completely blocked the effect of TGF-β1 while other chemical inhibitors or stimuli, including wortmannin, PD98059, AG-490, staurosporine, rapamycin, okadaic acid or 8-Br-cAMP (Sigma, USA) had no effect (Fig.3). In the same experiment we also found that the mRNA synthesis process interrupter actinomycin-D but not the protein synthesis process interrupter cycloheximide (CHX) completely blocked the TGF-β1 effect on SHARP-2.

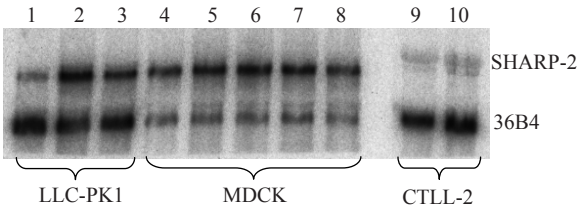

Fig.1 TGF-β1 up-regulates the expression of SHARP-2 mRNA in several cell lines

A representative result of three independent experiments. Northern blot analysis was carried out. 10 μg of total RNA from LLC-PK1, MDCK and CTLL-2 cells were subjected to 0.8% (w/v) denatured agarose gel electrophoresis and then transferred to a Biodyne membrane. The membranes were hybridized with ^{32}P-labeled probes, first for SHARP-2 probe, and then the membranes were de-probed and re-hybridized with 36B4 probe, which was used as the internal control to ensure that equal amount of total RNA was applied to each lane. Lanes 1~3, LLC-PK1 cells. Lane 1, untreated; Lanes 2 and 3, treated with 2 ng/ml recombinant human TGF-β1 for 2 and 3 h, respectively. Lanes 4~8, MDCK cells. Lane 4, untreated; Lanes 5~8, treated with 2 ng/ml TGF-β1 for 1, 2, 3 and 4 h, respectively. Lanes 9 and 10, CTLL-2 cell line. Lane 9, untreated; Lane 10, treated with 2 ng/ml TGF-β1 for 2 h

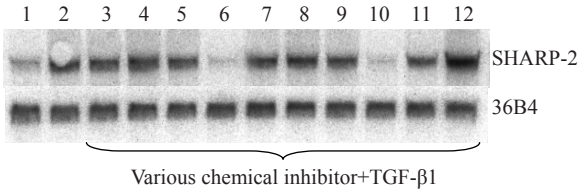

Fig.3 Primary signaling pathway study of TGF-β1 in inducing the expression of SHARP-2 mRNA in the LLC-PK1 cell line

A representative result of three independent experiments. Northern blot analysis experiment details were as described in Fig.1. Lanes 1 and 2 were used as the control; cells of other lanes were treated with various chemical signaling pathway inhibitors 20 min prior to treatment with 2 ng/ml TGF-β1. After 2 h, cells were harvested, total RNA was extracted and Northern blot analysis was carried out. 36B4 was used as the internal control to ensure that equal amounts of total RNA were applied to each lane. Lane 1, untreated; Lane 2, 2 ng/ml TGF-β1; Lane 3, 25 μmol/L PD98059; Lane 4, 200 μmol/L AG-490; Lane 5, 100 nmol/L wortmannin; Lane 6, 100 μmol/L LY294002; Lane 7, 100 nmol/L staurosporine; Lane 8, 100 nmol/L rapamycin; Lane 9, 10 nmol/L okadaic acid; Lane 10, 0.8 μmol/L actinomycin D; Lane 11, 10 μmol/L cycloheximide; Lane 12, 1 mmol/L 8-Br-cAMP. Concentrations specified above were all referred to as the final concentration

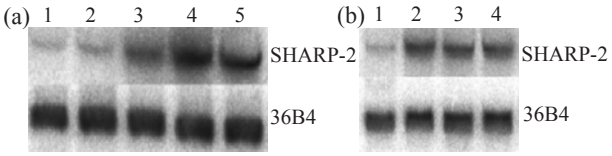

Fig.2 Northern blot analyses of time course and dose effect of TGF-β1 on SHARP-2 mRNA expression in LLC-PK1 cells

A representative result of three independent experiments. Northern blot analysis experiment details were as described in Fig.1. (a) Time-course study: Lane 1, untreated; Lane 2, cells were treated with solvent for TGF-β1; Lanes 3~5, cells were treated with 2 ng/ml TGF-β1 for 1, 2 and 3 h, respectively; (b) Dose-effect study: Lane 1, untreated; Lanes 2~4, cells were treated with 0.4, 2.0 and 5.0 ng/ml TGF-β1 for 2 h, respectively. After the above treatments, cells were harvested, total RNA was extracted and Northern blot analysis was carried out. 36B4 was used as the internal control to ensure that equal amounts of total RNA were applied to each lane

