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Antiapoptotic role of heme oxygenase (HO) and the potential of HO as a target in anticancer treatment*

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Heme oxygenase-1 (HO-1) is an inducible enzyme that catalyzes oxidative degradation of heme to form biliverdin, carbon monoxide (CO), and free iron. Biliverdin is subsequently reduced to bilirubin by the enzyme biliverdin reductase. Increasing evidence has indicated the critical role of HO-1 in cytoprotection and more diverse biological functions. Induction of HO-1 by various chemical inducers that are primarily cell stress inducers or by HO-1 gene transfection confers a protective capacity to cultured cells as well as to cells in several *in vivo* animal models. In addition, HO-1-deficient mice exhibit a significant increase in susceptibility to tissue injury. The cytoprotective action of HO-1 seems to be mainly a function of the antiapoptotic effects of the enzyme. HO-1 is believed to exert this antiapoptotic action by multiple mechanisms: (a) decreased intracellular pro-oxidant levels, (b) increased bilirubin levels, and (c) elevated CO production. CO may produce an antiapoptotic effect by inhibiting both expression of p53 and release of mitochondrial cytochrome *c*. HO-1 may also be a target in antitumor therapy because the growth of most tumors depends on HO-1. Our preliminary studies with an HO inhibitor showed a promising antitumor effect. This preliminary work warrants continued investigation for possible novel anticancer chemotherapy.

Keywords: anticancer target; apoptosis; bilirubin; cancer; carbon monoxide; heme oxygenase.

Introduction

Heme oxygenase (HO) plays a key role in regulating the intracellular heme level by catalyzing the initial and rate-limiting step of heme degradation, in which oxidative cleavage of the porphyrin ring results in formation of biliverdin, carbon monoxide (CO), and free iron.[1,2] Biliverdin is subsequently reduced by cytosolic biliverdin reductase to form the potent antioxidant bilirubin (Figure 1).[3] To date, three isoforms of mammalian HO have been identified: HO-1, HO-2, and HO-3.[1,2,4] Under physiological conditions, HO-1, the inducible 32-kDa isoform, is highly expressed in liver and spleen; HO-2 is the constitutive 36-kDa isoform and is expressed mainly in brain and testis. HO-3 is a recently cloned 33-kDa isoform that closely resembles HO-2: its mRNA has been detected in many organs including spleen, liver, thymus, prostate, heart, kidney, brain, and testis.[4] However, the physiological function of HO-3 remains unclear because of its very low enzyme activity compared with that of the other two isoforms.[4]

HO-1 is a member of the family of heat shock proteins (HSP32), and its expression is triggered by diverse stress-inducing stimuli including hypoxia,[5] heavy metals,[6] UV radiation,[7] reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2),[7] and reactive nitrogen oxides such as nitric oxide (NO).[8–12] The biological functions of HO-1 are thus believed to be associated with a fundamental adaptive and defensive response against oxidative stress and cellular stress.[7,13–18] Indeed, inhibition of HO-1 by using specific HO inhibitors such as zinc protoporphyrin (ZnPP) or tin protoporphyrin (SnPP) resulted in worsening of the diseases in which these stresses operate, such as graft rejection,[19] ischemia-reperfusion injury,[20] cisplatin nephrotoxicity,[21] and endotoxin-induced septic shock.[22] In contrast, HO-1 inducers, such as cobalt protoporphyrin, and selective overexpression of HO-1 by genetic manipulation produced beneficial effects in cultured cells and in a variety of animal models of various diseases in brain, heart, kidney, lung, and liver.[20,21,23–31] Accumulating evidence suggests a vital role for HO-1 in both cell growth and cell death, especially involvement of the enzyme in the regulation of apoptosis.[11,20,32–36] In this article, we review studies from our laboratory and from others that characterize the function of HO-1 and the effects of HO-1 on apoptosis. Furthermore, we describe the

