FEBS Letters 461 (1999) 334–338 FEBS 22877

Adenovirus E1A blocks oxidant-dependent ferritin induction and sensitizes cells to pro-oxidant cytotoxicity

Kouichi Orino[a,1], Yoshiaki Tsuji[b], Frank M. Torti[b,c], Suzy V. Torti[a,*]

[a] *Department of Biochemistry, and the Comprehensive Cancer Center, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA*
[b] *Department of Cancer Biology, and the Comprehensive Cancer Center, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA*
[c] *Department of Medicine, and the Comprehensive Cancer Center, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA*

Received 25 September 1999; received in revised form 16 October 1999

Abstract Ferritin is a protein that oxidizes and sequesters intracellular iron in a mineral core. We have reported that the E1A oncogene selectively represses ferritin H transcription, resulting in reduced levels of the ferritin H protein. Here we demonstrate that cells respond to pro-oxidant challenge by inducing ferritin mRNA and protein, and that this response is completely blocked by E1A. Concordantly, E1A sensitized cells to the cytotoxic effects of oxidative stress and enhanced the accumulation of reactive oxygen species in response to pro-oxidant challenge. These results demonstrate that expression of E1A impedes the cellular response to oxidative stress, including the induction of ferritin.

Key words: Ferritin; Oxidative stress; Adenovirus E1A

1. Introduction

Ferritin is a 24-subunit protein with a major role in the regulation of intracellular iron storage and homeostasis (reviewed in [1–3]). Ferritin is comprised of two subunit types, termed H and L. These subunits assemble to form an apoprotein of approximately spherical structure that encases an internal ferrihydrite mineral core. Ferritin is ubiquitously distributed in the animal kingdom, and is also present in plants and bacteria, suggesting that it performs functions vital to cell survival [4,5]. One of these functions is to limit iron availability for participation in reactions that produce oxygen free radicals. For example, the donation of electrons by reduced iron to endogenously produced oxygen species such as hydrogen peroxide can lead to the accumulation of oxygen free radicals, which have the potential to damage lipids, proteins and DNA [6]. Storage of iron as an oxidized species within the mineral core of ferritin minimizes the availability of iron for such reactions. Indeed, several reports have implicated ferritin in the protection from oxidant injury [7]. For example, pretreatment of cells with hemin, an iron source that induces ferritin, was shown to protect endothelial cells from the toxic effects of a subsequent challenge with hydrogen peroxide [8]. Antisense oligomers to ferritin H were able to partially block a similar response in leukemic cells, strongly implicating ferritin H in the protection against oxidative stress [9].

*Corresponding author. Fax: (1) (336) 7160255.
E-mail: storti@wfubmc.edu

[1] Present address: Laboratory of Biochemistry, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, Aomori 034-8628, Japan.

Transcription of the murine ferritin H gene is modulated by an enhancer element located approximately 4.1 kb 5′ to the transcriptional start site, termed FER-1 [10]. We have previously reported that the adenovirus early region 1A oncogene (E1A) selectively represses ferritin H transcription by targeting the FER-1 element [11]. The E1A gene is a potent oncogene that can reprogram normal regulation of cell growth, leading to cell immortalization (reviewed in [12]). E1A gene products modify the transcriptional program by directly binding to a number of host proteins involved in transcriptional control [13]. E1A targets ferritin H both by reducing the binding of AP-1 family members to FER-1, and by interacting with p300, a transcriptional co-activator involved in FER-1 enhancer activity [10,11]. As a consequence, cells that express E1A contain ferritin depleted in the ferritin H subunit [14].

These observations suggested that cells expressing E1A might exhibit a diminished ability to respond to oxidant challenge. Here we show that expression of E1A impedes the response to oxidative stress, including the induction of ferritin.

2. Materials and methods

2.1. Cell culture

NIH3T3 cells were transfected with an expression vector for E1A or with a control vector encoding hygromycin resistance, and stable transfectants were derived and cultured as described previously [14,15]. Both a clonal E1A transfectant (3T3/E1A clone F2) and a pool derived from over 100 independent clones (3T3/E1A pool) were analyzed. Expression of E1A was confirmed by Western blotting [14].

