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Sox-4 is a positive regulator of Hep3B and HepG2 cells' apoptosis induced by prostaglandin (PG)A_2 and Δ^{12}-PGJ_2

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Abbreviations: PG, Prostaglandin; HMG, high mobility group, Sox-4, Sry-HMG-box 4.

Abstract

We reported earlier that expression of Sox-4 was found to be elevated during prostaglandin (PG) A2 and Δ^{12}-PGJ_2 induced apoptosis in human hepatocarcinoma Hep3B cells. In this study, the role of Sox-4 was examined using human Hep3B and HepG2 cell lines. Sox-4 induction by several apoptotic inducer such as A23187 (Ca^{2+} ionophore) and etoposide (topoisomerase II inhibitor) and Sox-4 transfection into the cells were able to induce apoptosis as observed by the cellular DNA fragmentation. Antisense oligonucleotide of Sox-4 inhibited the induction of Sox-4 expression and blocked the formation of DNA fragmentation by PGA_2 and Δ^{12}-PGJ_2 in Hep3B and HepG2 cells. Sox-4-induced apoptosis was accompanied with caspase-1 activation indicating that caspase cascade was involved in this apoptotic pathway.
These results indicate that Sox-4 is involved in Hep3B and HepG2 cells apoptosis as an important apoptotic mediator.

Keywords: Sox-4, PGA_2, Δ^{12}-PGJ_2, apoptosis

Introduction

Many Sox (SRY-HMG box containing) proteins are assumed to be involved in the regulation of developmental stages of several distinct tissues (Uwanogho *et al*., 1995: Schilham *et al*., 1998: Southard-Smith *et al*., 1998) and nervous system in mammals (Prior and Walter, 1996: Jay *et al*., 1997). Unlike other Sox family proteins, Sox-4, a transcription factor, contains serine-rich trans-activation domain in the C-terminus and is selectively expressed in brain, heart and testis of fetus mice (van de Wetering *et al*., 1993: Schilham *et al*., 1997). In adult mice, Sox-4 is also known to be present in the thymus and gonads and is important for early B-cell differentiation (Schilham *et al*., 1997). Recently, the significant functions of Sox-4 gene have been reported by the gene disruption experiment. The mutation of Sox-4 in the germ line of mice leads to premature death specifically at 14th embryonic day due to improper development of the valves in the outflow tract of the heart (Schilham *et al*., 1996). However, little is known about the target genes of Sox-4 proteins and hence about their actual pathophysiological function.

Cyclopentenone prostaglandins(PGs) such as PGA_2 and Δ^{12}-PGJ_2 have antineoplastic effects on the growth of various tumor cells with characteristic morphological and biochemical findings of apoptosis (Fukshima *et al*., 1994: Lee *et al*., 1995: Ahn *et al*., 1998). The expression of genes and their products such as p53, c-myc and heat shock 70 are up- or down-regulated by presence of PGA_2 and Δ^{12}-PGJ_2 in human hepatocarcinoma cells. And these genes and their products serve as a positive or negative regulator in PGA_2 and Δ^{12}-PGJ_2 induced apoptotic pathway in human hepatocarcinoma cells (Lee *et al*., 1995: Ahn *et al*., 1998). When Hep3B cells were exposed to PGA_2 and Δ^{12}-PGJ_2, the expression of Sox-4 was specifically increased (Ahn *et al*., 1999). But the precise pathophysiological role of Sox-4 during tumor cells apoptosis is not well understood.

In this study, we found that the expression of Sox-4 induced by apoptotic inducers such as A23187 (Ca^{2+} ionophore) and etoposide (topoisomerase II inhibitor) in Hep3B and HepG2 cells and transient Sox-4 expression alone induced apotosis mediated by caspase-1. Antisense to Sox-4 cDNA blocked the DNA fragmentation by PGA_2 and Δ^{12}-PGJ_2 in Hep3B and HepG2 cells, supporting their involvement in PGA_2 and Δ^{12}-PGJ_2 induced apoptotic pathway.