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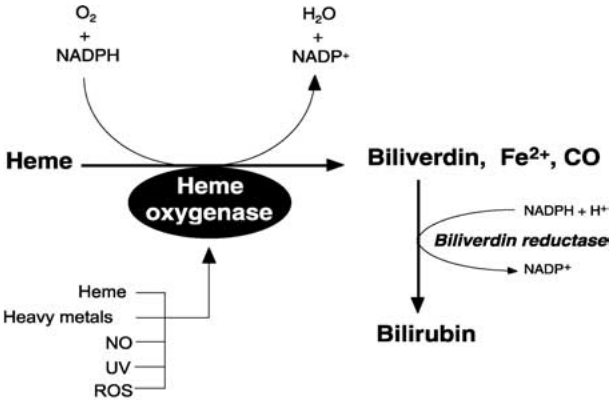
Figure 1. The reaction catalyzed by heme oxygenase (HO). Heme is cleaved by HO to yield equimolar amounts of iron (Fe^{2+}), carbon monoxide (CO), and biliverdin. Biliverdin is subsequently metabolized to bilirubin by the enzyme biliverdin reductase. The inducers of the inducible form of HO, or HO-1, include heme, heavy metals, UV radiation, nitric oxide (NO), and reactive oxygen species (ROS).



therapeutic potential of HO-1 inhibition, by targeting HO-1 in tumors, to induce tumor cell apoptosis.

HO-1 function: Implications for cell growth and cell death

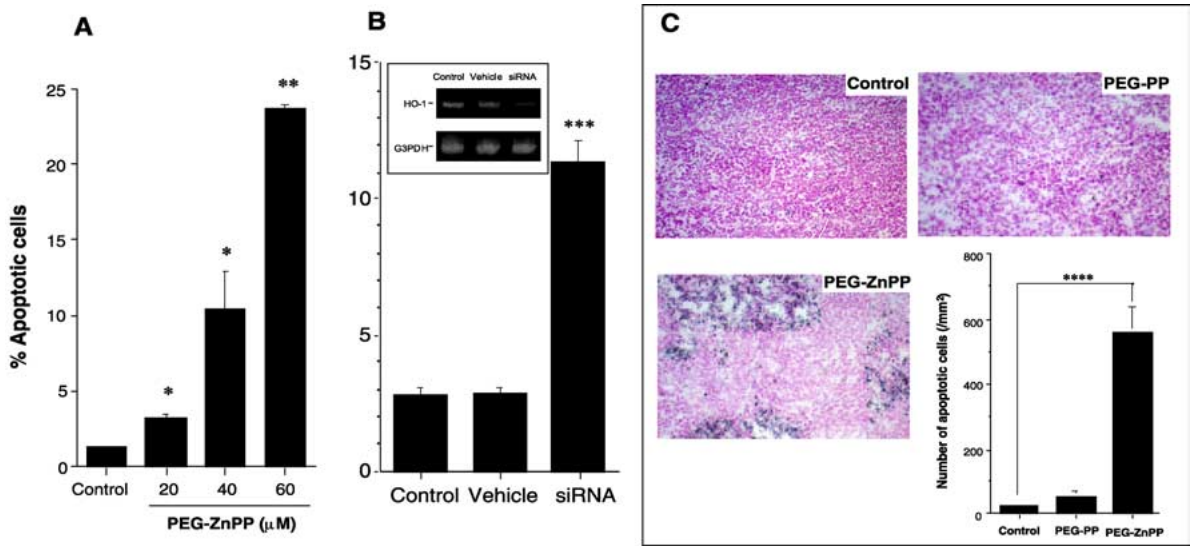
HO-1 has been reported to stimulate cell growth and the proliferation of various types of cells. HO-1 expression induced by NO donors enhanced the growth of epidermal keratinocytes, and administration of the HO-1 inhibitor SnPP totally nullified this proliferative effect.[37] A similar phenomenon was found in the vascular endothelium.[38,39] Of greater interest, transfection of the HO-1 gene into coronary endothelial cells promoted the formation of capillary-like tubular structures, which suggests a possible role for HO-1 in angiogenesis.[38] Consistent with these findings, high expression of HO-1 was observed in the hyperproliferating epidermis during cutaneous wound repair and in psoriatic skin lesions.[40] HO-1 was also extensively expressed in various tumor cells, which are the most rapidly proliferating cells, including adenocarcinoma, hepatoma, sarcoma, glioblastoma, melanoma, and squamous carcinoma cells.[10,41–44] In our laboratory, we studied the biological relevance of HO-1 in two experimental solid tumors, *i.e.*, rat hepatoma AH136B and mouse sarcoma S-180.[10,11,45,46] The levels of HO-1 expression in these two tumors were comparable to those in spleen and liver. Furthermore, administration of the HO-1 inhibitor ZnPP or its water-soluble derivative poly(ethyleneglycol) PEG-conjugated ZnPP (PEG-ZnPP) significantly suppressed the growth of these tumors, which suggests a critical role for HO-1 in tumor growth.[10,46] These data indicate the importance of HO-1 in cell growth, particularly tumor cell proliferation.

