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Quantitative Measurement of Heme Oxygenase-1 in the Human Renal Adenocarcinoma

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Abstract Heme oxygenase (HO-1) is the rate-limiting enzyme in heme catabolism. HO-1, a stress protein, has been suggested to be involved in defense mechanisms against agents that may induce oxidative stress. It has been proposed that renal HO gene expression regulates important hemoprotein(s) such as cytochrome P450 and may be essential to maintain homeostasis in the kidney. Because accurate assessment of HO-1 mRNA in normal and disease states in kidney were not available due to the limited number of cells, we developed a system to quantitate human HO-1 mRNA in samples limited in cell number and/or mRNA copies. Total RNA from human kidney was used to establish this technique; it was reverse-transcribed and then amplified by polymerase chain reaction (PCR) in a tube also containing an internal standard obtained by deleting 50 bp from the original human HO-1 gene. This allowed us to use the same primers for both the sample and internal standard. After amplification, templates were resolved by acrylamide gel electrophoresis and quantitated either by densitometry or radioactivity counted from the bands excised from the gel. When the internal standard is present in the reaction mixture, the ratio of amplified sample vs. the standard template is proportional to the amount of sample RNA, and it is therefore possible to calculate the number of specific mRNA molecules. We have used this approach to quantitate the number of HO-1 mRNA molecules in adenocarcinoma cells. Results show that reverse transcription (RT)/PCR methods were able to determine the number of HO-1 mRNA copies in biopsy samples of human adenocarcinoma cells. © 1996 Wiley-Liss, Inc.

Key words: heme oxygenase, stress protein, adenocarcinoma, cancer, RT/PCR

A variety of oxidative stress-inducing agents, such as viral and bacterial toxins, metals, heme, and hemoglobin, have been implicated in the pathogenesis of the inflammatory process. The cellular response to such agents involves the production of a number of soluble mediators, including acute-phase proteins, eicosanoids, and various cytokines.

The rate-limiting enzyme in heme catabolism, heme oxygenase (HO), is a stress protein, and its induction has been suggested to represent an important protective response against oxidative stress produced by heme and hemoglobin [Abraham et al., 1988; Maines 1988; Marks et al., 1991; Shibahara et al., 1987; Mitani et al., 1989; Keyse and Tyrrel, 1989; Taketani et al., 1989]. Induction of HO may decrease cellular heme (prooxidant) and elevate bilirubin (antioxidant) levels [Abraham et al., 1995; Nath et al., 1992; Neuzil and Stocker, 1994]. Elevation of HO in tumor-bearing rats [Schacter and Kurz, 1986] and in partial hepatectomized rats [Solangi et al., 1988] resulted in a decrease in the content of renal and liver hemoproteins such as cytochrome P450. Two HO isozymes, the products of distinct genes, have been described [McCoubrey et al., 1992; Shibahara et al., 1993]. HO-1 is ubiquitously distributed in mammalian tissues and is strongly and rapidly induced by many compounds that elicit cell injury. The natural substrate of HO, heme, is itself a potent inducer of the enzyme [Abraham et al., 1988]. HO-2, which is believed to be constitutively expressed, is present in high concentrations in such tissues as brain and testis [McCoubrey et al., 1992]. In human skin fibroblasts, both HO-1 and HO-2 genes contribute to the enzymatic activity in stressful states [Applegate et al., 1995].

Endotoxin, interleukin-1, and other stress agents cause a rapid (within 5–10 min) activation of the HO gene and subsequent accumulation of HO mRNA [Cantoni et al., 1991; Riz-

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zardini et al., 1993; Lutton et al., 1992]. This process involves transcriptional activation of several regulatory sites in the human HO promoter region [Shibahara et al., 1987; Lavrovsky et al., 1994]. A recent study from this laboratory demonstrated that the proximal promoter region of the human HO gene contains NF-κB and AP-2 binding sequences [Lavrovsky et al., 1994]. The finding of these binding sites on the HO promoter suggests the potential importance of HO in the stress response, as these transcriptional factors are known to be activated during this process [Lavrovsky et al., 1994].

