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Effects of Cadmium on Telomerase Activity, Expressions of TERT, C-myc and P53, and Apoptosis of Rat Hepatocytes*

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Summary: This study investigated the effect of cadmium on the telomerase activity, the expression of TERT, c-myc and p53 and the apoptosis of rat hepatocytes. The rats were administrated 5, 10 and 20 μmol/kg cadmium chloride intraperitoneally and sacrificed 48 h after the initial treatment. The telomerase activity of the rat hepatocytes was measured by the telomeric repeat amplification protocol (TRAP), and apoptosis was detected by flow cytometry. The mRNA expressions of TERT, c-myc and p53 were measured by reverse transcription-polymerase chain reaction (RT-PCR). C-myc and P53 proteins were determined by immunochemistry. The results showed that cadmium chloride increased the hepatocellular telomerase activity in a dose-dependant manner and induced the apoptosis of hepatocytes significantly. The value of relative coefficient between the telomerase activity and the apoptosis rate was 0.9398. RT-PCR revealed that specific bands corresponding to the TERT mRNA, c-myc mRNA, and p53 mRNA were displayed at 185, 342 and 538 bp respectively. Cadmium chloride could substantially increase the mRNA expressions of TERT, c-myc and p53 in rat hepatocytes, as compared with control. Moreover, cadmium chloride at the doses of 5, 10 and 20 μmol/kg could increase the content of P53 protein in rat hepatocytes obviously, but only that at the doses of 10 and 20 μmol/kg substantially promoted the c-myc protein level in rat hepatocytes. Our study herein suggested that cadmium may contribute to the carcinogenesis by activating telomerase, and overexpressing the mRNAs of TERT, c-myc and p53, and causing apoptosis of normal cells.

Key words: cadmium; telomerase; apoptosis; telomerase reverse transcriptase; c-myc; p53

Telomerase is a ribonucleoprotein enzyme that maintains the protective structures at the ends of eukaryotic chromosomes, called telomeres. In most human somatic cells, telomerase expression is repressed, and telomeres shorten progressively with each cell division. In contrast, most human tumors express telomerase, resulting in stabilized telomere length. Over 90% of tumors possess telomerase activity. A direct correlation has been found between the level of telomerase activity and tumor stage and aggressiveness. It is suggested that telomerase can be used as a cancer marker, a diagnostic/prognostic tool and a potential therapeutic target[1–3].

Cadmium is a heavy metal, which is widely used in industry, affecting human health through occupational and environmental exposure. In mammals, it exerts multiple toxic effects and has been classified as a human carcinogen by the International Agency for Research on Cancer. Cadmium exposure has been linked to human lung, prostate and renal cancers. In animals, cadmium has been proved to effectively induce cancers at multiple sites and by various routes[4]. Although most studies indicate cadmium is poorly mutagenic and probably acts through indirect or epigenetic mechanisms, the mechanisms of cadmium on carcinogenesis are poorly defined[4, 5].

Cadmium had been reported to cause a moderate induction of telomerase activity in ovarian epithelial cells, and increase hTERT mRNA levels in human breast cancer cells[6, 7]. In this study, the effects of cadmium on telomerase activity, expressions of TERT, c-myc and p53, and apoptosis of rat hepatocytes were examined.

1 MATERIALS AND METHODS

1.1 Animal Handling

Male Sprague-Dawley rats weighing approximately 200±30 g were used in this study. Rats were housed in polycarbonated cages with compressed fiber bedding. Commercial pelleted diet and water were provided *ad*

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libitum. The rats were divided into 4 groups with 5 in each group: Control group, in which the rats received no cadmium treatment; 3 cadmium chloride ($CdCl_2$) groups in which the rats were intraperitoneally administered $CdCl_2$ at the doses of 5, 10 and 20 μmol/kg respectively. $CdCl_2$ solution was prepared in 0.9% NaCl and animals in the control group received equal volume of 0.9% NaCl. All the rats were sacrificed 48 h after the initial treatment and the livers were removed immediately for later use.

