COMPARISON OF MANGANESE SUPEROXIDE DISMUTASE PRECURSOR INDUCTION ABILITY IN HUMAN HEPATOMA CELLS WITH OR WITHOUT HEPATITIS B VIRUS DNA INSERTION

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Summary. – In a previous study (Hajnická *et al., Acta virol.* **38**, 55–57 (1994)), we described synthesis of a 23 K protein in high amounts in the PLC/PRF/5 human hepatoma cell line after stimulation with sera of patients suffering from liver cirrhosis. In this study we identified this protein as manganense superoxide dismutase (Mn-SOD). When PLC/PRF/5 cells stimulated by various cytokines (interleukin-1α (IL-1α) , IL-1β, tumor necrosis factor -α (TNF-α) ,TNF-β, IL-6, tumor growth factor-α (TGF-α), TGF-β , and interferon-gamma (IFN-γ) were compared, the most effective was IL-1, followed by TNF-α and IL-6. Other cytokines had no effect on the stimulation of Mn-SOD. IL-1α was selected for stimulation of Mn-SOD production in four human hepatoma cell lines (PLC/PRF/5, Hep-3B, Hep-G2 and Sk-Hep 1). Maximum Mn-SOD production occured in PLC/PRF/5 cells. In other cell lines, Mn-SOD production was lower, reaching 35.7% and 31.5% in Hep-3B and Sk-Hep-1 cells, respectively, while it was only 4.3 % in Hep-G2 cells.

Key words: human hepatoma cells; manganese superoxide dismutase; 2D-electrophoresis; IL-1; IL-6; TNF-α

Introduction

Activated oxygen species are generated in aerobic cells as an intermediate of the reduction of molecular oxygen, occurring in a variety of physiological processes and in the immunological defence against invading agents (Gille and Sigler, 1995). High levels of activated oxygen species react with various molecules in the cell, causing injury of cells and leading to a wide range of pathological consequences (Martínez-Cayuela, 1994).

Organisms generate a large number of antioxidants to prevent or repair the damage caused by reactive oxygen species. In this way toxic oxygen radicals and non-radical molecules are converted into water.

Primary enzymatic defence includes a family of metalloenzymes, superoxide dismutases, scavengers of superoxide radicals (McCord and Fridovich, 1969). Within mitochondria a primary defense against oxidative damage is Mn-SOD (Fridovich, 1989). Human mitochondrial Mn-SOD is an inducible homotetrameric enzyme (Borgstahl *et al.*, 1992).

In many studies the abnormalities have been demonstrated in both the level and regulation of antioxidant enzymes in tumor cells. In most of these cases the activity of Mn-SOD was reduced (Oberley and Oberley, 1997), although in some tumors high Mn-SOD activity was described (Yang *et al.*, 1987).

Here we show a comparison of Mn-SOD production in four human hepatoma cell lines after induction with some cytokines. Irrespective of their common liver origin, these

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Abbreviations: PBMCs = peripheral blood mononuclear cells; Mn-SOD = manganese superoxide dismutase; IL = interleukin; TNF = tumor necrosis factor; TGF = transforming growth factor; PPO = 2,5-diphenyloxazol; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; IEF = isoelectric focusing; interferon-gamma = INF-γ; LPS = lipo-polysaccharide; HBV = hepatitis B virus; HCV = hepatitis C virus

cell lines differ in the degree of differentiation, morphology and the presence of hepatitis B virus (HBV) DNA. PLC/PRF/5 and Hep-3B are primary hepatocellular carcinoma cell lines (Alexander *et al.*, 1976; Aden *et al.*, 1979.,) with HBV-DNA integrated into the cellular DNA, and producing the HBV-coded surface antigen, HBsAg (Twist *et al.*, 1981). Hep-G2 is a hepatoblastoma-derived cell line (Knowles *et al.*, 1980) and Sk-Hep 1 is a hepatic adenocarcinoma cell line (Fogh *et al.*, 1977), probably of endothelial origin (Heffelfinger *et al.*, 1992).

Materials and Methods

Cytokine. Human recombinant TNF-α (3.2 x 10^7 U/mg), human recombinant TGF-α, and human recombinant TGF-β1 (all three from Serva), human recombinant TNF-β (1.0 x 10^7 U/mg, Sigma), human recombinant IFN-γ (>2.0 x 10^7 U/mg), human recombinant IL-1α (>1.0 x 10^7 U/mg), human recombinant IL-1β (>1.0 x 10^7 U/mg), human natural IL-6 (>1.0 x 10^8 U/mg) (all from Boehringer Mannheim), and human recombinant IL-6 (from Dr. Aarden, Blood Transfusion Service, Amsterdam, the Netherlands) were used. Following concentrations were used in the experiments: IL-1α and IL-1β:20 U/ml; TGF-α: 5 ng/ml; TGF-β1: 1 ng/ml; IL-6: 4000 U/ml; TNF-α:10 ng/ml; IFN-γ: 500 U/ml; TNF-β: 500 U/ml.

