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# Induction of glutathione synthetase by 1,10-phenanthroline

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**Abstract The differential display (DD) was employed to identify the gene(s) responsible for 1,10-phenanthroline (OP)-induced apoptosis in murine tumor cells (Sun, Y., Bian, J., Wang, Y. and Jacobs, C. (1997) Oncogene 14, 385–393 [1]). An OP-inducible gene was isolated which encodes mouse glutathione synthetase (GSS). The GSS mRNA level began to increase 6 h post OP treatment and remained at a high level thereafter up to 24 h tested. Induction of GSS was found not to be associated with p53 activation. No significant induction of DNA fragmentation was detected in two murine tumor lines upon GSS transfection. This is the first observation indicating that GSS is inducible rather specifically by a metal chelator and that induction of GSS, however, is not sufficient to induce apoptosis. It may merely reflect a cellular response to OP-induced redox disturbance.**



*Key words:* Glutathione synthetase; Phenanthroline; Apoptosis; Differential display; p53

## 1. Introduction

1,10-Phenanthroline (OP) is a typical metal chelating reagent. It chelates Fe(II) and prevents Fe(II)-mediated hydroxyl radical formation through the Fenton reaction [2,3]. By doing so, OP has been shown to prevent hydroxyl radical-induced DNA damage in a number of cellular systems [4–6]. It has recently been shown in a cell-free system that OP can inactivate DNA binding activity of p53, a zinc containing transcription factor and tumor suppressor [7]. We have extended this in vitro observation to intact cells and found that in contrast to in vitro results, OP actually activated p53 DNA binding and transactivation activity in two murine tumor lines, harboring endogenous wild-type p53 [1]. Activation of p53 was found to contribute to, but not to be required for subsequent apoptotic cell death. In an attempt to identify and characterize the critical genes responsible for OP-induced apoptosis, we employed the differential display technique and report here the cloning of an OP-inducible gene, which encodes glutathione synthetase (GSS), the enzyme involved in the last step of glutathione (GSH) synthesis [8].

## 2. Materials and methods

### 2.1. Cell maintenance and drug treatment

Mouse JB6 tumor line (L-RT101), an epidermal originated tumor line, was cultured in minimal essential medium with Earle's salts (BRL) containing 5% fetal calf serum (Sigma). The cell doubling time is about 16 h. H-Tx cells, a spontaneously transformed mouse liver line, were cultured in Dulbecco's modified Eagle medium containing 10% fetal calf serum and 1 mM sodium pyruvate. The doubling time is about 10 h for this line. Both cell lines harbor the wild-type p53 as assayed by DNA sequencing [9,10]. Subconfluent cells were exposed to DMSO vehicle control, or OP (150 μM, Sigma) or other DNA damaging reagents, camptothecin (10 μM, Sigma), etoposide (10 μM, Sigma), or adriamycin (10 μM, Sigma) for various periods of time up to 24 h and subjected to the differential display and/or Northern analysis. All compounds were dissolved in DMSO.

### 2.2. The differential display

L-RT101 cells were treated with 150 μM OP (Sigma) for 6 h and subjected to the differential display analysis, using DMSO-treated cells as controls. The analysis was performed using an RNAimage kit B (GeneHunter), according to the manufacturer's instruction with a slight modification [11]. Briefly, total RNA was isolated using RNAzol solution (Tel-Test) and subjected to reverse transcription (RT), followed by polymerase chain reaction (PCR). The downstream primers are poly dT (T11) with one nucleotide (G, A, or C) anchored at its 3′ end and a *Hin*dIII site at its 5′ end (used for both RT and PCR) and the upstream primers are a 13 mer (H-AP 9 to H-AP16 for PCR only), consisting of a *Hin*dIII site at its 5′ end and a random-made 7 nucleotides. The sets of primers which detected differential expression between the control and OP-treated cells were used again in another freshly prepared RNA to confirm the reproducibility. The fragments reproducibly showing differential expression were PCR amplified and used as probes for Northern analysis [12] of both L-RT101 and H-Tx cells treated with OP. The clones detecting differential expression by Northern analysis were then subcloned into TA cloning vector (Invitrogen) and sequenced by DNA sequenase version 2.0 (Amersham). The computer analysis was performed using the GCG program.

