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Paradoxical Enhancement of Oxidative Cell Injury by Overexpression of Heme Oxygenase-1 in an Anchorage-Dependent Cell ECV304

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Abstract There has been increasing evidence suggesting the potent anti-inflammatory roles of heme oxygenase-1 (HO-1) in protecting renal tubular epithelial cells, vascular endothelial cells, and circulating monocytes. Based on these findings, novel therapeutic interventions have been proposed to control the expression of endothelial HO-1 levels to ameliorate various vascular diseases. We evaluated the effect of *HO-1* gene transfer into an anchorage-dependent cell, ECV304. Effect of HO-1 production on the cell injury induced by hydrogen peroxide was evaluated after hemin stimulation and after *HO-1* gene transfection. Morphological changes and the induction of various anti-apoptotic proteins were examined at the same time. Levels of HO-1 expression were variable in different clones of HO-1-transfected ECV304 cells. Among these, the clones with moderate levels of HO-1 expression were significantly more resistant to oxidative stress. In contrast, those with the highest levels of HO-1 exhibited paradoxically enhanced susceptibility to oxidative injury. Interestingly, the cell survival after oxidative stress was in parallel with the levels of Bcl-2 expression and of fibronectin receptor, α5 integrin. It is suggested from these results, that excessive HO-1 not only leads to enhanced cell injury, but also prolongs the repair process of the injured endothelial tissue. However, HO-1 reduces the oxidative cell injury and protects the endothelial cells, if its expression is appropriately controlled. J. Cell. Biochem. 93: 552–562, 2004.

Key words: oxidative stress; gene transfection; Bcl-2; α5 integrin; apoptosis

Endothelial cell injury has been regarded as the critical event in the pathogenesis of cardiovascular diseases including atherosclerosis and hypertension, infectious diseases, inflammatory diseases, aging, neoplasms, and cerebral vascular diseases. Oxidative stresses including superoxide, hydrogen peroxide, hydroxyradical, and cytokines are known to induce such injury [Halliwell, 1994]. Heme oxygenase (HO) degrades heme into free iron, carbon monoxide (CO) and biliverdin [Maines, 1988]. Three different forms of HO have been described to date. Among these, HO-1 production is induced by various forms of stress, whereas HO-2 and HO-3 are expressed constitutively in multiple tissues [Elbirt and Bonkovsky, 1999]. Biliverdin and bilirubin are synthesized from heme by HO and exert a potent antioxidative function inhibiting stress-induced cell injury. On the other hand, CO, similar to nitrogen oxide, acts as gaseous mediator of vasodilatation and helps to control the peripheral circulation [Goda et al., 1998; Siow et al., 1999]. CO is also known to exert an anti-inflammatory function through its activation of MAPK pathway and induction of IL-10 [Brouard et al., 2000; Otterbein et al., 2003].

Although HO-1 production is induced de novo by oxidative stresses, it is constantly expressed at the same time within selected cell types in the

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absence of specific stimulations, indicating that these cells are constantly exposed to oxidative stresses in vivo. The tissue-specific expression of HO-1 has been demonstrated within atherosclerotic lesions [Wang et al., 1998], renal tubular epithelium [Morimoto et al., 2001], and circulating monocytes [Yachie et al., 2002a]. Considering the potent anti-inflammatory and anti-oxidative functions of HO-1, numerous studies have been reported to prove the effect of HO-1 in the prevention of vascular endothelial cell injury [Ohta and Yachie, 2001]. In particular, induction of HO-1 by hemin [Aizawa et al., 1999; Hayashi et al., 1999; Hangaishi et al., 2000], by local injection or oral administration of L-arginine [Schwarzacher et al., 1997; Vermeersch et al., 2001] and transfers of nitric oxide synthase gene [von der Leyen et al., 1995; Channon et al., 1998; Janssens et al., 1998] or *HO-1* gene [Abraham et al., 1995; Deramaudt et al., 1998; Yang et al., 1999; Zhang et al., 2002] have been performed to induce regression of atherosclerosic lesions or to prevent restenosis of coronary arteries after balloon dilatation or stent implantation. Furthermore, intratracheal administration of *HO-1* gene has been tried to prevent the lung injury induced by hyperbaric oxygen [Otterbein et al., 1999]. Modulation of HO-1 expression may also be useful to prevent rejection of transplanted organs as it has been reported that HO-1 and CO induced at the site of rejection act to reverse the inflammatory reaction [Soares et al., 1998].

Most of the studies regarding the anti-inflammatory roles of HO stress the importance of the enzyme in protecting against cell injury and the potential therapeutic application of *HO-1* gene transfer. However, there are also some reports indicating that excessive and prolonged exposure to the enzyme may have certain deleterious effects on cell function and survival [Suttner and Dennery, 1999; Suttner et al., 1999].

In this study, we stimulated an anchorage-dependent cell ECV304 with hemin to induce de novo production of HO-1, and examined the effect of HO-1 on stress-mediated cell injury [Heath-Engel and Lingwood, 2003]. We also established several clones of ECV304 cells transfected with different levels of HO-1 and compared the characteristics with regard to the levels of HO-1 expression. The results shed some light on the future application of *HO-1* gene therapy for the prevention of vascular diseases.