These data also lead to the question, How does HO-1 support cell growth? To clarify this, many researchers, including those in our laboratory, focused on the effect of HO-1 and its products on cell death and apoptosis, because cell growth is a result of the balance between cell proliferation and cell death. We found a clear antiapoptotic effect of HO-1 in rat AH136B hepatoma cells, as HO-1 protected cells against harmful oxidative stress.[11] Administration of the HO-1 inhibitor ZnPP resulted in a marked decrease in HO-1 activity and concomitantly induced a significant increase in apoptosis. More recently, we verified this antiapoptotic effect of HO-1 both *in vitro* and *in vivo*.[46] Human colon cancer SW480 cells receiving PEG-ZnPP treatment showed apoptotic changes in a dose-dependent manner; apoptosis was also observed in cells transfected with small interfering RNA (siRNA) for HO-1 to knock-down HO-1 expression (Figure 2A and B). *In vivo* studies provided additional evidence: administration of tumor-targeted PEG-ZnPP to sarcoma S-180 tumor-bearing mice induced significantly increased apoptosis of tumor cells (Figure 2C). Treatment with ZnPP also led to a considerable increase in caspase-3 activity, which was completely abrogated by simultaneous addition of a caspase-3 inhibitor.[11] These findings provide strong support for the proposal that HO-1 has potent antiapoptotic activity.

Many data suggest that the important cytoprotective effect of HO-1 operates not only in tumor cells but also in normal cells.[26,33,47–51] Studies with HO-1-deficient mice further support this benefit of HO-1 in various cells and organs. Poss and Tonegawa showed that HO-1 knockout mice suffered significant embryonic loss or subsequent death within 1 year of birth; HO-$1^{-/-}$ adult mice developed normochromic, microcytic anemia and demonstrated a progressive chronic inflammation in the kidney and liver.[52,53] Cultured HO-$1^{-/-}$ embryonic fibroblasts exposed to hemin, H_2O_2, paraquat, or cadmium chloride, produced high levels of ROS, and they were much more susceptible to cytotoxicity caused by hemin and H_2O_2 than were normal fibroblasts. Furthermore, young adult HO-$1^{-/-}$ mice exposed to endotoxin had increased mortality and were vulnerable to hepatic necrosis.[53] The first known human case of HO-1 deficiency exhibited similar characteristics, including growth retardation; persistent hemolytic anemia; severe, persistent endothelial damage; iron deposition; and extreme vulnerability to oxidative stress-related injury.[54]

Although most evidence corroborates the cytoprotective (*i.e.*, antiapoptotic) effect of HO-1, this is not a universal finding. Studies by Durante's group showed that high expression of transfected HO-1 in smooth muscle cells triggered apoptosis both *in vitro* and *in vivo*. This suggests that HO-1 may exert different effects on cell survival