We were interested in evaluating the levels of renal HO-1 in both normal and adenocarcinoma biopsies. Most clinical samples are of limited cell number and/or copy number of mRNA. Conventional methods of mRNA analysis such as Northern blot and nuclease protection mapping were insufficient in sensitivity and/or accuracy. The polymerase chain reaction (PCR) technique has been widely used to amplify cDNA copies of low levels of mRNA after reverse transcription (RT) [Becker-Andre and Hahlbrock, 1989; Wang et al., 1989; Gilliland et al., 1990]. We applied this technique to renal tissue and achieved reliable quantitation by including an internal standard of equivalent amplification efficiency in the reaction mixture. We chose competitive RT/PCR, as it has become the most widely used form of quantitative RT/PCR and also offered the greatest advantage because by using competitive PCR it is not necessary to assay PCR products exclusively during the exponential phase of the amplification procedure [Cross, 1995; Murphy et al., 1990]. In competitive PCR, serial dilutions of the sample were used with a fixed amount of competitor template (internal standard) cDNA, and this mixture was subjected to amplification. We have applied this approach to determine the levels of HO-1 in human kidney obtained from both normal tissue and adenocarcinoma. The results of this study demonstrated that the human kidney basal level of HO-1 mRNA copies was markedly elevated in adenocarcinoma cells. Increased cellular expression of HO-1 has been implicated as a defense mechanism against oxidative stress generated in cancerous tissues.

MATERIALS AND METHODS

Guanidinium isothiocyanate and Moloney murine leukemia virus reverse transcriptase (MMLV-RT) were obtained from Bethesda Research Laboratories (Gaithersburg, MD); restriction enzyme EcoRI and [α-^{32}P]dCTP (3,000 Ci/mmol; 1 Ci = 37 GBq) was from Amersham Corporation (Arlington Heights, IL). DNA molecular weight marker VI was from Boehringer Mannheim Biochemicals (Indianapolis, IN), deoxynucleotides (dNTPs) (100 mM in sterile deionized H_2O) from Promega Corporation (Madison, WI), Taq DNA polymerase and restriction enzymes PstI and PvuII from Stratagene (La Jolla, CA), and Nusieve GTG and SeaKem LE agarose from FMC BioProducts (Rockland, ME). All other reagents of the highest grade obtainable were purchased from Sigma Chemical Company (St. Louis, MO) or Fisher Scientific (Springfield, NJ).

Internal Standard and Oligonucleotides

The plasmid pCMV-HHO-1 [Yoshida et al., 1988] was linearized with Eco47 III (a single restriction site in human HO-1 cDNA at position 420 bp). The linearized plasmid was digested with Bal 31 nuclease for 5 min in order to digest nucleotides and create a mutation. After this digestion, the construct was ligated with T4 DNA ligase. After transformation into JM109 *E. coli* competent bacteria, several clones were analyzed for the size of the mutated human HO-1 cDNA (mHHO-1) which resulted from the digestion by Bal 31 and for the presence of the Eco47 III restriction site.

One clone was selected with approximately 50 bp truncated from the original cDNA of HHO-1. This clone was amplified and the insert excised from the vector with Hind III and purified. This insert was used as the internal standard in the PCR reactions. Two previously published oligonucleotides [Kutty et al., 1992] were used as primers for the RT/PCR reaction: 5′-CAG GCA GAG AAT GCT GAG TTC-3′ (sense) and 5′-GAT GTT GAG CAG GAA CGC AGT-3′ (antisense). They were obtained from NBI (Plymouth, MN). These primers were designed to amplify a 555 bp stretch (79–633 bp) of the published cDNA of the human HO-1 [Yoshida et al., 1988].

Reverse Transcription/Polymerase Chain Reaction (RT/PCR)

Total RNA from human kidney was isolated using the method of Chomczynski and Sacchi [1987] and quantified by spectrophotometry. The quality of RNA was checked by gel electrophore-

sis and ethidium bromide staining. RNA was reverse-transcribed using the first strand cDNA synthesis kit from Clontech Inc. (Palo Alto, CA). Briefly, RNA in 13.5 μl of DEPC-treated H_2O containing 1 μl of oligo $(dT)_{18}$ (final concentration 0.2 μM) was denatured at 70°C for 2 min. The denatured RNA was placed on ice, and 6.5 μl of the reverse transcription mixture containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM of each dNTP, 1 U/μl of RNase inhibitor, and 200 U MMLV-RT was added. The reaction tube was then incubated at 42°C for 1 h followed by heating to 95°C to stop the reaction and then placed on ice.

The PCR reaction was performed by adding the PCR mixture to a final volume of 100 μl to the RT reaction tube. The PCR mixture contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.001% gelatin, 1.5 mM $MgCl_2$, 250 μM of each dNTP, 1 μM of sense and antisense primers, 2.5 U of Taq DNA polymerase, and 1 μCi of ^{32}P-dCTP. The reaction mixture was overlaid with two drops of mineral oil and subjected to 40 cycles as follows: 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min. After the last cycle, a final extension was performed at 72°C for 10 min.

