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Heat shock protein 27 downregulates the transferrin receptor 1-mediated iron uptake

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Abstract

It has been reported that over-expression of human heat shock protein 27 (hsp27) in murine cells decreased the intracellular iron level [Arrigo, A. P., Virot, S., Chaufour, S., Firdaus, W., Kretz-Remy, C., & Diaz-Latoud, C. (2005). Hsp27 consolidates intracellular redox homeostasis by upholding glutathione in its reduced form and by decreasing iron intracellular levels. *Antioxidants & Redox Signalling*, *7*, 412–422]. However, the mechanism involved is unknown. In this study, the regulation of transferrin receptor 1 (TfR1)-mediated iron uptake by human hsp27 was investigated in CCL39 cells by overexpression of human hsp27 and its dominant-negative mutant (hsp27-3G). The results showed that overexpression of hsp27 diminished intracellular labile iron pool, increased the binding activity of iron regulatory protein (IRP) to iron responsive element (IRE) and the cell surface-expressed TfR1s. However, the increased surface-expressed TfR1s resulted in decrease rather than increase of iron uptake. Further study revealed that overexpression of hsp27 decelerated transferrin endocytosis and recycling, while overexpressed hsp27-3G had a reversal effect. Moreover, flowcytometric analysis showed an enhanced actin polymerization in the cells overexpressing hsp27. In particular, fluorescence imaging of cytoskeleton displayed highly stabilized microfilaments and preferential localization of hsp27 in cortical area of the actin cytoskeleton. In contrast, disruption of actin cytoskeleton by cytochalasin B resulted in acceleration of the endocytosis and recycling of Tf, as well as increase of iron uptake. Meanwhile, the possible involvement of ferroportin 1 in down-regulation of intracellular iron level by overexpression of hsp27 was checked. However, the outcome was negative. Our findings indicate that hsp27 down-regulates TfR1-mediated iron uptake via stabilization of the cortical actin cytoskeleton rather than the classical IRP/IRE mode. The study may also imply that hsp27 protects cells from oxidative stress by reducing cellular iron uptake.

Keywords: Heat shock protein 27; Transferrin receptor-mediated iron uptake; Transferrin recycling; Iron regulatory protein; Actin polymerization

1. Introduction

Iron is an element required for many metabolic processes in all eukaryotes and most prokaryotes. Besides synthesis of hemoglobin, iron is also essential for cell cycling, synthesis of DNA and some other important enzymes (Lieu, Heiskala, Peterson, & Yang, 2001). Iron-deficiency causes many diseases, such as anemia. On

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the other hand, iron overload in human body causes cell death and tissue damage through iron-catalyzed Fenton chemistry (Halliwell & Gutteridge, 1990). In particular, abnormally high levels of iron in the brain have been found in a number of neurodegenerative disorders, including Parkinson's and Alzheimer's disease (Aisen, Wessling-Resnick, & Leibold, 1999). While the epidemiological and clinical studies on whether iron is a risk factor for heart disease have demonstrated conflicting results, it is still highly speculated that iron might play a role in cardiac disease (Patricia & Alpert, 2004). Therefore, understanding the molecular mechanisms of cellular iron uptake and homeostasis is of physiological importance.

Cells acquire iron via transferrin (Tf)-dependent and -independent pathways. Although the intestine absorbs iron from food via divalent metal transport 1 (DMT1) (Gunshin et al., 1997) and the differentiating epithelial cells in the earliest stages of embryonic development take up iron via neutral gelatinase-associated lipocalin-mediated iron delivery (Yang et al., 2002), the transferrin receptor-dependent pathway is still considered to be the main route of cellular uptake after iron is exported from intestine (Kaplan, 2002). There are two types of transferrin receptor, TfR1 and TfR2. TfR1 is ubiquitously expressed receptor for Tf that delivers iron to cells (Richardson & Ponka, 1997). TfR2 is a type II transmembrane protein and homolog of TfR1, and has limited tissue distribution (Fleming et al., 2000; Kawabata et al., 1999). Like TfR1, TfR2 binds Tf at neutral pH. However, differences in the activity, regulation, and expression of TfR1 and TfR2, and in the pathophysiology of disorders caused by their deficiency, indicate that they have different roles in iron homeostasis. The affinity of TfR2 for Tf at pH 7.5 is approximately 25-fold lower than that of TfR1 (West et al., 2000).

Unlike TfR1, TfR2 mRNA expression does not change in cells treated with $Fe_2(NO_3)$ or the iron chelator desferrioxamine (Kawabata et al., 2000), nor does it change in iron-deficient or iron-overloaded mice (Fleming et al., 2000). In humans homozygous for the Y250X TfR2 mutation and mice transgenic for the orthologous Y245X mutation, the liver accumulates iron, despite an absence of membrane-bound TfR2 and a reduction in TfR1 suggesting that the uptake of Tf-bound iron for use by the hepatocytes is not the principal role of TfR2 (Fleming et al., 2002).

It has been established that the level of intracellular iron regulates the expression of many key molecules that participate in iron metabolism in cells via a feedback regulatory mechanism. Two iron regulatory proteins, IRP1 and IRP2, were found to regulate the expression of TfR1 and ferritin by binding to the iron responsive elements (IREs) in the 3′-untranslated region in the mRNA of TfR1 (Mullner, Neupert, & Kuhn, 1989) and the 5′-untranslated region in the mRNA of ferritin (Goossen, Caughman, Harford, Klausner, & Hentze, 1990). Iron depletion enhances the binding of IRPs to IREs, inhibits ferritin mRNA translation and stabilizes TfR1 mRNA. These changes resulting from iron depletion lead to decreased iron sequestration into ferritin and enhanced iron uptake through TfR1. On the other hand, iron repletion inactivates IRP-1 and leads to degradation of IRP-2 which results in an efficient translation of ferritin mRNA and rapid degradation of TfR1 mRNA. Besides TfR1 and ferritin, some other iron metabolism-related proteins that possess IREs in their mRNAs are also regulated through the IRE/IRPs mode. These proteins include mitochondria aconitase (Kim, LaVaute, Iwai, Klausner, & Rouault, 1996), the iron–sulfur subunit of succinate dehydrogenase (Kohler, Henderson, & Kuhn, 1995) and DMT1 (Fleming et al., 1998). However, it is unclear whether the IRP/IRE regulatory mode is ubiquitous and the determinant regulation mechanism for intracellular iron homeostasis.

Heat shock protein 27 (hsp27) belongs to the family of stress proteins. Its expression increases following heat shock (Gething & Sambrook, 1992), oxidative stress (Marini, Frabetti, Musiani, & Franceschi, 1996) or stimulation by cytokines including tumor necrosis factor (Mehlen, Briolay et al., 1995; Mehlen, Preville et al., 1995) and basic fibroblast growth factor (Kozawa et al., 2001). In general, hsp27 has been thought to serve multiple functions in a variety of cell types. It acts as an ATP-independent chaperone that interacts with misfolded or oxidized polypeptides (Jakob, Gaestel, Engel, & Buchner, 1993) and up-regulates several key enzymes involved in the reactive oxygen species (ROS) (Arrigo, 2001; Preville et al., 1999). It protects cells from apoptosis induced by various environmental factors such as heat and H_2O_2 (Mehlen, Briolay et al., 1995; Mehlen, Preville et al., 1995). In addition to enhancing stress tolerance, hsp27 also plays an important role in regulation of actin cytoskeleton by phosphorylation (Schafer, Clapp, Welsh, Benndorf, & Williams, 1999).

