行政院國家科學委員會專題研究計畫成果報告

計畫名稱：

中　文：細胞分化與自由基之研究

英　文：Study on the Effect of Free Radicals on Cell Differentiation

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中文摘要

自由基，尤其是指活性氧分子(ROS)，是細胞正常代謝過程所產生的高活性代謝物，ROS 可經由直接與生物分子作用導致細胞傷害，或者經由訊息傳遞過程促使細胞分化、增生、癌化甚至凋亡(apoptosis)。抗氧化酵素為細胞內活性氧分子的重要代謝系統，為了瞭解抗氧化酵素活性與細胞分化間的關聯性，本論文選用不同分化程度肝癌細胞進行系列性研究，包括分化程度較高的 Hep G2、Hep 3B，以及分化程度較低的 SK-Hep-1、HA22T/VGH、HA55T/VGH，分別比較不同分化程度的肝癌細胞，其內生性抗氧化酵素的活性，包括 catalase (CAT)、manganeses superoxide dismutase (MnSOD)、copper-zinc superoxide dismutase (CuZnSOD)、glutathione peroxidase (GPx)以及 glutathione reductase (GRx)等。實驗結果指出，分化程度較高的肝癌細胞株中，其 CAT、MnSOD、GRx 之活性明顯較分化程度低的肝癌細胞株高出許多，其平均值分別差了 5.1 倍、2.8 倍及 6.9 倍；而 GPx 與 CuZnSOD 則在不同分化程度的肝癌細胞株未出現明顯差異。以西方墨漬、放射線免疫沉澱及北方墨漬法進行分析，發現 MnSOD 及 CAT 的蛋白質及 RNA 表現量在分化程度較高的肝癌細胞株中亦被提升，為了解釋不同分化程度肝癌細胞株之抗氧化能力，實驗中以 50 μM H_2O_2 處理細胞，作為氧化壓力的來源，數據顯示，與控制組相比較，分化程度較低的肝癌細胞株 MnSOD 活性可被誘導增加約 1~2 倍，而分化程度較高的肝癌細胞株則無此情形。這結果顯示分化程度較低的肝癌細胞有較佳的氧化壓力耐受性，推測 MnSOD 的增加可能與訊息傳遞因子 NFκB 的活化有關，此假設需進一步證實。除此之外，在臨床上，CAT 以及 GRx 活性的差異應可用於肝癌細胞分化的指標。

Abstract

Free radicals, especially the reactive oxygen species (ROS) are highly reactive metabolites that are generated during normal cell metabolism. The ROS can cause cellular damages by direct interaction with bio-molecules or regulate the process of differentiation, proliferation, carcinogensis and apoptosis through the signaling pathway. In order to reveal the correlation of scavenging enzymes activities and cell differentiation, a panel of human hepatocellular carcinoma (HCC) cell lines, including three poor-differentiated (HA22T, HA55T and SK-Hep-1) and three well-differentiated (Hep 3B, Hep G2 and Chang liver cell) were used to examine the expression pattern of superoxide dismutase (MnSOD and CuZnSOD), catalase (CAT), glutathione reductase (GRx) and glutathione peroxidase (GPx). Results showed that the well-differentiated cells exhibited higher activities of antioxidant enzymes (MnSOD, CAT and GRx) than poor-differentiated ones for about 2.8, 5.1 and 6.9 folds in average, respectively. However, the activities of CuZnSOD and GPx were only minor differences in the HCC cell lines used. By immunoblotting, radio-immunoprecipitation and northern blot analysis, the protein and RNA level of CAT and MnSOD were also elevated in the well-differentiated cells. To elucidate the antioxidation ability of different differentiated HCC cells, we investigated the antioxidant enzyme activities after treatment with H_2O_2. The activity of MnSOD could be induced significantly in poor differentiated HCC cells (SK-Hep-1). However, the other scavenging enzymes show only minor differences compared with the control cells. These results suggested that poor-differentiated HCC cells might have better antioxidative ability than well-differentiated ones. It is possible that the inducible activity of MnSOD might be contributed by NF-κB signaling

pathway. Thereby, such phenomena could participate to the high metastasis frequency of poor-differentiated HCC cells clinically. This hypothesis will be investigated in future study. In clinical, the activities of CAT and GRx might be potential differentiation makers of human hepatocellular carcinoma.