Analysis of the Amplified Template

The amplied products were analyzed on a 6% acrylamide-bisacrylamide (37.5:1) gel in tris-glycine-EDTA buffer (1× 25 mM Tris, 200 mM glycine and 1 mM EDTA). After drying the gel, it was exposed for autoradiography. The autoradiogram was then analyzed by densitometry, or the radioactivity was counted after the bands were excised from the gel.

Quantitation of Human Adenocarcinoma

Normal human kidney RNA was obtained from either Clontech or the Westchester County Medical Center (Valhalla, NY). Biopsies of tumors obtained from pathological samples were processed for histological examination. The morphological characteristics of normal and carcinoma were assessed in six patients undergoing nephrectomy. The normal kidney samples were taken from a cortical portion of the kidney not in the vicinity of the tumor. Appropriate approval for the use of human kidney obtained from discarded samples was obtained from the New York Medical College Human Investigation Committee.

RESULTS

Two points had to be clarified before the quantitative nature of our RT/PCR system could be examined: 1) the amplified templates had to be clearly distinguished, and 2) the internal standard had to be amplified as efficiently as the sample templates.

To Distinguish Between the Amplified Templates

Several clones were analyzed for the size of the mutated cDNA (mHHO-1) resulting from the digestion by Bal 31 and for the presence of the Eco47 III restriction site. After transformation into competent bacteria by the mutated plasmid, PCR products obtained with our primers from several clones were analyzed on 6% acrylamide gel (Fig. 1). As a result, clone 2 (lane 3) was chosen for the experiments described below and designated pCMV-mHHO-1. This clone was also examined for size (Fig. 2). The difference in size between the two was approximately 50 bp (Fig. 2, lanes 5, 6), and in the mutated insert the Eco47 III site was missing (data not shown). To better evaluate the size of the PCR products, the two inserts (HHO-1 and mHHO-1) were amplified and analyzed on 2% agarose gel. A difference in 50 bp was demonstrated, as expected, from the previous results (Fig. 3).

Competitive vs. Noncompetitive Amplification

In order to achieve a reliable quantitation, it is critical to demonstrate that the internal standard and sample template(s) are amplified equally under our reaction conditions. We examined whether the 10% (50 bp) difference in the sequence between the two templates caused a

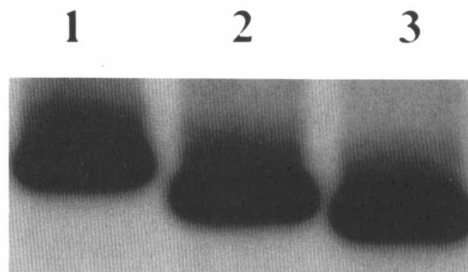

Fig. 1. PCR products obtained from the amplification of two clones (among 15 others) selected after modification of the Eco47 III site (Materials and Methods). *Lane 1:* Nonmutated HHO-1 as a positive control. *Lanes 2, 3:* Two different clones, 1 and 2, respectively. Clone 2 (lane 3) was chosen for the following experiments as the internal standard due to the presence of a larger deletion.

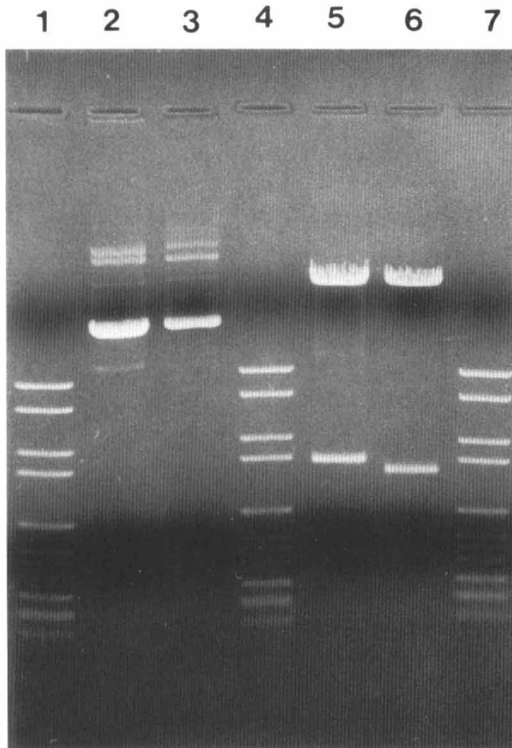

Fig
which was selected as an internal standard. *Lane 2:* pCMV-HHO-1. *Lane 3:* pCMV-mHHO-1. *Lane 5:* pCMV-HHO-1 digested by Hind III. *Lane 6:* pCMV-mHHO-1 digested by Hind III. *Lanes 1, 4, 7:* DNA molecular weight marker (pBR 328 DNA-Bgl I + pBR 328 DNA-Hinf I; marker VI [Boehringer Mannheim]).