MATERIALS AND METHODS

Cell Cultures

ECV304 [Takahashi et al., 1990] was obtained from the Human Science Research Resource Bank (Tokyo, Japan) and maintained in Medium 199 containing 10% FCS at 37°C with 5% CO_2 in culture flasks (Corning, Inc., Corning, NY). For immunohistochemistry and immunofluorescence, the cells were cultured in four-chambered slides (Lab-Tek II chamber slide, Nalge International, Naperville, IL) for 72 h until confluent monolayers were obtained.

Reagents

Concentrated stock of hemin (Sigma, St. Louis, MO) was prepared freshly in 0.01 N sodium hydroxide and used to achieve the final concentration by appropriate dilution with the culture medium. Tin protoporphyrin IX dichloride (SnPP, AFFINITY Research Products, Mamhead, UK) was used at 100 μM to inhibit HO activity. Anti-HO-1 monoclonal antibody was kindly provided by Prof. M. Suematsu (Keio Univ., Tokyo, Japan) [Goda et al., 1998].

Immunoblotting

Samples for immunoblotting were prepared as described previously [Yang et al., 2003]. Briefly, equivalent numbers of cells were treated with 10% TCA in PBS for 10 min on ice. The cells were spun down and suspended in 100 ml of lysis solution containing 9 mM urea, 2% Triton X and 10% 2-mercaptoethanol. The mixture was disrupted by sonication and neutralized with 1 M Tris solution. The lysates were applied to precast SDS–polyacrylamide gels, with 12 wells, 5–20% concentration, 10,000–200,000 molecular weight separation range (ATTO, Tokyo, Japan), and electrophoresis was carried out in SDS. The proteins were blotted onto nitrocellulose filters using a HORIZBLOT apparatus (ATTO). Blots were blocked in 5% skim milk in PBS for 1 h and reacted with rabbit anti-HO-1 (StressGen, Victoria, British Columbia, Canada), mouse anti-Bcl-2 (DAKO, Kyoto, Japan), rabbit anti Bcl-X (DAKO), mouse anti-Bax (Bcl-2-associated X protein, MBL, Nagoya, Japan) or mouse anti-actin monoclonal antibody (CHEMICON International, Temecula, CA) appropriately diluted in PBS containing 0.1% Tween 20 (PBS-T) for 1 h. The membranes were washed and further incubated with an appropriate dilution of peroxidase-conjugated

anti-rabbit IgG or anti-mouse IgG (DAKO Co., Carpinteria, CA) in PBS-T for 1 h at room temperature. Immunoblots were developed by using the ECL™ chemiluminescence signal generation system (Amersham Pharmacia Biotech, Inc., Buckinghamshire, UK) according to the manufacturer's instructions.

Immunohistochemistry

For immunohistochemistry the cultured cells were washed in PBS twice, air-dried, fixed in acetone, and rinsed in Tris buffer. After blocking with normal goat serum, the slides were stained with appropriate dilutions of anti-HO-1 rabbit antiserum for 1 h at room temperature. After washing three times in Tris buffer, alkaline phosphatase-conjugated EnVision (DAKO) was reacted for 30 min at room temperature. Alkaline phosphatase activity was visualized using Fast Red TR salt and Naphtol As-MX phosphate. Morphology of the cultured cells was examined after staining the cells with May–Grünwald and Giemsa.

Actin Staining With Fluorescein-Phalloidin

ECV304 cells in chamber slides were air-dried and fixed in 4% paraformaldehyde solution and permeabilized in 0.1% Triton X-100 in PBS. Subsequently, the slides were reacted with the optimal dilution of fluorescein-phalloidin (Molecular Probes, Inc., Eugene, OR) for 30 min in the dark. After washing three times in PBS, the slides were briefly exposed to 10 μg/ml of propidium iodide solution in PBS. After further washing in PBS, the slides were mounted with antifade glycerol, and fluorescent actin polymers were visualized by epi-fluorescent microscopy.

Cytotoxicity Assay

For the cytotoxicity assay, ECV304 cells were cultured at 1×10^5/ml in 96-well COSTAR microtiter plates (Corning) for 12 h in the presence of the following reagents. Hemin was used at 10 or 100 μM, and hydrogen peroxide was used at 20, 40, or 60 μM. Viabilities of these cells were determined with a Cell Counting kit 8 (Wako Chemicals, Osaka, Japan) after the cultures, as described previously [Yang et al., 2003]. Briefly, the plates were washed with PBS, fresh culture medium was added to each well and 10 μl of the reaction mixture containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide was added to each well. After 3 h incubation under 5% CO_2 at 37°C, the solubilized formazan product was quantified spectrophotometrically using a microplate reader. Cell viability was calculated by the following formula:

$$\% \text{ Survival} = \text{O.D. of stimulated culture} - \text{O.D. of culture medium} / \text{O.D. of control culture} - \text{O.D. of culture medium.}$$