2.2. Metabolic labeling and immunoprecipitation

Cells were labeled and ferritin immunoprecipitated and analyzed by SDS-PAGE, essentially as described [16]. Gels were analyzed on a PhosphorImager (Molecular Dynamics).

2.3. Northern blotting

RNA was isolated and expression of ferritin mRNA evaluated by Northern blotting using ferritin H and ferritin L cDNA probes as previously described [14].

2.4. Measurement of reactive oxygen species (ROS)

Cells were plated at a concentration of 5×10^4 cells/well in 96-well dishes. After incubation for 18–24 h, the growth medium was removed and the well was washed two times with 150 mM NaCl, 10 mM HEPES-Tris (pH 7.3) (HBS). The adherent cells were incubated with 100 μl of HBS containing 4 μM di(acetoxymethyl ester) of carboxy-2′,7′-dichlorofluorescin-diacetate (DCFH-DA Molecular Probes, Oregon) in the presence or the absence of H_2O_2 (300 μM). DCFH-DA is a non-fluorescent permeant molecule, which is acted upon by intracellular esterases to produce 2′,7′-dichlorofluorescin (DCFH) [17]. In the presence of intracellular ROS, DCFH is rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) [18]. The oxidative products were measured with a fluorescent microplate reader (Molecular Devices, California) using 485 nm and 538 nm as

PII: S0014-5793(99)01443-X

extinction and emission filters, respectively, at 37°C. Fluorescent units were converted to pmol product using a standard curve constructed with authentic DCF (Molecular Probes).

2.5. Cytotoxicity assays

Cells were plated in 96-well plates at 1×10^4 cells/well. After 18–24 h, hydrogen peroxide at various concentrations was added directly to the wells, and cytotoxicity assessed after 24 h further incubation essentially as described [15]. For measurement of the cytotoxic response to *tert*-butyl hydroquinone (*t*-BHQ), cells were plated at 2.5×10^5 cells/35-mm dish before exposure to various concentrations of *t*-BHQ (Sigma). Cells were washed twice with phosphate-buffered saline (PBS), fixed with ethanol, and stained with crystal violet. Dye was eluted with 50% ethanol in 0.1 M sodium citrate, and optical density measured at 595 nm.

2.6. Statistical analysis

ROS accumulation by 3T3/hyg and 3T3/E1A cells in the presence and absence of hydrogen peroxide was analyzed using repeated measures analysis of variance. Each experiment consisted of four experimental conditions: two cell types in the presence and absence of hydrogen peroxide. The interaction between cell type and hydrogen peroxide was first assessed to test if the effect of hydrogen peroxide differed between 3T3/hyg and 3T3/E1A cells. Paired *t*-tests were then used to assess the effect of hydrogen peroxide for each cell type. All *P* values reported are two-sided; *P* values less than 0.05 are considered statistically significant.

3. Results

3.1. E1A transfectants exhibit an enhanced accumulation of ROS in response to oxidant stress

In order to measure the effect of E1A on the response to oxidative stress, we first assessed whether expression of E1A modulated the accumulation of ROS. Cells were either exposed to 300 μM hydrogen peroxide or left untreated, and