### 2.3. GSS cloning and expression vector construction

RT-PCR [9] was performed to clone the mouse GSS cDNA flanking the entire coding region [13]. The primers used are GSHS.01 5′ GGAATTCATGGCTACCAGCTGGGG and GSHS.02 5′ GGAATTCAC ACAGGGTAGGGGTT. The resulting 1.4 kb fragment was *Eco*RI digested and subcloned into a pre-digested eukaryotic expression vector, pcDNA3 (Invitrogen). Both sense and antisense constructs were obtained and verified by DNA sequencing.

### 2.4. Transient transfection and DNA fragmentation assay

Five independent GSS expressing clones, along with the antisense construct and vector control, were transiently transfected into both L-RT101 and H-Tx cells by calcium phosphate method in a 10 cm dish. The DNA fragmentation assay was performed 40 h post transfection as detailed previously [1].

## 3. Results and discussion

### 3.1. Cloning of GSS as an OP-inducible gene by the differential display

The differential display (DD) technique was employed in an attempt to isolate genes responsible for or associated with OP-induced apoptosis in two murine tumor lines. Since OP-induced apoptosis occurs obviously at 12 h post exposure [1], we reasoned that gene(s) responsible for apoptosis induction should be up- or down-regulated prior to the appearance of

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*Abbreviations:* OP, 1,10-phenanthroline; DD, differential display; GSH, glutathione; GSS, glutathione synthetase; RT, reverse-transcriptase; PCR, polymerase chain reaction; MnSOD, manganese-containing superoxide dismutase; DMSO, dimethyl sulfoxide


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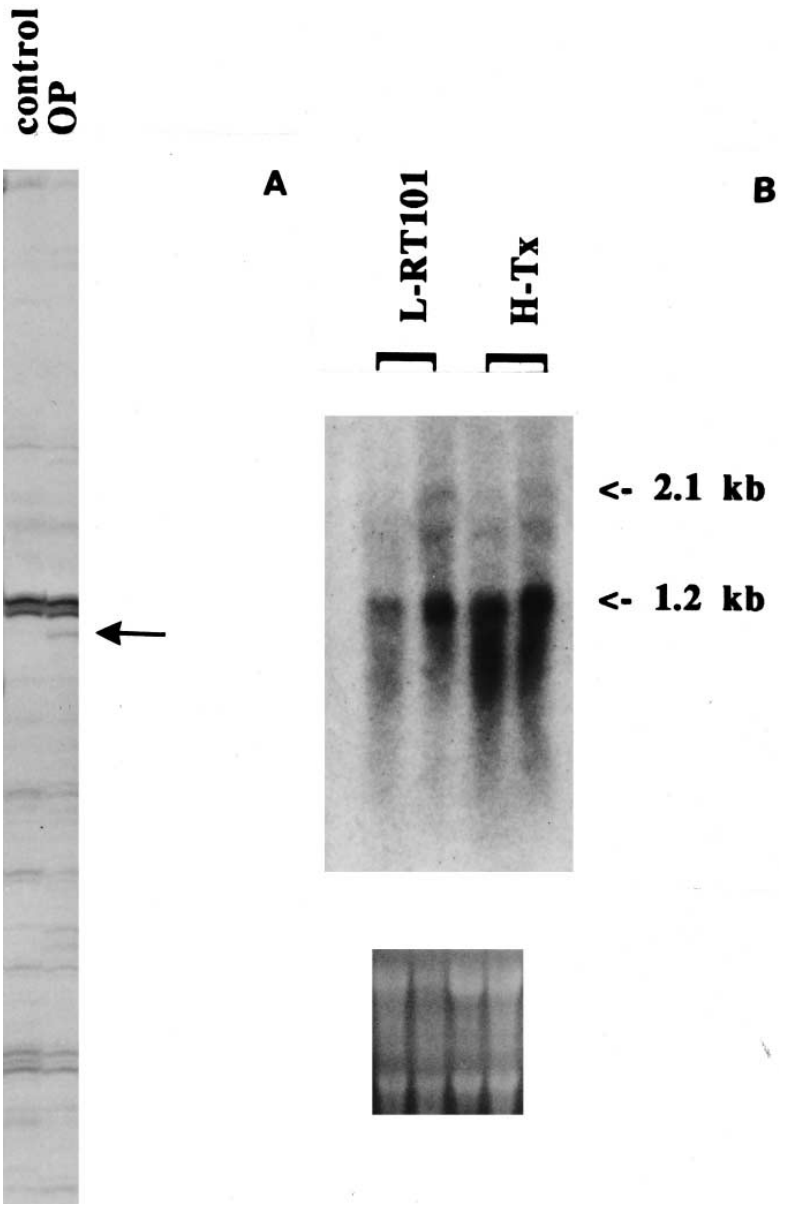


