# Endogenous production of tumour necrosis factor is required for manganese superoxide dismutase expression by irradiation in the human monocytic cell line THP-1

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Manganese superoxide dismutase (MnSOD) is a mitochondrial enzyme that scavenges superoxide ($O_2^-$) ions. We studied the regulation of MnSOD gene expression by irradiation and the mechanisms in human monocytic cell line THP-1. We found that irradiation induced expression of the MnSOD gene through the autocrine mechanism, involving the production of tumour necrosis factor (TNF). Irradiation increased TNF production in THP-1 cells, and TNF increased the levels of MnSOD transcripts. Supernatant from irradiated THP-1 cells induced the expression of MnSOD mRNA, and anti-TNF antibody blocked the induction of MnSOD mRNA. Irradiation also increased the levels of MnSOD mRNA in other myelocytic cell lines, HL60 and KG-1, and the ovarian cancer cell line SK-OV-3. Moreover, increased levels of MnSOD mRNA were observed in mature myeloid cells, including macrophages and granulocytes, as well as in immature cells. However, irradiation did not increase the level of MnSOD mRNA in THP-1 cells with prolonged exposure to PMA. We also found that irradiation increased the rate of MnSOD transcription, and irradiation stabilized MnSOD mRNA in THP-1 cells. Our results indicate that the endogenous production of TNF is required, at least in part, for the induction of MnSOD mRNA expression by irradiation in THP-1 cells, and the increased levels of MnSOD transcripts on irradiation occur through a pathway involving protein kinase C activation. Our results also indicate that the increase in MnSOD mRNA caused by irradiation is regulated by both transcriptional and post-transcriptional mechanisms.

## INTRODUCTION

Many of forms of inflammation are characterized by the accumulation of inflammatory cells, including monocytes/macrophages and granulocytes. Monocytes/macrophages increase their phagocytic activity and produce a variety of enzymes or factors in response to various stimuli and influence the physiology of many tissues [1]. These cells migrate out of the circulation and constitute a major element of the dynamic cellular system, with representation in all tissues and with the potential to exert a modulatory role in homoeostasis and immunological and inflammatory responses [1,2]. Activated macrophages are also highly microbicidal and tumoricidal [1]. Thus monocytes/macrophages have various functions against external insults.

Superoxide dismutases (SODs; EC 1.15.1.1) catalyse the dismutation of superoxide ions ($O_2^-$) to $H_2O_2$ and $O_2$ [3,4]; SODs are important as initial components in the cellular defence against $O_2^-$ [3]. Three forms of SOD with distinctive distributions characterized by their metal requirements are found: manganese SOD (MnSOD) is found in prokaryotes and in the mitochondria of eukaryotes, copper–zinc SOD is mainly in the cytosol of eukaryotes, and iron SOD is in the cytosol of prokaryotes [4]. Whereas copper–zinc SOD and iron SOD are constitutively expressed, production of MnSOD is inducible by various stimuli such as interleukin 1 (IL-1), tumour necrosis factor (TNF), lipopolysaccharide, interferon $\gamma$, lymphotoxin, hypoxia and irradiation [5–13].

Irradiation induces neoplastic transformation, and also causes bone marrow suppression that leads to either bleeding or infection. In the presence of $O_2$, irradiation increases the formation of $O_2^-$ [14,15]; the reaction of these radicals with DNA results in DNA strand breaks, which induces carcinogenesis [16]. These reactive oxygen species thus have an important role in the damage induced by irradiation [17,18]. Superoxide radicals ($O_2^{-\cdot}$) have been also implicated as important pathological mediators in various disorders, including cancer, inflammation or ischaemia [16,19]. In response to these stresses, cells induce or activate proteins that protect themselves from external insults. Previous studies have shown that cytokines such as granulocyte/macrophage colony-stimulating factor (GM-CSF), TNF and IL-1 were produced after irradiation in various cells [20–26]. Studies have also demonstrated that these cytokines have a radioprotective activity [27]. However, the roles of these cytokines in irradiation are not fully understood.

In the present study we examined the effects of irradiation on the expression of the MnSOD gene in the human monocytic cell line THP-1 and in other cells; we found that production of TNF is required for the accumulation of MnSOD transcripts by irradiation in THP-1 cells. We also explored the mechanisms for the regulation of MnSOD mRNA by irradiation.

## MATERIALS AND METHODS

### Cells and culture

Human monocytic leukaemia cells THP-1, promyelocytic leukaemia cells HL60, human myeloblastic cells KG-1 (Japanese Cancer Research Resources Bank, Tokyo, Japan) and ovarian cancer cells SK-OV-3 (American Type Tissue Culture Collection) were cultured in $\alpha$-medium (Cosmo Bio Co., Ltd., Tokyo, Japan) supplemented with 7% (v/v) fetal calf serum (Mitsubishi Kasei Co., Tokyo, Japan) in a humidified air/$CO_2$ (19:1) atmosphere.

Abbreviations used: GM-CSF, granulocyte/macrophage colony-stimulating factor; IL-1, interleukin 1; MnSOD, manganese superoxide dismutase; NBT, Nitro Blue Tetrazolium; RA, all-*trans* retinoic acid; SOD, superoxide dismutase; TNF, tumour necrosis factor; Vit $D_3$, 1$\alpha$,25-dihydroxyvitamin $D_3$.

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Human macrophages were derived from the differentiation of myeloid stem cells from bone marrow. Briefly, normal human bone marrow cells were obtained from informed normal volunteers by aspiration; the cells were centrifuged on Ficoll–Paque (Pharmacia, Piscataway, NJ, U.S.A.) to separate the mononuclear cells from granulocytes and red blood cells. The mononuclear cells were cultured with the culture supernatant of CHU-2 cells that produced GM-CSF [17]. After incubation for 2 weeks the non-adherent cells were removed; the remaining adherent monolayers were washed and cultured with fresh medium without the supernatant for 3 days before the experiments. Morphological and cytochemical studies with Wright Giemsa and $\alpha$-naphthyl esterase staining showed that more than 98% of the cells were macrophages.