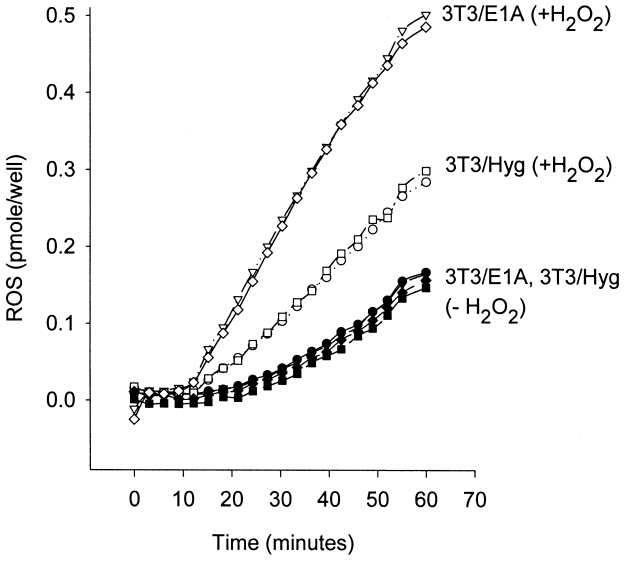

Fig. 1. Time course of ROS accumulation in 3T3/Hyg control cells and 3T3/E1A cells. Cells were incubated in the presence and absence of 300 μM hydrogen peroxide and the accumulation of reactive oxygen species measured continuously in a fluorescence microplate reader as described in Section 2. Fluorescence of growth medium alone was unchanged throughout the course of the experiment and was subtracted before calculating ROS accumulation. Experiments were performed multiple times with similar results; the results of two replicate experiments are shown. Cells retained full viability during the time course of this assay (not shown).

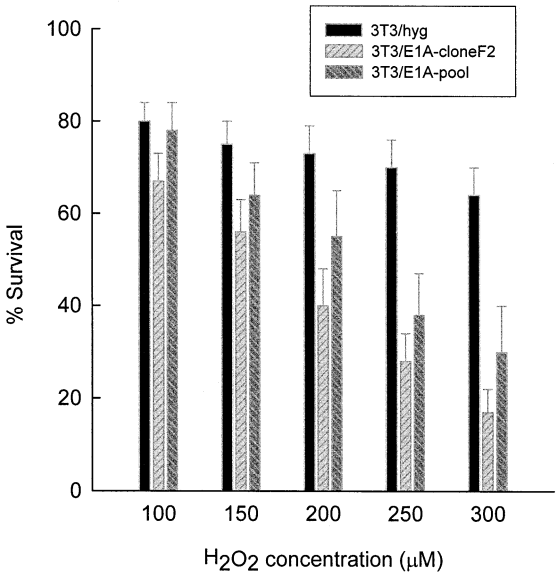

Fig. 2. E1A transfectants exhibit enhanced sensitivity to hydrogen peroxide over a wide range of hydrogen peroxide concentrations. Cells were plated in 96-well dishes and allowed to attach for 18–24 h before being treated with the indicated concentrations of hydrogen peroxide in growth medium for 24 h. Four replicate cultures were prepared for each time point. Surviving cells were assessed by staining with crystal violet; the staining intensity of untreated cells was defined as 100%. Data represent mean and standard error for seven independent experiments.

the accumulation of ROS measured continuously over a 1-h period at 37°C. As shown in Fig. 1, basal production of ROS was approximately equivalent in cells expressing E1A and in controls transfected with vector alone. Treatment of cells with hydrogen peroxide led to an increase in ROS in both cell types. However, following challenge with hydrogen peroxide, both clonal (not shown) and pooled E1A transfectants (Fig. 1) exhibited an increase in accumulation of ROS that was greater than that seen in control cells.

To quantitate the effect of E1A expression on the response to hydrogen peroxide, seven independent experiments comparing the response of control 3T3/hyg cells and 3T3/E1A cells to hydrogen peroxide were performed. Expression of E1A enhanced the accumulation of ROS in response to challenge with hydrogen peroxide: hydrogen peroxide increased ROS accumulation by 0.14 pmol/well after 1 h (0.13 ± 0.03 to 0.27 ± 0.04 s.d., $P=0.0001$) in control cells, compared to 0.30 pmol/well (0.12 ± 0.03 to 0.42 ± 0.08 s.d., $P=0.0001$) in E1A transfectants. The difference between ROS accumulation in 3T3/hyg and 3T3/E1A cells in response to hydrogen peroxide was highly statistically significant ($P\leq0.0001$).