Figure 2. Induction of apoptosis in SW480 cells by PEG-ZnPP. Cells were treated with increasing concentrations of PEG-ZnPP for 24 h (A) or were treated with siRNA for 48 h (B). The number of apoptotic cells was determined by a flow cytometric assay with Annexin V-FITC, and the percentage of apoptotic cells (per the total number of calculated cells) is shown. Values are means ± SE ($n = 3$ wells). In (A), $^{*}P < 0.05$ and $^{**}P < 0.00001$ versus control. In (B), vehicle indicates the result for cells treated with TransMessenger Transfection Reagent only (without siRNA); siRNA indicates the result for cells transfected with siRNA for HO-1 mRNA; $^{***}P < 0.0007$ versus control (no treatment). The inset in Fig. 3B shows reverse transcription-PCR analyses for HO-1 mRNA expression of the cells with or without siRNA (or vehicle) transfection. (C) gives results for *in vivo* induction of apoptosis in S-180 solid tumors by PEG-ZnPP. Apoptosis was detected by terminal deoxynucleotide transferase (TdT)-mediated dUTP-biotin nick endlabeling (TUNEL) staining of tumor specimens. TUNEL-positive cells were counted in four different fields per sample, and counts were calculated as the number of positive cells per mm^2. Data are means ± SE ($n = 3$ for each group). $^{****}P < 0.005$, PEG-ZnPP group versus control and PEG-PP groups. Modified from Ref. 46 with permission from American Association for Cancer Research.



depending on the level of HO-1 and the cell type.[55,56] As discussed later, high levels of bilirubin caused by overexpression of HO-1 may lead to toxicity, as in jaundice, which may explain the cytotoxicity of HO-1 when it is highly expressed.

Molecular mechanisms involved in the antiapoptotic effect of HO-1

With regard to the molecular mechanism by which HO-1 blocks apoptosis, three major pathways can be proposed: decreased intracellular pro-oxidant levels, increased bilirubin levels and elevated CO production.

Decreased intracellular pro-oxidant levels

HO-1 is the key enzyme in heme degradation; thus, increased HO-1 activity means rapid degradation of the heme moiety. Heme can induce the generation of various toxic ROS, such as lipid peroxyl radicals, as we reported previously.[57–59] Because of the high pro-oxidant potential of heme,[57–60] a decrease in heme content will lower the ROS levels in cells. Moreover, HO-1 induction is accompanied by increased synthesis of ferritin, which will sequester free iron and consequently result in reduced levels of free iron, a pro-oxidant.[61] HO-1 may also decrease cellular levels of free iron by increasing cellular iron efflux through up-regulation of an iron pump, which remains to be fully characterized.[62] High concentrations of cellular oxidants have induced apoptotic cell death in a variety of cell types,[63,64] and an increase in cellular oxidant production has often been found during apoptotic processes triggered by various stimuli including APO-1/Fas/CD95 ligands.[65–70] These findings suggest that the antiapoptotic effect of HO-1 depends, at least in part, on reduced intracellular pro-oxidant levels.

Increased bilirubin levels

Bilirubin, a yellow bile pigment, is another important mediator of the antiapoptotic effect of HO-1. It is a lipophilic tetrapyrrole, is abundant in blood plasma, is the final product of the cleavage of the heme ring that is catalyzed by HO and biliverdin reductase, and occurs uniquely in mammals. Bilirubin is reputed to be a potentially toxic agent at high concentrations (*i.e.*, >3 mg/dl in serum), particularly when it accumulates in the serum of neonates and causes jaundice, in which case substantial

deposits in the brain with the resultant kernicterus causing major brain damage.[71,72] However, bilirubin also appears to be one of the most abundant endogenous antioxidants in mammals and accounts for the major antioxidant activity in human serum.[73] Thus, bilirubin showed potent scavenging activity against various ROS including superoxide, peroxyl radical, and peroxynitrite.[73,74] More interesting, while the concentration of bilirubin in normal tissues is low (20-50 nM), this concentration operates in a redox recycling process catalyzed by biliverdin reductase, so that effective bilirubin concentrations are maintained in tissues. Consequently, biliverdin reductase protects against almost 10,000-fold excess concentrations of H_2O_2, as reported recently.[3] This finding explains the strong antioxidant potential of bilirubin, beyond a 1:1 chemical stoichiometry, and its physiological importance against toxic ROS. In fact, Dore *et al*. demonstrated that bilirubin derived from HO-2 played a neuroprotective role in H_2O_2-induced cytotoxicity in cultured rat primary neuronal cells.[74] Furthermore, mice with a genomic deletion of the HO-2 locus are more susceptible to stroke damage and the excitotoxitity of seizures.[75,76] Because ROS are believed to be potential mediators of apoptosis induction as described above,[63–70] it is reasonable to expect that the antioxidant activity of bilirubin may exert the antiapoptotic effect for HO-1 by scavenging toxic ROS.

