FEBS 27010 FEBS Letters 537 (2003) 187–192

Role of iron and ferritin in TNFα-induced apoptosis in HeLa cells

Anna Cozzi[a], Sonia Levi[a], Barbara Corsi[a], Paolo Santambrogio[a], Alessandro Campanella[a], Gianmario Gerardi[b], Paolo Arosio[b,*]

[a]*Dibit, Department of Biological and Technological Research, IRCCS H. San Raffaele, Milano, Italy*
[b]*Department M.T. and Biomedical Technologies, University of Brescia, Via Valsabbina 19, 25125 Brescia, Italy*

Received 7 January 2003; revised 24 January 2003; accepted 24 January 2003

First published online 7 February 2003

Edited by Barry Halliwell

Abstract We found that tumor necrosis factor α (TNFα)-induced apoptosis in HeLa cells was accompanied by a ∼2-fold increase in H- and L-ferritin and a decrease in transferrin receptor, two indices of increased iron availability. Iron supplementation and overexpression of H-ferritin or its mutant with an inactivated ferroxidase center reduced by about ∼50% the number of apoptotic cells after TNFα-treatment, while overexpression of L-ferritin was ineffective. The data indicate that H-ferritin has an anti-apoptotic activity unrelated to its ferroxidase activity and to its capacity to modify cellular iron metabolism.
© 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Iron regulatory protein; Ferritin; Iron metabolism; Oxidative damage

1. Introduction

Iron is an essential element for the synthesis of enzymes that are central to respiration, DNA synthesis and detoxification, but when in excess it is toxic to cells and parenchymal tissues [1,2]. The mechanism of its toxicity has not been fully clarified and it is probably related to the easy oxidation that releases a single electron with the production of damaging reactive oxygen species molecules, as it occurs in the Fenton reaction. Because of this property, iron is considered a pro-oxidant agent that participates in the damage of membrane, proteins and nucleic acids. Many oxidant agents are known to induce cell death and apoptosis [3,4], but curiously, we are not aware of reports showing that iron excess does it. On the contrary, iron supplementation to cells was found to reduce nitric oxide (NO)-induced apoptosis, an effect that was attributed to the scavenging capacity of the metal on the NO [5,6]. In addition, cell treatment with iron chelators, which are expected to reduce iron availability, induce apoptosis in HeLa and cancer cells through mechanisms that seem to involve mitochondrial activation [7–9]. Thus, iron deprivation rather than abundance seems to activate apoptotic pathways. Other evidence that iron can be protective to cells comes from the demonstration that exposure of cells to the pro-oxidant heme-iron plus hydrogen peroxide protects cells from a following oxidative insult [10,11]. Also upregulation of heme oxygenase in epithelial cells was shown to be protective from oxidative insults, and exert anti-apoptotic effects [12–14]. One of the products of this enzyme is the iron liberated from heme, and this was thought to contribute to the protective effect together with CO and biliverdin [13]. The protective role of iron may be secondary to the iron-induced ferritin upregulation, which in turn would sequester iron and reduce its pro-oxidant activity. However, ferritin expression is so tightly regulated by iron that it is difficult to distinguish between the two. In addition, ferritin is composed of two subunit types: the H- and L-chains which are similarly regulated by iron, although they have distinctive functional activities: the H-chain has ferroxidase activity and readily sequesters the excess of cytosolic iron, while the L, with a more efficient iron nucleation activity, assists the H-chain in the functionality of the hybrid molecules [1,15]. Thus, iron and ferritin seem to have complex and unclear roles in apoptosis. To clarify this, we first studied the effect of iron supplementation on tumor necrosis factor α (TNFα)-induced apoptosis in HeLa cells, and then we analyzed whether this was attributed to the H- or L-ferritins by using cell lines that overexpress the two proteins. The results indicate that under these conditions iron has an anti-apoptotic effect mediated by H-ferritin upregulation. Surprisingly, the effect was independent from the ferroxidase activity of the protein, suggesting that it is not related to the regulation of cellular iron availability. In addition, we found that during the course of apoptosis ferritin is upregulated, a possible protective response to apoptosis.