1.2 Telomerase Activity Assay

The telomerase activity was examined by the telomeric repeat amplification protocol (TRAP) by using a TRAPeze® XL telomerase detection kit (Chemicon international). Briefly, hepatocytes were separated by trypsinization, re-suspended in CHAPS lysis buffer and incubated for 30 min on ice. Hepatocytes (1.0×10^5) were used for each telomerase assay. After centrifugation at 12000 g for 30 min at 4°C, the supernatants were used as the cell extracts. The TRAP assay was performed with 1.0 μg of cell extracts. The assay specificity for active telomerase was determined by inclusion of heat-inactivated samples, in which the extracts were heat-inactivated at 85°C for 10 min before the TRAP assay. The experiments were repeated two to four times, and the telomerase activity was semiquantified by fluorescence measurements, as recommended by the manufacturer. The telomerase activity of the samples was defined as mean fluorescent values.

1.3 Flow Cytometry

The hepatocytes were washed, fixed and permeated with 70% ice-cold ethanol at 4°C for 2 h. Cells were then incubated with freshly prepared propidium iodide (PI) staining buffer (0.1% Triton X-100,200-μg/mL RNase A, and 20-μg/mL PI in PBS) for 15 min at 37°C, followed by flow cytometry analysis of 20000 cells in each group. The histogram was abstracted and the percentage of cells in the sub-G_1 phase was then calculated to reflect the percentage of apoptotic cells.

1.4 Isolation of RNA and Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was isolated from cells by using Trizol (GIBCO, Scotland), according to the manufacturer's recommendations. First strand cDNA synthesis with oligo (dt) primers was performed by using M-MLV (a reverse transcriptase). The primer sequences and PCR product sizes are shown in table 1. The PCR products were subjected to agarose gel electrophoresis and visualized by ethidium bromide staining. The relative value of detected mRNA normalized to β-actin was obtained.

Table 1 RT-PCR Primer sequence

Target cDNA	Primer sequence	Product size (bp)
TERT	F 5'-GAC ATG GAG AAC AAG CTG TTT GC-3'	185
	R 5'-ACA GGG AAG TTC ACC ACT GTC-3'	
C-myc	F 5'-AAC TTA CAA TCT GCG AGC CA-3'	342
	R 5'- AGC AGC TCG AAT TTC TTC CAG ATA T-3'	
P53	F 5'-TTC CCT CAA TAA GCT GTT CTG CC-3'	538
	R 5'-TGC TCT CTT TGC ACT CCC TGG-3'	
β-actin	F 5'- GAGACCTTCAAGACCCCAGCC-3'	404
	R 5'- TCGGGGCATCGGAACCGCTCA-3"	

Note: F, forward; R, reverse

1.5 Immunochemistry

The sections were deparaffinized in xylene and hydrated in graded ethanol continuously. Then the sections were covered with 3% hydrogen peroxide in PBS to block the endogenous peroxidase activity for 10 min. The sections were pretreated in citrate buffer (0.01 mol/L, pH 6.0) and antigen retrieval was then performed by microwave heating for 20 min. Normal goat serum was added to the slides and co-cultured for 30 min at room temperature. Afterwards, the sections were incubated overnight at 4°C with monoclonal primary antibodies including anti-c-myc, anti-P53 antibodies (Santa Cruez, USA) respectively. PBS (0.01 mol/L, pH 7.4) was used as the substitute for anti-c-Myc and anti-P53 antibodies in the control sections (blank controls). SP kit was purchased from Beijing Zhongsan Biotechnology Ltd (China). After the slides were washed three times in PBS for 5 min each, the biotinylated goat secondary antibody was added and incubated for 30 min, followed by addition of avidin-biotinylated peroxidase complex for culture for another 30 min at room temperature. After being washed with PBS, the slides were stained with diaminobenzidine (DAB) and hydrogen peroxide for 3 min, and stain reaction was stopped by tap water.