Materials and Methods

Reagents

PGA_2 was purchased from Sigma Chemical Co. (St. Louis, Mo, USA) and Δ^{12}-PGJ_2 was from BioMol (Plymouth

Meeting, PA, USA). The human hepatocarcinoma cell lines, Hep3B and HepG2 were obtained from American Type Culture Collection (ATTC, Rockville, MD, USA). Transient expression vector pCDM8 was from Invitrogen (Carlsbad, CA, USA). Antisera to Sox-4 were raised against synthetic peptides derived from the corresponding sequence to amino acid residues, 145-158.

Cell culture condition and treatments

Hep3B and HepG2 cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (Gibco Laboratories, Grand Island, NY, USA), penicillin (100 unit/ml) and streptomycin (100 mg/ml). Cells were plated at a density of 2 x 10^5 cells/ml on a 6-well culture plate and incubated in 37°C, 5% CO_2 incubator. Treatments were performed on exponentially growing subconfluent cells.

Determination of DNA fragmentation

DNA fragmentation was detected by agarose gel electrophoresis as described previously (Ahn *et al*., 1999). Cells were washed with phosphate buffered saline (PBS) and incubated in digestion buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 25 mM EDTA, 0.2 mg proteinase K, 1% sarkosyl) at 45°C for 3 h. After the addition of 2 ml RNase A (10 mg/ml), the DNA containing mixture was placed in 37°C water bath for 1 h and extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The isolated DNA was loaded into 1.8% agarose gel. After eletrophoresis, the gel was stained with ethidium bromide.

Immunoblot analysis

Proteins from control and PGs-stimulated cells were solubilized by gel-running buffer (0.25 M Tris-HCl, pH 6.8, 1% SDS, 20% glycerol, 5% β-mercaptoethanol, 0.02 % bromophenol blue). Samples were fractionated on 10 or 15% SDS-polyacrylamide gels and then transferred to nitrocellulose membrane by electroblotting. After blocking with 5% none-fat dry milk in PBS, the membrane was incubated for 2 h at room temperature with antibodies specific to Sox-4 and caspase-1. Following washing with PBS containing 0.2% Tween 20, membrane was incubated with the blocking solution containing the goat anti-rabbit IgG peroxidase conjugate. After washing with PBS (three times for 15 min each), the proteins were detected using ECL-western blot detection kit (Amersham, Buckinghamshire, England).

Cell death detection by ELISA

For evaluation of apoptosis, a cell death detection ELISA kit (Cell Death Detection ELISA^{PLUS}, Boehringer Mannheim, Mannheim, Germany) was used according to the manufacturers instruction. Briefly, 12, 24 and 48 h after transfection with Sox-4 vector (pCDM8), cells were washed with PBS. The cells were lysed in 200 μl of lysis buffer and centrifuged. Cell supernatants were assayed for apoptosis using the Cell Death Detection Kit. The reaction was developed with the anti-DNA-peroxidase system and development of color was measured at 405 nm with reference wavelength of 490 nm. Assays were performed at least three times.

Transfection

Hep3B and HepG2 cells were transfected with 5 μg of empty expression vector or pCDM8-Sox-4 (2.1 kb) using DOTAP transfection reagent (Boehringer Mannheim). Sox-4 antisense oligomer (5'-CGGCATTGTTGGTTTGC TGCACCAT-3') and sense oligomer (5'-ATGGTGCAGC AAACCAACAATGCCG-3') were synthesized from sequences complementary to the translation starting site. Eighty percent confluent cells were incubated for 6 h with plasmid DNA mixed with 20 μl of DOTAP reagent in serum-free medium at 37°C in 5% CO_2 incubator.