3.2. E1A augments cytotoxicity of hydrogen peroxide and t-BHQ

To determine whether the enhanced accumulation of ROS in response to hydrogen peroxide seen in E1A transfectants resulted in functional cellular consequences, we measured survival of cells after exposure to either hydrogen peroxide or another oxidant, *t*-BHQ [19,20]. As shown in Fig. 2, exposure of cells to an increasing concentration of hydrogen peroxide for 24 h led to a dose-dependent decrease in survival. At all but the lowest dose, survival of E1A transfectants was substantially reduced relative to vector controls. For example, at

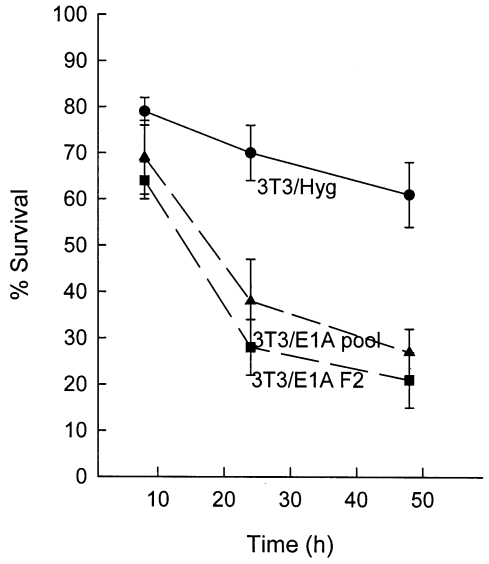

Fig. 3. Time-dependent sensitivity of 3T3 cells stably expressing E1A to hydrogen peroxide. Cells were plated in 96-well dishes and allowed to attach for 18–24 h before being treated with 250 μM hydrogen peroxide in growth medium. Four replicate cultures were prepared for each time point. At the end of the time intervals shown, surviving cells were assessed by staining with crystal violet. Data represent mean and standard error for seven independent experiments.

300 μM hydrogen peroxide, mean survival of vector transfectants at 24 h was 64%, whereas that of clonal and pooled E1A transfectants was 17% and 30%, respectively. A similar enhancement of hydrogen peroxide toxicity was observed at all times tested: as shown in Fig. 3, at 8, 24 and 48 h, vector transfectants exhibited substantially increased survival relative to E1A transfectants. This reflected a generalized increase in sensitivity to oxidants and not a particular response to hydrogen peroxide, since E1A transfectants also exhibited enhanced cytotoxicity when challenged with *t*-BHQ (Fig. 4).

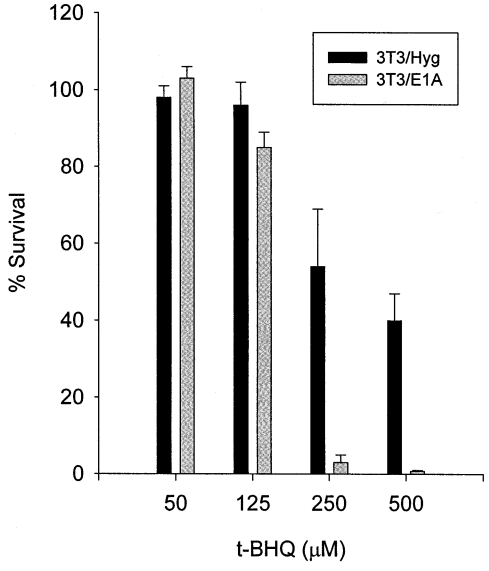

Fig. 4. E1A transfectants exhibit an enhanced sensitivity to *t*-BHQ. 3T3/Hyg and 3T3/E1A clone F2 cells were treated with the indicated concentrations of *t*-BHQ and survival measured after 24 h as described in Section 2. Shown are means and standard errors of three independent experiments.