Iron is a transition metal capable of generating $^{\bullet}OH$ radicals, the most potent reactive oxygen species. Based on epidemiological evidence citing excess iron as a risk factor for many diseases, the iron-induced oxidative stress has been considered as a key event in the pathogenic process of diseases (Reddy & Clark, 2004). Interestingly, tissue distribution comparisons demonstrated that high levels of hsp27 were present in heart and skeletal muscle (Voss et al., 2003). Very recently, it was

reported that over-expression of human hsp27 in murine L929 cells decreased the intracellular level of iron by almost 50% (Arrigo et al., 2005). However, the mechanism responsible for decreasing intracellular iron level is unknown. The investigators speculated that the responsible mechanism could be related to an inhibitory effect of hsp27 on the action of iron-regulatory proteins (Arrigo et al., 2005). Thus, the first goal of this investigation was to examine if the IRP/IRE regulatory mode is responsible for the decrease of intracellular iron level by hsp27. If it is not, is there any other mechanism involved in the hsp27-induced reduction of intracellular iron? Two cell model systems may be chosen to study the effect of hsp27 on iron uptake. One is myocardial or skeletal muscle cells because of their higher cellular level of hsp27. However, the study may face difficulty in choosing proper control that should be a similar cell type with low hsp27 expression. The other approach is to use a cell type that has lower or, at least normal, endogenous hsp27 level, and the same type of cells transfected with hsp27 as control. Thus, the Chinese hamster lung fibroblast CCL39 cells, which has relative low hsp27 content, transfected with human hsp27 or its functional-dead mutant were used as a model system to investigate how hsp27 downregulates the iron uptake and iron homeostasis.

2. Materials and methods

2.1. Reagents

Calcein acetoxymethyl ester (calcein-AM), biotin-XX-conjugated transferrin (BXX-Tf), rhodamine-phalloidin and Bodipy FL phallacidin were purchased from Molecular Probes. Human apo-transferrin, lysopalmitoylphosphatidylcholine (LPC), cytochalasin B and biocytin were obtained from Sigma. Goat anti-human transferrin serum was purchased from Bethyl Laboratories Inc. Salicylaldehyde hydrazone (SIH) was a gift from Prof. Prem Ponka (McGill University, Montreal). Recombinant human hsp27 is kindly provided by Dr. Lee A. Weber (Department of Biology, University of Nevada, Nevada). Avidin was bought from Rockland Immunochemicals Inc. Streptavidin-conjugated HRP was purchased from Vector Lab. AT7 Transcription Kit was purchased from MBI Fermentas. The ^{32}P-labeled uridine triphosphate and the ^{55}Fe (in the ferric chloride form) were obtained from Beijing Furui Bioengineering Company and Perkin-Elmer Life Sciences Inc., respectively. Rabbit anti-hamster hsp27 polyclonal antibody and rabbit anti-human hsp27 polyclonal antibody were purchased from Upstate and Santa Cruz, respectively. Anti-mouse ferroportin1 polyclonal antibody was obtained from Alpha Diagnostic Intl. Inc. (San Antonio). The plasmid pSPT-fer, containing the sequence corresponding to the IRE of the H-chain of human ferritin mRNA, was kindly provided by Dr. Gaetano Cario (University of Milan, Italy).

2.2. Cloning of stable Hsp27-expressing cell lines

The plasmid pcDNA3.1, pcDNA3.1-hsp27 and pcDNA3.1-hsp27-3G containing the neomycin resistance gene, the wild-type human hsp27 gene and mutated human hsp27 gene were transfected in Chinese hamster lung fibroblast cells (CCL39), respectively, using Lipofectamine 2000. Four monoclonal cell lines, namely the CCL39 cells expressing only neomycin, and the cells overexpressed and highly overexpressed hsp27, as well as the cells overexpressed hsp27-3G, were established by selecting the transfectants with G418 (400 μg/ml) for 4 weeks and screened by immunoblotting analysis of hsp27 expression. They were referred to as neo, hsp27-overexpressed, hsp27-highly-expressed and hsp27-3G-expressed cells, respectively. The cells were maintained at 37 °C in DMEM containing 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin.

2.3. Immunoblotting analysis of hsp27 and ferroportin 1

The cells were lysed with lysis buffer (10 mM HEPES, 3 mM MgCl2, 40 mM KCl, 5% glycerol, 1 mM DTT, 0.2% Nonidet P-40 and 1 mM PMSF, pH 7.5), followed by centrifugation at 12,000 × *g* for 5 min at 4 °C. Protein contents of the clear lysates were determined using the Bio-Rad protein assay kit. The lysate was mixed with equal volume of SDS buffer (2% SDS, w/v, 10% glycerol, 5% β-mercaptoethanol, 0.025% bromophenol blue, w/v, 0.125 mM Tris–HCl, pH 6.8), and then heated at 95–100 °C for 5 min. The cell extracts with equal protein content (10 μg proteins) were separated on a 12% SDS-polyacrylamide gel, and then transferred onto nitrocellulose membranes. The membranes were blocked in PBS containing 5% glycerol, 5% non-fat milk, 0.5‰ Tween 20 for 1 h at 37 °C, incubated with anti-human hsp27 antibody or anti-hamster hsp27 antibody for 4 h, and then with HRP-conjugated goat anti-rabbit IgG for 2 h. The resulting immune-complexes were visualized using a chemiluminescence detection kit. The same procedure was used for immunoblotting analysis of ferroportin 1 in the cell extract except for that instead of protein content assay immunoblotting detection of actin in the loaded samples was used as controls.

2.4. Detection of apoptotic DNA fragmentation

1×10^6 cells were seeded in 25-cm^2 flasks and exposed to 200 μM H_2O_2 for 1.5 h. After 30 h incubation with DMEM medium, the cells were harvested, washed twice with PBS, and lysed in 20 μl lysis buffer (100 mM Tris–HCl (pH 7.8), 20 mM EDTA and 0.8%, w/v, sodium dedecyl sulfate). The lysate was incubated at 37 °C for 30 min after addition of 10 μl of RNAse A (10 mg/ml), and then incubated for 2 h at 50 °C after addition of 10 μl proteinase K (20 mg/ml). Eight microliters of the mixture was subjected to 2% agarose gel electrophoresis in TBE buffer (45 mM Tris, 45 mM boric acid and 1 mM EDTA, pH 8.0). The DNA fragments were visualized by ethidium bromide staining.

2.5. Measurement of transferrin-bound ^{55}Fe uptake

The uptake of transferrin-bound iron by CCL39 cells and their derivatives was studied using ^{55}Fe-loaded human apo-Tf (Roy, Penny, Feder, & Enns, 1999). Cells were plated in dishes (3×10^5 cells/dish) and grown overnight in a complete DMEM medium. The cells were initially incubated with serum-free DMEM medium containing 0.1% BSA (referred as BSA-DMEM) at 37 °C for 1 h to remove endogenous Tf, and then with 5.0 μg/ml ^{55}Fe-Tf in the same medium at 37 °C for various durations. At indicated times, the cellular iron uptake was stopped by adding ice-cold acidic buffer (0.2 M acetic acid, 500 mM NaCl and 1 mM $FeCl_3$) for 3 min on ice. After centrifugation to remove cell surface-bound radioactive ^{55}Fe-Tf, the cells were washed again with ice-cold PBS, lysed in 1% SDS, and counted for 10 min in a scintillation counter (Perkin-Elmer). The counts represent the ^{55}Fe taken up by the cells.