Introduction

Free radicals, especially the reactive oxygen species (ROS) are highly reactive metabolites that are generated during normal cell metabolism. Cellular damage is usually caused by ROS through reaction with proteins, lipids, carbohydrates, and DNA, which consequently lead the cell to apoptosis. Cell differentiation is involved in many physiological and pathological events such as embryogenesis, cell renewal and neoplasia. Recent studies suggested that free radicals might play some regulatory roles throughout cell differentiation and proliferation. However, little information is available regarding the role of free radicals in cell differentiation. On the other hand, several diseases occurring in liver are correlated with free radicals. Moreover, the poorly differentiated human hepatocellular carcinoma (HCC) exerts higher frequency of metastasis and *vice versa*. Here, in this report, several human hepatocellular carcinoma (HCC) cell lines, including two categories of poorly and well-differentiated cell lines, were employed to reveal the correlation of scavenging enzymes activities and differentiation stages as well as their antioxidant ability under oxidative stress. Results showed that the expression of mRNA, protein and the specific activities of catalase (CAT), glutathione reductase (GRx) and manganese superoxide dismutase (MnSOD) were higher in the well-differentiated HCC cells. The activitise of CAT and GRx might be potential differentiation markers of human HCC. Nevertheless, only the activity of MnSOD in poorly differentiated HCC cells can be induced under oxidative stress. It may play pivotal role for the metastasis ability of poorly differentiated human hepatocellular carcinoma and will be discussed in the following text.

Specific Aims

1. To demonstrate the correlation between the activities of antioxidant enzymes and the differentiation stage of human hepatoma cell lines, including poorly differentiated HA22T/VGH, HA55T/VGH and SK-Hep-1 cells as well as the well-differentiated Hep 3B and Hep G2 cells.
2. To evaluate which antioxidant enzyme could be as a differentiation marker of human hepatoma cells.
3. To reveal the antioxidant ability of human HCC cells of different differentiation.

Materials and Methods

Cell culture and treatment

Human hepatoma cell lines, including HA22T/VGH, HA55T/VGH, SK-Hep-1, Hep 3B and Hep G2 were cultured in a 37°C humidified environment containing 5% CO_2 in air and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100μg/ml). Serum starvation was achieved by incubation in DMEM containing 0.1% FBS for at least 16h prior to the direct addition of H_2O_2 into this culture medium.

Activity assay of scavenging enzymes

1. Catalase (CAT) activity assay

Catalase activity was determined by monitoring the rate of decomposition of H_2O_2 from the decrease in absorbance at 240 nm. The reaction mixture contained appropriate amount of H_2O_2 and cell lysate (0.2~0.4 mg) in phosphate buffer (pH 7.0). Mix well after addition of H_2O_2 and monitor the decrease in 240 nm absorbance.

2. Superoxide dismutase (SOD) activity assay

SOD activity in the cell extract was assayed on the basis of the ability of the enzyme to inhibit the auto-oxidation of pyrogallol. Assay mixture contained Tris-cacodylate buffer (pH 8.4), 2 mM pyrogallol, and cell lysate. The auto-oxidation rate of pyrogallol is determined by the increase in absorbance at 420 nm. The activity of MnSOD was determined by

addition of NaCN to inhibit CuZnSOD activity.

3. *Glutathione reductase (GRx) activity assay*

GRx activity is measured by the decrease in the absorbance due to the oxidation of NADPH. The reaction mixture contains potassium phosphate buffer (pH 7.2), 0.5 mM EDTA (pH 7.2), 2 mM oxidized form of glutathione and appropriate amount of cell lysate. Incubate the mixture at 37℃ and then NADPH was added. Measure the absorbance at 340 nm.