Fig. 1. Identification of an OP-inducible fragment in L-RT101 tumor cells by the differential display: total RNA was isolated from L-RT101 murine epidermal tumor cells either treated for 6 h with DMSO or 1,10-phananthroline (OP, 150 μM, dissolved in DMSO), and subjected to the DD analysis as described in Section 2. The arrow points to a fragment which was reproducibly seen only in OP-treated cells (A). The fragment was cut out, PCR-amplified with primers H-AP11 and T11C (see Fig. 2), and used as a probe for Northern analysis of both L-RT101 and H-Tx cells (B). The fragment detected two transcripts with a size of 2.1 kb and 1.2 kb, respectively. The ribosomal 28S and 18S were shown on the bottom as loading controls.

apoptosis. Six hours of OP treatment was, therefore, conducted in one of these tumor lines, L-RT101, followed by the DD analysis. A total of 8 differential expressed fragments (4 for up-regulation and 4 for down-regulation) were identified between DMSO control and OP-treated cells after two independent DD analyses, using 8 upstream primers and 3 downstream primers (Fig. 1A and data not shown). All 8 fragments were PCR amplified and used as the probes for Northern analysis. Both OP-treated L-RT101 and H-Tx cells were examined. The gene(s) of interest will be those either being induced or repressed by OP in both cell lines since they both undergo apoptosis after exposure to OP. Only 1 of the 8 fragments (the one shown in Fig. 1A) detected differential expression by Northern analysis. It detected two transcripts with the size of 1.2 kb and 2.1 kb, respectively. Both transcripts express at a very low basal level and are induced dramatically by the OP treatment (Fig. 1B). As previously described [11], the DD was able to identify differentially expressed genes, but with a high false positive rate.

This fragment was then subcloned into a TA cloning vector and 13 resulting clones were sequenced. They fell into two different DNA sequences. Two clones (Cl.7 and Cl.9), representing these two different individual clones, were used individually as probes for Northern analysis and each of them detected only one transcript with a size of 1.2 kb (Cl.9) and 2.1 kb (Cl.7), respectively (data not shown). The computer analysis using the GCG program revealed that Cl.9 was a novel gene, while Cl.7, with a size of 312 bp (Fig. 2, top

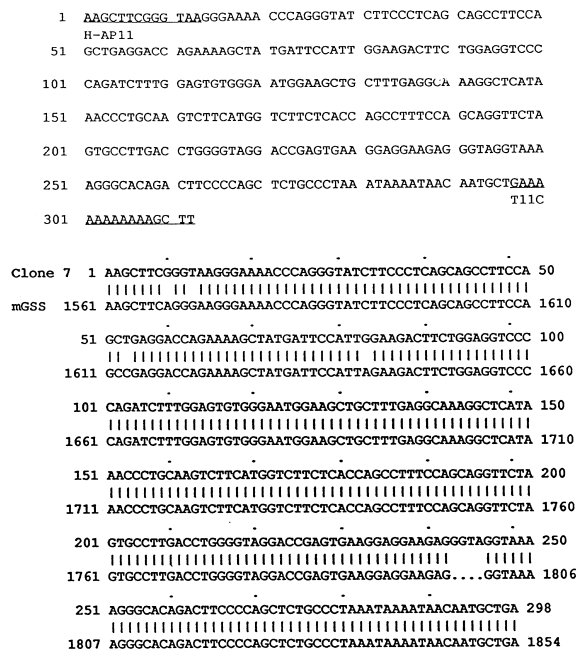


Fig. 2. Nucleotide sequence of clone 7 (A) and 98% DNA sequence identity of clone 7 with mouse glutathione synthetase (B): the fragment detected by the DD as shown in Fig. 1 was subcloned and 13 independent clones were sequenced. They fell into two independent DNA sequences. Shown is the DNA sequence of a representive clone 7 (A). The primers used for the DD-PCR cloning were underlined. The computer database analysis revealed that this fragment is part of the 3′ end untranslated sequence of mouse GSS (B).

panel), had 98% identity to the 3′ untranslated region of a newly isolated cDNA encoding mouse glutathione synthetase [13] (Fig. 2, bottom panel). The result indicated that one of OP-inducible genes is the mouse GSS.