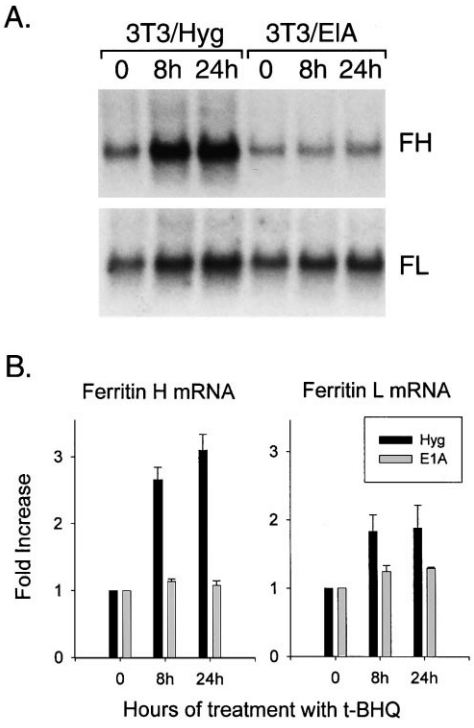

Fig. 5. A: Induction of ferritin mRNA by *t*-BHQ is blocked in E1A transfectants. 100 μM *t*-BHQ was added to cells in growth medium. After 0, 8 and 24 h cells were lysed, RNA was isolated, and Northern blots prepared. Blots were hybridized sequentially with mouse ferritin H and L cDNA probes. B: Data from three (ferritin L) or four (ferritin H) replicate experiments were scanned and band intensities quantitated by densitometry. For each cell type, the band intensity of untreated samples was assigned a value of 1.0. Standard errors are shown.

3.3. *Treatment with t-BHQ induces an increase in ferritin mRNA and protein that is blocked by E1A*

As shown in Fig. 5, treatment of 3T3/hyg cells with 100 μM *t*-BHQ dramatically increased levels of ferritin H mRNA. An elevation in ferritin L mRNA was also seen, although the increase was more modest (Fig. 5). Similar results were observed with hydrogen peroxide (not shown), suggesting that the induction of ferritin occurs in response to oxidative stress.

We previously showed that cells transfected with E1A exhibited a relative reduction in basal levels of ferritin H mRNA [14]. This is also seen in Figs. 5A and 6A. We now demonstrate that E1A has an additional effect: as shown in Fig. 5, E1A not only reduced basal levels of ferritin H, but completely blocked *t*-BHQ-mediated induction of ferritin H mRNA. E1A was also able to reduce induction of ferritin L. The E1A-dependent inhibition of ferritin mRNA induction in response to oxidants was reflected in an inhibition of ferritin protein synthesis: as shown in Fig. 6, treatment with *t*-BHQ led to a time-dependent increase in synthesis of ferritin protein that was blocked in cells expressing E1A.

4. Discussion

Results presented here demonstrate that ferritin mRNA and

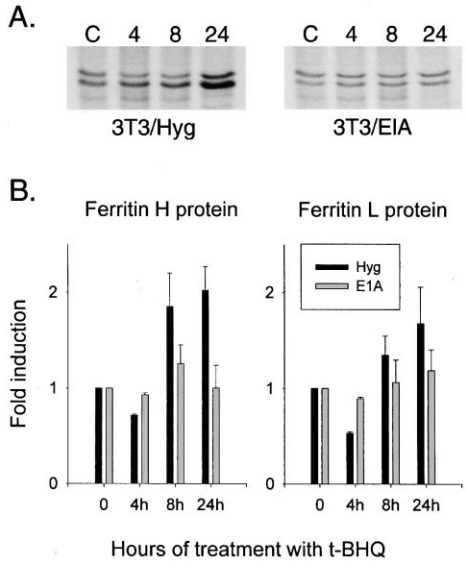

Fig. 6. A: Induction of ferritin protein by *t*-BHQ is blocked in E1A transfectants. Duplicate culture dishes were treated with 100 μM *t*-BHQ for 4, 8 and 24 h. Two hours prior to harvest, cells were incubated with ^{35}S-Translabel. Controls were incubated for 4 h in the absence of *t*-BHQ. Ferritin was isolated by immunoprecipitation and analyzed by SDS-PAGE as described in Section 2. B: Data from two (4 and 8 h time points) to three (24 h time points) replicate experiments were scanned and band intensities quantitated by densitometry. For each cell type, the band intensity of untreated samples was assigned a value of 1.0. Standard errors are shown.

protein are induced in cells exposed to a pro-oxidant such as *t*-BHQ (Figs. 5 and 6). Through its ability to enhance iron sequestration and reduce the availability of iron for participation in oxygen radical formation, ferritin induction may serve as one component of a cytoprotective response designed to minimize oxidative damage. Similarly, others have shown that increased expression of ferritin H reduces the labile iron pool [21,22], and suggested that ferritin induction may protect cells from the oxidative injury mediated by UV [23,24] and glutathione depletion [7].

