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A High Expression of Heme Oxygenase-1 in the Liver of LEC Rats at the Stage of Hepatoma: The Possible Implication of Induction in Uninvolved Tissue

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We have examined changes in the expression of heme oxygenase-1 (HO-1), an inducible isoform and HO-2, a constitutive isoform, in the liver of Long-Evans with a Cinnamon-like color (LEC) rat, a mutant strain which spontaneously develops acute hepatitis and hepatoma. HO-1 expression was highly enhanced in the LEC rat livers with jaundice, and then decreased slightly, but overall remained at a higher level than in the Long-Evans with Agouti color (LEA) control rats, as judged by Northern blotting analysis of the whole liver extract. The high expression of HO-1 in the LEC rat liver was, however, not due to the actual cancer lesion but, rather, due to the surrounding uninvolved tissues including hepatocytes. Immunohistochemical analysis also supported this conclusion. Among normal tissues, the expression of HO-1 but not HO-2 was high in only the spleen of both LEC and LEA rats.

The high expression observed in the stage of acute hepatitis and hepatoma stages in the LEC rat is probably due to the oxidative stress caused by the accumulation of free copper and free iron levels which has been reported earlier by our group (Suzuki *et al.*, *Carcinogenesis*, 1993, **14**, 1881–1884 and Koizumi *et al.*, *Free Radical Research*, in press) as well as by free heme levels. The inflammatory cytokines produced by the surrounding tissue at the hepatoma stage would also be expected to play a role in the induction mechanism. The physiological relevance of HO-1 induction might be an adaptive response to oxidative stress and vasodilatory effect of carbon monoxide on sinusoidal circulation.

Keywords: Heme oxygenase, LEC rats, hepatocarcinogenesis

INTRODUCTION

Reactive oxygen species have been implicated in the development and progression of cancer, inflammation and aging. Cells possess specific enzymes that act directly on the reactive oxygen species, as a protective defense mechanism. Changes in the expression of antioxidative

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enzymes, such as superoxide dismutase, glutathione peroxidase and catalase in hepatoma and normal liver have been noted and discussed in the past,[1–3] and, it is noteworthy that the level of all these antioxidative enzymes was found to be significantly lower in hepatoma, as compared to normal tissues.

Heme oxygenase (HO), located in the microsome is an initial and rate-limiting enzyme in heme catabolism.[4] Two isoforms of HO, HO-1 and HO-2, have been identified. HO-1 is inducible by heme,[5] as well as by a variety of stress-related stimuli, such as heavy metals,[6] UV irradiation,[7] inflammatory cytokines[8] and hyperthermia.[9] In addition, HO-2 is constitutively expressed in most tissues.[9] Both isoforms catalyze the cleavage of heme to biliverdin, carbon monoxide (CO), and iron. Biliverdin is subsequently converted to bilirubin by biliverdin reductase. CO is capable of binding to the heme moiety of soluble guanylate cyclase, elevating cGMP levels in a similar manner to nitric oxide (NO).[10] The resultant CO thus modulates sinusoidal tone in the perfused rat liver and may regulate vascular tone under physiological conditions.[11] Recently it has been suggested that HO also functions as a defense system against oxidative stress through eliminating heme[12] and producing bilirubin which functions as a natural antioxidant.[13]

The Long-Evans rat with a Cinnamon-like color (LEC) is a mutant strain which spontaneously develops acute hepatitis at 16–20 weeks of age, followed by chronic hepatitis and, eventually, after one year, hepatoma.[14] Hepatic changes in LEC rats are closely associated with copper accumulation, which, in turn, is caused by deletion of the coding region of the copper-transporting ATPase gene (Atp7b),[15,16] which is a rat homologue of the Wilson disease gene (ATP7B).[17,18] Thus, the LEC rat can be regarded as both a *bona fide* model for Wilson disease and a natural model for hepatocarcinogenesis from the genetic and biochemical points of view.[19]

LEC rats are under a higher than normal level of oxidative stress as a result of ongoing Fenton reactions which are catalyzed by copper and iron ions which accumulate in the liver.[3,20,21] Since heavy metals and inflammatory cytokines have been reported to be elevated in LEC rats[19] and the fact that HO functions as an antioxidative enzyme,[12,13] it would be of interest to investigate the role of HO in the development of hereditary hepatitis and hepatoma. This paper reports an investigation of the expression of both HO-1 and HO-2 mRNAs and HO-1 protein during hepatocarcinogenesis in LEC rats. This represents the first finding which implicates a physiological role for HO-1 induction under oxidative stress during hepatocarcinogenesis in *bona fide* model animals.