2.6. Determination of the labile iron pool (LIP)

Intracellular LIP was determined according to Epsztejn, Kakhlon, Glickstein, Breuer and Cabantchik (1997). Briefly, cells (2×10^6 cells/ml) were incubated with 0.25 μM calcein-AM for 10 min at 37 °C in a bicarbonate-free medium (pH 7.3) containing 1 mg/ml BSA, 0.1 M NaCl and 20 mM HEPES. After washing with PBS, the cells were re-suspended in the same medium at room temperature for 30 min to allow for sufficient de-esterification of the indicator. Prior to measurements, the cells were re-suspended in 2 ml of pre-warmed Hank's balanced salt solution in a thermostatic stirred cuvette (37 °C). Fluorescence of the calcein-loaded cells was monitored using a Hitachi F-4500 spectrophotometer (excitation 485 nm; emission 520 nm). Trypan blue (25 μg/ml) was added to quench the fluorescence of the extracellular calcein. After a dropped baseline was established, 100 μM SIH, the highly permeable iron chelator, was added. The fluorescence increase caused by SIH was recorded. Since calcein is insensitive to Ca^{2+} and Mg^{2+} up to 1 mM at physiological pH and the intracellular concentration of other calcein-binding molecules is very low, it is generally accepted that the SIH-induced fluorescence rise represents calcein-bound iron, or intracellular LIP level.

2.7. RNA–protein gel retardation assay of the IRP binding activity

The binding activity of IRP to IRE was determined as previously described (Kim & Ponka, 1999). Briefly, cytoplasmic extracts containing 20 μg proteins were analyzed for IRP binding activity by incubation with excess ^{32}P-labeled RNA transcripts. These RNA were transcribed in vitro from a linearized plasmid pSPT-fer template using T7 RNA polymerase in the presence of [α-^{32}P] UTP. After 10 min incubation at room temperature, 5 mg/ml heparins were added to prevent non-specific binding. IRP-RNA complexes were resolved in 6% non-denaturing polyacrylamide gels and radioactivities of the complex bands were quantified with a PhosphorImager system (Amersham Pharmacia Biotech). In parallel experiments, samples were routinely treated with 2% β-mercaptoethanol prior to the addition of the IRE probe to allow full expression of IRP binding activity.

2.8. Analysis of actin polymerization

The content of filamentous actin (F-actin) was analyzed by flow cytometry after staining with F-actin specific fluorescent dye, Bodipy FL phallacidin. In brief, cells were detached by PBS containing 0.25% Trypsin and 20 mM EDTA, washed twice with PBS, and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. The cells (1×10^6) were then simultaneously permeabilized with LPC (50 μg/ml) and loaded with Bodipy FL phallacidin (1.5 units/ml) for 30 min at room temperature in the dark. The cells were washed twice more and re-suspended in PBS. 10^4 cells of each sample were analyzed on a Coulter Epics Flow Cytometer. The actin polymerization was measured as cell number distribution against the fluorescence of the F-actin probe per cell.

2.9. Assay for continuous internalization of transferrin

Cells were detached with PBS containing 5 mM EDTA and incubated in BSA-DMEM for 1 h to remove endogenous transferrin. After washing, the cells were suspended in ice-cold PBS containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2% BSA and 5 mM glucose (referred as PBS-plus buffer) at 3×10^6 cells/ml. After addition of BXX-Tf (8 μg/ml), the cell suspension was split into aliquots of 100 μl volume containing 3×10^5 cells, and then rapidly warmed up to 37 °C to initiate internalization of BXX-Tf. At indicated times, the samples returned to ice to stop endocytosis. The internalized BXX-Tf was quantified using a slightly modified version of the ELISA-based avidin inaccessibility measurement (Carter, Redelmeier, Woollenweber, & Schmid, 1993).

Tetramethylbenzidine-substituted o-phenylenediamine in substrate solution, and absorbance of the colored reaction mixtures was read at 450 nm. The internalized BXX-Tf was normalized as the percentage of total surface-bound BXX-Tf.

2.10. Assay of transferrin exocytosis

Cells were collected with PBS containing 5 mM EDTA and incubated in BSA-DMEM for 1 h at 37 °C to remove endogenous transferrin. The cells were incubated with 16 μg/ml BXX-Tf in the PBS-plus buffer at 37 °C for 2 h to reach equilibrium between endocytosis and exocytosis of the transferrin. Thereafter, the cells were kept on ice and washed three times with ice-cold PBS. The cell surface-associated BXX-Tf were masked by addition of 50 μg/ml avidin, followed by shaking for 15 min at 4 °C. The excess avidin was quenched by adding 50 μg/ml biocytin. The cells were then re-suspended in the PBS-plus buffer containing 20 μg/ml unlabeled Tf, and incubated at 37 °C for indicated durations. The BXX-Tf that returned to the cell surface during recycling was again masked with avidin. The BXX-Tf remaining in the cells was quantified by utilizing the same procedure as that used in the continuous internalization assay. Results are expressed as the percentage of the initial intracellular BXX-Tf that still remains inaccessible to avidin during the incubation.

2.11. Determination of the transferrin receptors on cell surface

Cells were incubated with 16 μg/ml BXX-Tf on ice for 2 h, and then washed three times with ice-cold PBS-plus buffer to remove excess ligands. The cells were then lysed with 0.1 ml blocking buffer (10 mM Tris–HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100 and 0.2% BSA). The total cell-associated BXX-Tf was quantified by ELISA as described in literature (Carter et al., 1993).

2.12. Immunofluorescence imaging of the spatial distribution of human hsp27 and F-actin

The hsp27-overexpressed cells were seeded in dishes and cultured in DMEM until 80% confluence. The cells were then fixed with 3.7% paraformaldehyde in PBS for 30 min. After three washes with PBS, the cells were permeabilized for 10 min with 0.2% Triton X-100 in PBS, and then blocked with PBS containing 10% fetal calf serum and 0.5% non-fat milk for 1 h. The cells were then incubated with 2 μg/ml rabbit anti-human hsp27 polyclonal antibody overnight at 4 °C. After washing with PBS, the cells were incubated with 100-fold diluted FITC-labeled secondary antibody for 1 h and again washed with PBS. Hoechst 33342 (2 μg/ml) and rhodamine-phalloidin (1 U/ml) were added to stain nuclei and actin filaments. The stained cells were imaged on an IX/71 type Olympus microscope equipped with AquaCosmos Microscopic Image Acquisition and Analysis System (Hamamatsu Photonics K.K. Japan).

The sequential images of the Hoechst 33342-labeled cell nuclei, the FITC-labeled human hsp27 and the rhodamine-labeled F-actin in the cells were taken on 10 focal planes moving from the lowest to highest Z-position by a Z-focus unit with a step of 1 μm at the excitation of 360, 460 and 540 nm, respectively. Deconvolution software was applied to remove optical blur and haziness in fluorescence images usually caused by fluorescence coming from locations other than the focal plane. With the software, the images were improved as pseudo-confocal images.

2.13. Statistics

Student's *t*-test was applied for comparison of the data that are expressed as means ± S.D.