Human granulocytes were obtained from the peripheral blood of consenting healthy donors. The blood was centrifuged on Ficoll-Paque and contaminating erythrocytes in the pelleted cells were eliminated by hypotonic lysis in 0.2% NaCl. Morphological examination indicated that the cell preparation consisted of over 95% granulocytes.

### Irradiation

Cells were irradiated with $\gamma$-rays by a $^{137}$Cs source emitting a fixed dose rate of 12 Gy/min as determined by dosimetry.

### Reagents

The polyclonal sheep anti-(human MnSOD) antibody was purchased from The Binding Site (Birmingham, U.K.). Alkaline phosphatase-conjugated rabbit antibody against sheep IgG was purchased from Cappel Organon Teknika (Durham, NC, U.S.A.). The neutralizing antibody against human TNF [polyclonal rabbit anti-(human TNF$\alpha$)] was purchased from Genzyme (Cambridge, MA, U.S.A.); 1 $\mu$l of this antibody neutralizes 1000 i.u. of TNF. The antibody against human IL-1$\beta$ was a polyclonal rabbit antibody and was kindly provided by Dr. Tsutomu Nishida (number 297; Otsuka Pharmaceutical Co., Tokushima, Japan). Human recombinant TNF and IL-1$\beta$ were also from Genzyme, and their specific activities were $1.08 \times 10^8$ i.u./mg of protein and $5 \times 10^8$ i.u./mg protein respectively. Actinomycin D, Nitro Blue Tetrazolium (NBT), PMA and all-*trans*-retinoic acid (RA) were purchased from Sigma Chemical Co. 1$\alpha$,25-dihydroxyvitamin $D_3$ (Vit $D_3$) was purchased from Duphar (Weesp, the Netherlands).

### Western blot analysis

Cells were lysed in buffer 50 mM Tris/HCl, pH 8.0, containing 150 mM NaCl, 0.02% $NaN_3$, 0.1% SDS, 100 mg/ml PMSF, 1 $\mu$g/ml aprotinin, 1% (w/v) Nonidet P40 and 0.5% sodium deoxycholate. After centrifugation, the protein concentration in each sample was determined by the method of Bradford [28]. Samples, each containing 20 $\mu$g of cell lysate in SDS/PAGE loading buffer, were electrophoresed in 12% (w/v) polyacrylamide gels and transferred to a PVDF membrane (Immobilon; Millipore, Bedford, MA, U.S.A.). Then immunoblotting was performed with anti-(human MnSOD) antibody (final concentration 80 $\mu$g/ml protein). After the blots had been washed, alkaline phosphatase-conjugated rabbit antibody against sheep IgG, diluted 1:2000, was added to the blots. Immunoreactivity on the blots was detected by NBT/5-bromo-4-chloroindol-3-yl phosphate (Life Technologies). For a quantitative analysis, the relative densities of the bands of hybridization of MnSOD in different lanes were scanned by an LKB UltroScan XL laser densitometer.

### Assay for MnSOD activity

MnSOD activity was assayed essentially as described previously [29]. Cells were sonicated in ice-cold 0.05 M potassium phosphate buffer, pH 7.8, containing diethylenetriamine penta-acetic acid after irradiation. After centrifugation the protein concentration in each sample was determined [28]. An equal amount of each sample, 50 $\mu$g of proteins, was electrophoresed in a 10% (w/v) non-denaturing polyacrylamide gel. The gel was stained with 2 mg/ml of NBT for 15 min in the dark, then photopolymerized with 10 $\mu$g/ml of riboflavin in 36 mM potassium phosphate buffer, pH 7.8, for 15 min. The gel was then illuminated until it became blue except for the SOD bands; the SOD activity was detected as an inhibition of NBT reduction.

The MnSOD activity was also determined by NBT methods with the xanthine/xanthine oxidase system as the source of $O_2^-$ as previously described [30]. Cells were collected, and ice-cold 0.05 M potassium phosphate buffer, pH 7.8, containing diethylenetriamine penta-acetic acid, was added to them. After sonication on ice, the homogenates were centrifuged to obtain the supernatants. The supernatants were analysed for MnSOD activity, and a competitive inhibition was performed that used xanthine/xanthine oxidase-generated $O_2^-$ to reduce NBT at a constant rate (0.015–0.025 absorbance units/min). The rate of NBT reduction was monitored spectrophotometrically at 560 nm. One unit of SOD was defined as the amount of enzyme activity that inhibited the NBT reduction rate by 50%. The activity of MnSOD was assayed in the presence of 5 mM NaCN to inhibit copper–zinc SOD. The endogenous activity of NBT reductase was subtracted from the total activity of NBT reduction [30].

### Assay for TNF

The concentration of TNF in the culture supernatants or cell lysates was measured by ELISA (Genzyme). Conditioned medium from cultures of either the control or irradiated THP-1 cells were prepared by centrifuging the supernatants at 1000 ***g*** for 10 min. Cell lysates were made by sonicating the cells in ice-cold PBS after irradiation, after which the debris was removed by centrifugation. The content of protein in the cell lysates was measured [28]. The standard curve of TNF protein was plotted by using purified recombinant human TNF as a standard. The detection limit of this assay is 10 pg/ml. This assay was specific and did not detect other cytokines, including GM-CSF, granulocyte colony-stimulating factor, macrophage colony-stimulating factor, IL-1, IL-3, transforming growth factor $\beta$, IL-6 and interferon $\gamma$.

### DNA probes

The human MnSOD full-length cDNA probe (1.0 kb) was a gift from Dr. Madsushi (National Cancer Institute, Bethesda, MD, U.S.A.). The human TNF cDNA fragment (0.8 kb, *Eco*RI) was from pSPl42-2 [31], and the IL-1$\beta$ cDNA was from pA-26 [32]. The $\beta$-actin DNA probe (0.7 kb, *Eco*R1–*Bam*H1) was from the pHFb A-3′ untranslated plasmid [33]. These probes were $^{32}$P-labelled by a random priming method [34]. The specific activity of each probe was $2 \times 10^8$ c.p.m./$\mu$g of DNA.