2. Materials and methods

2.1. Cells culture and induction of apoptosis

The stable clones that express ferritin H-wild type and H222 mutant (E62K, H65G) under the control of the tetracycline promoter (named H- and H222 clones) were described in ref. [16]. The one expressing L-ferritin under the same promoter (L-clone) was produced as described in ref. [16]. The cells (HeLa-TET off, Clontech, Palo Alto, CA, USA) were maintained in DMEM (Life Technologies Invitrogen, Inc., Paisley, UK, which contains 0.25 μM ferric nitrate) supplemented with 10% fetal bovine serum (Clontech, Palo Alto, CA), 100 mg/ml G418 (geneticin, Sigma, St. Louis, MO), 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mM L-glutamin (Sigma, St. Louis, MO) supplemented with 150 mg/ml hygromycin (Clontech, Palo Alto, CA). To repress or induce ferritin expression the cells were grown for more than 7 days in the presence (Dox+) or the absence (Dox−) of 2 ng/ml doxycycline (Sigma, St. Louis, MO). Apoptosis was induced with 25 ng/ml of recombinant TNFα (kindly donated by Dr. A. Corti) and 1 μg/ml of actinomycin D (ActD) (Sigma, St. Louis, MO).

2.2. Western blotting

The cells were suspended in lysis buffer (20 mM Tris–HCl, pH 8,

*Corresponding author. Fax: (39)-30-307251.
E-mail address: arosio@med.unibs.it (P. Arosio).

0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00114-5

200 mM LiCl, 1 mM EDTA, 0.5% NP-40) and protein concentration was determined with the BCA assay (Pierce) calibrated on bovine serum albumin. Samples of 20 μg of total soluble proteins were loaded on 12% SDS–PAGE. After transfer, the nitrocellulose filters were incubated with rabbit anti-PARP p85 fragment (1:500) (Promega, Madison, WI) or with mouse-anti TfR antibody (1:500) (Zymed, San Francisco, CA) followed by secondary peroxidase-labeled antibody (Sigma, St. Louis, MO). Bound activity was revealed by ECL (Amersham, Uppsala, Sweden).

2.3. Ferritin quantification

Extracts of cells were analyzed for ferritin content by using ELISA assays based on monoclonal antibodies specific for the H-ferritin (rH02) and the L-ferritin (L03) calibrated on the corresponding recombinant homopolymers expressed in *Escherichia coli* [16].

2.4. Nuclear fragmentation

Nuclear fragmentation was analyzed using DAPI staining: cells (6×10^4) were seeded onto 24-well plates and allowed to attach for 18 h. After incubation with TNFα/ActD the cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 and stained with DAPI (4,6-diamidino-z-phenylindole) for 2 min [17]. The nuclei were visualized using fluorescence microscopy.

3. Results and discussion

To set up the conditions to induce apoptosis we incubated HeLa cells for 6 h with various concentrations of TNFα in the presence or absence of the transcription repressor ActD which inhibits the anti-apoptotic response elicited by the cytokine. Cells were monitored for nuclear fragmentation using the fluorescent dye DAPI [17], while cell viability was evaluated by trypan blue exclusion. In the absence of ActD, the proportion of apoptotic cells with fragmented nuclei was below 1%, as expected, while it was about 34% in the presence of ActD at TNFα concentration >25 ng/ml (Fig. 1). Cell viability was above 81%. Longer incubation time (18 h) in the presence of ActD resulted in massive cell death (>95% trypan blue positive). We chose 6 h incubation with 25 ng/ml TNFα/ActD as suitable conditions to study apoptosis. Blotting showed that the caspase product p85 PARP peptide [18], which was absent in the untreated cells, accumulated after treatment (Fig. 2A). This was accompanied by a more than two-fold decrease of the level of transferrin receptor (TfR) (Fig. 2B). To verify whether the TfR downregulation was related to modification iron availability we analyzed the levels of H- and L-ferritin types with specific ELISAs. This revealed that both ferritin types increased following the TNFα/ActD treatment (Fig. 2C). As a control, the cells were incubated for 24 h with 100 μM ferric ammonium citrate (FAC). This had no effect on p85 PARP accumulation (Fig. 2A), while downregulated TfR (Fig. 2B) and upregulated H- and L-ferritins (Fig. 2C), as expected. The mRNAs of H- and L-ferritins and of TfR have iron responsive elements (IREs) necessary for a post-transcriptional iron-dependent regulation mediated by the binding activity of the iron regulatory proteins (IRPs), and therefore the coordinate modification of these proteins during apoptosis is very likely the response to increased iron availability [19].