3. Results

3.1. Expression of human hsp27 in the studied cells

The endogenous hsp27 expression in Chinese hamster cell lines was reported as about 1 ng/μg of the

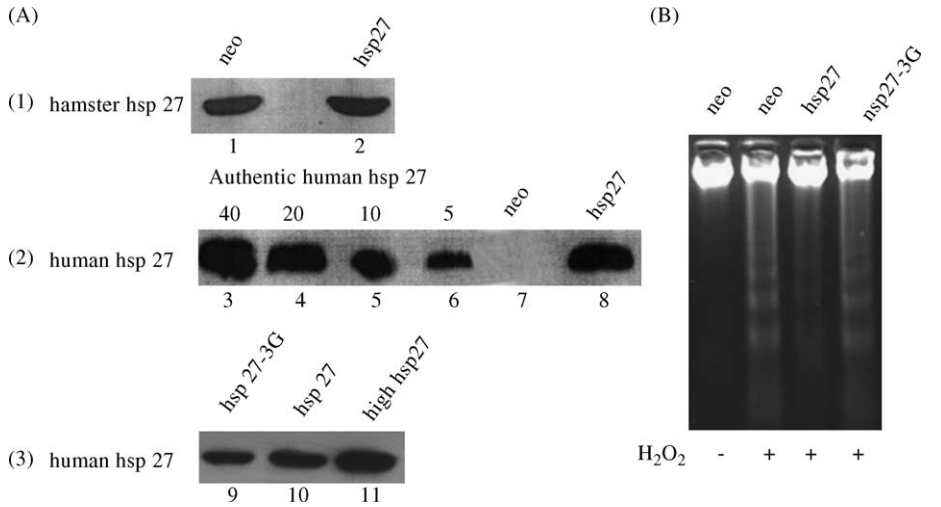

Fig. 1. Expression of hsp27 and H_2O_2-induced apoptosis in the neo, the hsp27-overexpressed, the hsp27-highly-overexpressed and the hsp27-3G expressed CCL39 cells. (A) Immunoblot of human and hamster hsp27 content in cell extracts containing 10 μg of total proteins. (1) Immunoblot of the endogenous hamster hsp27 probed by anti-hamster hsp27 antibody in the lysate of the neo and the hsp27-overexpressed cells, respectively. (2) Immunoblot of the human hsp27 probed by anti-human hsp27 antibody in the lysate of the neo and the hsp27-overexpressed cells and the authentic samples containing 40, 20, 10 and 5 ng of recombinant human hsp27, respectively. (3) Immunoblot of the protein probed by anti-human hsp27 antibody in the lysate of the cells overexpressing hsp27-3G, moderately and highly overexpressing the wild-type human hsp27, respectively. The three immunoblots were obtained in separate experiments. Owing to difference in exposure to chemiluminescence from immune-complex and development of photo film, the densities and sizes of the blots are only comparable within same immunoblot. (B) The H_2O_2-induced DNA fragmentation in the neo, the hsp27-overexpressed and the hsp27-3G-expressed cells. The neo cells without exposure to H_2O_2 were used as a control. The DNA fragmentation patterns are representative of three independent determinations.

total protein (Lavoie, Hickey, Weber, & Landry, 1993). As immunoblotting analysis shows, the endogenous hamster hsp27 contents in the neo and the hsp27-overexpressed cells were almost same (see Fig. 1A-1). About 2 ng/μg of total proteins was estimated for human hsp27 in the hsp27-overexpressed cells by comparison with the authentic samples. However, no human hsp27 was detected in the neo cells, suggesting that the used anti-human hsp27 antibody does not cross-react with hamster hsp27 (see Fig. 1A-2). The protein level of the mutant hsp27 in hsp27-3G-expressed cells was also about 2 ng/μg of total protein, while the human hsp27 content in the highly-overexpressed cells was about 1.5-fold of that in the hsp27 overexpressed cells (see Fig. 1A-3). To determine whether the overexpressed hsp27 is functionally active, the H_2O_2-induced apoptosis was observed in the neo, the hsp27-overexpressed and hsp27-3G-expressed cells. The apoptotic DNA fragmentation patterns in these three types of cells show that the transfected wild-type human hsp27 but not its mutant can well protect the cells from H_2O_2-induced apoptosis (Fig. 1B). This indicates that overexpressed wild-type human hsp27 in these cells is functionally active, while the mutant hsp27-3G is functional-dead.

3.2. *Hsp27 reduces the transferrin receptor (TfR1)-mediated iron uptake*

Many investigators have used human transferrin to study TfR1-mediated iron uptake in Chinese hamster cells. This is not only because hamster transferrin is not commercially available, but also because of the similar affinity of human transferrin to either human or hamster TfR1 (Harikumar et al., 2005; Soulet et al., 2002; Szászi et al., 2002; Waheed et al., 2002). Thus, iron uptake was investigated using ^{55}Fe-bound human Tf in the neo cells, the cells expressing human hsp27 at two different levels, and the cells expressing hsp27-3G. Fig. 2 shows that the cells overexpressing and highly-overexpressing human hsp27 took up 18% and 33% less ^{55}Fe, respectively, within 2 h, while the cells expressing functional-dead mutant hsp27-3G took up 19% more ^{55}Fe as compared with the neo cells. The up-regulation of iron uptake by hsp27-3G may imply that only phosphorylated hsp27 down-regulates the TfR1-mediated iron uptake. The mutant hsp27-3G, in which the Ser-15, Ser-78 and Ser-82 on phosphorylation site were mutated to Gly, is unable to be phosphorylated, and even suppresses phosphorylation of the endogenous hamster hsp27 by competition for the kinase

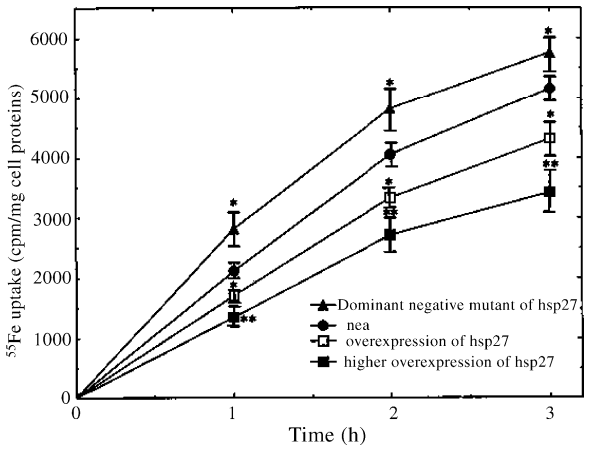

Fig. 2. Effect of hsp27-overexpression on the uptake of the transferrin-bound ^{55}Fe. 5 μg/ml ^{55}Fe-transferrin (1.5×10^4 cpm/μg Tf) was incubated with the neo (●), the hsp27-overexpressed (□), the hsp27-highly-overexpressed (■) and the hsp-3G-expressed (▲) CCL39 cells at 37 °C for various durations. The entered radioactivity in cells was measured by liquid scintillation counter. The amount of iron taken up by the cells is expressed as the counts of ^{55}Fe per milligram of total cell protein. Results are expressed as means ± S.D. of three independent experiments, and each experiment was performed in triplicate. '*' and '**' indicate the data are significantly ($p < 0.05$) and very significantly ($p < 0.01$) different from the data obtained from the neo cells.

that phosphorylates hsp27 in the cells overexpressing hsp27-3G.

To verify that the uptake of the transferrin-bound ^{55}Fe by the cells is really through the binding to transferrin receptors, 100-fold excess non-radioactive Tf was added in the cells-perfused medium prior to addition of the ^{55}Fe-Tf. It was found that pre-occupation of TfRs by non-radioactive Tf dramatically reduced the cellular uptake of Tf-bound ^{55}Fe (data not shown), indicating that the iron uptake is really TfR-mediated.

3.3. Hsp27 reduces LIP, increases IRP/IRE binding activity and TfR1 expression on cell surface

The intracellular labile iron pool (LIP) is a pool of chelatable and redox-active iron, which is transitory and serves as a crossroad for cell iron metabolism. Depletion of intracellular iron leads to a decrease in LIP (Kakhlon & Cabahtchik, 2002), which will be sensed by IRPs, resulting in a decrease of ferritin expression and an increase of surface transferrin receptors. To determine if less iron was taken up by the hsp27-overexpressed cells due to regulation by IRP, the LIP, IRP/IRE binding activity and the surface expression of the TfR1s were examined.