Our experiments with E1A lend further support to a cytoprotective role for ferritin in response to oxidative stress. These experiments demonstrated that E1A not only repressed basal transcription of ferritin H, but blocked oxidant-dependent augmentation of ferritin mRNA and protein as well (Figs. 5 and 6). This result is consistent with reports demonstrating a reduction in antioxidant defenses in transformed cells [25], a response that has been suggested to confer a selective advantage to these cells [26]. Cells that express E1A were not appreciably altered in growth rate when compared to vector-transfected control cells (unpublished observations), and both controls and E1A transfectants exhibited a similar accumulation of ROS under basal conditions (Fig. 1). However, in response to oxidant challenge, E1A expression led to an increase in ROS accumulation relative to control cells (Fig. 1). Since ROS accumulation has been correlated with enhanced cytotoxicity [27–29], augmented accumulation of intracellular ROS may explain the heightened sensitivity of E1A transfectants to the cytotoxic effects of oxidants such as *t*-BHQ (Fig. 4) and hydrogen peroxide (Figs. 2 and 3).

We have previously shown that E1A represses ferritin H gene transcription [14]. E1A exerts its inhibitory effect on the ferritin H gene by targeting FER-1, an enhancer element located approximately 4.1 kb 5′ to the transcriptional start site [10]. Taken together with results presented here, these experiments suggest that proteins binding to FER-1 may contribute to oxidant-dependent enhancement of ferritin H transcription, and that E1A may block this response by interfering with the assembly of a functional transcription factor complex at this site. Although aspects of this model require further testing, our recent observation that the induction of ferritin H by oxidants is mediated by an electrophilic response element that overlaps FER-1 (Tsuji et al., submitted) is consistent with this model.

In addition to induction of ferritin H, induction of ferritin L mRNA and protein in response to the pro-oxidant *t*-BHQ was also observed (Figs. 5 and 6). Although E1A does not reduce basal levels of ferritin L [14] (Fig. 5), we observed a partial inhibition of oxidant-dependent induction of ferritin L by E1A (Fig. 5). Thus, mechanisms regulating the response of ferritin L and H to oxidants may share some overlap. The electrophilic response element recently identified in the ferritin L gene [30] may represent a plausible target for such a regulatory event.

Cells transformed by E1A have been used to model pathways involved in tumor cell killing by chemotherapeutic agents, particularly DNA damaging agents [31]. Recent results have shown that APAF-1, a cofactor required for caspase 9 activation, may be an important component of E1A-mediated sensitization to such agents [32]. Our results demonstrate that E1A sensitizes cells not only to DNA damaging cytotoxic drugs, but to oxidative stress. Pathways of cell injury induced by these diverse agents may therefore share common elements.

Acknowledgements: We gratefully acknowledge Dr. D. Case for statistical analysis and Dr. S. Bailey for valuable assistance in developing the assay for reactive oxygen species. This work was supported by Grant DK-42412 from the National Institute of Health. Phosphorimaging analysis was performed in a facility supported by Grant CA12197 from the National Institute of Health and Grant 9510-IDG-1006 from the North Carolina Biotechnology Center.