### 3.2. OP, but not other DNA damage/apoptosis-inducing reagents induces mGSS

We have shown that GSS is subjected to OP induction. We next conducted a time course study of the GSS induction. Both L-RT101 and H-Tx cells were treated with 150 μM OP for 1, 2, 6, 12, and 24 h, along with the DMSO control, and subjected to Northern analysis using Cl.7 as the probe. As shown in Fig. 3 (top panel), induction of GSS occurs 6 h post treatment and induced level remains up to 24 h. The same result was seen when a cDNA encoding the entire coding region of mouse GSS (generated by RT-PCR, see below) was used as the probe (data not shown). The result indicates that OP induces expression of the mouse GSS gene prior to the appearance of obvious apoptosis (which requires 12 h treatment [1]) and maintains a high level during apoptotic cell death.

OP is a redox sensitive reagent, it may disturb intracellular redox balance and trigger the cellular response, such as induction of GSS, the enzyme catalyzing the synthesis of an important intracellular thiol, GSH. To test whether other redox sensitive enzymes were also subjected to OP induction, we chose to examine possible induction by OP of manganese superoxide dismutase (MnSOD). The MnSOD, a mitochondrial enzyme catalyzing the dismutation of superoxide anion to form hydrogen peroxide and water, was previously found to be inducible by OP in anaerobic *Escherichia coli* [14]. As shown in Fig. 3 (bottom panel), OP treatment did not, however, induce MnSOD expression up to 24 h, suggesting that induction of GSS expression is rather specific by OP.

We have previously shown that OP activates p53 and this activation contributes to apoptosis [1]. To examine whether GSS induction by OP falls into the p53 signal pathway, we treated cells with adriamycin, a DNA damage reagent and free radical producer, camptothecin, a topoisomerase I inhibitor and etoposide, a topoisomerase II inhibitor. All of these drugs were p53 activators and apoptosis inducers in a number of cell models, including L-RT101 [1,15–17]. As shown in Fig. 4, unlike OP, these drugs did not cause the induction of GSS. Instead, camptothecin or adriamycin treatment caused inhibition of GSS expression in L-RT101, but not in H-Tx cells. The result suggests that GSS induction might not be associated with p53 activation and subsequent apoptosis. It further suggests that the induction of GSS by OP is rather specific.

### 3.3. No induction of apoptosis/DNA fragmentation by GSS transfection

To examine the association of GSS induction and OP-induced apoptosis, a cDNA fragment flanking the entire open reading frame of mouse GSS was generated by RT-PCR [9] and was constructed into a CMV driven eukaryotic vector, pcDNA3. Five individual GSS expressing clones, along with an antisense clone and vector control, were transiently tranfected into both L-RT101 and H-Tx cells. After 40 h of transfection, morphological appearance of cell detachment and shrinkage, characteristic of apoptosis, was examined under a microscope and was not observed in L-RT101 cells, but was seen in all transfected H-Tx cells (data not shown). Both detached and attached cells were harvested and subjected to DNA fragmentation assay. As shown in Fig. 5, transient transfection of mouse GSS did not induce apoptotic DNA fragmentation in L-RT101 cells. Calcium transfection, however, induces apoptosis in H-Tx cells, since all transfectants, including the vector control, undergo apoptotic death as shown both in morphology and DNA fragmentation. These results suggested that OP-induced GSS expression is not casually related to apoptosis induction. It is worth noting, however, that unlike OP treatment by which all cells were virtually exposed to the drug due to its cellular permeability, transient transfection only caused expression of the introduced gene in 10% of the L-RT101 cells, as determined by β-galactosidase staining [1]. The lack of apoptosis might be due to the poor transfection efficiency. Nevertheless, the fact that (a) DNA damaging/apoptosis-inducing reagents fail to induce GSS and (b) transient transfection of GSS fails to induce apoptosis strongly suggests that GSS is not a p53 downstream target gene to mediate apoptosis and that GSS expression alone is not sufficient to induce apoptosis. We, however, cannot rule out the possibility that GSS expression may be necessary for apoptosis. OP has been shown to induce expression of a number of genes including *Waf*-1, *Mdm*2 in p53 pathway [1], GSS and a novel gene mentioned in this report. Expression of many other genes, yet to be identified, may also be induced or repressed in the process of OP-induced apoptosis. Biological consequence of apoptosis may result from interactions among many OP-inducible/repressible genes and the expression of any of the single genes may not be sufficient. In fact, transient transfection of *Waf*-1 (a p53 downstream target gene