In our laboratory, we investigated the potential role of bilirubin in the HO-1-induced antiapoptotic effect by use of rat hepatoma AH136B primary cells, which express a substantial amount of HO-1 during growth.[11] AH136B primary cells treated with the HO-1 inhibitor ZnPP exhibited a marked increase of apoptosis. In contrast, the number of apoptotic cells decreased dramatically during coincubation of bilirubin and ZnPP; this decrease occurred in a dose-dependent manner, in the range of 1–100 nM.[11] These results, along with those from others,[74–76] clearly indicated that bilirubin plays a major role in the antiapoptotic effect of HO-1. However, in our studies we also found no clearly dose-dependent inhibitory effect of bilirubin with concentrations higher than 100 nM.[11] Although this incomplete inhibition of apoptosis by bilirubin may be attributed to the cytotoxic effect of bilirubin itself rather than its antioxidant activity (as in the case of jaundice), pathways other than bilirubin may also contribute to the antiapoptotic effect of HO-1, such as decreased intracellular pro-oxidant levels, as mentioned above, and a CO-mediated pathway, which is discussed next.

Elevated CO production

CO is an important modulator molecule that in some respects has functions similar to those of NO, including acting as a neurotransmitter and modulating vascular perfusion.[77] CO generated by HO-1 during heme degradation has been suggested to be involved in the cytoprotective mechanism of HO-1.[11] For example, researchers in different laboratories have reported that exogenous administration of CO inhibits apoptosis of various cells including fibroblasts, endothelial cells, and vascular smooth muscle cells.[78–80] Liu *et al*. also showed that survival of cultured vascular cells is mediated by production of CO because cytoprotection obtained from HO-1 is reversed by the CO scavenger hemoglobin.[80] Moreover, a low concentration of CO can provide protection against hyperoxic lung injury *in vivo*.[81]

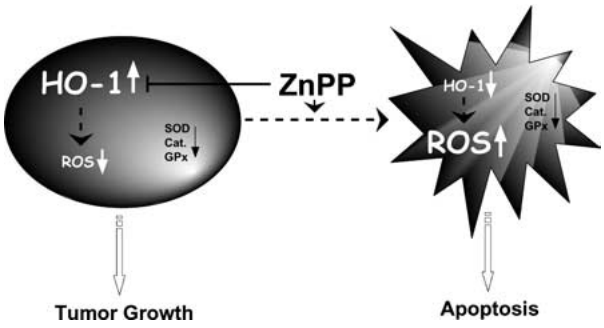
Inhibition of apoptosis afforded by CO may be mediated by several distinct mechanisms, although more detailed investigations are needed for full characterization of these mechanisms. In endothelial cells, the antiapoptotic action of CO was reported to result from activation of p38 mitogen-activated protein kinase (MAPK).[79] Liu *et al*. demonstrated in vascular smooth muscle cells that activation of soluble guanylate cyclase by CO contributed to inhibition of apoptosis;[80] however CO was much less potent than NO in activating soluble guanylate cyclase. With regards to the mechanisms by which CO functions in suppression of the apoptotic cascade, the same group also showed that CO not only blocked release of the mitochondrial cytochrome *c*, this factor being essential for apoptosis induction, but also inhibited expression of the proapoptotic protein p53. In addition, Inguaggiato *et al*. suggested that up-regulation of p21 by overexpression of HO-1 may have a role in marked resistance to apoptosis by suppressing proliferative cell growth.[82]

HO-1 as a target in clinical applications, especially cancer chemotherapy

Because of the potent cytoprotective role of HO-1, clinical application of the enzyme has also been suggested in various disorders, including atherosclerosis,[15,83,84] hypertension,[85] acute renal injury,[86] toxic nephropathy,[21] transplant rejection,[12,19,20,23,49] endotoxic shock,[22] chronic obstructive lung disease,[87] gastrointestinal diseases,[88] Alzheimer's disease,[89,90] and others.[26,91–97] Inhibition of HO-1 by a specific HO inhibitor such as ZnPP or SnPP has resulted in the worsening of the disease,[19–22] whereas administration of HO-1 inducers such as cobalt protoporphyrin, hemin, and trinitrobenzene sulfonic acid or HO-1 gene transfer had beneficial effects in certain other diseases.[20,21,23–31,88]