Results

PGs induced the Sox-4 expression in Hep3B and HepG2 cells

Previously we reported that when Hep3B cells were treated with 20 μg/ml of PGA_2 for 24 h, the expression of Sox-4 increased in a dose and time dependent manner. Sox-4 transcription level was elevated closely 10-fold in comparison with the control in Hep3B cells (Ahn *et al*., 1999). Consistent with these results of the Sox-4 expression (Figure 1), the immunoblot with the anti-Sox-4 antibody showed the increased Sox-4 protein (approximately 48 kDa) in both Hep3B(Figure 1A) and HepG2 cells treated with PGA_2 and Δ^{12}-PGJ_2. (Figure 1B). The Sox-4 protein began to increase after 12 h and reached the maximum level at 24 hr in both cell lines. The expression of Sox-4 was also induced by several apoptotic agents such as A23187 (Ca^{2+} ionophore, 1 ug/ml) and etoposide (topoisomerase II inhibitor, 5 ug/ml) exposure to Hep3B(Figure 2A) and HepG2 cells(Figure 2B).

Effect of Sox-4 expression on apoptosis

To investigate the possible role of Sox-4 in modulating the PG-mediated apoptosis, we have developed human hepatoma cell lines expressing the Sox-4 protein. The cells were transiently transfected with the pCDM8-Sox-4 plasmid based up on CMV(cytomegalovirus) immediated early gene promoter. After transfection, the expression of Sox-4 was multiplied by five fold at 36 h in Hep3B cells (Figure 3). HepG2 cells also showed an increase in the expression of Sox-4 which developed up to eight folds at 36 h under the same condition (Figure 3).

The relative level of apoptotic activity in Hep3B and

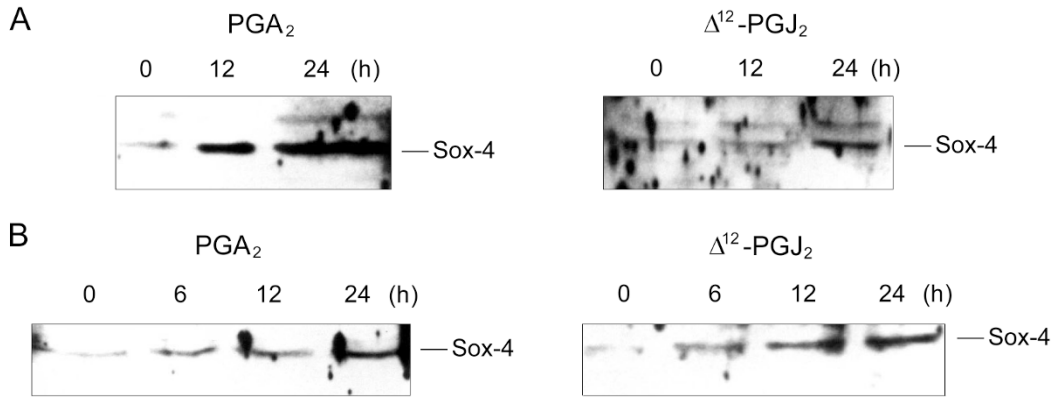

Figure 1. Immunoblot analysis of Sox-4 in Hep3B and HepG2 cells exposed to PGA_2 and Δ^{12}-PGJ_2. The cells were exposed to PGA_2 (20 μg/ml) and Δ^{12}-PGJ_2 (5 μg/ml) for indicated time periods, and the expression levels of Sox-4 were studied by immunoblot analysis. Control lane(0 h) is untreated cells. A, Hep3B cells; B, HepG2 cells.