### Isolation and blotting of RNA

Total RNA from cells was obtained by the guanidinium/hot phenol method as described previously [35,36]. Cells were lysed in a guanidinium isothiocyanate mixture [4 M guanidinium isothiocyanate/50 mM Tris/HCl (pH 7.0)/20 mM EDTA/2%

(v/v) sodium lauryl sarcosinate/140 mM 2-mercaptoethanol]. The lysed cells were treated with proteinase K, after which their total RNA was extracted by the phenol/chloroform method. After denaturation at 65 °C, the RNA was electrophoresed in a 1% (w/v) agarose/formaldehyde gel and transferred to a nylon-membrane filter (Amersham) [37]. The filters were hybridized with $^{32}$P-labelled probe for 16–24 h at 42 °C in 50% (v/v) formamide/2 × SSC (where SSC is 150 mM NaCl/15 mM sodium citrate)/5 × Denhardt's solution/0.1% SDS/10% (w/v) dextran sulphate/100 $\mu$g/ml salmon sperm DNA. Blots were sequentially hybridized with $^{32}$P-labelled MnSOD cDNA, TNF cDNA and IL-1$\beta$ cDNA. Filters were washed to a stringency of 0.1 × SSC for 10 min at 65 °C and exposed to X-ray film (RX; Fuji Photo Film Co., Kanagawa, Japan). Autoradiograms were developed at different exposures. The relative densities of the bands of hybridization of MnSOD mRNA in different lanes were scanned by an LKB UltroScan XL laser densitometer with multiple exposures of the blot.

### Transcriptional run-on assay

THP-1 cells were irradiated; the nuclei were then isolated by suspending the cells in an ice-cold hypotonic buffer [10 mM Tris/HCl (pH 7.4)/10 mM KCl/3 mM $MgCl_2$] and then lysed in the same buffer containing 0.5% NP40. Nuclei were harvested by centrifugation (500 ***g***, 5 min), washed in a hypotonic buffer containing 0.5% NP40, and then resuspended in nuclear storage buffer [40% (v/v) glycerol/50 mM Tris/HCl (pH 8.3)/5 mM $MgCl_2$/0.1 mM EDTA]. Nuclei were incubated for 30 min at 30 °C in a reaction buffer containing 150 mM KCl, 3 mM $MgCl_2$, 5 mM Tris/HCl, pH 8.0, 0.25 mM ATP, 0.25 mM GTP, 0.25 mM CTP and 200 $\mu$Ci of [$\alpha$-$^{32}$P]UTP (3000 Ci/mM). The reaction was terminated by adding DNase I for 10 min at 30 °C. The reaction mixture was digested by 400 $\mu$g/ml of proteinase K in a solution containing 10 mM EDTA and 1% (w/v) SDS, followed by extraction with phenol/chloroform. The aqueous phase was precipitated at −70 °C with 50% (v/v) isopropyl alcohol in the presence of 0.3 M sodium acetate, and the precipitate was collected by centrifugation and then dissolved in TE buffer [10 mM Tris/HCl (pH 8.0)/1 mM EDTA). After denaturation in ice-cold 0.3 M NaOH and then neutralization in 0.25 M Hepes, nuclear RNA was run through a Sephadex G50 spun column to remove unincorporated [$^{32}$P]UTP. Plasmid DNA containing the cDNA coding inserts was denatured by heat and alkali treatment (0.3 M NaOH). Denatured plasmids (10 $\mu$g for MnSOD and 2 $\mu$g for $\beta$-actin) were bonded to nylon membranes (Hybond-N) with Bio-Dot SF (Bio-Rad) and immobilized by a UV cross-linker. Newly elongated nuclear RNA was hybridized to the filters containing plasmids. Hybridizations were performed with $10^7$ c.p.m./ml $^{32}$P-labelled RNA in a buffer containing 3 × SSC, 0.1% SDS, 10 × Denhardt's solution, 50% (v/v) formamide, 0.2 mg/ml yeast tRNA, 10 mM $NaH_2PO_4$, pH 7.0, and 100 $\mu$g/ml of salmon sperm DNA for 3 days at 42 °C. After hybridization, the filters were rinsed in 2 × SSC at room temperature and then in 2 × SSC and 0.1 × SSC at 42 °C.

## RESULTS

### Increased levels of MnSOD protein and its activity following irradiation in the human monocytic cell line THP-1

We determined whether irradiation affected the levels of MnSOD protein in THP-1 cells by Western blotting with an MnSOD antibody (Figure 1, upper panel). The THP-1 cells were cultured for 8 h after exposure to irradiation at different doses (0, 10, 20, 40 and 80 Gy). The cells were harvested and the levels of MnSOD in the total cellular protein were examined. Unirradiated THP-1 cells constitutively expressed a very low level of MnSOD protein. An increased level of MnSOD was observed at 10 Gy of irradiation; this reached a plateau at a dose of 20 or

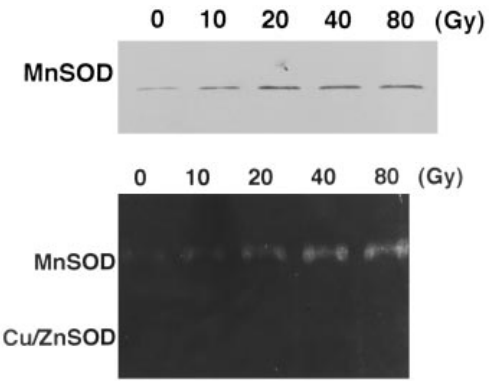


**Figure 1 Levels of MnSOD protein and its activity in THP-1 cells exposed to irradiation**

Upper panel: cells were cultured for 8 h after irradiation at various doses as indicated. The cells were harvested and 20 $\mu$g samples of whole cell protein were electrophoresed in an SDS/12% (w/v) polyacrylamide gel, transferred to a PVDF membrane and analysed for MnSOD protein as described in the Materials and methods section. Lower panel: to determine the activity of MnSOD, THP-1 cells were sonicated in ice-cold potassium phosphate buffer and 50 $\mu$g samples of protein were electrophoresed in non-denaturing 10% (w/v) polyacrylamide gel followed by staining with NBT.