MATERIALS AND METHODS

Animals

Animals were maintained at the Institute of Experimental Animal Science, Osaka University Medical School. Two LEC rats which had a hepatoma were purchased from Charles River Japan Inc. All research procedures complied with the ethical standards of the Helsinki Declaration in 1975. Rats were anesthetized with diethylether and then dissected. Samples were then immediately immersed in liquid nitrogen and preserved at −80°C until used. Long-Evans with Agouti color (LEA) rats, which are a sibling line of LEC rats but do not develop hepatitis or hepatoma, were used as controls in some experiments.

Materials

[α-^{32}P] dCTP and an oligolabeling kit were obtained from Amersham Corp. Polyclonal antibodies against HO-1 were obtained either from Stressgen Biotechnologies Corp. or raised in a rabbit as described below. All other reagents were of the highest analytical grade available.

Preparation of Anti-HO-1 Antibodies

A 20-mer peptide corresponding to residues 266–285 from human HO-1[15] was synthesized using an automated peptide synthesizer (Applied Biosystems, 432A Peptide Synthesizer SYNERGY). Female rabbits were immunized subcutaneously with the peptide, which was conjugated to ovalbumin (0.1 mg) in complete Freund's adjuvant. The rabbit was bled 2 weeks after the last injection and the anti-sera was used as an anti-HO-1 sera without purification.

cDNA Probes

cDNA probes for rat HO-1 and HO-2 for Northern blot analysis were prepared by the reverse transcriptase–polymerase chain reaction (RT–PCR) from total RNA of rat aortic smooth muscle cells in a primary culture,[22] using specific primers synthesized according to the reported sequences.[23,24] The expected size of RT–PCR products were subcloned, and confirmed by sequencing. Insert cDNA were excised by BamHI and HindIII restriction digests, labeled with the oligolabeling kit, and used for Northern hybridization.

RNA Preparation and Northern Blotting

Total RNA was prepared from rat tissues by the method of Chomczynski and Sacchi,[25] and quantitated by measuring the absorbance at 260 nm. Twenty μg of total RNA were heat-denatured at 68°C for 15 min in the presence of 50% formamide and the running gel buffer (20 mM morpholinopropanesulphonic acid (Mops), 5 mM sodium acetate, and 1 mM EDTA, pH 7.0), and then electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde. The size-fractionated RNAs were transferred onto Zeta-Probe membranes (Bio-Rad) for 15–40 h by capillary action, and the blotted RNAs were then immobilized on the membranes by incubation for 2 h at 80°C. After hybridization with the ^{32}P-labeled HO-1 cDNA probe at 42°C in the presence of 50% formamide for 12–20 h, the membranes were washed twice at 55°C with $2 \times$ SSC containing 0.1% SDS for 80 min. The Kodak XAR films were exposed for 1–7 days with an intensifying screen at −80°C. The blots were stripped and reprobed with a rat HO-2 cDNA using the hybridization and washing conditions described above.

SDS-PAGE and Immunoblotting of the Protein

Various tissue samples including liver with hepatoma were homogenized in 4 volumes of PBS with a polytron homogenizer. After centrifugation at $4300 \times g$ for 20 min, the supernatant fraction was used for further analyses. Protein concentrations were determined by the Bradford method.[26] Proteins were fractionated by 12% SDS-PAGE according to Laemmli[27] and then transferred onto nitrocellulose membranes (Schleicher & Schiell) using a Trans-Blot SD semidry transfer cell (Bio-Rad) for 80 min. After blocking in 4% non-fat powder milk for 80 min at room temperature, the nitrocellulose membranes were washed four times for 10 min each in washing buffer (TBS containing 0.05% Tween 20 and 0.1% bovine serum albumin), the blots were incubated for 60 min at room temperature with a 1 : 1000 dilution of anti-rat HO-1 polyclonal antibody. After washing the nitrocellulose membranes four times for 10 min each in washing buffer, the blots were incubated with peroxidase-conjugated goat anti-rabbit IgG (Organon Teknika) diluted 1 : 2000, for 45 min at room temperature. The immunoblots were again washed three times in washing buffer and twice in TBS and the signal detected by an ECL western blotting analysis system (Amersham).