Flowcytometry

Confluent ECV304 cells were harvested from the culture flasks using plastic cell scrapers without trypsinization. For HO-1 staining, the detached ECV304 cells were washed in PBS twice and fixed in 1% paraformaldehyde in PBS at room temperature for 20 min. They were further washed in PBS twice, and permealized in 0.2% saponin in PBS with 3% FCS and 0.1% sodium azide (saponin buffer) for 20 min at room temperature. After washing once in saponin buffer, the cells were incubated with mouse anti-HO-1 mAb or with control isotype-matched IgG antibody for 20 min on ice. After washing, these cells were further incubated with FITC-conjugated goat anti-mouse antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) at the optimal dilution for 15 min on ice. HO-1 expression by each cell population was evaluated independently with a Cytoron Absolute flow cytometer (Ortho Diagnostic Systems, Tokyo, Japan). Mean fluorescence intensity (MFI) was expressed as ΔMFI by subtracting MFI obtained with control antibody from MFI obtained with specific antibody. For the analysis of ICAM-1, the cell suspension was reacted with appropriate dilutions of PE-conjugated ICAM-1 (Pharmingen, San Diego, CA) or with PE-conjugated control antibody (Pharmingen). Expression of α5β1 integrin was determined by staining with anti-CD49e monoclonal antibody (Pharmingen), followed by PE-conjugated goat anti-mouse IgG.

HO-1 Gene Transfer

The human HO-1 cDNA was cloned into pGCsamEN (kindly provided by Dr. F. Candotti) so that the HO-1 translational start site was positioned precisely where the env translational initiation site is located in the wild type virus [Onodera et al., 1998; Yachie et al., 2002a]. pGCsamEN is a retrovirus-based plasmid containing Moloney murine leukemia virus LTRs

with cistronic insertion of internal ribosomal entry sites (IRES) from encephalomyocarditis (EMC) virus and neomycin phosphotransferase (*Neo*) gene. The vector containing *HO-1* gene was first transfected into the ecotropic fibroblast packaging cell line GP&E86 by the calcium phosphage method using the MBS mammalian Transfection Kit (STRATAGENE, La Jolla, CA). The obtained virus supernatant was further transfected into amphotropic packaging cells PG13 by infection. PG13 transfected with *HO-1* gene was subcloned by G418 selection to obtain high production, and the supernatant with concentrated virus was used to infect ECV304. ECV304 successfully transfected with pGCsamEN were selected by G418. HO-1 expression was confirmed by a flow cytometry and by immunoblotting. HO-1 transfected ECV304 were cloned by the limiting dilution method, and sublones with variable levels of HO-1 were established. ECV304 transfected with neomycin resistant (*NeoR*) gene served as a control.

Statistical Analysis

Statistical analysis was performed by the Mann–Whitney test. All results were considered statistically significant at $P < 0.05$.

RESULTS

Dose-Dependence and Kinetics of HO-1 Induction in ECV304 by Hemin

Expressions of actin and HO-1 were examined sequentially by immunoblotting after hemin stimulation (Fig. 1A). HO-1 expression was detectable but very low before stimulation (lane 1), started to increase 2 h after stimulation with 100 μM hemin (lane 3), peaked at around 8 h (lane 5), and declined after 24 h (lane 6). The levels of actin remained constant throughout the cultures. Dose-dependence of HO-1 induction was examined after 8 h of culture with different concentrations of hemin. Low concentraion of hemin (10 μM) induced a significant level of HO-1 in ECV304 (Fig. 1B, lane 2), and the levels increased in a dose-dependent manner with the maximum induction at 300 μM of hemin (lane 5). Actin levels did not change with hemin stimulation.

Cytotoxicity Induced by Hydrogen Peroxide

Cell injury was induced in ECV304 by hydrogen peroxide in a dose-dependent manner.

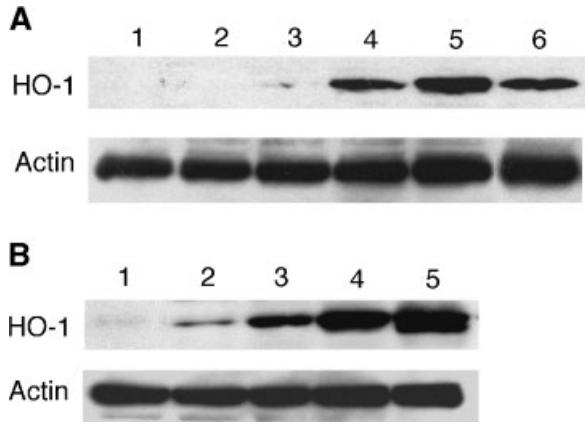

Fig. 1. Hemin-induced heme oxygenase-1 (HO-1) production by ECV304 cells. ECV304 cells were cultured in the presence of 100 μM hemin for various periods of time (**A**). HO-1 production was evaluated by immunoblotting before (**lane 1**) and 1, 2, 4, 8, and 24 h after stimulation (**lanes 2–6**, respectively). Dose dependence of hemin stimulation was examined after 8 h of stimulation with 0, 10, 30, 100, and 300 μM of hemin (**B**, **lanes 1–5**, respectively).