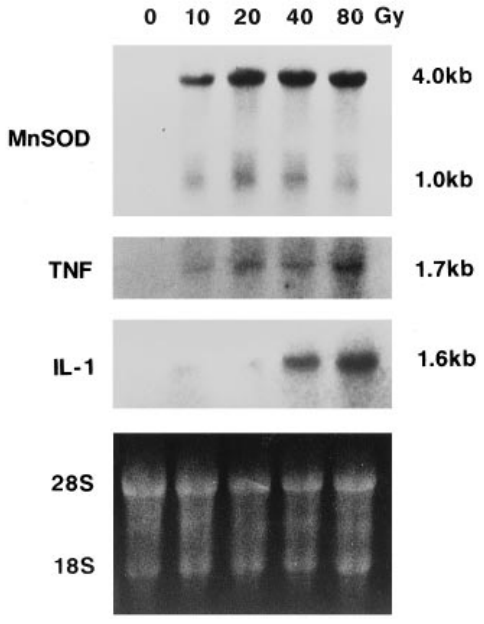


**Figure 2 Dose-dependent effect of irradiation on levels of MnSOD mRNA in THP-1 cells**

The cells were cultured for 4 h after irradiation. Total RNA (15 $\mu$g per lane) was prepared and analysed by formaldehyde/agarose gel electrophoresis and transferred to a nylon membrane as described in the Materials and methods section. Hybridization was performed with $^{32}$P-labelled MnSOD cDNA (1.0 and 4.0 kb bands of hybridization), TNF cDNA (1.7 kb band) and IL-1$\beta$ cDNA (1.6 kb band). The bottom panel shows the picture of the ethidium bromide-stained formaldehyde gel before Northern blotting; levels of 28 S and 18 S ribosomal RNA were comparable in each lane.

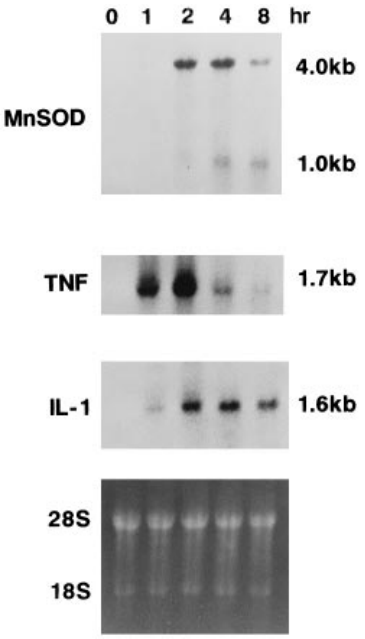


**Figure 3 Time-dependent effect of irradiation on levels of MnSOD mRNA in THP-1 cells**

The cells were cultured for various durations (0–8 h) after irradiation at a dose of 40 Gy. Northern blot analysis of mRNA was performed by blotting the total RNA (15 $\mu$g per lane).

40 Gy of irradiation. At 40 Gy, the level of MnSOD was approx. 3-fold that of the unirradiated cells.

We also studied the MnSOD activity in these cells by polyacrylamide gel fractionation of the lysate followed by NBT staining (Figure 1, lower panel). As reported previously, eukaryotes have two types of SOD: the bands at high molecular mass are MnSOD and the very faint bands at low molecular mass correspond to copper–zinc SOD. Irradiation increased MnSOD activity in an almost dose-dependent fashion until 40 Gy and then reached a plateau, whereas the levels of copper–zinc SOD were not changed. Irradiation at 10 Gy induced a significant increase in MnSOD activity and 40 Gy of irradiation increased the MnSOD activity by 4-fold compared with that of untreated cells. Studies of MnSOD activity with the method of NBT reduction and xanthine/xanthine oxidase were also performed. The results obtained were almost identical with those described above (results not shown).

### Dose-dependent effect of irradiation on levels of MnSOD, TNF and IL-1$\beta$ mRNA in monocytic cells

THP-1 cells were cultured for 4 h after exposure to various doses of irradiation as indicated. To determine the effect of irradiation on MnSOD gene expression we performed a Northern blot analysis of the total RNA with a $^{32}$P-labelled MnSOD cDNA probe (Figure 2). Northern blot analysis showed that RNA from these cells had two species of MnSOD transcripts at 1.0 and 4.0 kb. Irradiation with a dose of 10 Gy markedly induced MnSOD gene expression. At 20 Gy the level of MnSOD RNA reached a plateau. Irradiation also induced TNF and IL-1$\beta$ mRNA expression in these cells; a significant increase in the level of TNF mRNA was observed at a dose of 10 Gy, and the induction occurred in an almost dose-dependent fashion. In contrast, the induction of IL-1$\beta$ gene expression was observed at