Immunohistochemical Study

Tissue samples dissected from LEC rats were embedded in OCT compound. Cryosections

were made at a thickness of 7 μm and mounted on silane-coated glass slides. The sections were washed three times for 15 min at room temperature in PBS to remove the OCT compound, and postfixed with Zamboni fixative (2% paraformaldehyde, 0.21% picric acid). After rinsing with PBS, the sections were treated with 10% normal goat serum for 30 min to block nonspecific binding, followed by treatment with rabbit anti-human HO-1 antiserum (1 : 5000–1 : 10000) for 12 h. After washing for 15 min, they were reacted with biothinylated-goat anti-rabbit IgG for 30 min and then with peroxidase-conjugated streptavidin for 30 min using a peroxidase-conjugated streptavidin–biotin immunoglobulin detection system (Histofine SAB-PO kit; Nichirei, Tokyo). For the detection of peroxidase activity, the slides were immersed in a medium containing 40 mg/100 ml of 3,3′-diaminobenzidine and 0.01% H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.6, and monitored by light microscopy for the localization of peroxidase activity.

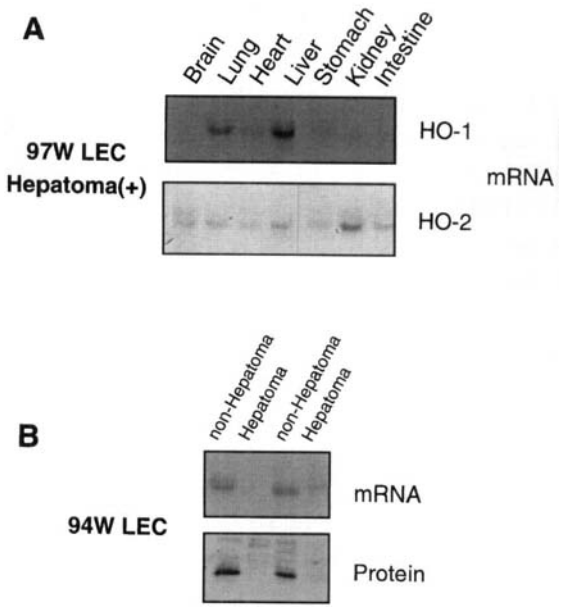

FIGURE 1 A: Tissue distribution of HO-1 and HO-2 mRNAs in LEC rats with hepatoma. The same membrane was hybridized with HO-1 and HO-2 cDNA fragments as probes. B: HO-1 expression in cancerous lesions and uninvolved adjacent tissues of LEC rats. Total RNA (20 μg), isolated from a cancerous lesion and uninvolved tissues of two LEC rats at 94 weeks of age, were analyzed by Northern blotting. Proteins (40 μg) from the same tissues were analyzed by immunoblotting using an anti-HO-1 antibody.

RESULTS

A High Expression of HO-1 in the Involved Tissues but not in Hepatoma Tissues

We compared the expression of HO-1 and HO-2 mRNAs by Northern blot analysis. Total RNAs were isolated from tissues of the 97 weeks old LEC rats bearing a hepatoma and used specific cDNA probes (Figure 1A). The HO-1 mRNA expression was high in the liver and lung. However, the level of expression of HO-2 mRNA was low and virtually the same among the organs, which is consistent with the reported nature of the gene.[9]

We then separated the hepatoma tissue from the uninvolved tissue in tumor bearing, 94 week old LEC rat liver and examined the expression of HO-1 in these samples (Figure 1B). The pathologically involved and uninvolved tissues were carefully excised macroscopically, and then assayed separately. The expression of both HO-1 mRNA and protein was below the detectable levels in hepatoma tissues under these conditions, but relatively high in the surrounding, uninvolved tissue. Thus the elevation of HO-1 mRNA in the whole liver of tumor bearing rats (Figure 1A) appears to be largely due to an enhanced expression in the surrounding, uninvolved tissue.