4. *Glutathione peroxidase (GPx) activity assay*

Gpx activity is determined using a coupled assay in which enzyme activity is proportional to the rate of nadph oxidation. The reaction is started by adding t-butylhydroperoxide and the decrease in absorbance at 340 nm is monitored for 5 min.

Western-blot and Radioimmunoprecipitation

40 μg of cell lysate were electrophoresed in a SDS-PAGE and then transferred to nitrocellulose paper. Western-blot are performed with the following antibodies and detected by the enhanced-chemiluminescence method (Amersham Co.). For RIP, cells are lysed by mild detergent (NP40) and specific proteins are precipitated with antibody-conjugated protein A sepharose. Anti-CuZnSOD antibody (cat. no. 8474-9504, Biogenesis Co.) and anti-catalase antibody (cat. no. K50805R, Biodesign Co.) are suitable for Western-blotting analysis. However, anti-MnSOD antibody (cat. no. 8474-9524, Biogenesis Co.) was suitable for RIP, but not for Western-blot.

RNA preparation and Northern-blot

RNA was extracted by RNeasy spun column (QIAGEN; cat. no. 74104). The RNA samples (20 μ g) were electrophoresed in a formaldehyde-agarose gel and then transferred to nylon paper. Hybridization was performed using nick-translated DNA probe.

Results and Discussion

The activities of antioxidant enzymes in hepatoma cells

As shown in Tab. 1, the specific activities of CAT, MnSOD, CuZnSOD, GPx and GRx were determined. The activities of MnSOD, CAT and GRx of the well-differentiated cells (Hep G2 and Hep 3B) were higher than those of poorly differentiated ones (HA22T/VGH, HA55T/VGH and SK-Hep-1) at about 2.5, 5.1 and 6.9 folds in average, respectively. Hence, the H_2O_2 scavenging enzymes (CAT and GRx) were positively correlated to the differentiation stage of hepatoma cells and which might be differentiation markers of human hepatoma cells. According to the convenience of assay methods, CAT might be a better marker than GRx. In clinical, poorly differentiated HCC implicated higher metastasis frequency and which is an important prognosis for human HCC patient. Here, we provided another option to determine the differentiation stage of human HCC by a simple analysis of CAT activity.

Expression of mRNA and protein of antioxidant enzymes in hepatoma cells

By Northern-blot (Fig. 1) and Western-blot or radioimmuno-precipitation (Fig. 2), the expression level of mRNA and protein were determined. Almost the same phenomena as demonstrated in the enzyme activity (above section) were observed. The relative magnitude for poorly and well-differentiated cells was showed as Tab. 2. These data implied that probably the regulation of antioxidant enzymes was the gene transcription level.

The activities of antioxidant enzymes under oxidative stress

To elucidate the antioxiant ability of HCC cells with different degree of

differentiation, we assayed the antioxidant enzymes activities after treatment with 50 μM H_2O_2, which is a powerful and well-studied reactive oxygen species (ROS) and can be derived to produce other radicals *via* cellular metabolism. Furthermore, H_2O_2 can freely diffuse across cell membrane and its half life is longer than 10 min in culture medium. Based on these reasons, H_2O_2 was used as a source of oxidative stress. Since H_2O_2 can be scavenged by catalase, two negative control experiments were performed: without treatment of H_2O_2 and addition of catalase before treatment of H_2O_2. As shown in Fig. 3, there were no significant changes for the specific activities of CAT, CuZnSOD, GPx and GRx in both poorly (SK-Hep-1) and well-differentiated (Hep G2) cells. Only the activities of MnSOD was elevated and reached to the plateau at 72 hr time point in poorly differentiated SK-Hep-1 cells after treatment of H_2O_2 (panel B,c). The induction magnitude was about 3 folds at 72 hr time point. The same phenomena were obtained for HA22T/VGH and Hep 3B cells (data not shown). Hence, we suggested that only the activity of MnSOD in poorly differentiated HCC cells could be induced under oxidative stress of H_2O_2, i.e., the antioxidant ability of poorly differentiated human HCC cells was much more better for well-differentiated ones. Recent investigations have demonstrated that MnSOD was located in the mitochondria membrane to scavenge free radicals produced by mitochondria. Induction of the expression of MnSOD can protect cells from apoptosis. Thereby, since poorly differentiated HCC cells exhibited better antioxidant ability, we proposed that the metastasized HCC cells will be challenged by oxidative stress and the survival rate of poorly differentiated cells will be higher than well-differentiated ones. Moreover, the expression of MnSOD can be regulated by transcription factors AP-1 and NF κB. H_2O_2 can stimulate the activity of AP-1 and NF κB through signaling pathway. Therefore,