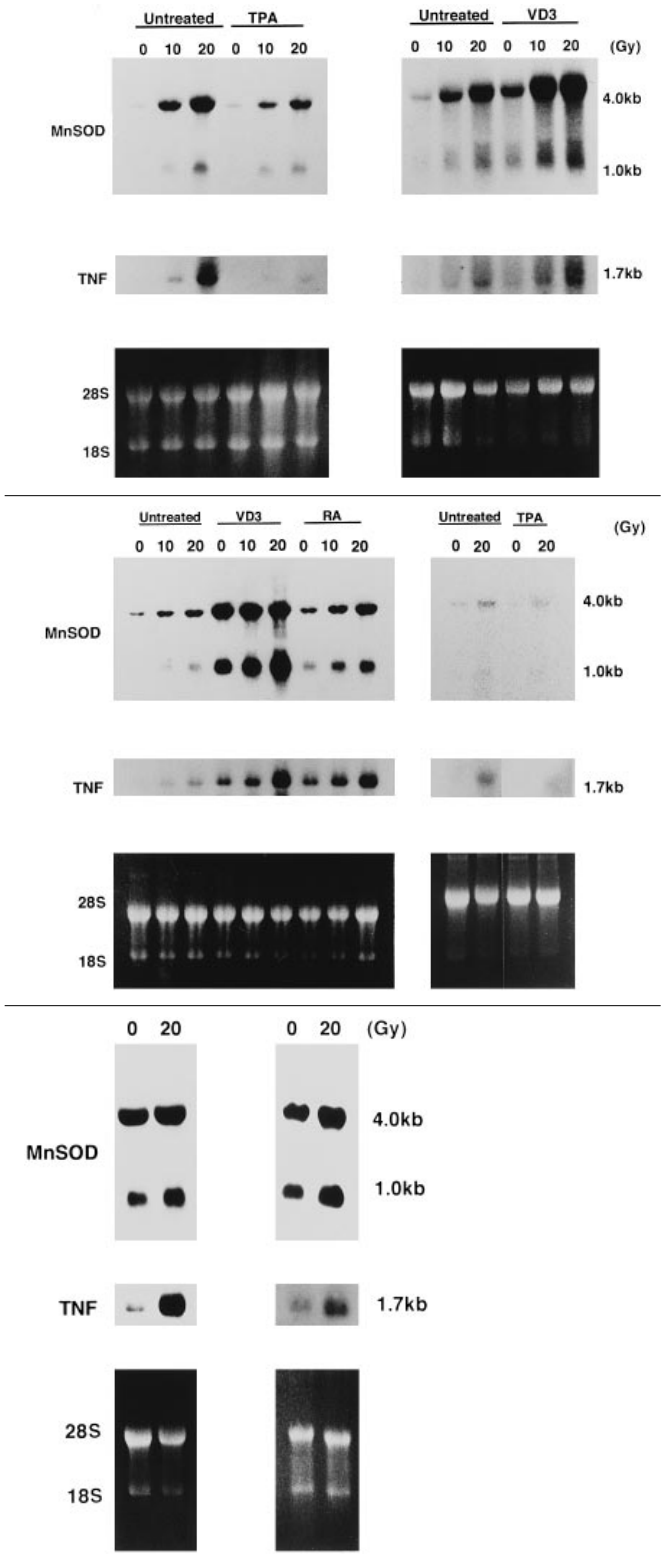


**Figure 4 Effect of irradiation on MnSOD mRNA expression of differentiated cells**

Top panel: THP-1 monocytic cells were cultured with either 20 nM PMA or 100 nM Vit $D_3$ for 3 days, washed with medium and then irradiated at 10 or 20 Gy. Middle panel: HL60 promyelocytic cells were treated with 20 nM PMA, 100 nM Vit $D_3$ or 1 $\mu$M RA for 3 days. Bottom panel: human macrophages from bone marrow and granulocytes from peripheral blood were also irradiated at 20 Gy. At 2 h after irradiation, these cells were harvested and analysed by Northern blotting.

**Table 1 Increased levels of TNF production in THP-1 cells exposed to irradiation**

Cells were cultured for 8 h after irradiation at various doses. Cells were harvested; the conditioned medium and cell lysate were assayed for TNF by ELISA. Results are means ± S.E.M. for triplicate assays. Results with the same footnote marker are significantly different at the $P < 0.001$ level.

| Dose (Gy) | Conditioned medium (pg per $10^6$ cells) | Cell lysates (pg/mg of protein) |
|---|---|---|
| 0 | < 2* | 50 ± 1.2‡ |
| 10 | < 2 | 71 ± 0.8‡ |
| 20 | 22 ± 0.3*† | 107 ± 0.1 |
| 40 | 102 ± 8.2 | 304 ± 0.3 |
| 80 | 206 ± 2.7† | 330 ± 0.4 |

40 Gy. To study whether irradiation was capable of inducing MnSOD mRNA expression in other cells, we tested two other cell lines, KG-1 and SK-OV-3. Irradiation also increased the level of MnSOD mRNA in a dose-dependent manner in these cell lines (results not shown).

### Time-dependent effect of irradiation on levels of MnSOD, TNF and IL-1$\beta$ mRNA

THP-1 cells were irradiated at 40 Gy, cultured and then harvested sequentially at different times. Northern blot analysis showed that an increase in the MnSOD mRNA level was observed 2 h after irradiation; the level was maximal at 4 h, and then it decreased to almost the baseline level at 8 h. Similar results were observed for IL-1$\beta$ gene induction. In contrast, irradiation induced the expression of the TNF gene at 1 h after irradiation; this reached a peak at 2 h. This increase in TNF mRNA level was transient, and the level of TNF transcripts decreased by 8 h (Figure 3).

### Effects of irradiation on MnSOD mRNA expression in differentiated myeloid cells

PMA and Vit $D_3$ are known to induce the differentiation of immature myeloid cells into mature monocytes/macrophages [38]. RA is also a potent inducer of differentiation of HL60 towards granulocytes [39]. To investigate the effects of irradiation on the expression of MnSOD mRNA in mature cells, myeloid leukaemia cell lines were treated with these factors and monitored for their expression of MnSOD mRNA. THP-1 monocytic cells and HL60 promyelocytic cells were cultured with either 20 nM PMA or 100 nM Vit $D_3$ for 3 days, washed with medium and then irradiated at a dose of 10 or 20 Gy. The cells were harvested after 2 h, and the levels of MnSOD mRNA were determined. The induction of differentiation with PMA did not affect the levels of MnSOD mRNA in THP-1 and HL60 cells (Figure 4, top and middle panels). Irradiation also increased the accumulation of MnSOD mRNA in these differentiation-induced cells and macrophages from bone marrow, but to a smaller extent than in native leukaemic cells (Figure 4, bottom panel). In contrast, treatment of both cell lines with Vit $D_3$ resulted in increased levels of MnSOD mRNA (Figure 4, top and middle panels); irradiation increased MnSOD mRNA levels in these cells.

