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Expression of Heme Oxygenase and Inducible Nitric Oxide Synthase mRNA in Human Brain Tumors

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Heme oxygenase-1, a key enzyme in heme catabolism, and inducible nitric oxide synthase (iNOS) are responsible for production of carbon monoxide and nitric oxide (NO), respectively. Expression of each enzyme has been shown to be modulated by heme and NO, raising a possibility for the coordinated regulation of the two enzymes. We therefore analyzed the expression levels of both mRNA in humans using brain tumors. Either heme oxygenase-1 or iNOS mRNA was expressed at higher levels in brain tumors compared to the brain tissue, but their expression levels were not apparently correlated. In the brain tumor cell lines, treatment with cytokines increased the expression of iNOS mRNA but not heme oxygenase-1 mRNA, whereas treatment with an NO donor increased the expression of heme oxygenase-1 mRNA but not iNOS mRNA. These results suggest the separate regulation of expression of both enzyme mRNA in humans.

Heme oxygenase-1, an essential enzyme in heme catabolism, cleaves heme to release carbon monoxide (CO), iron, and biliverdin (1, 2), and is induced by various stimulants (3, 4), such as hemin (5) and cadmium (6-8). The induction of heme oxygenase-1 has been considered as a defense system against oxidative stress, because bilirubin, derived from biliverdin, could function as a radical scavenger (9, 10). Nitric oxide (NO), a short-lived free radical gas, is synthesized from L-arginine by NO synthase (NOS) and functions as a messenger molecule in various cells and tissues (11, 12). Inducible NOS (iNOS) is characterized by its inducibility in response to combination of certain cytokines (13) and is responsible for the production of a large amount of NO (14, 15). By analogy with the functions of NO, CO has been proposed to be an active signaling molecule (16-18) and is also known to inhibit the NOS activity by binding the heme moiety (19). In the brain, CO and NO have been reported to be involved in long-term potentiation (16, 18, 20, 21), which is however still controversial (22-24).

It is noteworthy that heme degradation products and NO have both beneficial and detrimental effects for the host. Recent studies have shown that the expression of heme oxygenase-1 mRNA and protein was increased in the cerebral cortex and cerebral vessels in patients with Alzheimer's disease (25, 26), suggesting that heme oxygenase-1 is involved in the pathophysiology of certain neurodegenerative diseases. NO was also suggested to be involved in pathophysiological features of various neurological diseases (27-29). It is therefore of vital importance for humans that expression of heme oxygenase-1 and iNOS is properly regulated.

Several lines of evidence suggest the possible correlation in the regulation of expression of heme oxygenase-1 and iNOS. Heme, the substrate of heme oxygenase-1, is required for the

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assembly of an iNOS monomer to form a functional dimeric enzyme (30, 31) and also functions as a potent inducer of heme oxygenase-1 (3, 4). NO may function as a negative modulator of the iNOS activity by interacting with its heme moiety (32) and by inhibiting iNOS mRNA expression (33). On the other hand, expression of heme oxygenase-1 mRNA was increased by the treatment with NO donors in rat hepatocytes (34) and in T98G human glioblastoma cells (35), suggesting that NO may increase the expression of heme oxygenase-1 mRNA under certain conditions. Furthermore, in porcine vascular endothelial cells, both heme oxygenase-1 and iNOS activities were induced in parallel by the treatment with cytokines (36).

This study was therefore designed to examine the regulation of expression of the two inducible enzymes generating CO and NO in humans. As a first step, we examined the expression levels of both heme oxygenase-1 and iNOS mRNA in primary brain tumors and in the brain tumor cell lines expressing both enzymes.

EXPERIMENTAL PROCEDURES

Materials. Interferon-γ (IFN-γ) was a gift from Shionogi (Osaka, Japan), interleukin-1β (IL-1β) was from Ostuka Pharmaceutical (Tokushima, Japan) and tumor necrosis factor-α (TNF-α) was purchased from Genzyme (Boston, MA).

Human brain tissues. Pathological specimens of brain tumors were obtained from eight patients at surgery performed at the Department of Neurosurgery, Tohoku University Hospital, and were placed immediately in liquid nitrogen and stored at -80°C until RNA extraction (37). Tumor type and grade are summarized in Table 1. Human control brain tissues were obtained from a patient (66 years old, male) without neurological disorders (35, 37). Postmortem examination was performed within 3 hours of death at the Department of Pathology, Tohoku University Hospital. This study has been approved by the Ethics Committee on human study of Tohoku University School of Medicine.