More important, it is now well known that expression of high levels of HO-1 occurs in various tumors,[10,41–46] and that HO-1 has an important role in rapid tumor growth because of its antioxidative and antiapoptotic effects.[10,11] On the basis of this information, we hypothesized that tar-

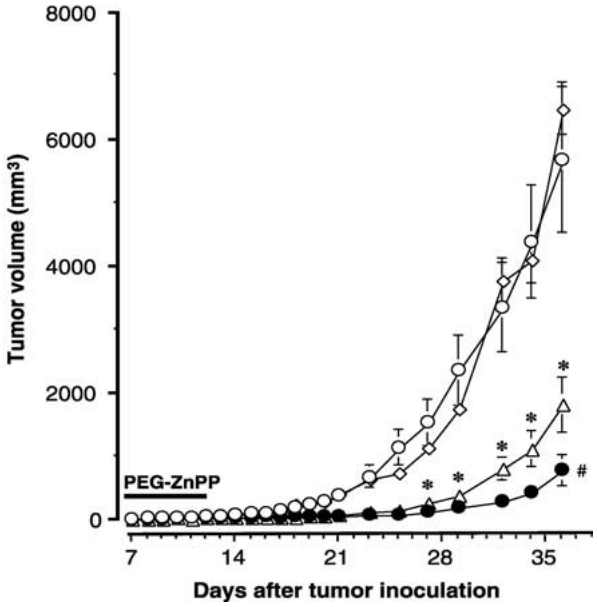
Figure 3. Schematic of a new anticancer tactics, that of targeting HO-1 in tumor cells. Tumor cells rely mostly on HO-1 for defense against toxic ROS, which supports rapid tumor growth (left). When HO-1 is inhibited by ZnPP, the intracellular ROS levels greatly increase because of the lack of antioxidative defense, which results in apoptosis. SOD, Cat., and GPx represent superoxide dismutase, catalase, and glutathione peroxidase, respectively.



geted inhibition of intratumor HO-1 activity by use of the inhibitor ZnPP may be a new anticancer treatment tactics. As shown in Figure 3, HO-1 or its products (*e.g.*, bilirubin and CO) render tumor cells protected against toxic ROS generated by host cells such as macrophages, thus sustaining tumor growth. However, antioxidative enzyme systems such as superoxide dismutase, catalase, and glutathione peroxidase are highly down-regulated in tumor cells.[98–101] Thus, the HO system appears to be a major antioxidative system in tumor cells. Therefore, inhibition of HO-1 activity by ZnPP in tumor cells should lead to increased ROS production in the tumor, and tumor cells would die through induction of apoptosis. As we expected, rats with AH136B hepatoma solid tumor that received an intraarterial injection of ZnPP via a tumor-feeding artery showed significant suppression of tumor growth.[10]

More recently, we prepared a water-soluble ZnPP derivative, PEG-ZnPP, that has not only the same HO-1 inhibitory activity as native ZnPP but also improved pharmacokinetic parameters in terms of drug targeting to solid tumor. We investigated its antitumor potential both *in vitro* and *in vivo*.[45,46] Human colon cancer SW480 cells treated with PEG-ZnPP exhibited a dose-dependent increase in intracellular oxidative stress, which finally led to apoptosis. Transfection of SW480 cells with siRNA for HO-1, to reduce HO-1 expression, resulted in a similar induction of apoptosis.[46] More important, systemic administration of PEG-ZnPP to sarcoma S-180-bearing mice dramatically suppressed tumor growth, which continued even after cessation of treatment (Figure 4). This result was comparable to, and even better than, the effect of the conventional anticancer drug doxorubicin: the maximum tumor inhibition of doxorubicin to S-180 tumor was 72%,[102] and our treatment using PEG-ZnPP showed a tumor inhibition of more than 80% with no or very few side effects.[46] Furthermore, our recent studies showed that PEG-ZnPP may enhance the chemotherapeutic response