Cells. Human hepatoma cells PLC/PRF/5, Hep-3B, Hep-G2 and Sk-Hep-1 were routinely grown in the Leibovitz L-15 medium supplemented with 5% of bovine fetal serum and antibiotics at 37°C.

Stimulation of PBMCs. PBMCs were isolated by a modified Boyum's method (Boyum, 1976) using Ficoll-Hypaque density gradient centrifugation of heparinized blood samples from healthy volunteers. PBMCs (1.0 x 10^6/ml) were incubated for 18 hrs in RPMI 1640 medium supplemented with 10% of heat-inactivated human serum and 1 μg/ml lipopolysaccharide (LPS). The cultivation medium containing a cytokine was used to induce Mn-SOD.

Radiolabeling of cellular proteins and preparation of cell lysates. Cells were grown for 3 days to confluence in 6-cm Petri dishes with 5 ml of the Leibovitz L-15 medium. The cultivation medium was then replaced by a fresh medium containing an inducer and ^{14}C-labeled protein hydrolysate (ÚVVVR, Prague, Czech Republic, 1 MBq/dish). After incubation for 48 hrs cell monolayers were washed 3 times with phosphate-buffered saline (PBS), solubilized in 100 ml of the buffer for isoelectric focusing (IEF) (9 mol/l urea and 4% Triton X-100), and incubated at room temperature for 2 hrs. Samples were then centrifuged at 50,000 x g for 2 hrs (Biofuge 28 RS, Heraeus). The solubilized samples were kept at -70°C until used (Dunbar, 1987).

Isoelectric focusing (IEF) and two-dimensional polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (2D-SDS-PAGE) were performed according to O'Farell (1975). IEF gels (1.5 mm in diameter and 10.5 cm in length) were prepared with 2% ampholines of pH range 3–10 (Servalyt 3–10). A 25 μl sample containing 2% β-mercaptoethanol and 2% ampholines was loaded per gel. Samples were focused at 700 V for 17 hrs. After IEF, the gels were equilibrated for 10 mins in a buffer containing 0.0625 mol/l Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and applied onto SDS-polyacrylamide gels. A linear gradient of 7.5–12.5% acrylamide was used in preparation of these gels. For analytical investigation the gels were impregnated with PPO scintillator and dried. Du Pont X-ray films were used for auroradiography of the dried gels at -70°C for 5 weeks. The gels were scanned by the SHARP JX-330 scanner and examined using the ImageMaster 2D Elite software (Pharmacia Biotech).

Electroblotting and amino acid sequencing. For micropreparative purposes, IEF and SDS-polyacrylamide gels were only 3-mm thick. After 2D-electrophoresis proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon-P transfer membrane, Millipore). A spot of about 24 K protein was sequenced on a pulsed liquid amino acid sequencer (Applied Biosystems) as described in detail elsewhere (Proost *et al.*, 1993).

Results

Effect of cultivation medium from cytokine-stimulated PBMCs on protein pattern of PLC/PRF/5 cells

Hepatoma PLC/PRF/5 cells growing for 4 days were treated with cultivation medium from cytokine-stimulated PBMCs. Comparison of radiolabeled protein patterns of control and treated cells in 2D-SDS-PAGE demonstrated a marked increase of the protein with Mr of 23–25 K in the treated cells (Fig. 1A, B). The protein could be detected also in the control cells. In the cells grown in culture only for 1 day, the Mn-SOD precursor was undetectable in the control cells while it was present in the treated cells in the same amount as in 4-day-old culture (Fig. 1C, D).

Identification of Mn-SOD precursor in protein pattern of stimulated PLC/PRF/5 cells

After 2D-SDS-PAGE of the lysate from non-labeled stimulated cells, the gel was electroblotted on a PVDF membrane and stained with Coomassie Brilliant Blue. The N-terminal sequence of the corresponding spot was analyzed with this result:

KHSLPDLPYDYGALEPHINA

According to this sequence the protein was identified as the Mn-SOD precursor.