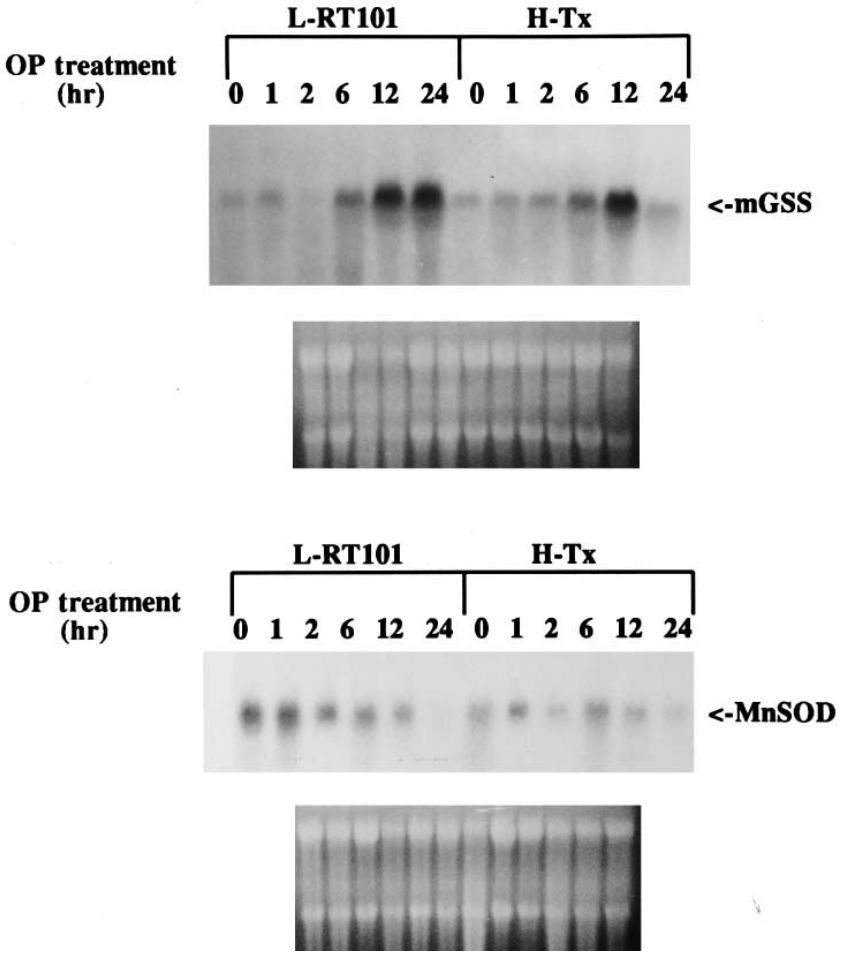


Fig. 3. Time course of GSS induction by OP: total RNA was isolated from L-RT101 and H-Tx cells after exposure to OP (150 μM) for a various time period up to 24 h, as indicated, and subjected (15 μg) to Northern analysis using clone 7 (A) or MnSOD cDNA (B, see [23]) as probes. The ribosomal 28S and 18S were used for loading controls.