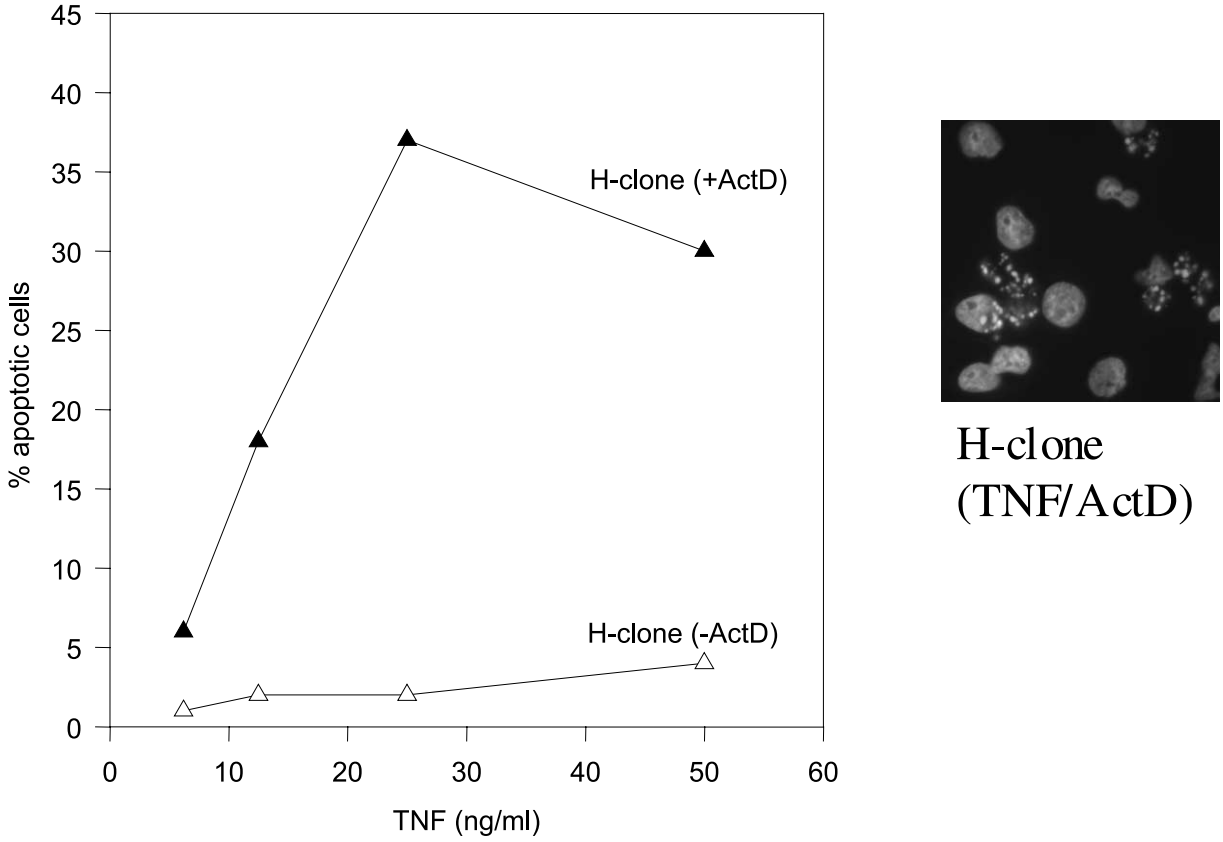

Fig. 1. Apoptosis in HeLa cells induced by TNFα. The HeLa cell clone with H-ferritin cDNA under the control of Tet promoter was grown in the presence of 2 ng/ml doxycycline to repress H-ferritin synthesis, and incubated for 6 h with various concentrations of TNFα in the presence or the absence of ActD, as indicated. The cells were harvested and stained with the fluorescent DAPI dye to evidentiate nuclear fragmentation, as shown in the insert. The proportion of apoptotic cells (with fragmented nuclei) was calculated for each TNFα concentration and reported in the plot. Data are representative from three independent experiments. Similar results were obtained with untransfected HeLa cells.