AP-1 and/or NF κ B might play some pivotal roles during the induction of MnSOD by H_2O_2. However, this notion warrants further investigation by EMSA (electrophoretic mobility shift assay).

Summary

1. In human HCC cells, the well-differentiated cells express higher expression level of antioxidant enzymes (CAT, MnSOD and GRx) than the poorly differentiated ones.
2. The transcription level might be a pivotal point for regulation of antioxidant enzymes expression in human HCC cells with different stage of differentiation.
3. The specific activity of CAT enzyme might be a potential differentiation marker of human HCC cells.
4. After treatment of H_2O_2, only the activity of MnSOD could be induced in poorly differentiated HCC cells, but not in well-differentiated ones.

Our results could partially explain the clinical observations that poorly differentiated human HCC cells exhibited higher frequency of metastasis.

Tab. 1. The specific activities of antioxidant enzymes in different differentiated

cell	Antioxidant Enzvme					
	CAT *	T-SOD *	MnSOD *	CuZnSOD *	GPx #	GRx #
HA22T/ VGH	27.7 ± 3.3	12.1 ± 1.4	3.6 ± 1.2	8.3 ± 1.4	10.6 ± 2.1	24.8 ± 2.5
HA55T/ VGH	39.7 ± 2.4	10.7 ± 1.2	4.0 ± 0.2	6.7 ± 1.4	37.7 ± 2.3	27.9 ± 2.1
SK-Hep-1	48.8 ± 6.7	10.2 ± 0.1	3.7 ± 1.1	6.5 ± 1.1	7.7 ± 1.5	33.5 ± 9.4
Hep G2	215.9 ± 7.1	22.0 ± 1.2	11.1 ± 1.4	12.2 ± 1.6	25.7 ± 3.2	195.3 ± 18.7
Hep 3B	182.6 ± 10.4	19.6 ± 0.8	9.8 ± 0.4	8.5 ± 1.9	14.3 ± 2.0	198.9 ± 8.6

Cells were serum starved for 24 hr before lysis and then lysate were subjected to analysis of catalase (CAT), manganese superoxide dismutase(MnSOD), copper-zinc superoxide dismutase (CuZnSOD), glutathione peroxidase (GPx) and glutathione reductase (GRx). The values are means and standard deviations of three independent experiments. *, unit/mg protein; #, unit/g protein.

Tab. 2. The relative expression level of CAT, MnSOD and GRx in poorly and well-differentiated human HCC cells

Differentiation Stage	Antioxidant Enzyme						
	CAT			MnSOD			GRx
	enzyme activity	protein amount	RNA amount	enzyme activity	protein amount	RNA amount	enzyme activity
poor	1.0	1.0	1.0	1.0	1.0	1.0	1.0
well	5.1	6.5	4.1	2.8	2.9	4.6	6.9

HA22T/VGH, HA55T/VGH and SK-Hep-1 cells are poorly differentiated human hepatocellular carcinoma (HCC) cells. The well-differentiated category includes Hep G2 and Hep 3B cells.