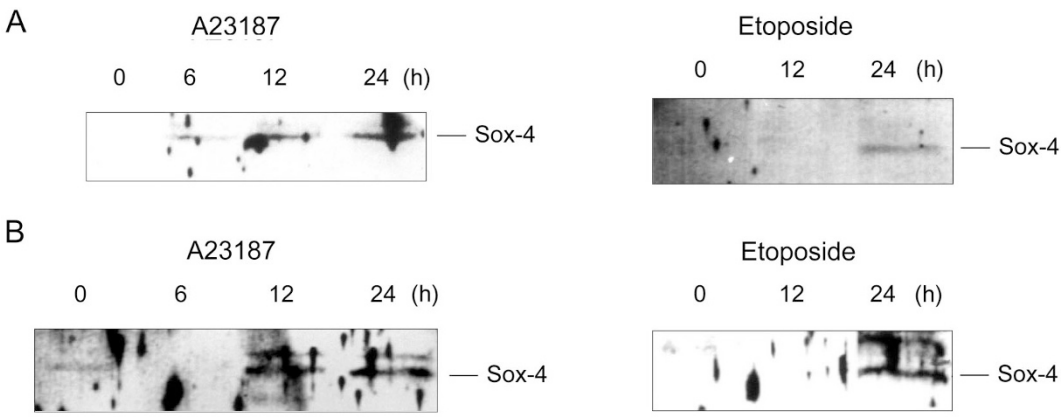

Figure 2. Immunoblot analysis of Sox-4 In Hep3B and HepG2 cells exposed to A23187 and etoposide. The cells were exposed for each time point with presence or absence of A23187(1 μg/ml) and etoposide(5 μg/ml) and the expression levels of Sox-4 were detected by immunoblot analysis. Control lane(0 h) shows untreated cells. A, Hep3B cells; B, HepG2 cells.

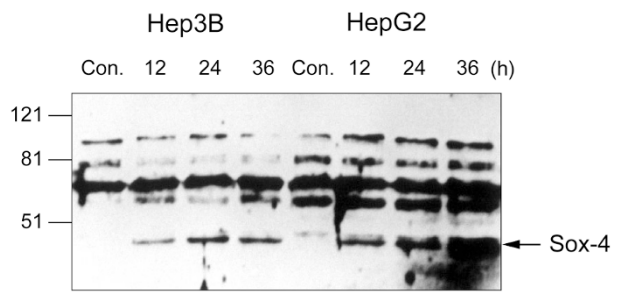

Figure 3. Western blot analysis of Hep3B and HepG2 cells transient transfected with pCDM8-Sox-4. Coding region of Sox-4 cDNA (1.34 kb) was inserted into pCDM8 expression vectors. Purified DNA (5 μg) of pCDM8 expression vector containing Sox-4 cDNAs was transfected into Hep3B and HepG2 cells using DOTAP as described. Whole cells were harvested at 12 , 24 and 36 h after the start of the transfection and analyzed by Western blot.

HepG2 cells transfected with the pCDM8-Sox-4 or control vector was quantified by cell death detection ELISA kit and determined by counting the numbers of apoptotic cells. Approximately 40% of the transfected cells with the pCDM8-Sox-4 were dead at 24 h post-transfection. As shown in Figure 4A, only 40% of Hep3B/Sox-4 cells were sustained upon overexpression of Sox-4 at 24 h, and similarly, only 62% of HepG2/Sox-4 cells survived upon overexpression of Sox-4 at 24 h (Figure 4B). In contrast, pCDM8 vector alone did not have an impact on Hep3B and HepG2 cell viability under the same experimental condition (Figure 4A, 4B).

The ability of Sox-4 to induce apoptosis was confirmed by the DNA fragmentation. Total cellular genomic DNA isolated from pCDM8-Sox-4-transfected Hep3B and HepG2 cells were separated on 1.8% agarose gels. The transfected cells with the pCDM8-Sox-4 plasmid caused DNA fragmentation at 24 h post-transfection in Hep3B cells, while the genomic DNA of HepG2 showed significant DNA degradation after 48 h later (Figure 5).