Fig. 3A shows the LIP in the neo and the hsp27-overexpressed cells. As compared with the neo cells, a significantly less rise of the calcein fluorescence was observed when SIH was added in the suspension of the calcein-loaded hsp27-overexpressed cells, indicating that a smaller LIP exists in the cells overexpressing human hsp27. The right columns and data of dequenched fluorescence represent the relative size of LIP in both type of cells calculated based on three independent measurements.

The IRP/IRE binding activity was determined as a complex of IRP with ^{32}P-laeled IRE probe in the reaction mixture containing the ^{32}P-laeled IRE probe and the cell extract from the hsp27-overexpressed cells and the neo cells, respectively. The results are shown in Fig. 3B. Only a single band of RNA–protein complex was observed, which is probably due to inability of the performed band shift assay to separate the IRP1 from the IRP2. Statistical analysis of the data from three independent band shift assays showed that the IRP/IRE binding activity was enhanced by 25% in the cells overexpressing hsp27 (see Fig. 3C), implying that more transferrin receptors would be expressed in the hsp27-overexpressed cells if the IRP/IRE regulatory mode works. To confirm that the difference in the binding activity of IRP1 was not due to lower expression of the IRP1 but lower IRP1 binding activity, the cell extracts of equal protein content were treated with 2-mercaptoethanol, which can fully activate latent IRP1 (Schalinske et al., 1997), to see if the densities of IRP/IRE complex band become identical in both type of cells. It was found that the difference in the complex band between the neo cells and the hsp27-overexpressing cells disappeared (see the left two lanes in Fig. 3B). These results suggest that overexpression of human hsp27 leads to an increase of the binding activity of IRP1 to IRE in the cells.

Because hsp27 enhances IRP/IRE binding activity, we predicted that the number of the surface-expressed TfR1 might increase in the cells overexpressing hsp27. The steady-state level of TfR1 expression at the cell surface was determined. A study on TfR1 saturation kinetics was performed and showed that saturation of specific transferrin-binding sites on 2×10^5 cells was found to occur at a transferrin concentration of about 0.16 μg/ml (data not shown). Thus, the concentration of 16 μg/ml BXX-Tf used in this investigation guaranteed a full occupation of the TfR1s on the cell surface by BXX-conjugated transferrin. The results in Fig. 3D show that overexpression of hsp27 results in a 30% increase of TfRs at the cell surface. The increased surface-expressed TfR is in good agreement with the reduced LIP and increased IRP/IRE binding activity previously observed. Unfortunately, it could not explain less iron uptake in the hsp27-overexpressed cells. These results suggest that

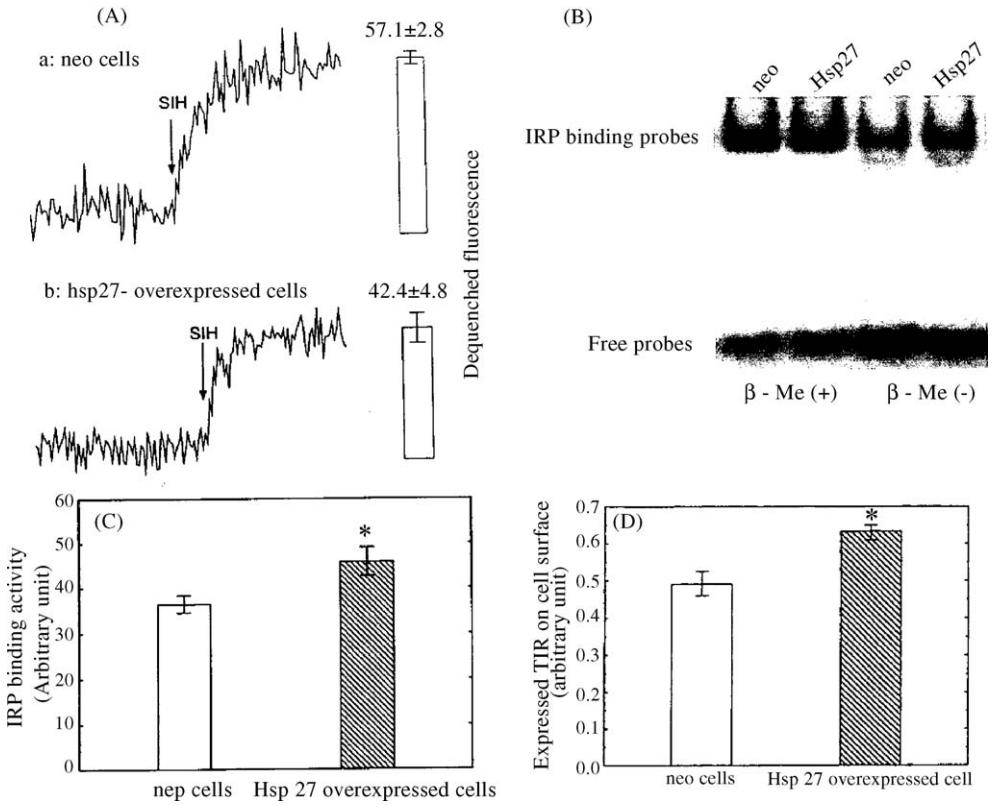

Fig. 3. The effects of hsp27-overexpression on intracellular LIP, IRP/IRE binding activity and the surface-expressed transferrin receptors in the hsp27-overexpressed and the neo CCL39 cells. (A) LIP was measured as the dequenched fluorescence of calcein by addition of the membrane-permeant iron chelator SIH in the calcein-loaded cell suspension. The two curves on the left side show the typical fluorescence dequenching in the two types of cells. The columns and data on the right side are the calculated dequenched fluorescence based on three independent measurements. (B) The IRP/IRE binding activities in the neo and hsp27-overexpressed CCL39 cells in the presence and absence of β-Me. (C) Statistic analysis of the IRP/IRE binding activities in the two types of cells above. (D) The surface-expressed transferrin receptors in the neo and hsp27-overexpressed CCL39 cells. Vertical columns represent the means ± S.D. ($n = 3$). '*' indicate the data are significantly ($p < 0.05$) different from the data obtained from the neo cells.

hsp27 does regulate the binding activity of IRP to IRE and consequently increase surface-expression of TfR1s via reducing LIP, but regulates the TfR1-mediated iron uptake probably through a different mechanism that is independent of the number of TfR1s on cell surface.

3.4. Hsp27 down-regulates the endocytosis and recycling of transferrin

TfR1-mediated cellular iron uptake depends not only on the number of TfR1s on cell surface, but also on the rate of endocytosis and recycling of the Tf-TfR1 complex (Sainte-Marie et al., 1997). To determine the effect of hsp27 on the rate of transferrin endocytosis, cells were incubated with BXX-Tf on ice to allow full binding of the ligands to the TfR1s. The cells were then rapidly warmed up to 37 °C to initiate endocytosis of the BXX-Tf. As Fig. 4A shows, the rate of the Tf endocytosis was decreased by 20% and 35% in the hsp27-overexpressed and hsp27-highly-overexpressed cells, respectively, if compared with the neo cells. However, overexpression of its mutant hsp27-3G resulted in a 25% increase in the rate of Tf endocytosis. It was also found that the rate of Tf endocytosis was significantly increased when the neo CCL39 cells were pre-incubated with cytochalasin B (CB), a blocker of the microfilaments assembly by depolymerizing F-actin (Jesaitis, Tolley, & Allen, 1986).