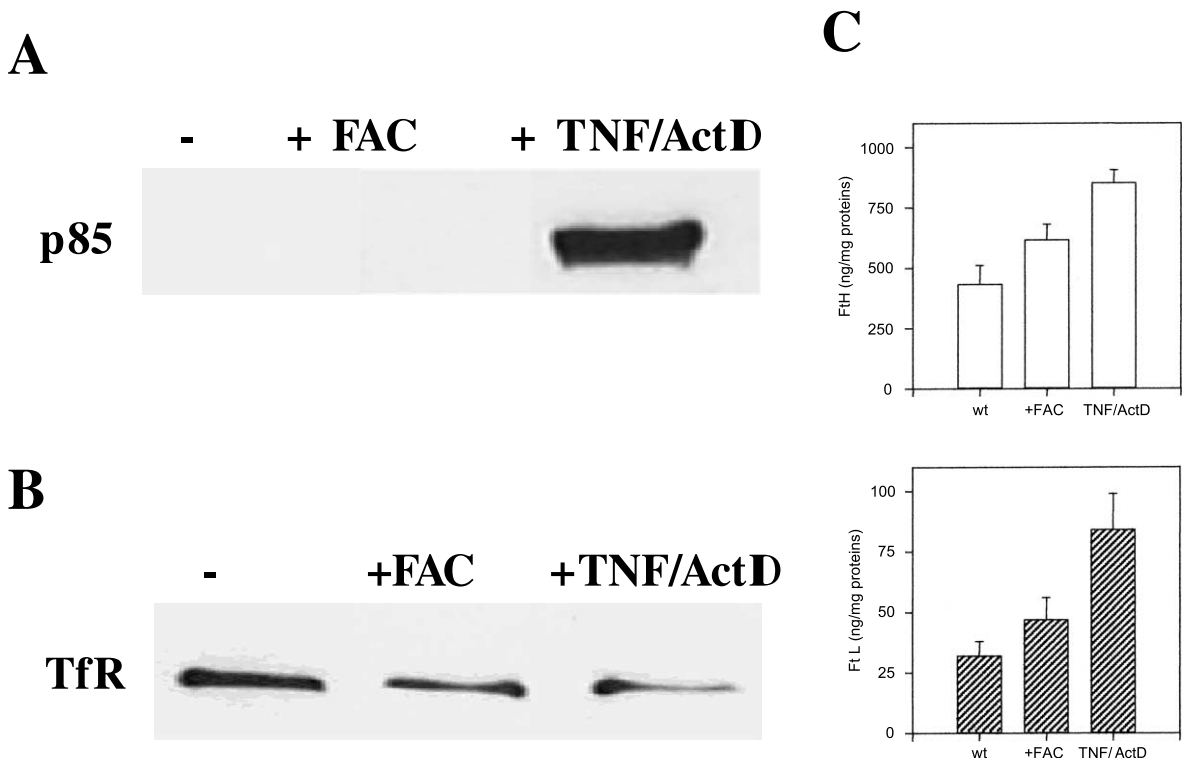

Fig. 2. Panel A: Cellular extracts from untreated HeLa cells (-), incubated for 24 h with 100 μM FAC or for 6 h with 25 ng/nl TNFα and 1 μg/ml ActD (TNF/ActD) were separated on SDS–PAGE and blotted with a specific antibody for the p85 peptide of PARP. Panel B: Same as in panel A, except that anti-TfR antibody was used in blotting. Panel C: Evaluation of ferritin levels with ELISAs specific for H-ferritin (upper) and L-ferritin (lower) in the extracts of cell treated as in panel B. The data are means of three experiments and are expressed as ng of ferritin per mg of total proteins.

However, EMSA assays did not show an evident decrease in the IRE-binding activity of IRPs following the TNFα/ActD treatment, while it was reduced by about 30% following iron supplementation (not shown). To explore this further, we analyzed H-ferritin and p85 PARP accumulation during the course of the incubation with TNFα/ActD. This showed that the caspase product became visible after 3 h incubation and strongly increased at 5 and 6 h (Fig. 3A), while H-ferritin level decreased in the first 2 h of incubation and then increased in parallel with p85 PARP (Fig. 3B). This suggests that in the early phase of the incubation, preceding the appearance of p85 PARP, some iron decompartmentalization occurred which caused ferritin upregulation. This, in turn, could sequester the excess iron and restore IRPs activity to background level at later times, thus explaining the lack of differences observed at 6 h. It should be noticed that this biphasic pattern of ferritin accumulation has been already observed during post-ischemic reperfusion of rat liver, a model of oxidative damage [20], and that ferritin is induced by H_2O_2 [21].