1.6 Statistical Analysis

Data were expressed as $\bar{x}\pm s$. Statistical analysis was performed with analysis of variance (ANOVA) by using SPSS 12.0 software package. A $P<0.05$ was considered statistically significant.

2 RESULTS

2.1 Effects of Cadmium on Telomerase Activity and Apoptosis of Hepatocytes

$CdCl_2$ at the doses of 5, 10 and 20 μmol/kg increased hepatocellular telomerase activity in a dose-dependent manner (r=0.9723, $P<0.05$), and statistical analysis yielded significant difference in the telomerase activity between cadmium groups and control group ($P<0.05$). Table 2 showed the effects of $CdCl_2$ on hepatocellular apoptosis by using flow cytometry. There were statistically significant differences in the apoptotic rate between cadmium groups and control group ($P<0.05$). Additionally, statistical analysis yielded a relative coefficient value of 0.9447 ($P<0.01$). The value of relative coefficient between telomerase activity and apoptosis rate was 0.9398 ($P<0.01$).

Table 2 Effects of cadmium on telomerase activity and apoptosis in hepatocytes ($\bar{x}\pm s$, n=5)

Groups	Telomerase activity	Apoptosis rate (%)
Control	42.14±6.09	4.16±0.74
$CdCl_2$ (5 μmol/kg)	86.56±12.98*	11.73±0.95**
$CdCl_2$ (10 μmol/kg)	94.13±8.79*	20.10±2.28**
$CdCl_2$ (20 μmol/kg)	139.21±8.95*	23.85±1.65**

*P<0.05，**P<0.01 as compared with the control group

2.2 Effects of Cadmium on TERT, C-myc and P53 Expressions in Hepatocytes

RT-PCR revealed that the bands of TERT, c-myc and p53 mRNAs in rat hepatocytes were expected to display at 185, 342, and 538 bp. The relative value of mRNA was expressed as absorbance (A) by using densitometry.

$CdCl_2$ at the doses of 5, 10 and 20 μmol/kg increased the mRNA expressions of TERT, c-myc and p53 in rat hepatocytes significantly, as compared with control (table 3, figs. 1–3).

Table 3 Effects of cadmium on TERT, c-myc and p53 expressions in hepatocytes ($\bar{x}\pm s$, n=5)

Groups	TERT/β-actin	c-myc/β-actin	p53/β-actin
Control	0.332±0.026	0.325±0.016	0.296±0.031
$CdCl_2$ (5 μmol/kg)	0.424±0.045**	0.501±0.057**	0.382±0.018*
$CdCl_2$ (10 μmol/kg)	0.526±0.038**	0.597±0.077**	0.515±0.110**
$CdCl_2$ (20 μmol/kg)	0.557±0.073**	0.645±0.060**	0.565±0.089**

*P<0.05, **P<0.01 as compared with the control group

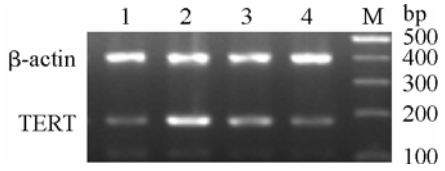

Fig. 1 Expression of TERT and β-actin mRNA in rat hepatocytes after exposure to $CdCl_2$
1: Control group, 2–4: Cadmium groups at the doses of 20, 10 and 5 μmol/kg respectively; M: DNA Marker

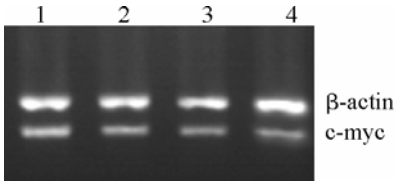

Fig. 2 Expression of c-myc and β-actin mRNA in rat hepatocytes after exposure to $CdCl_2$
1–3: Cadmium groups at the doses of 20, 10 and 5 μmol/kg respectively; 4: Control group