References

[1] Harrison, P.M. and Arosio, P. (1996) Biochim. Biophys. Acta 1275, 161–203.
[2] Theil, E.C. (1990) Adv. Enzymol. Relat. Areas Mol. Biol. 63, 421–449.
[3] Chasteen, N.D. (1998) Metal Ions Biol. Syst. 35, 479–514.
[4] Proudhon, D., Wei, J., Briat, J. and Theil, E.C. (1996) J. Mol. Evol. 42, 325–336.
[5] Grossman, M.J., Hinton, S.M., Minak-Bernero, V., Slaughter, C. and Stiefel, E.I. (1992) Proc. Natl. Acad. Sci. USA 89, 2419–2423.
[6] Buettner, G.R. and Jurkiewicz, B.A. (1996) Radiat. Res. 145, 532–541.
[7] Cairo, G., Tacchini, L., Pogliaghi, G., Anzon, E., Tomasi, A. and Bernelli-Zazzera, A. (1995) J. Biol. Chem. 270, 700–703.
[8] Balla, G., Jacob, H.S., Balla, J., Rosenberg, M., Nath, K., Apple, F., Eaton, J.W. and Vercellotti, G.M. (1992) J. Biol. Chem. 267, 18148–18153.
[9] Lin, F. and Girotti, A.W. (1998) Arch. Biochem. Biophys. 352, 51–58.
[10] Tsuji, Y., Akebi, N., Lam, T.K., Nakabeppu, Y., Torti, S.V. and Torti, F.M. (1995) Mol. Cell. Biol. 15, 5152–5164.
[11] Tsuji, Y., Moran, E., Torti, S.V. and Torti, F.M. (1999) J. Biol. Chem. 274, 7501–7507.
[12] Moran, E. and Mathews, M.B. (1987) Cell 48, 177–178.
[13] Dyson, N. and Harlow, E. (1992) Cancer Surv. 12, 161–195.

[14] Tsuji, Y., Kwak, E., Saika, T., Torti, S.V. and Torti, F.M. (1993) J. Biol. Chem. 268, 7270–7275.
[15] Tsuji, Y., Ninomiya-Tsuji, J., Torti, S.V. and Torti, F.M. (1993) J. Immunol. 150, 1897–1907.
[16] Torti, S.V., Kwak, E.L., Miller, S.C., Miller, L.L., Ringold, G.M., Myambo, K.B., Young, A.P. and Torti, F.M. (1988) J. Biol. Chem. 263, 12638–12644.
[17] Bass, D.A., Parce, J.W., Dechatelet, L.R., Szejda, P., Seeds, M.C. and Thomas, M. (1983) J. Immunol. 130, 1910–1917.
[18] LeBel, C.P., Ischiropoulos, H. and Bondy, S.C. (1992) Chem. Res. Toxicol. 5, 227–231.
[19] Schilderman, P.A., van Maanen, J.M., Smeets, E.J., ten Hoor, F. and Kleinjans, J.C. (1993) Carcinogenesis 14, 347–353.
[20] Yamane, Y., Furuichi, M., Song, R., Van, N.T., Mulcahy, R.T., Ishikawa, T. and Kuo, M.T. (1998) J. Biol. Chem. 273, 31075–31085.
[21] Picard, V., Renaudie, F., Porcher, C., Hentze, M.W., Grandchamp, B. and Beaumont, C. (1996) Blood 87, 2057–2064.
[22] Picard, V., Epsztejn, S., Santambrogio, P., Cabantchik, Z.I. and Beaumont, C. (1998) J. Biol. Chem. 273, 15382–15386.
[23] Applegate, L.A. and Frenk, E. (1995) Photodermatol. Photoimmunol. Photomed. 11, 95–101.
[24] Applegate, L.A., Scaletta, C., Panizzon, R. and Frenk, E. (1998) J. Invest. Dermatol. 111, 159–163.
[25] Oberley, T.D., Schultz, J.L., Li, N. and Oberley, L.W. (1999) Free Radical Biol. Med. 19, 53–65.
[26] Clement, M.-V. and Pervaiz, S. (1999) Free Radical Res. 30, 247–252.
[27] Sokoll, L.J. and Dawson-Hughes, B. (1992) Am. J. Clin. Nutr. 56, 1045–1048.
[28] Shen, H.M., Ong, C.N. and Shi, C.Y. (1995) Toxicology 99, 115–123.
[29] Shen, H.M., Shi, C.Y., Shen, Y. and Ong, C.N. (1996) Free Radical Biol. Med. 21, 139–146.
[30] Wasserman, W.W. and Fahl, W.E. (1997) Proc. Natl. Acad. Sci. USA 94, 5361–5366.
[31] Samuelson, A.V. and Lowe, S.W. (1997) Proc. Natl. Acad. Sci. USA 94, 12094–12099.
[32] Fearnhead, H.O., Rodriguez, J., Govek, E.E., Guo, W., Kobayashi, R., Hannon, G. and Lazebnik, Y.A. (1998) Proc. Natl. Acad. Sci. USA 95, 13664–13669.