Differentiation–induction of HL60 cells along a granulocytic pathway with 1 $\mu$M of RA resulted in almost the same level of

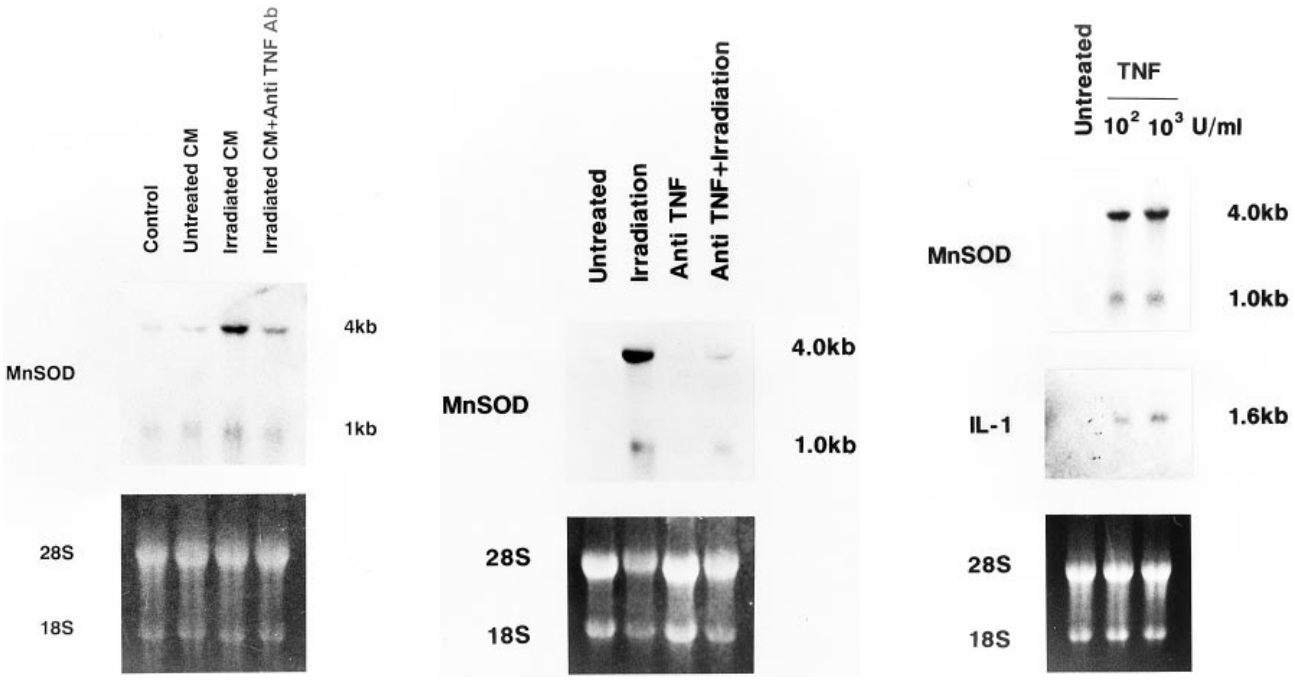


**Figure 5 Role of TNF production in irradiation-induced MnSOD expression**

Far-left panel: cells were incubated in culture dishes (Falcon® 3002) for 8 h after irradiation at a dose of 20 Gy; the conditioned medium (CM) was harvested and then added to cultures of THP-1 cells either with or without pretreatment with anti-TNF antibody (Ab) for 1 h at 37 °C (50%, v/v). As control, unirradiated medium was used (50%, v/v). Middle panel: cells were pretreated for 1 h with anti-TNF antibody, 1 ml of which neutralized 1000 i.u. of TNF, and then these cells were irradiated at 20 Gy in the presence of the antibody and cultured for 4 h. Far-right panel: cells were exposed to different concentrations of TNF (100 or 1000 i.u. (U)/ml) for 4 h. Untreated and treated cells were harvested, and the levels of MnSOD and IL-1 mRNAs were determined.

MnSOD mRNA as that in untreated cells (Figure 4, middle panel). Irradiation also increased the levels of MnSOD mRNA in native and differentiated HL60 cells as well as in granulocytes. Irradiation increased the levels of TNF mRNA almost in parallel with MnSOD mRNA in both native and mature cells, including macrophages and granulocytes.

### Induction of TNF production by irradiation

To investigate whether irradiation affected TNF production, THP-1 cells were cultured for 8 h after exposure to irradiation at different doses (10–80 Gy). As a control, unirradiated cells were cultured for 8 h. Conditioned media and cells were harvested; levels of TNF in conditioned media and cell lysates were determined by ELISA (Table 1). The TNF protein in the conditioned medium of untreated cells was below the level of detectability. Irradiation increased the levels of TNF in a dose-dependent manner; a significant increase in TNF production was observed at an dose of 20 Gy ($P < 0.001$). At 80 Gy the level of TNF was approx. 9-fold that from the 20 Gy-irradiated cells ($P < 0.001$). The study of TNF in cell lysates showed that untreated THP-1 cells constitutively contained low levels of TNF; irradiation markedly increased the TNF levels in a dose-dependent manner. At a dose of 10 Gy the levels of intracellular TNF were 1.5-fold those in untreated cells ($P < 0.001$); at 80 Gy they were 6-fold greater.

### Irradiation induces MnSOD expression through TNF production

To determine the role of TNF produced by irradiation, THP-1 cells were cultured for 8 h after irradiation at a dose of 20 Gy and the conditioned media were harvested and added to THP-1 cells (50 %, v/v). The conditioned media induced MnSOD expression in THP-1 cells compared with those from unirradiated cells, and anti-TNF antibody, 1 ml of which neutralizes 1000 i.u. of TNF, inhibited MnSOD mRNA induction by the conditioned media almost completely (Figure 5, far-left panel). Furthermore cells were preincubated with antibody against human TNF, 1 ml of which neutralizes 1000 i.u. of TNF, for 1 h. Then the cells were irradiated at 20 Gy in the presence of anti-TNF antibody. After 4 h the cells were harvested and their levels of MnSOD mRNA were compared with those in control cells that had been exposed to irradiation alone. Treatment with anti-TNF antibody blocked the increase in irradiation-induced MnSOD transcripts by 70 % (Figure 5, middle panel). In parallel, cells were cultured for 4 h after exposure to TNF at different concentrations (100 and 1000 i.u./ml). As control, untreated cells were cultured for 4 h without TNF. The cells were harvested and Northern blot analysis was performed (Figure 5, far-right panel). Exogenously added TNF markedly increased the levels of MnSOD and IL-1$\beta$ mRNA species.