Cell survival increased significantly when 1 μM hemin was added to the cytotoxicity assay. However, the use of a higher concentration (100 μM) of hemin had little protective effect, but instead accelerated cell death (Fig. 2A). Addition of hemin alone up to 100 μM did not have any significant effect on cell survival (data not shown). The protective effect of hemin was completely abolished when the cytotoxicity assay was performed in the presence of a specific inhibitor of HO activity, SnPP (Fig. 2B).

HO-1 Expression After Gene Transfection

After *HO-1* gene transfection, clones of HO-1-transfected ECV304 were obtained by limiting dilution. HO-1 expression in these HO-1-transfected ECV304 clones was determined by a flowcytometry. ECV304 before HO-1-transfection (wild ECV304 line) expressed low levels of HO-1 (Fig. 3A), whereas representative HO-1-transfected clones (clones No. 2 and No. 9) expressed variable levels of HO-1 within their cytoplasm (Fig. 3B,C, respectively). Wild ECV304 line stimulated with hemin expressed a significantly high level of HO-1 (Fig. 3D).

Effect of *HO-1* Gene Transfection on Hydrogen Peroxide-Induced Cell Injury

To further confirm that HO-1 has a protective role in hydrogen peroxide-induced cell injury, we compared the cell survival among HO-1-transfected ECV304 clones with different levels

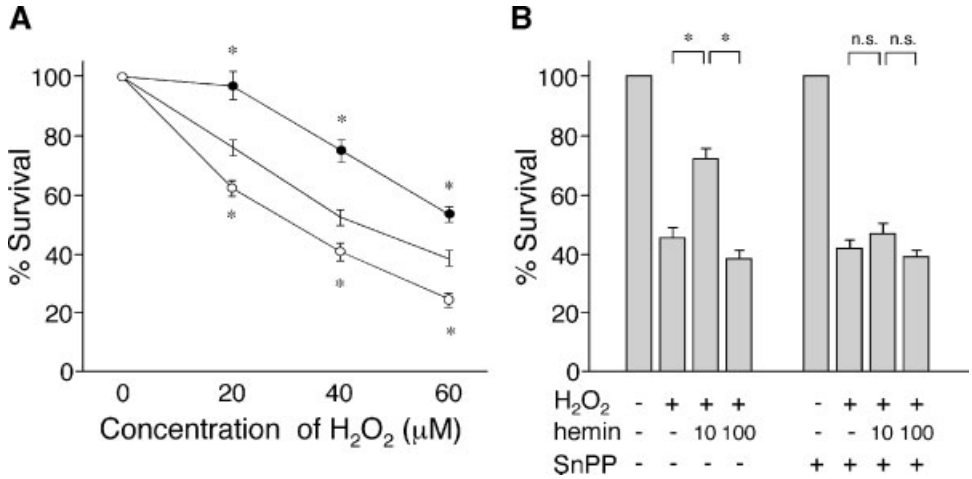

Fig. 2. Effect of HO-1 on cytotoxicity induced by hydrogen peroxide. ECV304 cells (no symbol) were cultured for 12 h in the presence of various concentrations of hydrogen peroxide (**A**). The cultures were performed at the same time with 10 μM (closed circles) or 100 μM (open circles) of hemin. In different experiments, SnPP was added at 100 μM to the cultures to see if hemin effect was via induced HO activity (**B**). Hydrogen peroxide was added at 40 μM and hemin was added at 10 or 100 μM in these cultures. Asterisks indicate the statistical significance ($P < 0.01$), and n.s. denotes ''not significant.''

of HO-1 expression. We first compared the cell survival of the representative clones No. 2 and No. 9 with the wild ECV304 line. The cell survival of clone No. 9, which expressed a moderately increased level of HO-1, improved significantly over a wide range of hydrogen peroxide concentrations (Fig. 4, panel A). In marked contrast, the cell survival of clone No. 2, which expressed a high level of HO-1, was lower than that of wild ECV304 line (Fig. 4, panel A). These results suggested that the levels of HO-1 have critical roles in determining cell survival after hydrogen peroxide stimulation. To see the dose-dependent differences in the HO-1 effect, HO-1-transfected ECV304 clones were classified into four different subgroups based on the levels of HO-1 (ΔMFI less than 10, 10–20, 20–30, and more than 30). Clones No. 2 and No. 9 belonged to the groups with ΔMFI 20–30 and more than 30, respectively. Survival after hydrogen peroxide treatment increased significantly when the cells expressed HO-1 at a mild level (ΔMFI = 10–20), with survival decreasing significantly when the level of HO-1 was high (ΔMFI > 30) (Fig. 4, panel B).