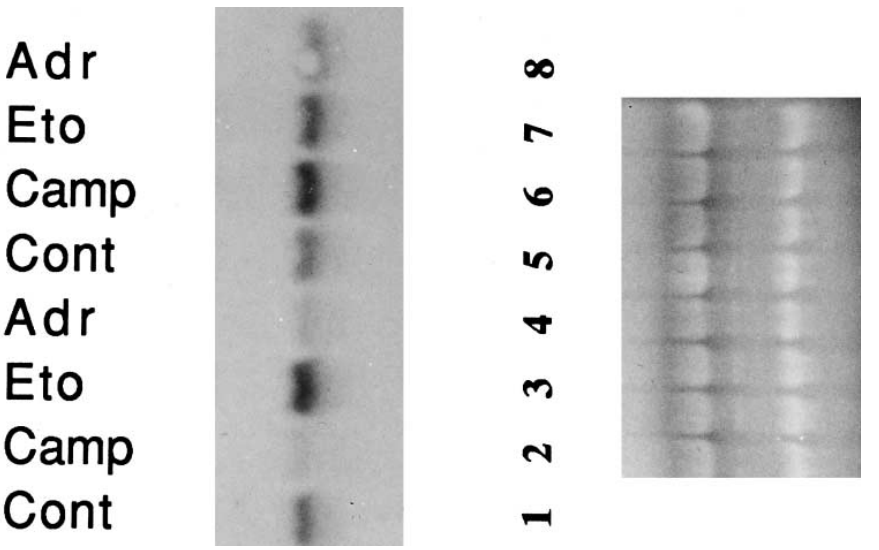


Fig. 4. Lack of induction of GSS by DNA damaging/apoptosis-inducing reagents: total RNA was isolated from L-RT101 and H-Tx cells after 6 h exposure to DMSO (Cont), camptothecin (Camp 10 μM), etoposide (Eto 10 μM), or adriamycin (Adr 10 μM), and subjected (15 μg) to Northern analysis using 1.4 kb GSS cDNA flanking the entire open reading frame as a probe. Again, the ribosomal 28S and 18S were used for loading controls. Lanes 1–4 were RT-101 cells, while lanes 5–8 were H-Tx cells.

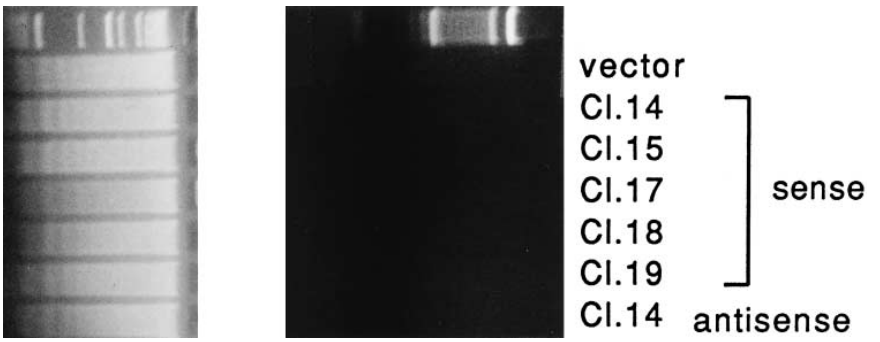


Fig. 5. GSS transfection is not sufficient to induce apoptosis: subconfluent L-RT101 and H-Tx cells in a 10 cm dish were transiently transfected with 5 independent GSS expressing cDNA clones, along with the vector and antisense controls. Both attached and detached cells were harvested 40 h post transfection and subjected to DNA fragmentation assay as detailed (Fig. 1). Top panel: L-RT101 cells. Bottom panel: H-Tx cells. The molecular weight markers were loaded in the first lane of each gel.

and inducible by OP, see [1]) did not induce apoptotic DNA fragmentation in L-RT101 cells (data not shown).

GSS catalyzes the ATP-dependent synthesis of GSH from γ-L-glutamyl-L-cysteine and glycine [8]. Human disease with GSS deficiency has been described. The patients with this disorder have the symptoms of massive 5-oxoprolinurine, metabolic acidosis, hemolytic anemia and central nervous system damage leading to mental retardation [8]. The gene encoding GSS has recently been cloned from humans, mice and rats [13,18,19]. Mutation inactivation of GSS has recently been documented in 5-oxoprolinuria patients [20]. We report here for the first time, to our knowledge, that GSS is an inducible gene. It is induced rather specifically by OP, a metal chelating reagent. The biological consequence of OP-induced GSS expression is not clear at the present time and is a subject for future investigation. It is likely that OP, as a redox sensitive compound, changes intracellular redox status by its metal chelating activity. As a result, the level of intracellular thiol, such as GSH may decrease which could trigger a series of cellular responses including the induction of GSS. It is therefore of interest to examine the expression of other genes involved in GSH metabolism after OP exposure. The examples include γ-glutamylcysteine synthetase and multidrug resistance-associated protein (MRP), both are inducible by heavy metals and cytokines in a number of cell models [21,22]. In summary, we have shown that GSS is inducible by OP and this induction is not sufficient to cause apoptosis. The finding reported here may, however, serve as the first step in the development of specific GSS-inducing drug(s) for the treatment of these human diseases caused by GSS deficiency.

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