Immunohistochemical Detection of HO-1 in the Uninvolved Tissues of LEC Rat Liver

In order to determine which types of cells actually express HO-1 in LEC rat liver, an immunohistochemical analysis was carried out using the HO-1 specific antibody. Although the

antibody used for immunoblot gave a clear staining of hepatocytes, the background was slightly high (data not shown). We then raised a new polyclonal antibody in rabbits against the synthetic HO-1 peptide, which corresponds to the carboxyl-terminal domain of human HO-1. The antibody specifically recognized HO-1 on an immunoblot of cultured rat aortic smooth muscle cell homogenates (Figure 2). Immunohistochemistry of the same sample with this antibody clearly showed that hepatocytes surrounding the tumor lesion were specifically stained with a quite low background staining (Figure 3). The hepatocytes were sparsely distributed in the adjacent normal tissues, probably because of affects by the developing cancer cells. The cancer lesion, as well as other types of cells were, however, less stained by this antibody, and consistent with data from Northern blotting and Western blotting. Thus all data are consistent with HO-1 gene expression being augmented only in the surrounding, normal appearing hepatocytes in the tumor bearing LEC rat liver.

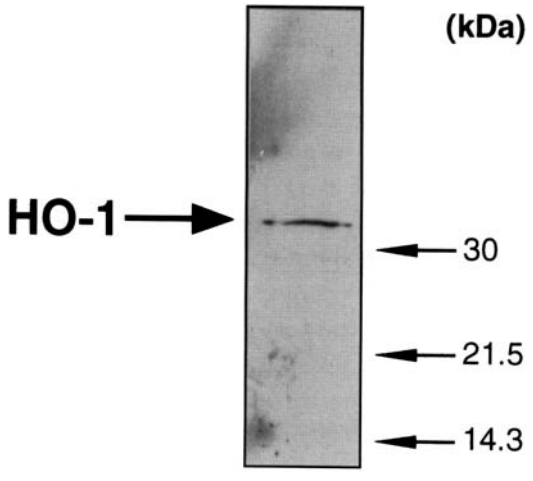

FIGURE 2 Immunoblot analysis of rat HO-1 protein using a rabbit polyclonal antibody against the HO-1 peptide corresponding to the carboxyl-terminal domain of human HO-1. Proteins (20 μg) from cultured rat aortic smooth muscle cell were subjected to immunoblot analysis. Analyses were carried out at the same conditions using a commercially available HO-1 antibody, obtained from Stressgen as described in Materials and Methods.

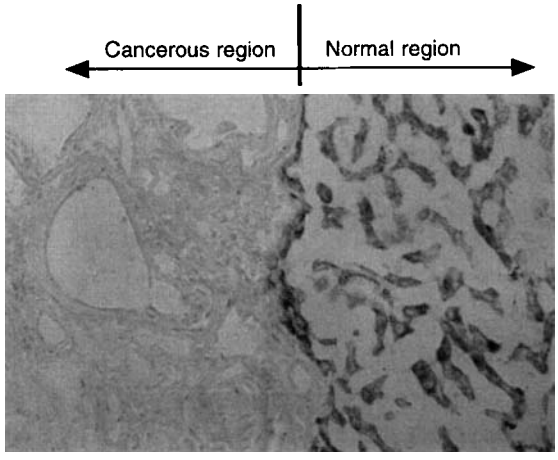

FIGURE 3 Immunohistochemistry of HO-1 protein in LEC rat liver with hepatoma tissue using an anti-HO-1 antibody (1:10000). HO-1 specific immunoreactivity of 7 μm-thick sections were visualized using a peroxidase-conjugated streptavidin–biotin immunoglobulin detection system. Magnification for the panel is ×80.