Effect of different cytokines on Mn-SOD precursor production by PLC/PRF/5 cells

To identify the cytokine responsible for the marked increase in the production of Mn-SOD precursor in PLC/PRF/5 cells, stimulation with IL-1α, IL-6, TNF-α, TNF-β, IFN-γ, TGF-α, and TGF-β was tested. Maximum stimulation

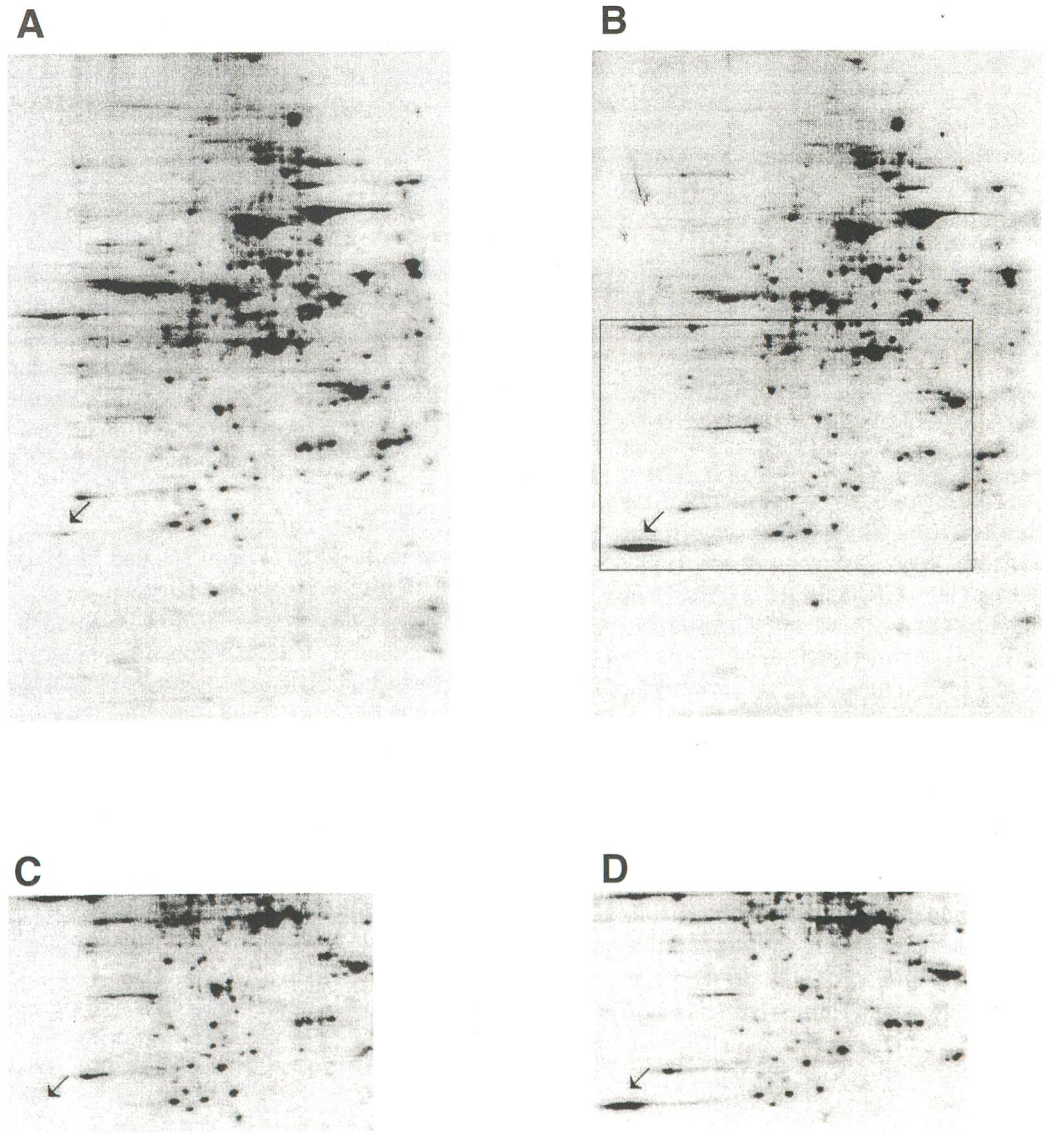

Fig. 1
Effect of cultivation medium from stimulated PMBCs on protein pattern of hepatoma PLC/PRF/5 cells grown in culture for 1 day and for 4 days
Protein pattern determined by 2D-gel electrophoresis. A. Control 4-day-old cell culture. B. Treated 4-day-old culture. C. Control-1-day-old culture. D. Treated 1-day-old culture. Arrows indicate positions of Mn-SOD precursors.

occurred after treatment with IL-1α and IL-1β, while TNF-α was less effective (data not shown). Interestingly, some differences in Mn-SOD production were observed between natural and recombinant IL-6. Other cytokines tested did not stimulate the Mn-SOD production. The quantitative comparison of the amounts of Mn-SOD protein precursor induced by different cytokines in PLC/PRF/5 cells is shown in Table 1.