Effects of Sox-4 antisense oligonucleotide on PGA_2-induced apoptosis

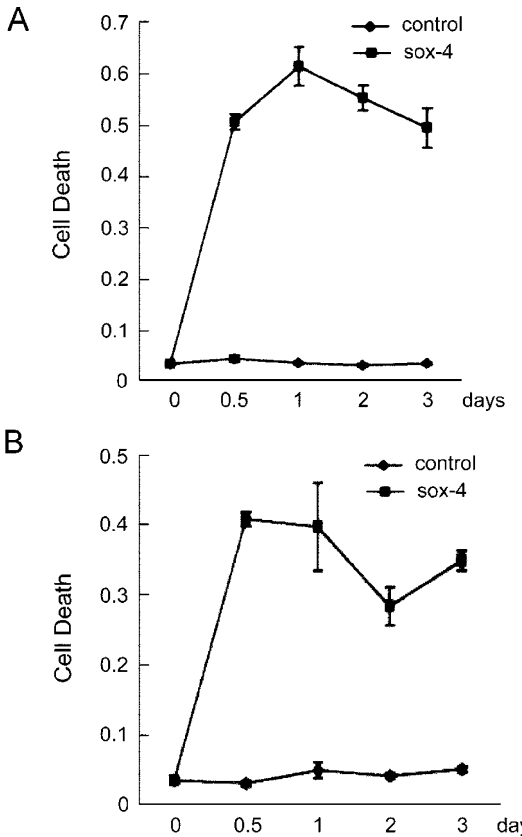

Figure 4. Sox-4 induced apoptosis in Hep3B and HepG2 cells after transfection of pCDM8-Sox-4. 1 x 10^3 cells/well were seeded into 96 well culture dishes and incubated at 37°C, 5% CO_2 after pCDM8-Sox-4 transfection for 12, 24, 48 and 72 h, respectively. Percentage of apoptotic cells were measured by modified Cell Death Detection ELISA at 405 nm-490 nm. A, Hep3B cells; B, HepG2 cells.

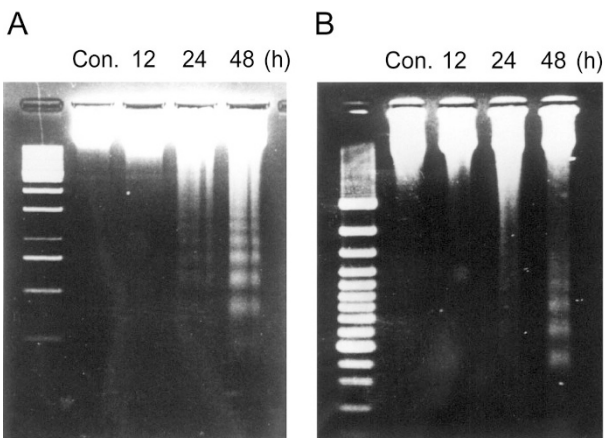

Figure 5. Time course of DNA fragmentation in Hep3B and HepG2 cells by Sox-4 overexpression. Whole cells were harvested at 12, 24 and 48 h, respectively after the start of the pCDM8-Sox-4 transfection and genomic DNA were extracted from the cells. A, Hep3B cells; B, HepG2 cells.

To determine whether Sox-4 was directly involved in the induction of apoptosis, we used antisense oligonucleotide to diminish the function of this gene. Whereas the sense

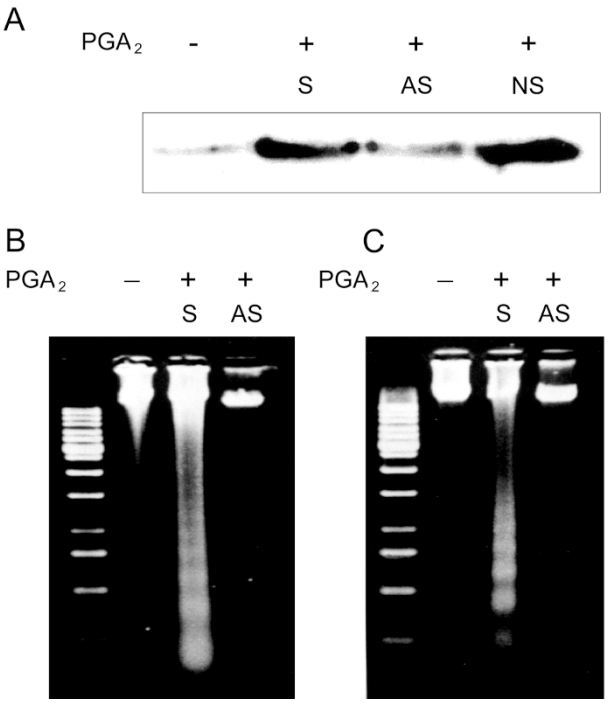

Figure 6. Inhibition of DNA fragmentation by treatment of antisense oligonucleotide corresponding to Sox-4. A. Western blot analysis for the production of Sox-4 in Hep3B cells treated with the sense, antisense and nonsense Sox-4 oligonucleotides. B and C. Hep3B and HepG2 cells were treated with either antisense or sense oligonucleotides corresponding to the translation start region of human Sox-4 and DNA fragmentation was induced by PGA_2 (20 μg/ml). DNA fragmentation was analyzed on agarose gels (1.8%) stained with ethidium bromide. B, Hep3B cells; C, HepG2 cells.