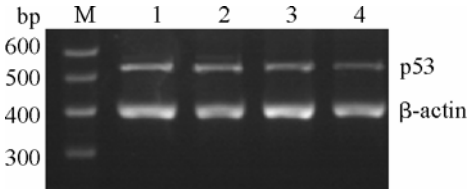

Fig. 3 Expression of p53 and β-actin mRNA in rat hepatocytes after exposure to $CdCl_2$
M: DNA Marker; 1–3: Cadmium groups at the doses of 20, 10 and 5 μmol/kg respectively; 4: Control group

2.3 Effects of Cadmium on C-myc and P53 Proteins in Rat Hepatocytes

Immunohistochemistry showed that the proteins of c-myc and P53 were mainly located in the cytoplasm of hepatocytes. The expression of these proteins was defined as absorbance (A) by densitometry. $CdCl_2$ at the doses of 5, 10 and 20 μmol/kg could increase the protein expression of P53 in rat hepatocytes obviously, but that only at the doses of 10 and 20 μmol/kg could promote the protein expression of c-myc in rat hepatocytes significantly (table 4).

Table 4 Effects of cadmium on protein expressions of c-myc and P53 in rat hepatocytes ($\bar{x}\pm s$, n=5)

Groups	c-myc	P53
Control	0.057±0.020	0.073±0.014
$CdCl_2$ (5 μmol/kg)	0.096±0.031	0.125±0.022*
$CdCl_2$ (10 μmol/kg)	0.297±0.109*	0.154±0.038*
$CdCl_2$ (20 μmol/kg)	0.376±0.089*	0.204±0.019*

*P<0.05 as compared with the control group

3 DISCUSSION

The role of telomerase, a ribonucleoprotein responsible for maintaining the ends of chromosomes, has been the subject of intensive investigation in recent years due to its potential role in carcinogenesis, cellular aging and immortalization[8]. Cancer development occurs during a critical transition from a telomerase-negative to a telomerase-positive state. During this transition, the telomere shortening that accompanies cell division may either prevent or facilitate tumorigenesis by activating checkpoints and impairing chromosomal stability. In mature cancers, telomerase probably serves a crucial role in tumor progression and maintenance by stabilizing telomeres and supporting the immortal growth of cancer cells[9]. Telomerase activation is an early step in the de-

velopment of many tumors[10]. Luzar *et al* reported that telomerase reactivation is an early event in laryngeal carcinogenesis[11], and Youssef *et al*'s study confirmed that telomerase is expressed in most hepatocellular cancers, and focal telomerase reactivation is an early event during human liver carcinogenesis[12]. Thus, the activation of telomerase may be a useful marker for the early detection of malignant progression in liver disease[13]. In the present study, we found that telomerase activity can be obviously induced by cadmium in rat hepatocytes in a dose-dependent manner.

Telomerase enzyme complex has two major subunits contributing to the enzymatic activity: an RNA component (hTER) that serves as a template for the polymerase activity of this enzyme and a catalytic subunit with reverse transcriptase activity (hTERT). While hTER is widely expressed in embryonic and somatic cells, hTERT is tightly regulated and is not detectable in most somatic cells. hTERT mRNA expression temporally parallels changes in telomerase activity during cellular differentiation and neoplastic transformation[14, 15]. Thus TERT is the key component for the control of telomerase activity[16]. Some studies have shown that overexpression of human telomerase RNA is an early event in oesophageal and stomach cancers[17, 18]. In this study, TERT expression can be increased significantly by cadmium at the doses of 5, 10 and 20 μmol/kg in rat hepatocytes. Because in normal somatic cells, telomerase activity and TERT expression are repressed, our results suggested that telomerase activation and TERT overexpression may be a useful biomarker for cadmium toxicity.