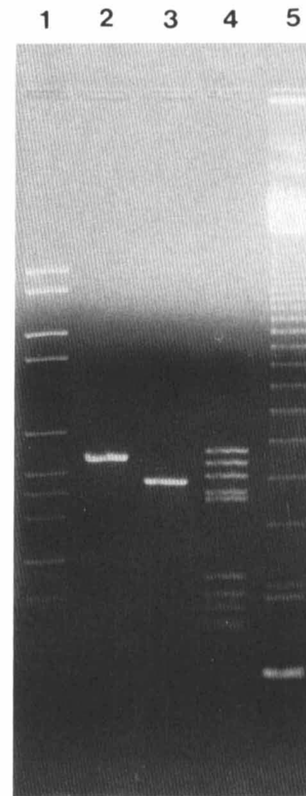

Fig. 3. Analysis of 2% agarose gel of the PCR products obtained from the original insert (HHO-1) compared to the mutated one (mHHO-1). *Lane 2:* PCR product from HHO-1 insert (555 bp). *Lane 3:* PCR product from mHHO-1 insert (500 bp). *Lane 1:* DNA molecular weight marker (marker VI [Boehringer Mannheim]). *Lane 4:* DNA molecular weight marker (pBR 322 DNA-Hae III; marker V [Boehringer Mannheim]). *Lane 5:* DNA molecular weight marker (123 bp DNA ladder [Gibco-BRL]).

discrepant amplification efficiency. Various amounts of total RNA from human kidney were subjected to RT/PCR alone or as a mixture with a fixed amount of the mutated insert (mHHO-1). In the combined PCR, the mHHO-1 was added at the PCR step, and RT was performed using oligo(dT)$_{18}$ primers as described in Materials and Methods. Respective templates were identified by their difference in size examined by electrophoresis on acrylamide gel and were quantified by densitometry of the bands on the autoradiogram. As can be seen in Figure 4, when the internal standard (mHHO-1) was included in the PCR mixture (the same amount was added to each reaction), the amount of amplified standard decreased as the amount of kidney RNA sample increased. These results indicate that the sample and the internal standard templates were both amplified in the same manner and were competing for the amplification; as the amount of one template increased, the chance of the other template being amplified declined (eventually, the portion of the other template became too small to be significant). It should be noted that, in the presence of the internal standard, the amplification of the kidney template does not have a linear relationship with its input. This has been noted by other investigators the saturation of the system [Becker-Andre and Hahlbrock, 1989; Wang et al., 1989]. However, the amount of specific target present at input can be determined from the ratio of the amplified kidney template to the internal standard.

Quantitation of Human Heme Oxygenase (HHO-1) mRNA

Once our RT/PCR system had been shown to be specific for designated targets and the sample and internal standard templates could be amplified with an equivalent efficiency, the quantitative nature of the system could be examined. In the course of testing for the competitiveness of the sample and the internal standard templates

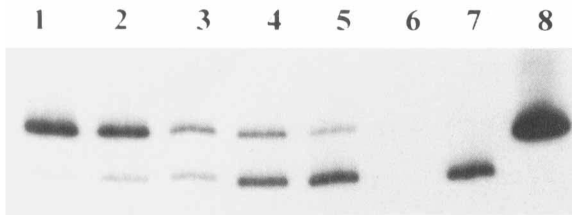

Fig. 4. Competitive amplification of human kidney total RNA and mutated insert (mHHO-1). Internal standard (mHHO-1) at 10 fg was mixed with human kidney total RNA at 200 ng, 100 ng, 50 ng, 25 ng, and 5 ng, respectively (*lanes 1–5*). *Lane 8:* Positive control of the PCR reaction, amplification of the HHO-1 insert (555 bp). *Lane 7:* Positive control of the PCR reaction, amplification of the mHHO-1 insert (500 bp). *Lane 6:* negative control (no RNA was used in the reaction).