### Effect of prolonged exposure of cells to protein kinase C activator on MnSOD mRNA expression by irradiation

Cells exposed for prolonged durations to high concentrations of PMA show a decrease in their protein kinase C activity, thus making them resistant to repeated exposure to PMA [37,38]. THP-1 cells cultured with PMA (50 nM) for 4 h had a markedly increased accumulation of MnSOD mRNA compared with the untreated cells (Figure 6). Prolonged exposure (24 h) of cells to PMA (100 nM) did not increase the level of MnSOD mRNA. In addition, exposure of the cells for 24 h to PMA attenuated the response to PMA by more than 90 %. Furthermore the accumulation of MnSOD by irradiation was also attenuated in cells exposed to PMA for 24 h. Similar results were observed in the expression of the TNF gene by irradiation in cells subjected to prolonged PMA exposure.

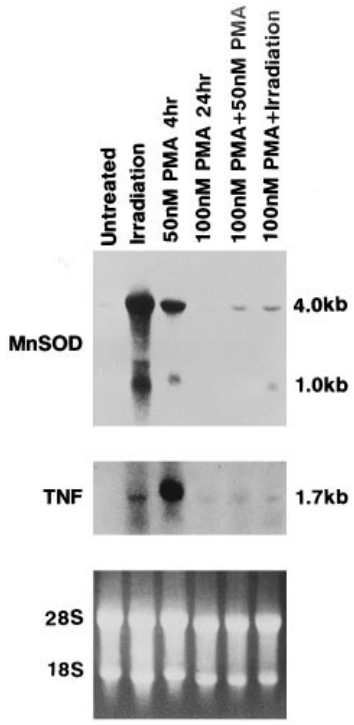


**Figure 6 Effect of prolonged exposure to a phorbol diester on the expression of MnSOD mRNA induced by irradiation**

THP-1 cells were pretreated with PMA (100 nM, 24 h), washed, and treated with either PMA (50 nM) or irradiation (40 Gy). After a further 4 h the total RNA was extracted and Northern blotting was performed. As controls, cells were cultured with PMA alone (50 nM, 4 h) or irradiated alone (40 Gy, 4 h).

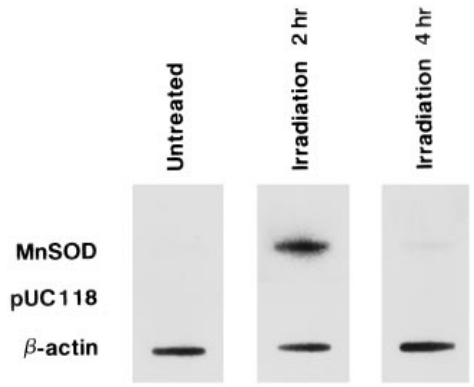


**Figure 7 Transcriptional run-on analysis of MnSOD in irradiated THP-1**

Cells were either untreated or irradiated at 20 Gy; 2 or 4 h later their nuclei were isolated as described in the Materials and methods section. Newly elongated $^{32}$P-labelled transcripts were hybridized to the plasmid containing the insert of MnSOD, $\beta$-actin or the control plasmid pUC118. Lanes of MnSOD and $\beta$-actin in untreated or irradiated cells were determined by densitometry; the relative density of MnSOD was compared by the ratio of MnSOD to $\beta$-actin.

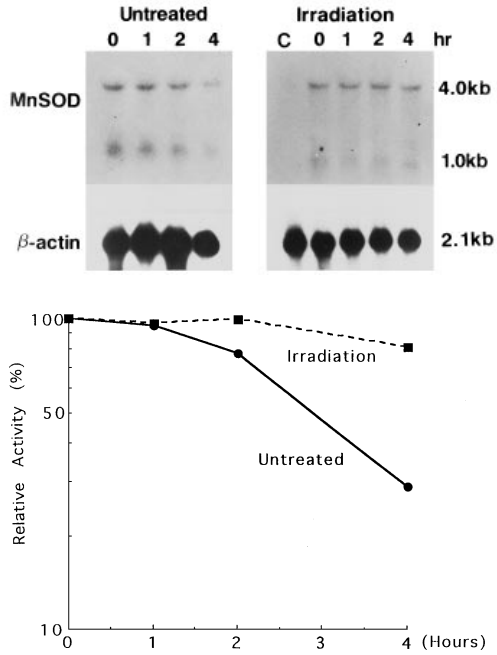


**Figure 8 Stability of steady-state MnSOD mRNA in THP-1 cells exposed to irradiation**

Untreated cells or cells irradiated at 40 Gy were cultured with actinomycin D (5 $\mu$g/ml) for 1–4 h. Total RNA (30 $\mu$g per lane in untreated cells and 15 $\mu$g per lane in irradiated cells) was extracted and analysed by RNA blotting as described in the Materials and methods section. The intensity of hybridization was determined by densitometry of autoradiograms obtained at several different exposures. Each lane was compared with respect to the intensity of the $\beta$-actin band; the results are expressed as the ratio of MnSOD to $\beta$-actin. Untreated cells were assumed to have 100% activity.

### Transcriptional regulation of MnSOD in irradiated THP-1 cells

Transcriptional run-on assays were performed to estimate the mechanisms responsible for the accumulation of MnSOD transcripts by irradiation (Figure 7). The actin gene was constitutively transcribed in untreated THP-1 cells, whereas there was no detectable level of pUC118 gene transcription (negative control for non-specific hybridization). Exposure of the cells to an irradiation dose of 20 Gy increased the transcriptional rate of MnSOD by 7-fold after 2 h; at 4 h after irradiation the rate decreased to almost the level of unirradiated cells.

### Stability of steady-state MnSOD mRNA in irradiated monocytic cells

To examine post-transcriptional regulation of MnSOD mRNA in irradiated THP-1 cells, unirradiated or 40 Gy-irradiated THP-1 cells were cultured for 4 h, after which actinomycin D (5 $\mu$g/ml) was added to the cultures. The cells were then cultured for a further 1–4 h and were sequentially harvested and examined for their level of MnSOD mRNA (Figure 8). The half-life ($t_{\frac{1}{2}}$) of steady-state MnSOD mRNA in unirradiated THP-1 cells was less than 3 h, whereas the $t_{\frac{1}{2}}$ of MnSOD mRNA after irradiation was more than 4 h.