One oncogene that might activate TERT in the natural setting is c-myc. Myc genes are frequently deregulated in human tumors and myc overexpression may cause telomerase reactivation[19]. The myc gene family encodes transcription factors which upon dimerization with Max protein, bind to the DNA sequence 5'-CACGTG-3', termed E-box. Genetic analysis of the hTERT locus revealed 29 canonical and non-canonical E-box sequences in the hTERT promoter region and nine in the second intron. This number by far exceeds the frequency of E-box sequences found in other myc-regulated genes. The E-boxes in the TERT locus are preferred target sites for Myc/Max heterodimers and TERT becomes upregulated by overexpressed c-myc. The activation of TERT by c-myc has been shown to be independent of protein synthesis, and c-myc can induce telomerase activation through TERT expression. It can be concluded that TERT is a direct target of c-myc[20, 21]. But different results about telomerase and c-myc are also reported. hTERT mRNA regulation was not significantly associated with c-myc level[22]. The correlation between hTERT and c-myc in endometrial hyperplasia and carcinoma were not found[23]. We found previously that c-myc overexpression can be induced by cadmium in rat hepatocytes and in cadmium-transformed human bronchial cells[16, 24], and in this study, our results indicated that c-myc overexpression may be related with telomerase activation and TERT expression.

The p53 tumor suppressor controls cell growth and survival through transcriptional regulation of gene expression. Previous research found that the human telomerase reverse transcriptase (hTERT) gene is downregulated by p53[25]. Beliveau *et al* concluded that telomerase reduced the basal level of activated p53 and raised cellular tolerance for other p53-dependent signals[26]. Wisman *et al* found that telomerase activity was positively related to hTERT mRNA, p53 and c-myc expression, and p53 expression (e.g. p53 mutation) as well as c-myc expression may have a role in the regulation of telomerase activity in ovarian tumours[27]. Another study also showed that p53 gene mutation and high telomerase activity cooperate to induce tumorigenesis[28]. Treatment with 10 μmol/L cadmium resulted in a transient increase in c-myc and p53 mRNA levels that peaked by 2-fold and 1.4-fold, respectively, compared to control 2 h after the treatment[29]. In the present study, cadmium was found to not only induce the overexpression of c-myc and p53, but also increase the TERT expression and the telomerase activity. As some study reported that telomerase expression is not correlated with p53 overexpression[30], the relationship between telomerase and p53 will be further clarified.

Apoptosis induced by cadmium has been exclusively demonstrated in animal and in human hepatocytes[24, 31]. In this study, we further found that there was statistically significant difference between cadmium groups and control groups in apoptotic rates. We also noted that the relative coefficient between the telomerase activity and the apoptosis rate was significant ($P<0.01$). Caspases are crucial mediators of apoptosis, but Bermudez's study indicated that telomerase may promote cellular survival in epithelial cells by suppressing JNK-dependent caspase-mediated apoptosis[32]. The inhibition of telomerase activity not only correlated with several parameters of apoptosis, but also enhanced the apoptosis via mitochondrial pathway[33, 34]. These results suggest that telomerase plays an important role in the induction of apoptosis. Mondello *et al* summarized that the maintenance of telomere integrity and telomerase protects cells from apoptosis. Disruption of the telomere capping function and (or) telomerase inhibition elicit an apoptotic response in cancer cells, while restoration of telomerase activity in somatic cells confers resistance to apoptosis[35]. However, in our study, positive relationship was observed between apoptosis and telomerase activity. The p53 pathway has been shown to mediate cellular stress responses; p53 can initiate DNA repair, cell-cycle arrest, senescence and, importantly, apoptosis[36]. hTERT has antiapoptotic activity and can antagonize p53-induced apoptosis independent of telomerase activity[25]. Cadmium induced the overexpression of p53 and also increased the expression of TERT in rat hepatocytes in this study.

In conclusion, the present study indicated that cadmium can increase the telomerase activity and the TERT expression obviously, and the expressions of c-myc and p53 and induce apoptosis of rat hepatocytes. Further study is needed to examine the exact mechanisms on relationship among telomerase, apoptosis and expression of TERT, c-myc and p53 in cadmium-induced toxicological effects.

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