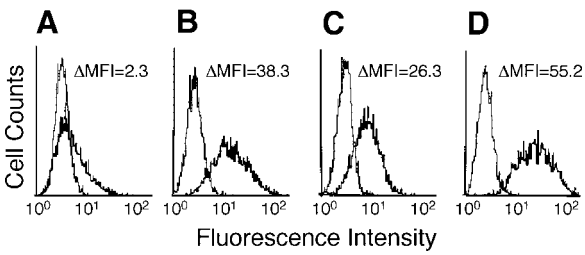

Fig. 3. Expression of HO-1 by HO-1 transfected ECV304 cells. Levels of HO-1 expression were compared by a flow cytometry among wild ECV304 line (**A**), No. 2 (**B**), and No. 9 (**C**) of HO-1-transfected ECV304 clones and hemin-stimulated ECV304 cells (**D**). The fine lines indicate the profiles obtained with control antibody and the bold lines the profiles obtained with anti-HO-1 antibody. ΔMFI denotes the differences in mean fluorescence intensities (MFI) obtained with anti-HO-1 antibody and the control antibody.

Expression of Apoptosis-Related Proteins in ECV304

Expression of proapoptotic proteins Bax and Bcl-x remained constant even after hemin stimulation (Fig. 5, panel A). In contrast, the levels of one of the anti-apoptotic proteins, Bcl-2, increased significantly after hemin stimulation in a dose-dependent manner, reaching a maximum at 300 μM of hemin (Fig. 5, panel A). When the levels of apoptosis-related proteins were compared between clone No. 2 and clone No. 9, the expression of Bax and Bcl-x did not change significantly (Fig. 5, panel B). The level of Bcl-2 was higher in clone No. 9 with an intermediate level of HO-1 (Fig. 5, panel B, lane 3) than in clone No. 2 with a higher level of HO-1 (Fig. 5, panel B, lane 2).

Morphological Characteristics of ECV304 Clones

Peculiar changes in morphology were observed in clone No. 2 of HO-1-transfected ECV304. Whereas wild ECV304 line exhibited distended cytoplasm with numerous protrusions and

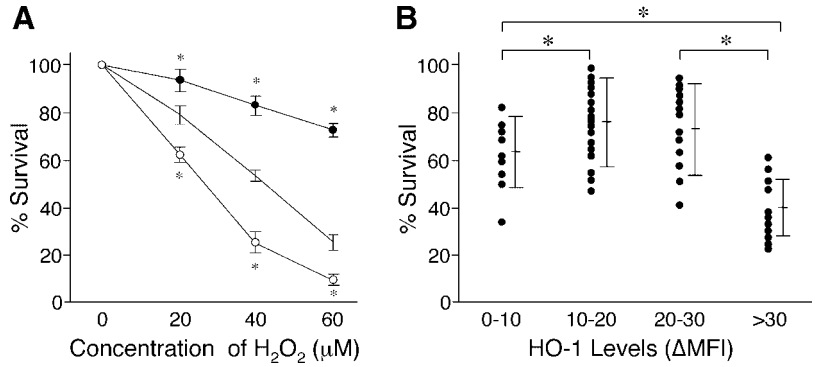

Fig. 4. Effect of HO-1-transfection on cytotoxicity induced by hydrogen peroxide. In **panel A**, wild ECV304 lines (no symbol), No. 2 (open circles) and No. 9 (closed circles) of HO-1-transfected ECV304 clones were cultured for 12 h in the presence of various concentrations of hydrogen peroxide. In **panel B**, different clones of HO-1-transfected ECV304 were classified into four different groups based on the levels of HO-1 expression, and the cytotoxicity induced by hydrogen peroxide was compared among these groups. Hydrogen peroxide was added at 40 μM in these cultures. Asterisks indicate the statistical significance ($P < 0.01$).

contact surfaces (Fig. 6A), clone No. 2 showed membrane ruffling and elongated cytoplasm with less contact surface (Fig. 6E). In confluent cultures, wild line showed a characteristic cobblestone appearance, similar to endothelial cells (Fig. 6B). In contrast, clone No. 2 showed accumulation of elongated cells (Fig. 6F) with less prominent cell to cell adhesion. As shown by flow cytometry, clone No. 2 expressed a much higher level of HO-1 than wild ECV304 line within their cytoplasm (Fig. 6G,C, respectively). Actin polymerization was visualized using FITC-conjugated phalloidin. As shown in Figure 6D, wild ECV304 line exhibited numerous intracytoplasmic stress fibers, whereas actin polymerization was concentrated at the anchorage points and few stress fibers were observed within clone No. 2 (Fig. 6H). These morphological characteristics of clone No. 2 were similar to those of wild ECV304 line after hemin stimulation (data not shown).