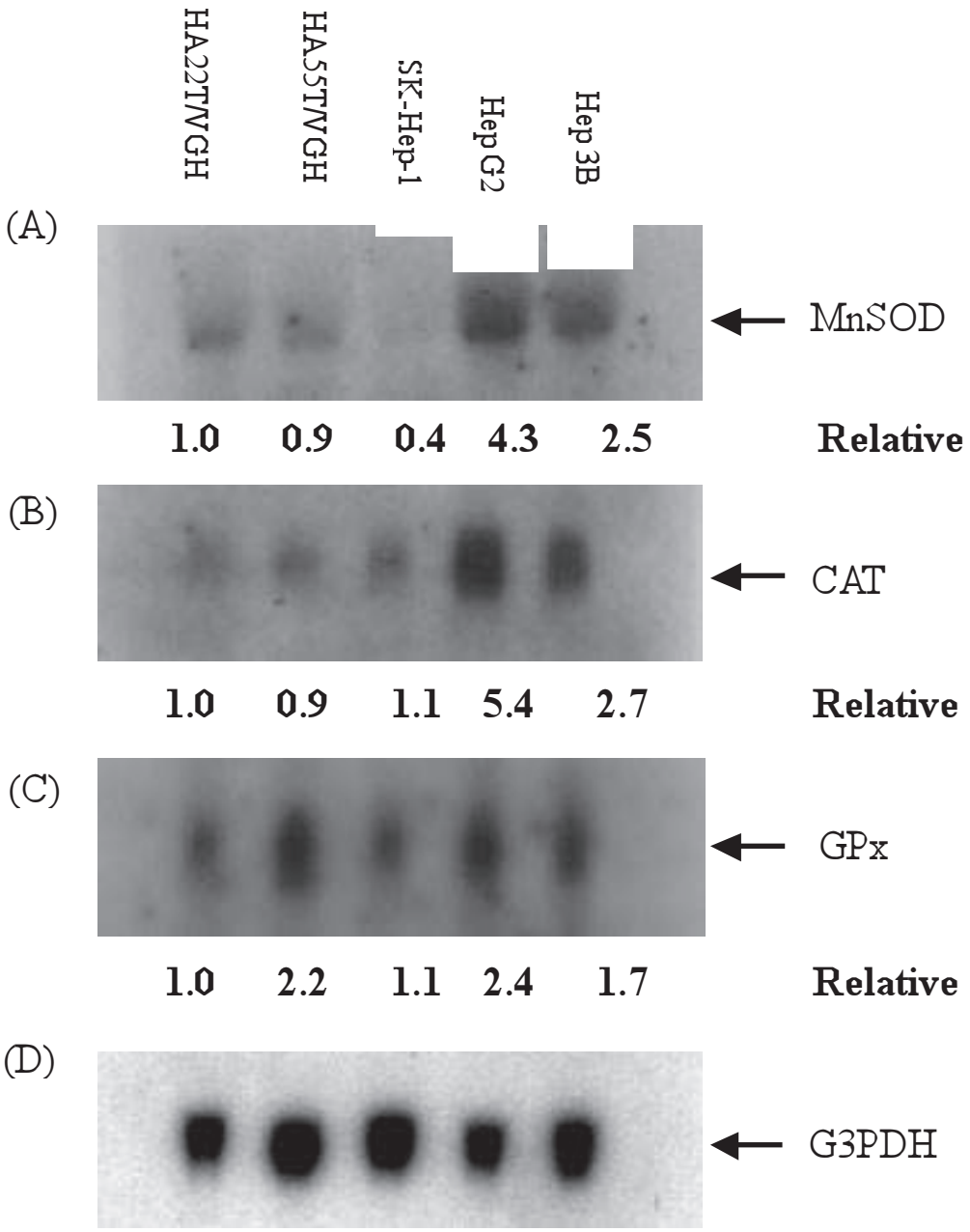

Fig. 1. Northern-blot analysis of manganese superoxide dismutase (MnSOD)、catalase (CAT)、glutathione peroxidase (GPx) transcripts synthesis in various human hepatocellular cell lines.

Total cellular RNA samples (20 μ g) were electrophoresed in a formaldehyde -agarose gel and then transferred to nylon paper. The same blot was hybridized with nick-translated MnSOD (A), CAT (B), GPx (C) or G3PDH (D). The relative fold was indicated at the bottom of each panel and was normalized with the of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) transcript.