oligonucleotide showed no effects on the induction of Sox-4 expression, antisense oligonucleotide of Sox-4 inhibited the induction of Sox-4 expression as well as the DNA fragmentation in PGA_2-treated Hep3B cells (Figure 6A, B). The similar result was obtained from the HepG2 cell (Figure 6C). These results demonstrates that the induction of Sox-4 may directly modulate the PGA_2- mediated apoptosis in Hep3B and HepG2 cells.

Sox-4 induced apoptosis is inhibited by the Caspase-1 like inhibitor Ac-YVAD-CHO

To test the ability of the caspase inhibitors in Sox-4-induced apoptosis, we incubated the Hep3B and HepG2 cells overexpressing Sox-4 with different concentrations of ICE inhibitor Ac-YVAD-CHO, Ich-1 inhibitor z-DVAD-FMK and CPP32/Yama inhibitor Ac-DEVD-CHO.

As shown in Figure 7A, approximately 40% and 90% of the DNA fragmentation was inhibited by 50 μM of Ac-YVAD-CHO and 100 μM of Ac-YVAD-CHO, respectively. Also, 100 μM of z-DVAD-FMK inhibited 80% of the DNA fragmentation appeared by Sox-4-induced apoptosis in Hep3B cells. Meanwhile, 100 μM of Ac-DEVD-CHO showed no significant inhibition of Sox-4-induced

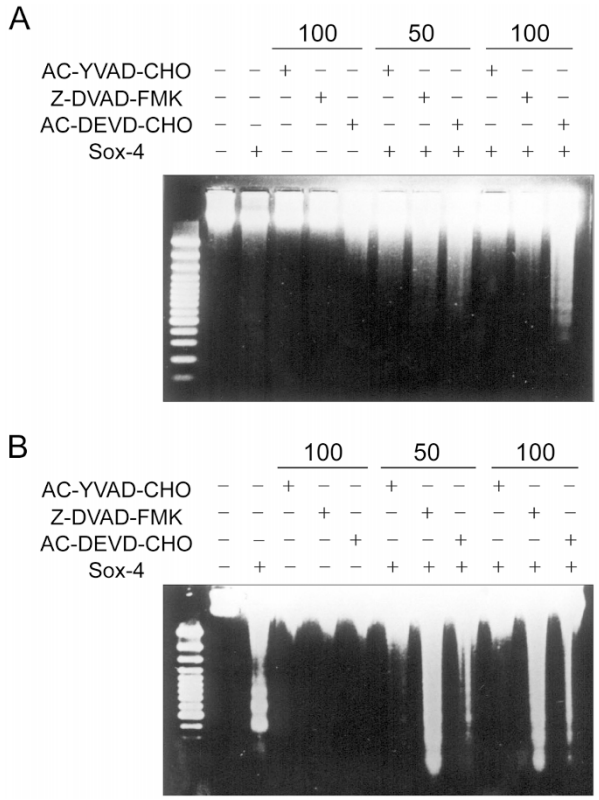

Figure 7. Inhibition of Sox-4-induced apoptosis in Hep3B and HepG2 cells by caspase inhibitors. Whole cells (1 x 10^5 cells/ml) harvested at 48 h after the start of the transfection, in presence of caspase inhibitors (each 50 μM and 100 μM) and genomic DNA samples were extracted from the cells. The concentrations of each caspase inhibitor tested are indicated in the figure. A, Hep3B cells; B, HepG2 cells

apoptosis in Hep3B cells. In contrast, the Sox-4-induced DNA fragmentation in HepG2 cells was not nearly inhibited by 100 μM z-DVAD-FMK and partially inhibited by up to 100 μM of Ac-DEVD-CHO (Figure 7B). Similarly, as shown in Hep3B cells, the Sox-4-induced DNA fragmentation was almost completely inhibited by 100 μM of Ac-YVAD-CHO(Figure 7B). Each caspase inhibitor had no effects on cell viability (>95%) as a result of MTT assay measurement (data not shown).