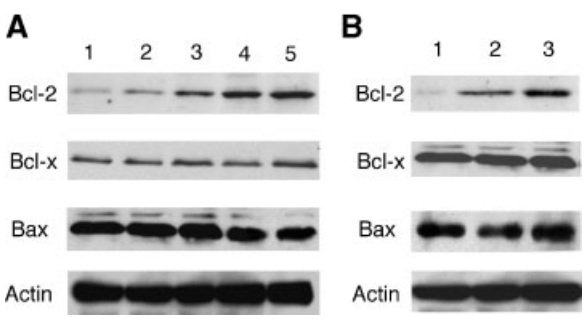

Fig. 5. Expression of apoptosis-related proteins. In **panel A**, expression of Bcl-2, Bcl-x, and Bax was examined after hemin stimulation. Actin was used as a control. Hemin was used at 0, 10, 30, 100, or 300 μM (**lanes 1–5**, respectively). In **panel B**, expression of these proteins was compared among wild ECV304 line (**lane 1**), No. 2 (**lane 2**), and No. 9 (**lane 3**) of HO-1-transfected ECV304 clones without hemin-stimuation.

Expression of α5β1 Integrin by ECV304 Clones

To see if loss of contact surface in clone No. 2 was associated with any change in surface molecule expression, we examined changes in ICAM-1 and α5β1 integrin by ECV304 clones. As compared to wild ECV304 line, clone No. 2 showed a siginificantly reduced level of α5β1 integrin (Fig. 7, panel A). In contrast, the levels of ICAM-1 did not differ significantly. After hemin stimulation of wild ECV304, the expression was reduced transiently but returned to a normal level within 3 days (Fig. 7, panel B).

DISCUSSION

The protective functions of HO-1/CO seem to be multifold. First, HO-1 acts as a potent anti-inflammatory agent through the production of CO [Brouard et al., 2000]. This is particularly important in protecting organ transplants from inflammatory immune reactions and rejection [Soares et al., 1998; Ke et al., 2002; Tsui et al., 2003]. Absence of HO-1 promotes graft rejection in an animal model of HO-1 deficiency, while pretreatment of the donor organs and the recipients with CO prevented rejection, indicating that the effect of HO-1 is mediated through production of CO [Otterbein et al., 2003]. In these experimental systems, CO acts to regulate the production of proinflammatory cytokines, thereby reducing the inflammatory reaction.