for amplification, we found that 10 fg of standard was the appropriate amount required in the reaction mixture for human kidney RNA at the range of 1–200 ng to be amplified and unambiguously quantified. Because the internal standard and sample templates have been shown to be amplified as efficiently under the conditions described, it is therefore possible to determine the human HHO-1 mRNA content. The amount of HHO-1 cDNA was calculated by extrapolating from the intersection of the curve with a ratio of 1.0 down to the x-axis. Because the number of molecules in the standard is known, the actual number of target DNA molecules added to the PCR reaction can be calculated. In turn, the number of mRNA molecules can be calculated in the RNA sample used for RT, assuming that the efficiency of cDNA synthesis is 100%. The actual efficiency is less than that value; thus, such calculation gives the minimum number of mRNA molecules. As described previously, there are 500 bases in the standard that account for a molecular weight of approximately 1.7×10^5. Thus, 10 fg of standard has an equivalency of 4.02×10^4 molecules. The ratio was calculated taking into account the difference in base composition between the two templates. The number of molecules of HHO-1 in human kidney was estimated to be between 1,100 and 1,300 per nanogram of total RNA from normal human kidney. Once the method was established for kidney, we examined the expression of HHO-1 in biopsies of adenocarcinoma RNA. Adenocarcinoma samples from six different donors were used for detection of HO-1 mRNA copies, and results indicated that the mean of HO-1 mRNA copies was 6,780 ± 1,125 molecules/mg of total RNA. This is about five- to sixfold higher than that measured in control biopsies.

DISCUSSION

Due to its powerful amplification capacity, PCR has been widely used to detect DNA and mRNA species which are present in low levels. Various groups have employed this technique to trace residual malignant cells in leukemic patients after bone marrow transplantation [Cross et al., 1993; Frenoy et al., 1993; Radich et al., 1995; Lion et al., 1995].

The great power of amplification of PCR presents a challenge when the technique is used to quantify copy numbers of genes and mRNAs. Minute differences in any of the variables which affect the efficiency of amplification can dramatically alter product yield. The problem has been circumvented by coamplifying a target sequence with a standard template so that the reaction is completed in the presence of an internal control [Becker-Andre and Hahlbrock, 1989; Wang et al., 1989; Gilliland et al., 1990]. Accordingly, our system to evaluate the genetic expression of HHO-1 was developed with the following considerations in mind. First, the internal standard used was identical to the sample template in the target region; the difference was 10% in length due to the modification described in Materials and Methods. Second, the primers were derived from regions where the standard and the original templates have identical sequences. Consequently, differences in the melting temperatures of cDNA templates and primer/template duplexes which can greatly influence the amplification were of no consequence. Furthermore, the standard and sample templates can be easily distinguished on an acrylamide gel due to their difference in size (50 bp).

As expected, the great sequence similarity between the standard and sample templates rendered them good candidates for amplification. The input ratio of internal standard vs. sample RNA was therefore critical for a clear-cut measurement of the amount of each template amplified; this required that each sample be individually determined. When the ratio was appropriate, a linear relationship existed between the amount of input sample RNA and the amount of amplified sample template after the latter had been normalized against the amplified internal standard. We estimated that there are approximately 1,130 HHO-1 mRNA molecules in each nanogram of total RNA from human kidney using this technique.

In the present report, we also demonstrated that HO-1 mRNA in adenocarcinoma is elevated severalfold compared to normal tissues. Therefore, elevated HO activity (data not shown) in adenocarcinoma may be attributed to higher steady-state mRNA levels due to an increase in mRNA stability or decrease in degradation. The exact mechanism by which HO-1 mRNA increased in adenocarcinoma needs further clarification. However, renal adenocarcinoma is not the only malignancy in which an alteration of HO activity has been observed. It has been reported that in lymphosarcoma-bearing rats [Schacter and Kurz, 1986] HO-1 levels were severalfold higher than in normal rats. Therefore, a relation between malignancy and alteration of HO-1 may exist. This alteration of HO-1 gene expression may be the result of local or circulating factors released from malignant cells. In addition, the elevation of HO-1 in renal adenocarcinoma is in agreement with the reported finding that induction of HO-1 is a general response to oxidative stress [Abraham et al., 1988; Maines, 1988; Marks et al., 1991; Shibahara et al., 1987].

Elevated HO levels in renal adenocarcinoma may be an attempt by cells to provide a protective mechanism against oxidative stress by promoting bilirubin formation and causing a depression in the levels of heme and hemoproteins. The latter may be responsible for the generation of free radicals leading to membrane and cell damage as well as the formation of endogenous substances that promote tumor growth. In summary, it is believed that after all proper normalizations, this competitive RT/PCR method will be a sensitive and truly quantitative way to measure the level of HO-1 mRNA copies not only in adenocarcinoma but also in other systems where HO-1 is an indicative for stress conditions.

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