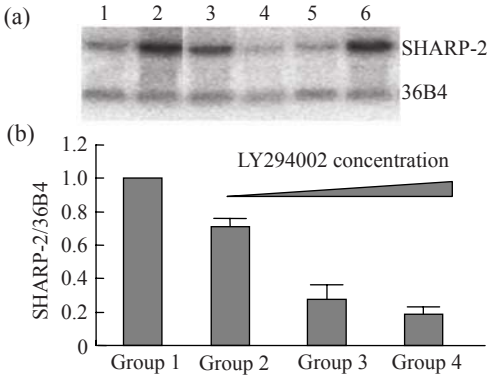

Fig.4 LY294002 blocks the effect of TGF-β1 on the expression of SHARP-2 mRNA in a dose-dependent manner in the LLC-PK1 cell line

Northern blot analysis experiment details were as described in Fig.1. (a) A representative result of three independent experiments. Lane 1 was used as the control, and cells of other lanes were treated with various concentrations of chemical signaling pathway inhibitors 20 min prior to treatment with 2 ng/ml TGF-β1 (Lanes 3~6) or TGF-β1 alone as a positive control (Lane 2). After 2 h, cells were harvested, total RNA was extracted and Northern blot analysis was carried out. Lanes 3~5, 10, 50 and 100 μmol/L LY294002, respectively. Lane 6, 50 μmol/L PD98059; (b) Densitometry quantitative results. LLC-PK1 cells were treated with 2 ng/ml TGF-β1 alone as a positive control (Group 1) or various concentrations (10, 50 and 100 μmol/L, respectively) of LY294002 20 min prior to treatment of 2 ng/ml TGF-β1 (Groups 2~4). After 2 h, cells were harvested, total RNA was extracted and Northern blot analysis was carried out. The values are the ratio of band densitometry of SHARP-2/36B4. The value of 2 ng/ml TGF-β1 stimulation only was arbitrarily set as 1 (Group 1). The mean values and their standard deviations from three independent experiments were plotted (Groups 2~4)

We then investigated two lower concentrations of LY294002, 50 and 10 μmol/L, respectively, and found that the 10 μmol/L LY294002 had a partial effect, and the 50 μmol/L LY294002 showed the full blocking effect as 100 μmol/L LY294002 (Fig.4).

DISCUSSION

In this study, we first tested our hypothesis that SHARP-2 mRNA expression can be induced by TGF-β1 just as in the case of its human conterpartner-DEC1 (Zawel *et al*., 2002). The effect of the same stimulation on different cells can vary greatly. This could be explained at least partly by different base levels of mRNA expression in different cells and the extreme complexity of gene transcription regulatory network. To take account of this potential variable response, we began our experiments using three different kinds of cells. Our results provide solid evidence in favor of our hypothesis at least in LLC-PK1 cells. Although the effect of TGF-β1 stimulation on SHARP-2 mRNA expression in MDCK and CTLL-2 cell lines was only marginal, it is possible that by using a higher concentration of TGF-β1 stimulation, with a longer stimulation period, or just switching to a more sensitive detection method, e.g., quantitative polymerase chain reaction (PCR), an effect could be clearly demonstrated. However, this was not the major objective of the current study.

We then tried to investigate the signaling pathway from TGF-β1 to induce SHARP-2 mRNA expression using several specific chemical inhibitors in LLC-PK1 cells. The TGF-β1 signal transduction pathway is involved in numerous biological processes (Zawel *et al*., 2002) including those regulating cell birth, cell death, differentiation, invasion, angiogenesis, and immunity. The Smad pathway is a current focus of interest in this regard. TGF-β1 ligands bind to receptor kinases on the cell surface, leading to phosphorylation of the receptor-phosphorylated Smad proteins (R-Smads). Once phosphorylated, these Smads interact with Smad 4 and translocate to the nucleus where the Smad complex binds to specific DNA sequences in conjunction with other nuclear proteins that regulate gene expression (Zawel *et al*., 2002). However, the ever-increasing complexity of TGF-β1 signaling has recently emerged, because it was found that although Smad is the signaling pathway in most cases, other pathways including Ras, RhoA, MAPK, Akt/PKB, etc. may also be involved (Roberts, 2002). We have also discovered recently that the PI-3 kinase pathway mediates the signaling of insulin up-regulation of the expression of SHARP-2 (Yamada *et al*., 2003).

Therefore, we used several specific pathway inhibitors including those for MARP, PI-3 kinase, Jak/stat etc. to investigate the possibilities. Our data showed that one of the PI-3 kinase inhibitors, LY294002 (Wu *et al*., 2009), blocked the effect of TGF-β1 in LLC- PK1 cells. It is unlikely that this was a systemic inhibitory effect of LY294002, or a non-specific effect, because it took effect even at 10 μmol/L, a very low concentration that generally has no systemic effect on mRNA expression.

Another PI-3 kinase inhibitor, wortmannin, showed no effect in blocking the induction effect of TGF-β1 on SHARP-2. Wortmannin and LY294002 are both PI-3 kinase specific inhibitors, and in signaling pathway studies they are usually used simultaneously to ensure the correctness of the results. However, they have different mechanisms of action and this may explain why only LY294002 blocked the effect of TGF-β1 in this pathway.

Finally, although our data indicated the involvement of the PI-3 kinase pathway, we have no reason to exclude the possibility that Smad may play a role simultaneously, independently or with cross-talk in LLC-PK1 cells.

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