Figure 4. Antitumor effect of PEG-ZnPP in the S-180 solid tumor model. S-180 cells (2×10^6 cells) were implanted subcutaneously in ddY mice. Seven days later, mice were treated with different doses of PEG-ZnPP (●, 5 mg/kg; △, 1.5 mg/kg) or PEG-PP (◇, 5 mg/kg) daily for 6 days. Control mice (○) received injections of physiological saline. Data are means ± SE ($n = 6$–8). $^*P <$ 0.001, PEG-ZnPP groups versus PEG-PP and control groups. #, Complete regression of tumor growth was observed in two of eight tumors after treatment with PEG-ZnPP (5 mg/kg). PEG-PP, PEG-conjugated protoporphyrin, a PEG-ZnPP analogue without HO inhibitory activity. Modified from Ref. 46 with permission from American Association for Cancer Research.



of tumor cells when it is used together with conventional anticancer drugs.[103]

Many conventional anticancer drugs such as doxorubicin and camptothecin have been reported to exert their antitumor effects by inducing production of toxic ROS.[104] Thus, our hypothesis was that reduced HO-1 activity may make tumor cells more susceptible to ROS-induced apoptosis, which thus would enhance the antitumor capacities of these anticancer drugs. In *in vitro* experiments, we found that PEG-ZnPP significantly reduced the median lethal dose of H_2O_2, *t*-butyl hydroperoxide, doxorubicin, and camptothecin in SW480 cells.[103] *In vivo* studies also clearly illustrated the synergistic effect of PEG-ZnPP plus an H_2O_2-generating anticancer agent, PEG-conjugated D-amino acid oxidase[105] (see Ref. 103 for detail). These findings strongly suggest the potential use of PEG-ZnPP, by targeting HO-1 in tumor cells, in anticancer treatment, especially when PEG-ZnPP is combined with other ROS-generating anticancer drugs. This possibility warrants further investigation.

In a related development in the area of effective and tumor-targeted anticancer treatment, we previously reported that biocompatible macromolecular drugs and lipids accumulate and are retained much more selectively

in solid tumor than in healthy tissue because of the unique characteristics of the tumor vasculature and the impaired lymphatic clearance system. We named this phenomenon the "EPR (enhanced permeability and retention) effect."[106–110] The EPR effect is now recognized as a universal characteristic of rapidly growing solid tumors, and the concept is regarded as one of the most rational strategies for designing anticancer drugs so that they selectively target solid tumors.[106–110] With regard to this EPR effect, PEG-ZnPP, a polymer conjugate with a molecular size larger than 70 kDa, exhibited particular accumulation in tumor tissue,[46] which indicated that tumor-selective inhibition of HO will be possible. In additional studies, such tumor-selective targeting finally did cause the marked suppression of tumor growth without apparent side effects.[46]

Conclusions

HO-1 was initially known as an enzyme needed for heme degradation. However, increasing evidence showed that HO-1 is a part of vital enzyme systems required for survival of mammals and has various physiologically by important functions including regulation of oxidative stress, anti-inflammation, and antiapoptosis. The antiapoptotic action of HO-1 derives mostly from its heme degradation products, including bilirubin, a potent antioxidant, and CO, a gaseous molecule that can trigger a series of signal transductions, with resultant significant antiapoptosis and anti-inflammation activities.

HO-1 is associated with various disorders, including atherosclerosis, acute renal injury, transplant rejection, endotoxic shock, respiratory disease and Alzheimer's disease. A new concept for treatment of these diseases involves elevation of HO-1 expression by means of a variety of HO-1 inducers or HO-1 gene therapy. Of greater interest, tumor cells usually have highly up-regulated HO-1 as a survival defense against host-derived ROS. Therefore, because tumor cells rely on HO-1 for rapid growth and circumvention of ROS, targeted delivery of HO-1 inhibitors into tumors may afford a new tactics in cancer therapy. Indeed, administration of the HO inhibitor, ZnPP or its polymeric derivative PEG-ZnPP significantly suppressed tumor growth and, more important, enhanced the antitumor efficacy of various conventional anticancer drugs, most of which depend on ROS generation for cytotoxic activity. Thus, studies of HO-1 as a new target molecule will open a new area in the control of various diseases including cancer.

Acknowledgments

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