The next question was whether the changes of iron metabolism in response to TNFα/ActD could have an effect on apoptosis. To test this hypothesis we increased cellular iron availability by incubating HeLa cells for 24 h with 100 μM FAC before the treatment with TNFα/ActD. Pre-exposure to iron reduced by about 40% the number of cells with fragmented nuclei and decreased by about 50% the accumulation of p85 PARP peptide, the two indices of apoptosis used in this work (Fig. 4A,B). To explore whether the anti-apoptotic effect caused by iron supplementation was due to increased ferritin expression, we used the previously described HeLa cells clones that overexpress H-ferritin wild type, the H-mutant (H222) with an inactivated ferroxidase activity [16], or L-ferritin. The cDNAs are under the control of Tet promoter and the expression of the exogenous ferritin is induced 4–6-fold after more than 7 days of growth in the absence of doxycycline (Dox−) (Table 1). The three repressed and unrepressed clones were treated with TNFα/ActD, and the fraction of cells with fragmented nuclei and the accumulation of p85 PARP peptide was analyzed as above. The results showed that the artificial increase of L-ferritin had no visible effect on the two indices of apoptosis; this also indicated that the presence or absence of Dox was without effect. On the other hand, the upregulation of H-ferritin reduced both the number of cells with fragmented nuclei and the accumulation of p85 PARP (Fig. 4C,D). Surprisingly, this occurred also with the clone that expresses the mutant H222 with an inactivated ferroxidase center. Iron treatment, which caused a 1.5-fold increase in H-ferritin level reduced the two indices of apoptosis by less than 50%, while the 4–6-fold increase in H- and H222 levels reduced the proportion of fragmented nuclei by 50–60% and the accumulation of p85 PARP by more than 70%.

It is known that TNFα activates anti-apoptotic transcriptional responses (Fig. 1). Our data indicate that TNFα induces also protective post-transcriptional responses that involves H-ferritin, which is upregulated by the cytokine and has a protective effect on apoptosis. The observation that H-ferritin induction is parallel to that of the L-ferritin and accompanied by a downregulation of TfR suggests that it is