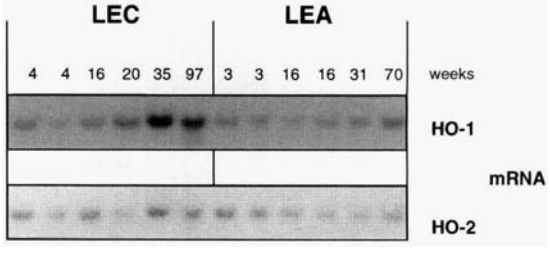

FIGURE 4 Northern blot analysis of total RNA from livers of LEC and LEA rats at different stages. The same membrane was hybridized with HO-1 and HO-2 cDNA fragments as probes.

Age-related Changes of HO Gene Expression in LEC and LEA Rat Livers

We then investigated age-related changes of HO mRNA expression in LEC and LEA rat livers as a function of weeks of age (Figure 4). The expression level of HO-1 mRNA was the highest in the liver of LEC rats with jaundice, which was diagnosed based on the appearance of a yellowish color in the coat and the bilirubineurea in the same manner as for the diagnosis of human jaundice. After the age of 16 weeks, HO-1 expression slightly decreased, but then increased

again, reaching the high level of the cancer stage. Only slight changes of HO-1 mRNA levels in LEA rat livers were observed with aging. The expression levels of HO-2 mRNA were nearly constant, regardless of age in both groups of rats.

High Expressions of HO-1 in Various Tissues of LEC Rats with Jaundice

Since LEC rats develop hepatitis at 16–20 weeks of age followed by spontaneous hepatoma,[14] we then compared the HO expression in several tissues of the LEC rats with jaundice to those without jaundice at the same 20 weeks of age. The HO-1 mRNA expression was highly induced in the liver and kidney of LEC rats with jaundice (Figure 5A,B). Lung also showed an induction in HO-1 mRNA expression, but to a lesser extent. However, HO-2 mRNA expression was virtually unaffected, even in rat tissues with jaundice, which is consistent with the reported nature of the gene.[9]

We also compared LEA rat tissues at 16 weeks of age as the control (Figure 5C). There was essentially no difference in the levels of HO-1 and HO-2 mRNAs between LEC rats without jaundice and LEA rats at this stage. HO-1 mRNA was highly induced in spleen as has been previously reported, but to a much lesser extent in other tissues, including liver, in both strains. The HO-2 mRNA was ubiquitously expressed in all tissues examined. We also confirmed a significant expression of HO-1 protein only in the spleen.

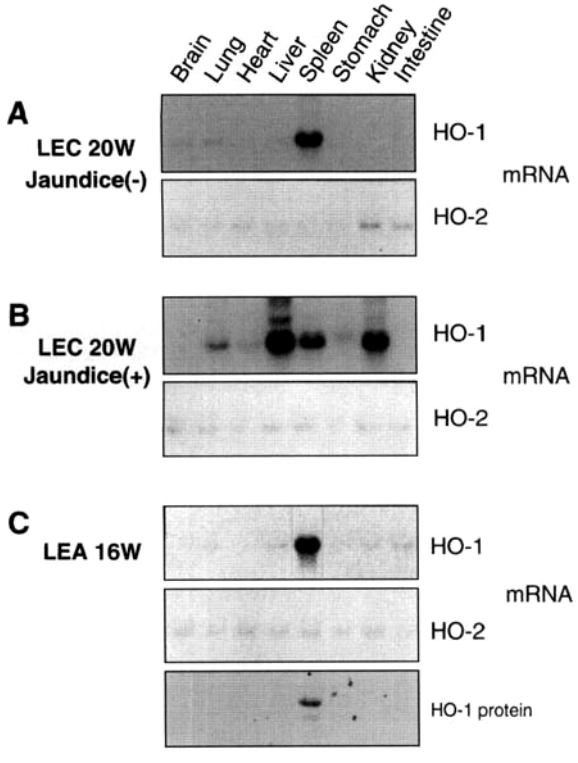

FIGURE 5 Tissue distribution of HO-1 and HO-2 mRNAs in LEC and LEA rats. A: LEC rats without jaundice at 20 weeks of age, B: LEC rats with jaundice at 20 weeks of age, and C: LEA rats at 16 weeks of age as control. The same membrane was hybridized with HO-1 and HO-2 cDNA fragments as probes.