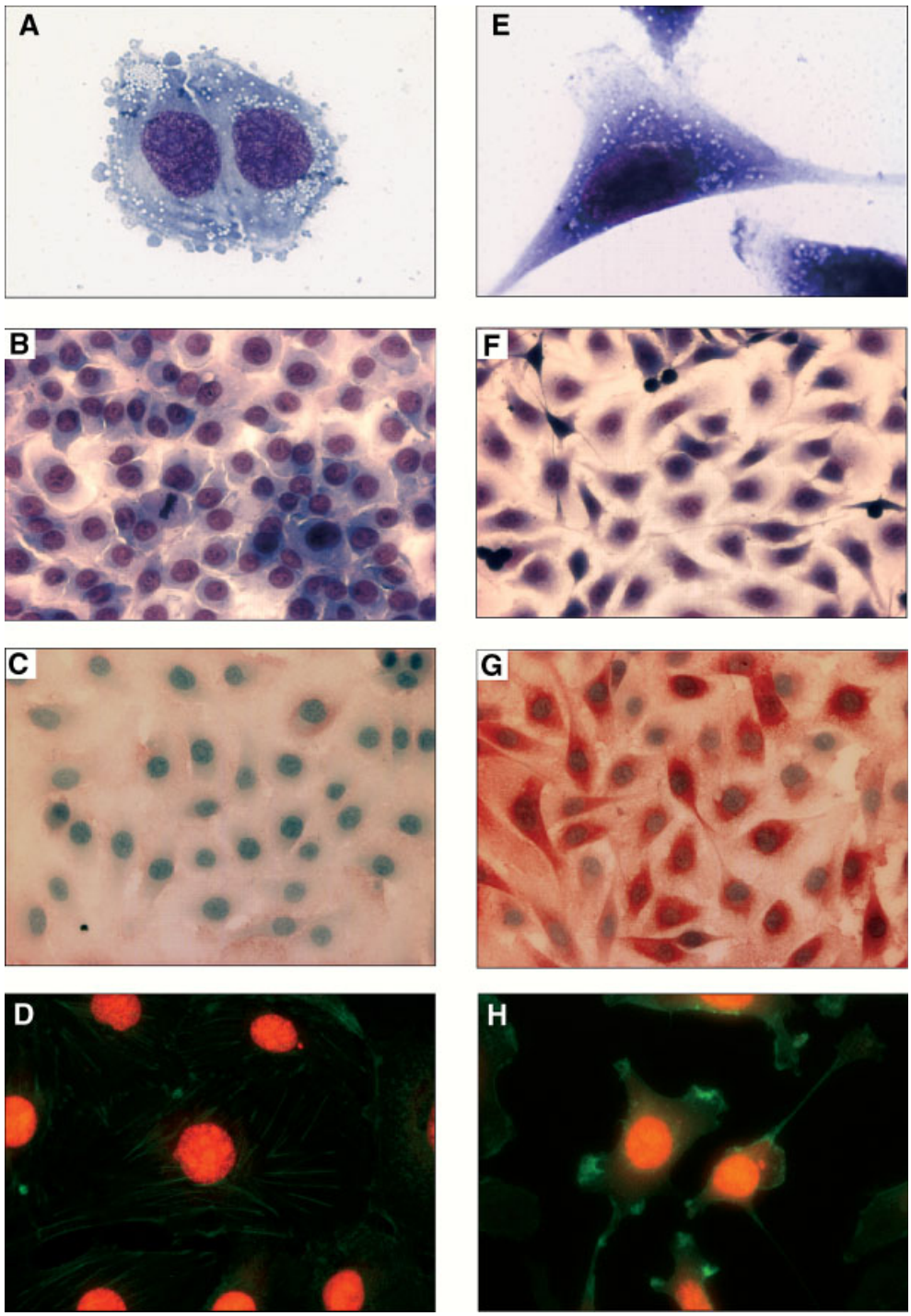

Fig. 6. Morphological changes of ECV304 cells after HO-1 transfection. Wild ECV304 line (**A**–**D**) and clone No. 2 of HO-1-transfected ECV304 (**E**–**H**) were compared. May–Giemsa Grünwald staining of low density sparse cultures and high density confluent cultures of wild ECV304 line (A and B) and clone No. 2 (E and F) are shown. Immunohistochemical study of HO-1 expression is shown in C and G. D and H show the actin staining with FITC conjugated phalloidin and propidium iodide.

Second, HO-1 may also exert its cytoprotective effect directly within a particular cell type, either through prolongation of cell survival or through prevention of cell injury under oxidative stresses [Lee et al., 1996; Pileggi et al., 2001; Liu et al., 2002]. This has been proved in various experimental systems. HO-1 transfected lung epithelial cells are significantly more resistant

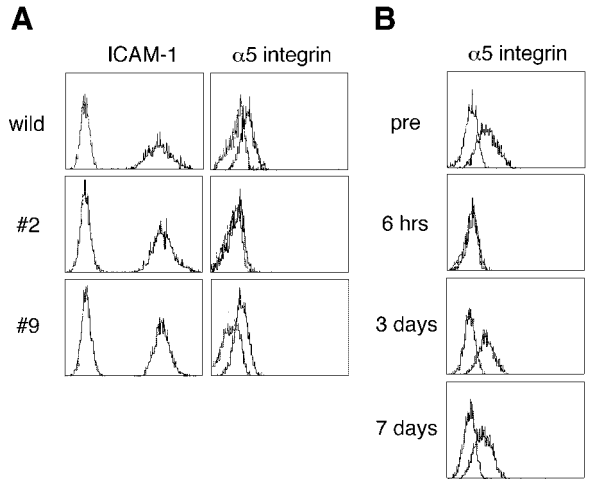

Fig. 7. Modulation of α5 integrin expression by HO-1. Expressions of α5 integrin and ICAM-1 were compared among wild ECV304 lines and No. 2 and No. 9 of HO-1-transfected clones (**A**). The fine lines indicated the profiles obtained by control antibody and the bold lines indicate the profiles obtained by the relevant antibodies. Expression of α5 integrin on wild ECV304 cells was compared after hemin stimulation (**B**).

to oxygen toxicity than control cells [Lee et al., 1996]. Renal tubular epithelial cells pretreated with hemin became resistant to H_2O_2-induced cell injury [Yang et al., 2003]. We have previously reported that a lymphoblastoid cell line (LCL) established from the first case of human HO-1 deficiency is particularly vulnerable to oxidative stresses. The enhanced cell injury seen in HO-1 deficient LCL was significantly reversed by *HO-1* gene transfection [Yachie et al., 2002b].

The cytoprotective effect of HO-1 is further confirmed by the studies of HO-1 promotor polymorphisms. Individuals with shorter GT repeats within HO-1 promotor region are known to have a reduced risk of restenosis after angioplasty [Exner et al., 2001]. GT repeat polymorphism is also associated with the frequency of lung fibrosis seen in patients with chronic obstructive lung disease [Yamada et al., 2000]. To support these clinical findings, in vitro studies indicate that shorter GT repeats within the promotor region have much higher promotor activity than longer GT repeats [Yamada et al., 2000; Hirai et al., 2003].

These anti-inflammatory and cytoprotective effects of HO-1/CO represent promising features of the gaseous mediators and provide a novel therapeutic approach for the treatment of vascular diseases. There have been numerous articles describing the effects of artificially induced HO-1 in the protection of vascular endothelial cells [Abraham et al., 1995; Deramaudt et al., 1998; Yang et al., 1999; Juan et al., 2001; Tulis et al., 2001; Melo et al., 2002]. Promotion of HO-1 activity either through gene transfer or by pharmacological induction plays important roles in the prevention of vascular diseases in these studies.