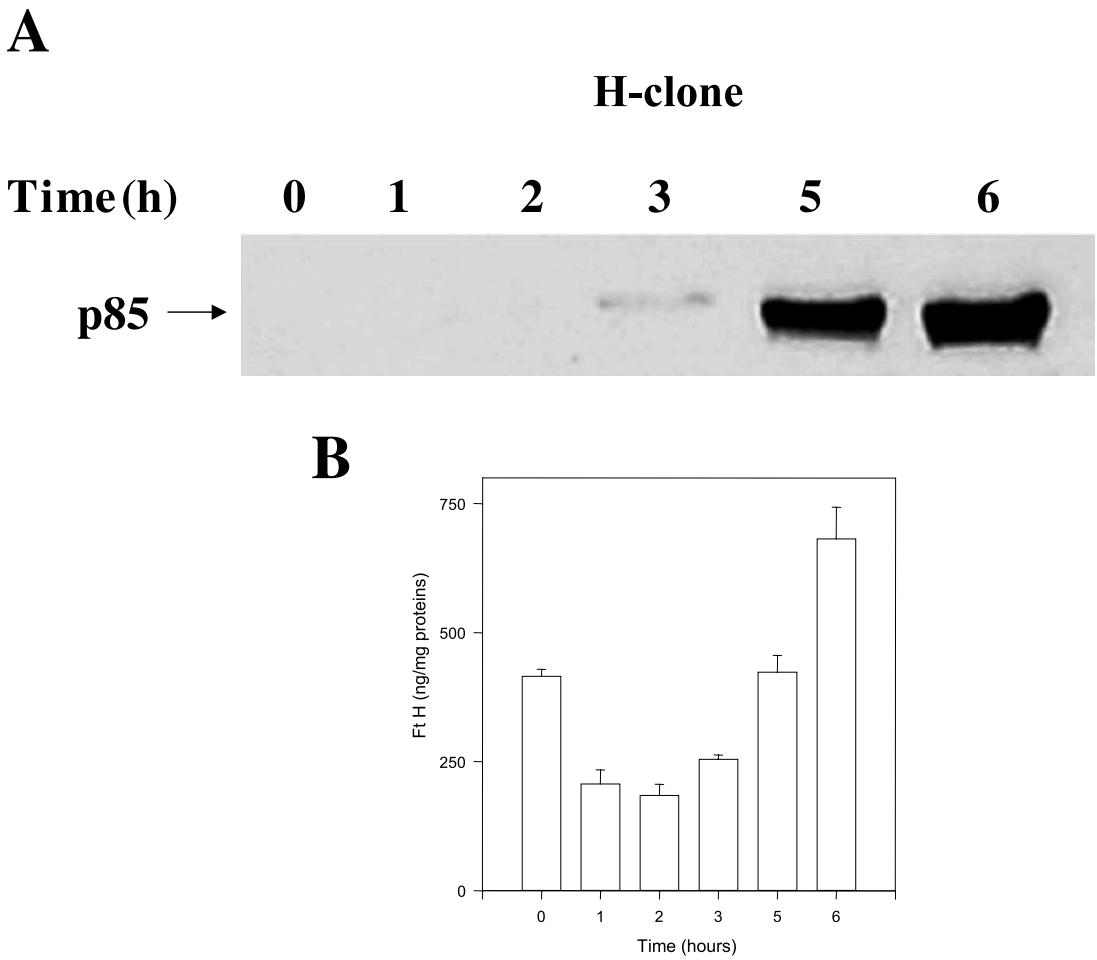

Fig. 3. Time course evaluation of p85 PARP and H-ferritin during incubation with TNFα/ActD. Panel A: Blotting analysis of p85 PARP peptide on the cell extracts of the cells harvested at the time indicated. Panel B: H-ferritin concentration of the same cell extracts. The data are means of three experiments and are expressed as ng of ferritin per mg of total proteins.

secondary to an increased iron availability, since these three proteins are tightly controlled by the IRE/IRPs machinery [19]. We postulate that the iron may originate from ferritin, which is degraded in the first hours of incubation with TNFα (Fig. 3B). Ferritin half-life in HeLa cells was found to be 12–14 h [16], therefore the finding that the amount of ferritin was halved just 1 h after exposure to the apoptotic stimuli implies the activation of a degradative mechanism. This was already observed in the rat liver during post-ischemic reoxygenation and proposed to be a consequence of the enhanced proteolytic activity that accompanies liver oxidative damage [20].

Probably, the major finding of this work was the observation that H-ferritin has an anti-apoptotic effect and that this is unrelated to its ferroxidase activity. The overexpression of mutant H222 in HeLa cells did not affect iron metabolism, and the protein was unable to incorporate iron in vivo [16]. Therefore its anti-apoptotic activity is unrelated to modifications of cellular iron availability.

Present findings are consistent with the reported cytoprotective activities of H-ferritin. It has been shown that the proliferative effect of c-myc requires the downregulation of H-ferritin [22], and that hemin-induced upregulation of H-ferritin protects cells from oxidative damage [10,11]. More recently, it has been shown that antisense ferritin oligonucleotides reduce ferritin levels in MCF-7 breast carcinoma cells and induce apoptosis [23]. It is possible that the anti-apoptotic activity of heme oxygenase in epithelial cells involves ferritin, which is induced by the iron arising from heme degradation [12,13]. In addition, we consider that the repression of ferritin synthesis caused by iron-chelating agents may have a role in the apoptosis they induce in HeLa cells [7–9]. Based on our

Table 1
H- and L-ferritin levels in the repressed and derepressed transfectant clones

Cells	H-ferritin (ng/mg proteins)	L-ferritin (ng/mg proteins)
H-clone, Dox+	433 ± 79	32 ± 6
H-clone, Dox−	1623 ± 47	13 ± 2
L-clone, Dox+	235 ± 44	25 ± 6
L-clone, Dox−	245 ± 42	161 ± 44
H222 clone, Dox+	320 ± 16	36 ± 4
H222 clone, Dox−	1464 ± 80	8 ± 2

The clones were grown for more than 7 days in the presence (Dox+) or the absence of doxycycline (Dox−) to regulate the expression of the exogenous ferritin, and the homogenates were analyzed for H-ferritin and L-ferritin content using specific ELISA assays. Data are expressed as ng of ferritin per mg of total proteins. Means and S.D. from at least three independent experiments. The induced cells contain 4–6-fold more exogenous ferritin than the non-induced ones.