Since ICE inhibitor Ac-YVAD-CHO showed strong inhibition effects on Sox-4-induced DNA fragmentation, we examined the expression of caspase-1 (ICE) in PGs-treated and Sox-4-transfected hepatoma cells using specific antibodies to ICE homologs. The caspase-1 processing was increased during 12-48 h in both cases in contrast to the control cells (Figure 8). As shown in Figure 8A and B, the 33 kDa processed intermediate form of caspase-1 was markedly increased after 12 h during PGA_2 and Δ^{12}-PGJ_2 treatment in Hep3B cells. Also, the caspase-1 cleaved to a smaller active fragment(33 kDa) in overexpressed Sox-4 Hep3B and HepG2 cells(Figure 8C and 8D). Therefore, the apoptotic activity of Sox-4 is correlated to the activation of caspase-1.

Discussion

The expression of acute phase reactants such as C-reactive protein, α_1-acid glycoprotein, haptoglobin (Hp) and heat shock protein (HSP) are increased especially by PGs and other inflammatory stimuli *in vitro* as well as *in vivo* (Santoro *et al*., 1989: Holbrook *et al*., 1992: Kim *et al*., 1995: Molle *et al*., 1997). In addition α_1-acid glycoprotein and α_1-antitrypsin inhibited TNF-α induced apoptosis (Molle *et al*., 1997). Hp, after complex formation with hemoglobin, induces apoptosis of Hep3B cells (Kim *et al*., 1995). The overexpression of HSP shows an increasing resistance to apoptosis by PGA_2 and Δ^{12}-PGJ_2 and other apoptotic inducing agents in hepatoma cells (Ahn *et al*., 1998). These results suggested that up-regulated genes and their products induced by PGA_2 and Δ^{12}-PGJ_2 in tumor cells may serve

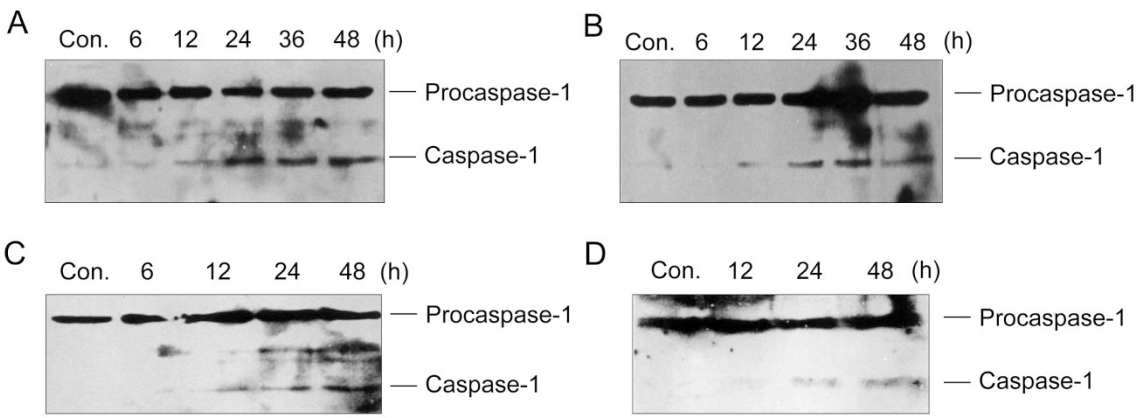

Figure 8. Cleavage of caspase-1 protein in Hep3B and HepG2 cells during Sox-4-induced apoptosis. Hep3B cells were prepared at different incubation times after PGA_2 (20 μg/ml, Figure 8A), Δ^{12}-PGJ_2 (5 μg/ml, Figure 8B) treatment and Sox-4 overexpression (Figure 8C). Under overexpression of Sox-4, an activation of the caspase-1 was also observed in HepG2 cells(D). Proteins were separated in SDS-PAGE and subsequently immunobloted with anti-caspase-1.

as a positive or negative mediator in PGA_2 and Δ^{12}-PGJ_2 induced apoptosis in tumor cells.