After Tf-TfR1 complex is internalized, the Tf-bound ferric ions are dissociated, reduced, and enter particular locations in cytoplasm. Transferrin itself then returns to cell surface. The rate of TfR1-mediated iron uptake depends not only on transferrin internalization but also on transferrin recycling. For this reason, we studied the recycling of TfR1. The results shown in Fig. 4B demonstrate that the rate of Tf recycling decreased by 40% and 60%, respectively, in hsp27-overexpressed and hsp27-

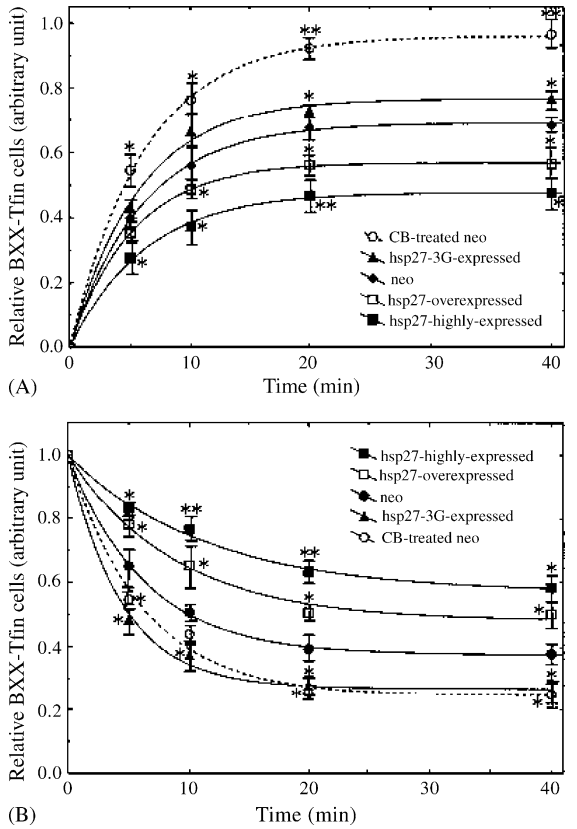

Fig. 4. Endocytosis and recycling of transferrin in the neo, the hsp27-overexpressed and the hsp27-highly-overexpressed, the hsp27-3G-expressed and CB-treated CCL39 cells. (A) Endocytosis of BXX-Tf by 3×10^5 cells. The internalized transferrin is expressed as the percentage of initial total surface-bound BXX-Tf. (B) The exocytosis of the internalized BXX-Tf after equilibrium between endocytosis and exocytosis of the transferrin is reached in the cells and measured as a percentage of the initial internalized BXX-Tf. Results are expressed as means ± S.D. of three independent experiments, and each experiment was performed in triplicate. '*' and '**' indicate the data are significantly ($p < 0.05$) and very significantly ($p < 0.01$) different from the data obtained from the neo cells.

highly-overexpressed cells, but enhanced by 20% in the cells overexpressing its mutant hsp27-3G. In addition, pre-incubation of the neo cells with CB results in a 20% increase in the rate of Tf recycling. Taken together, these results indicate that overexpression of hsp27 decelerates both the rate of Tf internalization and the rate of Tf recycling, which may explain why the cells overexpressing hsp27 take up more transferrin-bound iron.

3.5. Role of the actin cytoskeleton in regulating cellular iron uptake

As Fig. 4 shows, pretreatment with 1 μM CB enhances the rate of transferrin endocytosis and recycling in cells. It clearly demonstrates the important role of actin polymerization in TfR1-mediated iron uptake. Therefore, the actin polymerization was observed in the neo cells, the hsp27-overexpressed cells, and the CB-pretreated neo cells. The results show that the overexpression of hsp27 increases, while pre-treatment with CB decreases actin depolymerization (Fig. 5A and 5B). In addition, the assay of the uptaken ^{55}Fe-Tf in the cells pre-incubated with 1 μM CB showed an enhanced TfR1-mediated iron uptake by CB-treatment (Fig. 5C). Together with previous data on the CB-treatment caused acceleration of endocytosis and recycling of transferrin, these results indicate that a loosing actin cytoskeleton in cells enhances the TfR1-mediated iron uptake. Therefore, down-regulation of TfR1-mediated iron uptake by hsp27 in the hsp27-overexpressed cells might be due to enhanced actin polymerization and a more rigid actin cytoskeleton.

To identify which part of the actin cytoskeleton is preferentially subject to the hsp27-enhanced polymerization, the spatial distribution of human hsp27 and actin filaments in hsp27-overexpressed cells was observed by fluorescence microscopy. The immunofluorescence image of the human hsp27 protein and the fluorescence image of rhodamine-phalloidin-labeled filaments in the hsp27-overexpressed cells were taken separately on a same focused field and are shown in Fig. 6. In order to show the position of nucleus in each imaged cell, the nuclei were stained with Hoechst 33342, and their images were merged with immunofluorescence and fluorescence images of the cells, respectively. As Fig. 6A shows, the human hsp27 protein has a general cytoplasmic distribution with an enhanced concentration near the inner-side of the cell membrane (see the locations indicated by arrows). Correspondingly, the highest intensive fluorescence of the F-actin-associated rhodamine-phalloidin appears in the same area, the cortical regions of the actin cytoskeleton immediately below the cell membrane (see the same area indicated by arrows in Fig. 6B). By comparison of the two images, a clear correlation between the highest hsp27 content and the highest rhodamine fluorescence can be seen in the same cortical regions. The enhanced distribution of human hsp27 and the highest polymerization of actin in the cortical area provide a structural evidence for the possible regulation of actin cytoskeleton by hsp27.

3.6. Effect of hsp27 on the expression of ferroportin 1 in the CCL39 cells

To know if ferroportin 1, which releases iron from cells, is involved in the hsp27-regulation of the