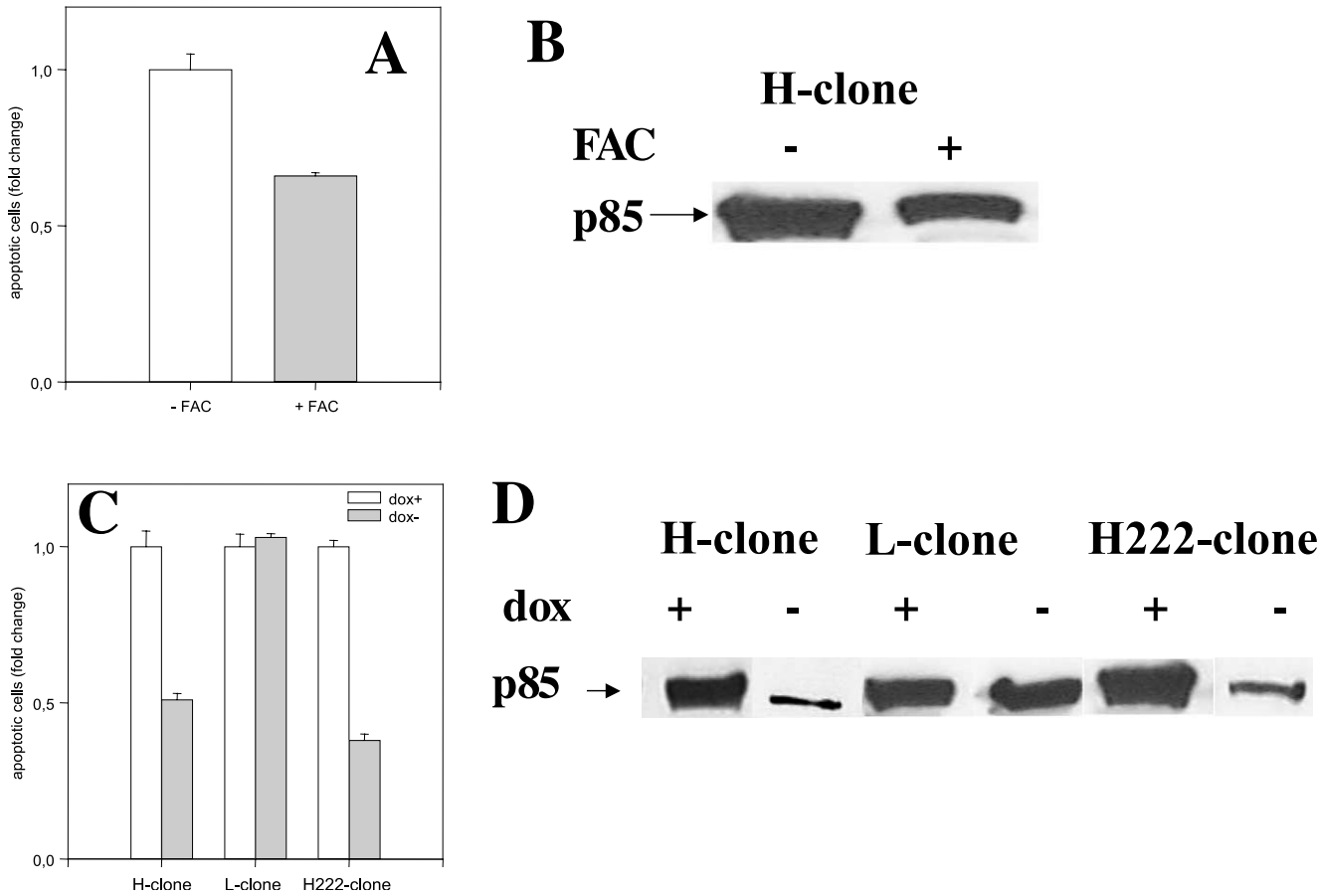

Fig. 4. Iron and ferritin effects on a TNFα-induced apoptosis. Panels A,B: Iron effects: HeLa cells were incubated first for 24 h with 100 μM FAC and then for 6 h with TNFα/ActD. The fraction of apoptotic cells was determined by nuclear fragmentation using DAPI stain (panel A) and by blotting of the cell homogenates with anti-p85 PARP antibody (panel B). Panels C,D: Ferritin effects. The HeLa cells clones that express H-ferritin (H-clone), L-ferritin (L-clone) and the mutant H222 (H222-clone) were grown on the presence of doxycycline (Dox+) to repress the synthesis of the exogenous ferritins, or in the absence of it (Dox−) to induce ferritin expression to the levels reported in Table 1. The cells were then incubated for 6 h with TNFα/ActD and the level of apoptosis detected by nuclear fragmentation using DAPI stain (panel C) or by blotting with p85 PARP antibody (panel D).

present data, we suggest that the anti-apoptotic activity of ferritin observed in these different experimental models is unrelated to the capacity of ferritin to regulate cellular iron availability, but rather is due to the presence of the H-ferritin subunit 'per se'. The substituted amino acids in the H222 variant are buried inside the subunit structure and the molecular surfaces of H-wild type and mutants are probably identical, but differ from that of L-ferritin that has no effect on apoptotic pathways. This suggests that the H-ferritin activity may involve a physical interaction with molecules of the apoptosis pathway. A physical association between H-ferritin and Bax has been recently indicated [24], and its role needs to be further clarified.

In conclusion, the present study indicates that the apoptosis pathways elicited by TNFα in HeLa cells involve modifications of cellular iron homeostasis, and that H-ferritin participates in the pathways independently from its ferroxidase activity.