Recent studies revealed that the mutation of Sox-4(*Sox-4*$^{-/-}$) in the embryo of mice leads to premature death at 14th embryonic days due to impaired development of the endocardial ridges (Schilham *et al*., 1996: Ya *et al*., 1998). In 1998, Southard-Smith *et al*. reported that premature termination of Sox-10, a member of the Sox family, disrupted neural crest development due to apoptosis in Dom Hirschsprung mouse models. These results add the possibility that Sox genes, including Sox-4, may involved in tumor cell apoptosis by various apoptosis inducing agents.

As previously mentioned, the induction of apoptosis in hepatoma cells by PGs showed characteristic morphological and biochemical findings of apoptosis (Lee *et al*., 1995: Ahn *et al*., 1998). Here, we described the increased expression of Sox-4 by PGA_2 and Δ^{12}-PGJ_2 treatment in Hep3B and HepG2 cells. The expression of Sox-4 was also increased by several apoptotic inducer such as A23187(Ca^{2+} ionophore) and etoposide (topoisomerase II inhibitor). During apoptosis of Hep3B cells and HepG2 cells Sox-4 were observed to be overexpressed. When Hep3B and HepG2 cells were transiently transfected with pCDM8-Sox-4, only 40% of Hep3B/Sox-4 cells and 62% of HepG2/Sox-4 cells survived upon overexpression of Sox-4 at 24 h, as well. The transfected cells with pCDM8-Sox-4 plasmid caused DNA fragmentation at 12 h and 24 h in Hep3B and HepG2 cells respectively and antisense oligonucleotide corresponding to the Sox-4 translation starting site completely inhibited the PGA_2 and Δ^{12}-PGJ_2 induced apoptosis.

There are two kinds of apoptotic pathway known in tumor cells induced by chemotherapeutic agents. One is AIF-dependent and the other is cytochrome C-dependent pathway (Hunot S. and Flavell R.A. 2001). Examination of the Sox-4-induced apoptosis path showed that Sox-4-induced DNA fragmentation was inhibited by Ac-YVAD-CHO, a universal inhibitor of caspase-1 like protease and processing of caspase-1 from its inactivate to active form. Such result suggests that the caspase cascade, specially caspse-1 is involved in Sox-4-induced apoptosis. Gagliardini *et al*. hypothesized that caspase-1 is involved in apoptosis in Dorsal root ganglion neurons in vertebrates (Miura *et al*., 1993: Gagliardini *et al*., 1995). Caspase-1 was found to be involved in the apoptosis of mammary epithelial cells and interferon regulatory factor-1-dependent T-lymphocyte apoptosis (Boudreau *et al*., 1995: Tamura *et al*., 1995). These results provide evidence that caspase-1 like protease plays a critical role in Sox-4-induced apoptosis. Alternatively, caspase-1 and the rest of caspase family may act as an activator of caspase-2 and caspase-3.

However, an inhibitor of caspase-3 like protease (Ac-DEVD-CHO) did not inhibit Sox-4-mediated apoptosis in Hep3B cells. We also could not detect any proteolytic activity capable of cleaving pro-capase-3 in Hep3B cell. But, an inhibitor of caspase-3 like proteases partially inhibited Sox-4-mediated apoptosis in HepG2 cells under the same condition. Furthermore, we found that the pro-caspase-3 is processed after PGA_2 and Δ^{12}-PGJ_2 treatment and Sox-4 overexpression (data not shown). These results demonstrated that a Sox-4-mediated activation of caspase-3 is not observed in Hep3B cells, suggesting that Sox-4-mediated caspase-3 activation is a cell-type specific.

Conclusively, our data demonstrated that Sox-4 is a positive regulator of Hep3B and HepG2 cells apoptosis induced by PGA_2 and Δ^{12}-PGJ_2, and its pathway might go through activation of caspase-1.

Acknowledgements

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