However, there have been several reports indicating that excessive levels of HO-1 may exert a deleterious effect on cell survival [Suttner and Dennery, 1999; Suttner et al., 1999]. Cell injury is promoted, rather than inhibited after prolonged expression of HO-1 at high levels, as detected by enhanced release of LDH into the culture medium. Although ferritin is normally induced and acts as a scavenger of the oxidative stress, a high level of free iron within cells may exert oxidative stress. In this study, HO-1 expression itself does not directly lead to significant ferritin production, as the intracellular ferritin levels within the HO-1-transfected clones are comparable to wild ECV304, as determined by immunoblotting. Or the levels of reactive oxygen species within these clones were similar to wild ECV304, as assayed by DHR123 (data not shown). Nevertheless, the fact that the expression of HO-1 is tightly regulated in vivo with regard to the cell types strongly indicates that the enzyme might exert adverse effects on cell survival and that it has to be degraded shortly after induction.

Our study clearly showed that there exists a dose-dependent effect of HO-1 on the cell survival of an anchorage-dependent cell ECV304. The cytoprotective effect of hemin was through production of HO-1, because the effect was completely abrogated when the culture was performed in the presence of SnPP, a specific inhibitor of HO activity.

In this culture system, higher hemin concentration resulted in accelerated, rather than diminished cell injury under oxidative stress. These results suggested that HO-1 may exert a detrimental effect on cell survival when present at high concentrations. This was also confirmed by using HO-1-transfected ECV304 lines. We utilized clones of ECV304 with different levels of HO-1 expression. The level of HO-1 expression remained relatively constant for several passages after gene transfection, and the experiments were performed during this period. The clone with a moderate level of HO-1 showed

enhanced cell survival under oxidative stress compared with the control wild ECV line, further suggesting that HO-1 itself but not hemin exerts the cytoprotective effect. The representative clone with the highest level of HO-1, however, exhibited enhanced cell injury, again confirming the previous experiment with hemin stimulation. Collectively, these data support the view that excessive levels of HO-1 have a paradoxical effect on cell survival and promote cell injury.

Apoptosis is known to be the major mechanism understanding cell injury upon oxidative stress. Various anti-apoptotic proteins are known to be induced in this context. Furthermore, these proteins are induced after *HO-1* gene transfer and play important roles in cell protection [Katori et al., 2002; Melo et al., 2002]. It is suggested therefore that the change in the level of HO-1 may alter the expression of apoptosis-related proteins within ECV304 cells. Among three apoptosis-related proteins, Bcl-2 expression was induced in a dose-dependent manner after hemin stimulation. In accord with this, HO-1-transfected ECV304 showed enhanced expression of Bcl-2 as compared to untransfected ECV304. Of interest was the finding that the clone with a high level of HO-1 showed less Bcl-2 expression than the clone with a moderate level of HO-1. These differences in Bcl-2 levels may explain the altered cell survival of ECV304 clones. However, the cell injury induced in ECV304 clones is not simple apoptosis. Our preliminary experiments showed that the cell injury induced after exposure to hydrogen peroxide is associated with simultaneous surface binding of Annexin V and intracellular uptake of propidium iodide, as determined by a flow cytometry (data not shown). It is postulated from these data that induction of HO-1 leads to secondary induction of Bcl-2 anti-apoptotic protein within ECV304, thereby contributing at least partly to the cytoprotective effect of HO-1.

The reason why the clone with high HO-1 level showed reduced Bcl-2 is not clear. However, the characteristic alteration in cell morphology of ECV304 is indicative of the different behavior of the clones upon oxidative stress. As shown in Figure 6, ECV304 exhibited characteristic morphological alterations after hemin treatment or after *HO-1* gene transfection. In contrast to the cobblestone appearance of wild type ECV304 cells with abundant contact surface along the periphery, hemin-treated ECV304 cells and the clone with a high level of HO-1 showed much less adherence to the contact surface with membrane ruffling and elongation of the cytoplasm. These morphological changes were associated with HO-1 expression and loss of stress fibers as shown by phalloidin staining. The attachment of anchorage-dependent cells to the contact surface is largely dependent on $\alpha5\beta1$ integrins [Davey et al., 1999]. As expected, the expression of $\alpha5\beta1$ integrins on ECV304 surface was reduced significantly either after hemin stimulation or by *HO-1* gene transfection. It has been reported that $\alpha5\beta1$ integrins play a significant role in the intracellular expression of Bcl-2 and therefore the protection of cells from apoptotic cell death [Zhang et al., 1995; Matter and Ruoslahti, 2001]. It is expected that this mechanism is particularly important for the cell survival of anchorage-dependent cells, such as ECV304.

These data provide significant hints as to how *HO-1* gene transfer should be performed for the treatment of vascular diseases. It is particularly important for anchorage-dependent cells, such as vascular endothelial cells and bronchial epithelial cells to have a tight regulatory system for the expression of transfected *HO-1* gene. Otherwise, overexpression or prolonged expression of HO-1 in vivo might lead to paradoxical cell injury and tissue damage, precluding the therapeutic potential of *HO-1* gene transfection.

Further study is necessary to determine if these mechanisms apply only to adherent cells. For the moment, it is more rational to use transient expression systems or to develop a regulatory system for the transfected genes in vivo.

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