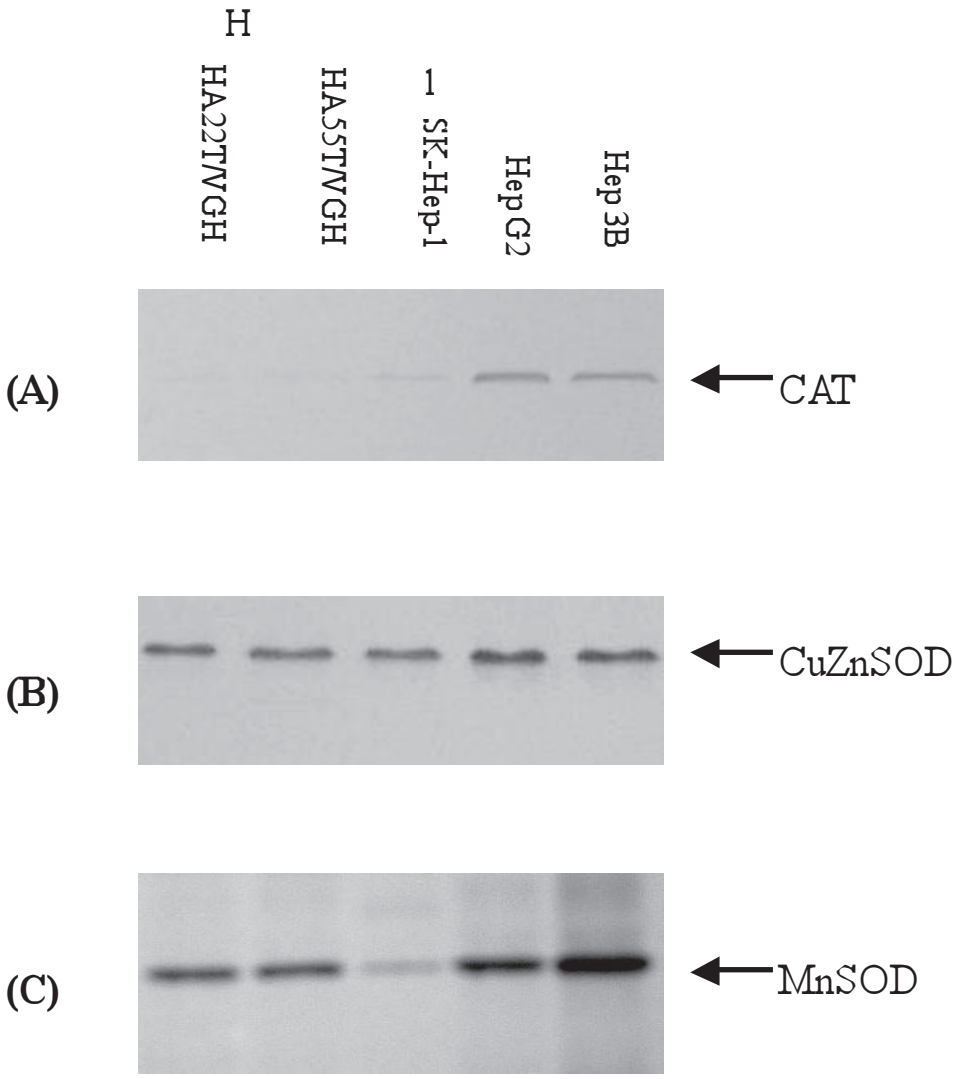

Fig. 2. Protein expression of anoxidant enzyme in human HCC cell lines
(A) and (B), Western-blot analysis with anti-catalase antibody and anti-CuZnSOD antibody, respectivly; (C), immunoprecipitated ^{35}S-labeled MnSOD protein