Effect of IL-1α on Mn-SOD precursor production by different human hepatoma cells

To compare the Mn-SOD production in four different human hepatoma cell lines (PLC/PRF/5, Hep-3B, Hep-G2, and Sk-Hep-1), IL-1α cytokine was chosen as it was the best inductor for the PLC/PRF/5 cell line. As shown in Table 2, the highest amounts of Mn-SOD were induced in PLC/PRF/5 cells and the lowest ones in Hep-G2 cells.

Table 1. Mn-SOD production in PLC/PRF/5 cells stimulated by different cytokines

Cytokines	Volume[a]
IL-1α	169,282
TNF-α	38,215
IL-6 natural	12,832
IL-6 recombinant	4,767

Spot measurement was performed by the Image Master 2D Elite software.
[a]The sum of intensities of every pixel in the spot.

Discussion

Although the concentrations of a variety of cytokines in serum are elevated in patients with liver diseases, their potential role is not clear (Simpson *et al.*, 1997). Some cytokines, such as TNF-α, IL-8, IL-10, and IL-12 may play a role in the immunopathogenesis and outcome of hepatitis C virus (HCV) infection (Kallinowski *et al.*, 1998; Masumoto *et al.*, 1998; Quiroga *et al.*, 1998; Zhu *et al.*, 1998), probably due to oxidative stress (Larrea *et al.*, 1998). Indeed, the absence of induction of antioxidant Mn-SOD in the liver during HCV infection suggests that this organ could be less protected against oxidative damage (Larrea *et al.*, 1998). The situation with HBV infection is more complicated because the expression of cytokines may differ at different phases of acute exacerbation of hepatitis B (Fukuda *et al.*, 1995), and some cytokines, namely TNF-α and IFN-γ may be important for the elimination of infected hepatocytes during acute HBV infection (Hohler *et al.*, 1998). In our previous study, changes in the protein pattern of the lysates of hepatoma PLC/PRF/5 cells after treatment with sera of cirrhotic patients were observed (Hajnická *et al.*, 1994). Apart from alpha-fetoprotein, another markedly induced protein of approximately 25 K was found. The purpose of the present study was to identify this protein and to determine whether these changes could be induced by cytokines. The protein maps and amino acid sequence analysis showed that the protein in question was a Mn-SOD precursor. When testing several cytokines in the hepatoma PLC/PRF/5 cell line, only pro-inflamatory cytokines IL-1α, IL-1β, TNF-α and IL-6 were found to induce the Mn-SOD precursor; IL-1α and IL-1β were the most efficient inducers. Differences were found also between the cell lines used, the most pronounced effect being observed in primary hepatocellular carcinoma PLC/PRF/5 cells and Hep-3B cells, while the weakest effect in the Hep-G2 hepatoblastoma cell line. Such differences can be explained by different tumorigenicity of the cell lines as found in nude mice and nude rats (Shouval *et al.*, 1998), different hepatocarcinogenesis, i.e. p53 gene abnormalities observed in PLC/PRF/5, Hep-3B, and Sk-Hep-1 cell lines but not in Hep-G2 cell line (Bressac *et al.*, 1990), and differences in the expression of HLA antigens and in the cytotoxicity after rIL-1 exposure (Yoshioka *et al.*, 1989). Whether the higher induction of the Mn-SOD precursor by cytokines in PLC/PRF/5 and Hep-3B cell lines is connected with integration of HBV-DNA into the DNA of these cells remains to be answered by further studies. A decrease by more than 90% of the Mn-SOD activity along with reduction of activities of other antioxidative enzymes in Hep-3B cell line is considered to be a carcinogenic mechanism through the accumulation of oxygen-free radicals and DNA damage (Bannister *et al.*, 1986).

Table 2. Production of Mn-SOD precursor in different human hepatoma cell lines

Cell line	Volume	Pure volume[a]	% of stimulation
PLC/PRF/5 control	9,450	–	–
PLC/PRF/5 + IL-1α	155,329	145,879	100
Hep-3B control	0	–	–
Hep-3B + IL-1α	52,176	52,176	35.7
SK-Hep-1 control	9,522	–	–
SK-Hep-1 + IL-1α	55,504	45,982	31.5
Hep-G2 control	28,355	–	–
Hep-G2 + IL-1α	34,713	6,358	4.3

Spot measurement was performed by the Image Master 2D Elite software.
[a]The volume of spot from control cells subtracted from the volume of spot from treated cells.

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