## DISCUSSION

Monocytes/macrophages have a variety of functions against external insults; many studies have shown that they have important roles in inflammation [1]. Many of the damaging effects of ionizing irradiation are mediated by reactive free radicals [15,17,40]. Irradiation increases the production of $O_2^-$, which causes DNA breakage, lipid peroxidation and protein modification [41]. However, little is known about the roles of monocytic cells in irradiated tissue. The results presented here demonstrate that the levels of transcripts of MnSOD, which is located in mitochondria, can be modulated by irradiation in human monocytic THP-1 cells, mature monocytes/macrophages and other myeloid cells including granulocytes; irradiation increases the accumulation of MnSOD transcripts in these cells. Studies have shown that the mitochondrion is a major direct intracellular target site for oxidant damage such as irradiation and chemical drugs [42–45]. It has been reported that there is preferential oxidative damage to mitochondrial DNA rather than to nuclear DNA after irradiation [44]. MnSOD is involved in resistance to irradiation [12,13], and overexpression of MnSOD promotes the survival of cells exposed to irradiation [17]. Our results therefore suggest that the induction of MnSOD might have an important biological role in the protection of haemopoietic cells against irradiation.

Previous studies have shown that stimulators of several pathways of signal transduction increase MnSOD levels; the induction of MnSOD in response to inflammatory mediators such as TNF has been implicated as being closely involved in the inflammatory response [5,7,8]. TNF and IL-1 are produced by various cells, including monocytes/macrophages and granulocytes [46]. In the present study, THP-1 cells produced TNF constitutively; this constitutive production of TNF was markedly augmented by irradiation. Other investigators have also demonstrated that irradiation induced TNF expression in monocytic cells [22–24]; our results are consistent with those studies. Incubation of THP-1 cells with conditioned media from irradiated cells resulted in the accumulation of MnSOD transcripts, and antibody neutralizing TNF inhibited the induction of MnSOD mRNA. Furthermore exogenously added TNF stimulated the expression of MnSOD mRNA and treatment of these cells with anti-TNF antibody blocked the induction of the MnSOD mRNA by irradiation in THP-1 cells. In contrast, irradiated THP-1 cells also had increased levels of IL-1$\beta$ mRNA. However, IL-1$\beta$ failed to induce MnSOD mRNA expression, and treatment with anti-IL-1 antibody did not affect the levels of MnSOD mRNA in irradiated THP-1 cells (results not shown). Our results indicate for the first time that irradiation might induce the expression of MnSOD mRNA through the autocrine pathway involving the production of TNF in THP-1 cells.

Our present studies showed that irradiation markedly increased the rate of MnSOD transcription in THP-1 cells. Previous studies, including our own, have reported that irradiation increases the transcriptional rates of several genes [20–22,24–26,47]. Moreover, HIV or Moloney sarcoma virus long terminal repeat-directed expression has been shown to be activated by X-irradiation [48,49]. The mechanism of the irradiation-induced transcription remains unknown. Recent studies have shown that irradiation increased transcriptional factors such as Fos, Jun and the early growth response family of genes [20,47]. The polypeptide product of the c-*jun* gene is a component of the AP-1 transcriptional factor complex [50] and several genes inducible by irradiation contain AP-1 *cis*-acting regulatory elements [51]. The AP-1-binding site has been identified in the promoter region of the human MnSOD gene [52]. Our results suggest that the induction of MnSOD mRNA by irradiation might also occur through a mechanism such as the induction of these transcriptional factors. The steady-state levels of mRNA species are dependent on both the rates of transcription and degradation; another mechanism of the accumulation is to increase the stability

of these transcripts. The $t_{\frac{1}{2}}$ of MnSOD RNA was less than 3 h in THP-1 cells; irradiation stabilized MnSOD mRNA ($t_{\frac{1}{2}} > 4$ h). It has been shown that irradiation stabilized GM-CSF mRNA via a pathway that requires the AU-rich sequences in the 3′ untranslated region [20,21]. However, RNA coding for MnSOD has no AU-rich sequences containing AUUUA repeats in the 3′ untranslated region [53]. How various extracellular signals can result in mRNA stabilization of these transcripts is unknown, and further studies are required.

Protein kinase C is involved in signal transduction by coupling receptor-mediated inositol phospholipid turnover with a variety of cellular functions [54]. To determine whether the activation of protein kinase C is required for the induction of MnSOD gene by irradiation, we took advantage of the fact that prolonged exposure of cells to PMA leads to the inactivation of protein kinase C [55,56]. Prolonged exposure to PMA (100 nM, 24 h) blocked the accumulation of MnSOD mRNA after re-exposure of the cells to PMA. Under the same conditions, the accumulation of MnSOD transcripts on irradiation was blocked in the PMA-treated cells. The AP-1 is activated on treatment with PKC activators [57]. A recent study has suggested that the activation of tyrosine-specific protein kinases is involved in the initiation of the transmembrane signal pathway induced by irradiation [58]. Our findings suggest that the activation of PKC might be also one of the mechanisms for the induction of MnSOD mRNA by irradiation.

Leukaemia cells can be induced to differentiate into more mature cells by various agents *in vitro*. The human monocytic leukaemia cell line THP-1 can differentiate into macrophages, and HL60 can terminally differentiate along either the monocytic or granulocytic pathway [39]. Monocytes/macrophages and granulocytes have various functions against external insults such as bacterial infection; acquisition of these functions is also a marker of differentiation in addition to changes in morphology and the appearance of cell-surface markers. In the present study, the induction of differentiation with PMA did not affect the levels of MnSOD mRNA in these cells, whereas the induction of differentiation with Vit $D_3$ along the monocytic pathway increased the levels of MnSOD mRNA in THP-1 and HL60 cells. HL60 cells differentiated towards granulocytes also had an MnSOD mRNA level almost equivalent to that of native cells. Asayama et al. [59] observed an increase in MnSOD activity with the functional maturation of monocytes. In contrast, other studies demonstrated that differentiated HL60 cells expressed mRNA coding for MnSOD at a lower level or a level nearly equivalent to that of native HL60 cells [60]. In our study, macrophages derived from bone marrow had higher levels of MnSOD mRNA than in Vit $D_3$-treated HL60 and THP-1 cells (results not shown). The reason for this discrepancy is unknown. The levels of MnSOD during differentiation–induction might be related to the increase in size and number of mitochondria, which are affected by individual inducers of differentiation.

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