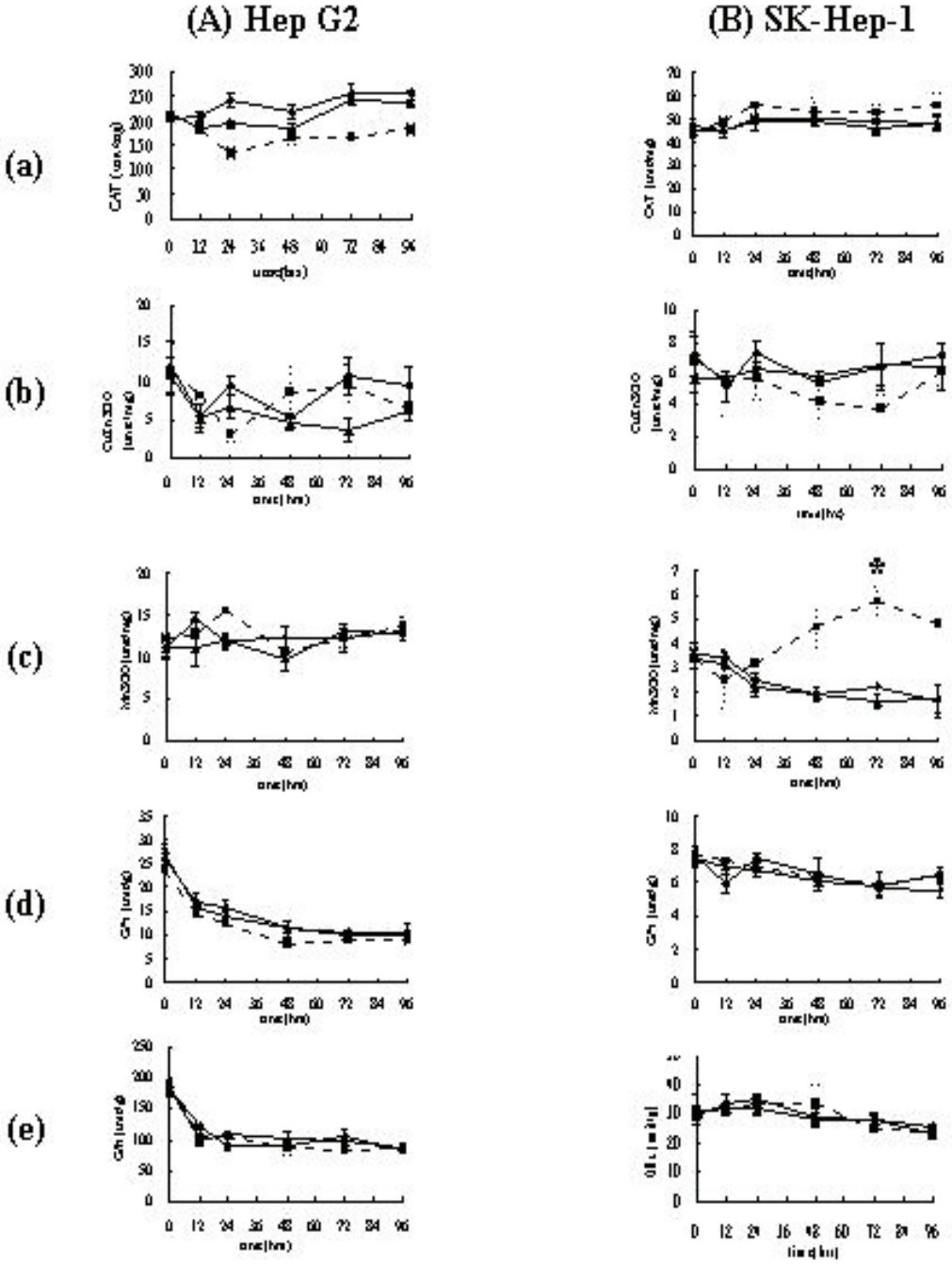

Fig. 3. Time course analysis of antioxidant enzymes expression under oxidative stress in various human HCC cell lines

Cells were serum starved for 24 hr before treatment of H_2O_2 and were lysed at indicated time point. Catalase (CAT), manganese superoxide dismutase(MnSOD), copper-zinc superoxide dismutase (CuZnSOD), glutathione peroxidase (GPx) and glutathione reductase (GRx) were determined as described in Materials and Methods. —◆— , control: without any treatment;—▲— , CAT + H_2O_2: addition of catalase before H_2O_2 treatment; - ■ - , treatment with 50 μM H_2O_2. Standard deviation bar was calculated from three independent experiments.