Acknowledgements: We are grateful to Dr. A. Corti for the kind gift of recombinant TNFα and to Prof. G. Cairo for helpful discussions. The work was partially supported by MURST-Cofin 2001 and 2002 to P.A. and by Telethon-Italy (Grant GP0075Y01) to S.L.

References

[1] Harrison, P.M. and Arosio, P. (1996) Biochim. Biophys. Acta 1275, 161–203.
[2] Torti, F.M. and Torti, S.V. (2002) Blood 99, 3505–3516.
[3] Ashkenazi, A. (2002) Nat. Rev. Cancer 2, 420–430.
[4] Gupta, S. (2001) Curr. Mol. Med. 1, 317–324.
[5] Feger, F., Ferry-Dumazet, H., Mamani Matsuda, M., Bordenave, J., Dupouy, M., Nussler, A.K., Arock, M., Devevey, L., Nafziger, J., Guillosson, J.J., Reiffers, J. and Mossalayi, M.D. (2001) Cancer Res. 61, 5289–5294.
[6] Kim, Y.M., Chung, H.T., Simmons, R.L. and Billiar, T.R. (2000) J. Biol. Chem. 275, 10954–10961.
[7] Abeysinghe, R.D., Greene, B.T., Haynes, R., Willingham, M.C., Turner, J., Planalp, R.P., Brechbiel, M.W., Torti, F.M. and Torti, S.V. (2001) Carcinogenesis 22, 1607–1614.
[8] Greene, B.T., Thorburn, J., Willingham, M.C., Thorburn, A., Planalp, R.P., Brechbiel, M.W., Jennings-Gee, J., Wilkinson, J., Torti, F.M. and Torti, S.V. (2002) J. Biol. Chem. 277, 25568–25575.
[9] Simonart, T., Boelaert, J.R., Mosselmans, R., Andrei, G., Noel, J.C., De Clercq, E. and Snoeck, R. (2002) Gynecol. Oncol. 85, 95–102.
[10] 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.
[11] Vile, G.F., Basu-Modak, S., Waltner, C. and Tyrrell, R.M. (1994) Proc. Natl. Acad. Sci. USA 91, 2607–2610.

[12] Ferris, C.D., Jaffrey, S.R., Sawa, A., Takahashi, M., Brady, S.D., Barrow, R.K., Tysoe, S.A., Wolosker, H., Baranano, D.E., Dore, S., Poss, K.D. and Snyder, S.H. (1999) Nat. Cell. Biol. 1, 152–157.
[13] Petrache, I., Otterbein, L.E., Alam, J., Wiegand, G.W. and Choi, A.M. (2000) Am. J. Physiol. Lung Cell. Mol. Physiol. 278, L12–L19.
[14] Brouard, S., Berberat, P.O., Tobiasch, E., Seldon, M.P., Bach, F.H. and Soares, M.P. (2002) J. Biol. Chem. 277, 17950–17961.
[15] Arosio, P. and Levi, S. (2002) Free Radic. Biol. Med. 33, 457–463.
[16] Cozzi, A., Corsi, B., Levi, S., Santambrogio, P., Albertini, A. and Arosio, P. (2000) J. Biol. Chem. 275, 25122–25129.
[17] Liu, X., Li, P., Widlak, P., Zou, H., Luo, X., Garrard, W.T. and Wang, X. (1998) Proc. Natl. Acad. Sci. USA 95, 8461–8466.
[18] Duriez, P.J. and Shah, G.M. (1997) Biochem. Cell. Biol. 75, 337–349.
[19] Cairo, G., Recalcati, S., Pietrangelo, A. and Minotti, G. (2002) Free Radic. Biol. Med. 32, 1237–1243.
[20] Tacchini, L., Recalcati, S., Bernelli-Zazzera, A. and Cairo, G. (1997) Gastroenterology 113, 946–953.
[21] Orino, K., Lehman, L., Tsuji, Y., Ayaki, H., Torti, S.V. and Torti, F.M. (2001) Biochem. J. 357, 241–247.
[22] Wu, K.J., Polack, A. and Dalla-Favera, R. (1999) Science 283, 676–679.
[23] Yang, D.C., Jiang, X., Elliott, R.L. and Head, J.F. (2002) Anticancer Res. 22, 1513–1524.
[24] Chang, C.M., Fitch, M.E., Yuan, M., Parslow, T.G., Sgu, H.G. (2002) Proceedings of the 43rd Annual Astro Meeting, New Orleans, LA, 6–10 Oct. 2002, p. 189. Abstract.