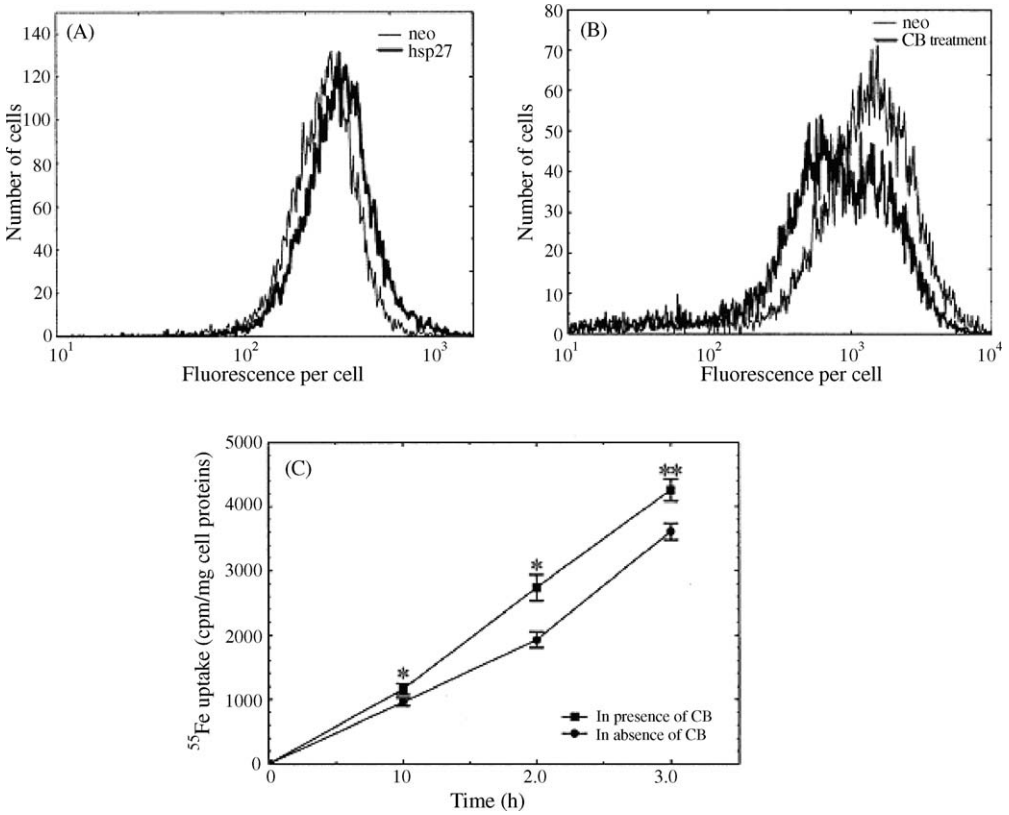

Fig. 5. The effects of hsp27-overexpression and cytochalasin B on actin polymerization and the effect of cytochalasin B on the uptake of transferrin-bound ^{55}Fe in CCL39 cells. Panels A and B show the distribution of cell numbers against the fluorescence per cell in the Bodipy FL phallacidin-loaded cells either over-expressing hsp27 or pre-incubated with cytochalasin B for 10 min. The same distribution in the Bodipy FL phallacidin-loaded neo CCL39 cells was used as a control in both cases. Total 10^4 cells were flow-cytometrically analyzed for each sample, and each distribution is representative of three independent analyses. Panel C: Uptakes of the transferrin-bound ^{55}Fe in the neo CCL39 cells pre-incubated with or without 1 μM cytochalasin B for 10 min. Results are expressed as mean ± S.D. of three independent experiments, and each experiment was performed in triplicate. '*' and '**' indicate the data are significantly ($p < 0.05$) and very significantly ($p < 0.01$) different from the data obtained from the cells without cytochalasin B treatment.

intracellular iron in CCL39 cells, the expression of ferroportin 1 was estimated by immunoblotting in the neo, the hsp27-overexpressed and the hsp27-highly-overexpressed cells. The results are shown in Fig. 7. Almost no difference could be found in the expression level of ferroportin 1 in the three different types of cells regardless of cellular content of hsp27.

4. Discussion

Hsp27, a marker of differentiation and proliferation, helps cells repair damage induced by environmental stress such as heat, UV-irradiation, and oxidative stress (Mehlen, Briolay et al., 1995; Mehlen, Preville et al., 1995). Hsp27 plays many important roles in regulating cell function such as inhibiting the death receptor mediated apoptosis (Ricci et al., 2001), promoting growth of human astrocytomas (Khalid et al., 1995), and characterizing a tumor as relatively benign and slow progressing (Bayerl et al., 1999). In this investigation, we found that overexpression of human hsp27 suppresses the TfR1-mediated iron uptake in Chinese hamster lung fibroblast cells and that the suppression cannot be explained by IRP/IRE regulatory mode. We found that overexpression of 2 and 3 ng human hsp27/μg of total proteins resulted in a dose-dependent decrease in TfR1-mediated iron uptake by about 18% and 33%, respectively, in the cells. Overexpression of the functional dead hsp27 mutant enhances TfR1-dependent iron uptake by 19%. These results indicate that hsp27 functions as a negative regulator of the TfR1-mediated iron uptake in cells. Since a relatively considerable background level of endogenous hamster hsp27 (1 ng/μg of total proteins) is not taken into account, the regulatory role of hsp27 in TfR1-mediated iron uptake may be even larger than that suggested by the above data.

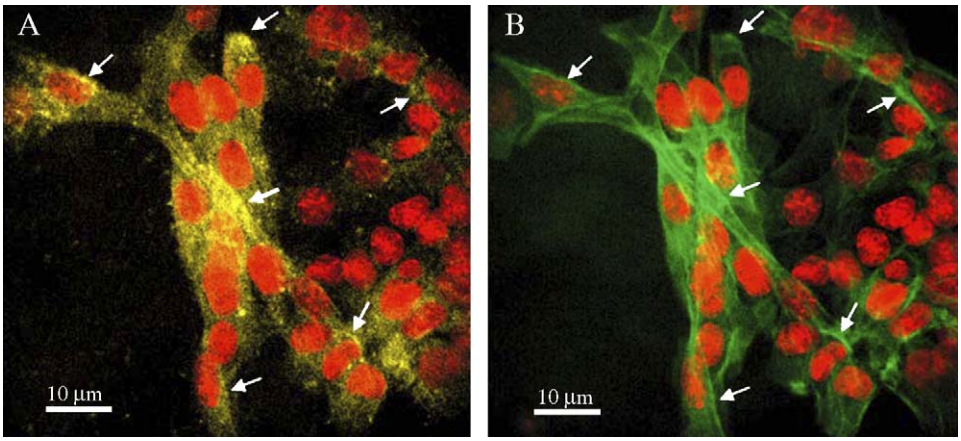

Fig. 6. Spatial distribution of hsp27 and actin filaments in the hsp27-overexpressed cells. The cells were grown on fibronectin-coated dishes, then fixed and permeabilized. Panel A: Distribution pattern of hsp27 in the cells. It was visualized using rabbit anti-human hsp27 polyclonal antibody and FITC-labeled goat anti-rabbit IgG as primary and secondary antibody, respectively. Panel B: Actin filaments in the same cells. They were visualized by rhodamine-phalloidin staining. The cell nuclei were stained with Hoechst 33342 and their images were superimposed onto each image in order to show the integrity of the cells. The cells were imaged on an IX/71 type Olympus microscope equipped with an AquaCosmos Microscopic Image Acquisition and Analysis System (including deconvolution software). Objective lens: Olympus UAPO40XOI3340 with N.A. of 1.35; magnification: $\times 10^3$; white arrows indicate the co-localization of hsp27 with cortical actin filaments.

The delivery of transferrin-bound iron into cells was recognized almost 50 years ago. In mammalian cells, the levels of intracellular iron via feedback regulatory mechanisms regulate the expression of many key molecules that participate in iron metabolism. At low intracellular iron levels, iron regulatory proteins, IRP1 and/or IRP2, bind to the iron responsive element, IRE, in transferrin receptor mRNA and ferritin mRNA with high affinity. This inhibits ferritin translation and stabilizes TfR1 mRNA, leading to the decrease of iron sequestration and the increase of iron uptake. In our investigation, a lower intracellular iron level, i.e. a smaller labile iron pool, was found in hsp27 over-expressed cells in comparison with that in the neo cells. It was also observed that in response to smaller LIP, the hsp27-overexpressing cells have higher IRP/IRE binding activity and more surface-TfR1s. These data suggest that IRP/IRE mode still works in the regulation of TfR1-mediated iron uptake by hsp27, but could not explain the inhibitory effect of hsp27 on cellular iron uptake. Therefore, IRP/IRE regulatory mode may not always play a central role in the regulation of iron metabolism. The regulation of iron uptake by heat shock protein 27 may represent a different mechanism.