DISCUSSION

The present study shows that the expression of HO-1, but not HO-2, is augmented in the liver of LEC rats after the onset of jaundice and remains at a high level through the cancer stages. In LEC rat liver with hepatoma, the uninvolved tissues expressed high levels of HO-1 mRNA and protein, while the hepatoma lesion itself exhibited only trace amounts of both. The LEC rat has a mutation with respect to the copper transporting ATPase gene (Atp7b),[15,16] which is the rat homologue of the human ATP7B gene and the defect responsible for Wilson disease.[17,18] Consequently, both copper which is bound to metallothionein and copper in the free state accumulate in the liver,[3,20,21] along with a concomitant increase in iron ion.[28] Free copper and iron ions constitute serious problems, since they mediate the production of the hydroxyl-radical, a harmful oxygen radical, which is involved in oxidative Fenton chemistry.[29] An increase in lipid peroxides and the production of reactive oxygen species have been reported in LEC rat livers,[30] thus supporting this view. Since HO-1 can be induced by reactive oxygen

species as well as heavy metals,[6] these stimuli are augmented in LEC rats, and would be expected to participate in the induction of the HO-1 gene. We[31] recently showed that levels of total copper in hepatoma lesions were significantly lower than the surrounding uninvolved tissues than is the case for hepatoma and hepatocytes of young-adults. This suggests that the accumulated copper ion in the tissues surrounding the hepatoma caused the induction of HO-1[32] in this lesion.

The strongest induction of HO-1 was observed in LEC rat liver with jaundice which is a consequence of hepatic failure by acute hepatitis. The release of free heme into the vascular vessels would be increased, as the result of the destruction of heme catabolism in hepatocytes. This would explain, in part, why HO-1 induction increased, not only in hepatocytes, but also in the kidney and lung. A number of genes which encode for enzymes with drug metabolizing activity,[30–32] and cancer-related genes[33–35] are also induced in liver with hepatoma, and the levels of these are generally higher in the uninvolved surrounding tissues, as well as in the cancer lesion. The promotor region of the HO-1 gene contains several regulatory elements for the binding of transcription factors, such as AP-1, AP-2, and NF-κB,[36,37] all of which are activated during inflammation. Since various cytokines including IL-1, TNF and IFN-γ are also produced in malignant cells, these may also affect the surrounding tissues causing the indirect induction of HO-1.

An important issue, therefore, is the exact role of overproduced HO-1 in liver of LEC rats with hepatitis and hepatoma. Bilirubinemia is associated with the risk of development of neurologic dysfunction as a consequence of the deposition of bilirubin in brain, suggesting that bilirubin is toxic to neuronal functions.[38] On the contrary, the antioxidative activity of bilirubin has also been demonstrated. Albumin-bound bilirubin can successfully eliminate the formation of peroxyl radicals and prevents the oxidation of low density lipoprotein and albumin-bound unsaturated fatty acids.[39] Thus, the role of bilirubin is obscure when produced in large amounts as in the case of jaundice.

Free iron ions, another product of heme degradation by HO, also induce the HO-1 gene expression.[40,41] Free iron ions which accumulate in the liver of LEC rats would be expected to induce the synthesis of ferritin and transferrin, which bind and detoxify ferrous ions.[29] These iron-carrier molecules synthesized in hepatocytes, however, would be in short supply due to liver dysfunction, resulting in an increase in the levels of unbound iron ion. As a result, the toxic and reduced form of iron, ferrous ion, exists at the stage of hepatitis and may participate in the production of hydroxylradicals through Fenton chemistry.

Carbon monoxide, another product of heme degradation by HO, modulates sinusoidal tone in liver via its NO like-vasodilatorial activity.[11] Accordingly, it is possible that the uninvolved, surrounding tissues in hepatoma might well gain an additional blood supply due to elevated CO levels. This increased circulation would be helpful for the remaining tissues in retaining normal function and in maintaining homeostasis, although it also would aid tumor cell growth by supplying increased oxygen and nutrition. Thus HO may exert its cytoprotective action through the production of CO and the elimination of potentially toxic heme on the one hand, and to increase the fraction of free iron ion and bilirubin on the other hand. In order to understand the physiological relevance of HO-1 induction in LEC rat liver, the use of specific enzyme inhibitors, such as Sn-protoporphyrin would be useful.

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