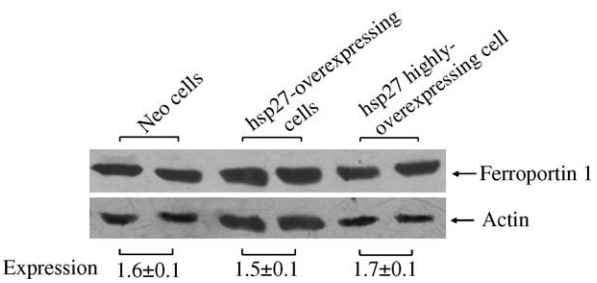

Fig. 7. Hsp27-independent expression of ferroportin 1 in CCL39 cells. The immunoblot of ferroportin 1 probed by anti-mouse ferroportin 1 antibody in lysate of the neo cells, the hsp27-overexpressed cells and the hsp27-highly-overexpressed cells, respectively. Two parallel detections were performed for each cell lysate, and the data below the blots represent the mean ± S.D. of the actin-normalized ferroportin 1 expression in corresponding cells indicated above the blots.

Tf-bound iron uptake is not only controlled by the number of TfR1s at cell surface, but also relies on the rate of endocytosis and recycling of transferrin. In this investigation, a slowdown in the internalization and recycling of transferrin was observed in hsp27-overexpressed cells. It may account for the down-regulation of cellular iron uptake by hsp27. The two findings that disruption of the actin cytoskeleton with CB resulted in an acceleration of transferrin endocytosis and recycling and the enhancement of actin polymerization by overexpression of hsp27 led to a slowdown in the transferrin recycling reveal a possible role for the actin cytoskeleton in hsp27-regulated iron uptake.

Genetic analysis links the actin cytoskeleton to endocytosis in lower eukaryotes such as yeast. However, in mammalian cells, the link is not so convincing (Jeng & Welch, 2001). The endocytic process can be broken down into several steps: membrane invagination, coated pit formation, coated pit sequestration, detachment of the formed vesicle, and movement of this new

endocytic compartment away from plasma membrane into the cytosol (Jeng & Welch, 2001). Actin cytoskeleton might be involved in each step via different molecular interactions at the interface of endocytosis and cytoskeleton organization (Qualmann, Kessel, & Kelly, 2000). It seems that the cortical actin cytoskeleton may play a vital role in regulating the internalization of transferrin by hsp27. The double-labeling fluorescence micrographs showed that the human hsp27 is localized in cytoplasm, but preferentially in the area near the inner side of cell membrane in hsp27-overexpressed cells. The heaviest F-actin staining occurred almost exclusively in the cell periphery as a narrow band immediately below the cell membrane. These two observations suggest that the highly polymerized cortical actin caused by preferentially localized human hsp27 might be responsible for down-regulation of TfR1-mediated iron uptake, since a rigid cortical actin cytoskeleton functions as a barrier to the formation and traffic of endocytic vesicles on the membrane (Trifaro & Vitale, 1993). A number of papers deal with the modulation of actin microfilament by hsp27. It is commonly believed that the stabilization of microfilaments is a major function of hsp27 to protect cells from heat, oxidative-stress or stimulation by various cytokines (Lavoie, Lambert, Hickey, Weber, & Landry, 1995). Overexpression of wild-type human hsp27 increases the stability of the actin microfilaments during heat shock or oxidative stress, and contributes to the faster recovery of disrupted microfilaments after treatments (Lavoie et al., 1993). Phosphorylation-induced conformational change in hsp27 oligomers is generally considered to enhance the stability of actin filaments (Guay et al., 1997). Because hsp27 is a ubiquitous target of phosphorylation upon cell stimulation, more phosphorylation of hsp27 is expected to occur upon binding of Tf to TfR1. We have found that p38 MAP kinase is activated upon binding of transferrin to transferrin receptor (submitted elsewhere). It has been well established that activated p38 kinase phosphorylates MAP kinase-activated protein kinase-2, which in turn phosphorylates heat shock protein 27 (Freshney et al., 1994; Rouse et al., 1994). The p38 MAP signaling induced phosphorylation of heat shock protein 27 might also account for the down-regulation of the TfR1-mediated iron uptake via regulation of actin filament dynamics.

Similar to the endocytosis, the rigid cortical actin cytoskeleton may also act as a barrier to block off moving out of the transferrin-containing cytoplasmic vesicles to the cell surface. This concept has been supported by other investigations. For example, it was reported that hyperstability of the cortical cytoskeleton coupled with microtubule perturbation would be responsible for the depressed pattern of mast cell exocytosis (Candussio, Crivellato, Decorti, Bartoli-Klugmann, & Mallardi, 1999). Chowdhury et al. also reported that the subcortical actin network functions as a barrier of the cytoskeleton for exocytosis and attenuates the rate of secretary activity in rat melanotrophs (Chowdhury, Popoff, & Zorec, 2000).

Although the major function of TfR2 may be involved in iron regulation rather than iron uptake (Kawabata et al., 2001), the report that heterologous expression of TfR2 in Chinese hamster ovary (CHO) cells lacking transferrin receptors (TRVb cells) increases the uptake of Tf-bound iron suggests that TfR2 is also able to mediate the uptake of transferrin-bound iron (Kawabata et al., 1999). Thus, the involvement of TfR2 in the reported regulation of cellular uptake of transferrin-bound iron (^{55}Fe) by hsp27 may not be excluded. However, TfR2 expression is specific for liver and normal erythroid precursor cells and lesser extent has been found in spleen, lung, muscle, prostate and peripheral mononuclear cells (Kawabata et al., 1999, 2001). Despite of lacking available data, it may be expected that the expression of TfR2 should be much lower than the TfR1expression in the studied hamster lung fibroblast cells. On the other hand, due to 25-fold lower affinity of TfR2 to transferrin in comparison with TfR1, the TfR2-mediated uptake of ^{55}Fe-Tf within 3 h after the cells exposed to Tf, or the TfR2-mediated endocytosis and recycling of the biotin-XX-conjugated transferrin within 40 min after the cells were warmed up to physiological temperature and initiated their endocytosis and exocytosis, which could occur in this investigation, would be much less important even if TfR1 and TfR2 had similar expression levels. Thus, the presence of TfR2 may not be able to substantially alter the conclusion made in the present study.

Ferroportin (Fpn1) is an iron transporter and considered to play roles in intestinal iron absorption and cellular iron release. Fpn1 has been also implicated in turnover of iron recovered from scavenged red blood cells in reticuloendothelial macrophages of the splenic red pulp and hepatic Kupffer cells (Abboud & Haile, 2000). The essential role of Fpn1 in iron homeostasis has been revealed by the iron overload resulted from mutations in Fpn1 in the reticuloendothelial system (Montosi et al., 2001). Therefore, besides regulation of actin cytoskeleton, a question whether overexpression of hsp27 would regulate the intracellular iron level by affecting the Fpn1 expression, which results in a change of iron release from cell, in the cells should be solved. The observed hsp27-independent expression of ferroportin 1 in the neo, the hsp27-overexpressed and hsp27-highly-overexpressed cells suggests that ferroprtin 1 is

not involved in the regulation of the cellular iron uptake by hsp27.

In summary, our findings suggest that hsp27 regulates TfR1-mediated iron uptake through at least two mechanisms: IRP/IRE regulation mode and increased stabilization of cortical actin microfilaments. However, the latter is determinant. Hsp27 modulates the ability of cells to respond to oxidative stress. Since increased intracellular iron leads to oxidative stress and the oxidative stress induced expression of hsp27, our findings may reveal a mechanism through which hsp27 protects cells against oxidative stress by down-regulating the TfR1-mediated cellular iron uptake for decreasing intracellular level of iron. The high level of hsp27 in the heart and the down-regulation of iron homeostasis by hsp27 in cells may imply that heat shock protein 27 may play a role in protecting the heart from the iron-induced oxidative stress.

Acknowledgments

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