Northern blot analysis. Total RNA was extracted from tissues by guanidium thiocyanate-cesium chloride method, and was subjected to Northern blot analysis. Total RNA (15 μg/lane) was electrophoresed on a 1.0% agarose gel containing 2M formaldehyde, transferred to a nylon membrane filter (Zeta-probe membrane, Bio-Rad, Richmond, CA), and fixed with a UV-linker (Stratalinker 1800, Stratagene). The filter was prehybridized at 42°C in a solution consisting of 5× SSC (0.75 M sodium chloride, 0.075 M sodium citrate), 1% sodium dodecyl sulfate (SDS), 50% formamide, 5× Denhardt's solution and 0.2 mg/ml salmon testes DNA for 2 hours, and was hybridized with radiolabeled cDNA probe at 42°C for 16 hours. The hybridized filter was extensively washed at 65°C with 1× SSC and 0.1% SDS. Radioactive signals were detected by exposing the filters to X-ray films (X-AR5, Kodak, NY) or with a Bioimage Analyzer (BAS 2000, Fuji Film Co., Ltd., Tokyo). Each cDNA probe was labeled with [α-^{32}P]dCTP (3000 Ci/mmol) using a random primer labeling kit (Takara, Tokyo, Japan). The hybridization probes for heme oxygenase-1 and constitutively expressed heme oxygenase-2 mRNAs were the XhoI/XbaI fragment (-64/923) derived from the human heme oxygenase-1 cDNA, pHHO1 (5) and the HinfI/HinfI fragment derived from the human heme oxygenase-2 cDNA, pHHO2-2 (38), respectively. A β-actin probe was prepared as described previously (38).

Preparation of cDNA probe for human iNOS. A human colorectal adenocarcinoma cell line, DLD-1, was cultivated at 37°C under 5% CO_2 in RPMI medium 1640 supplemented with 5% heat-inactivated fetal calf serum (FCS). RNA was prepared from DLD-1 cells treated for 6 hours with both IFN-γ (100 U/ml) and IL-1β (0.5 ng/ml), and was reverse transcribed into cDNA. Polymerase chain reaction (PCR) was performed to amplify an iNOS cDNA fragment. A forward primer, 5′-ATGGCCTGTCCTTGGAAATT-3′ (nucleotide positions 0 to 19), and a reverse primer, 5′-GGTAGGTTCCTGTTGTTTCT-3′ (positions 518 to 537), were based on the published iNOS cDNA sequence (39). Denaturation, annealing, and elongation were carried out at 94°C for 0.5 min, 50°C for 1 min, and 72°C for 1.5 min, respectively, and the reactions were repeated for 30 cycles. The 538-bp PCR product was isolated from a polyacrylamide gel, filled in at both ends with Klenow fragment (Toyobo, Tokyo, Japan), and subcloned into a pBluescript II (KS) vector (Stratagene, Cambridge, CA), yielding SpHiNOS1. The identity of its cDNA insert was confirmed by determining its nucleotide sequence using an automated DNA sequencer (model 373A, Applied Biosystems/Perkin-Elmer Co., Foster City, CA).

Cell culture. A human glioblastoma cell line, A172, was obtained from the American Type Culture Collection (Rockville, Maryland, USA). A172 cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% FCS. Cells were incubated at 37°C under 5% CO_2. To examine the expression of heme oxygenase-1 and iNOS mRNA, cells were cultivated in the fresh medium for 24 hours, and were exposed for 3-24 hours to a cytokine mixture, containing IFN-γ (500 U/ml), IL-1β (5 ng/ml), and TNF-α (500 U/ml). A172 cells were also treated for 5 h with an NO donor, sodium nitroprusside (SNP), in the presence or absence of 0.4 mM ascorbate. The treated cells were harvested for RNA extraction.

TABLE 1
Clinical Characteristics of Eight Patients with Brain Tumors

No.	Age	Sex	Histology of tumors	Location	HO-1 mRNA	iNOS mRNA
1	60 y	M	glioblastoma multiforme (grade IV)	temporal lobe	+++	−
2	2 m	M	choroid plexus carcinoma	ventricle	+	+++
3	28 y	M	anaplastic astrocytoma (grade II)	parietal lobe	+∼++	−
4	59 y	M	anaplastic astrocytoma (grade III)	frontal lobe	+∼++	+
5	39 y	M	anaplastic astrocytoma (grade III)	parietal lobe	++	+
6	66 y	M	anaplastic astrocytoma (grade III)	frontal lobe	++	+
7	51 y	F	anaplastic astrocytoma (grade III)	temporal lobe	+	++
8	24 y	M	glioblastoma multiforme (grade IV)	frontal lobe	+	+
9	66 y	M	control subject		+	−

Relative expression levels of heme oxygenase-1 and iNOS mRNA detected in Fig. 1 are indicated with either symbol ''+'', ''++'' or ''+++'', in which a symbol ''+++'' indicates the highest contents among the tumors examined. The symbol ''−'' represents undetectable expression levels. Case 9 represents a subject without neurological disorders and the brain tissue was derived from the frontal cortex. Abbreviations: HO-1, heme oxygenase-1; y, years; m, months; M, male; and F, female.

RESULTS AND DISCUSSION

The eight brain tumors examined are summarized in Table 1. Northern blot analysis showed a single species of 1.7-kilobases (kb) heme oxygenase-1 mRNA in the RNA samples of all eight brain tumors and the control brain tissue (Fig. 1). The highest levels of heme oxygenase-1 mRNA were detected in one case of a glioblastoma multiforme (lane 1). Expression of non-inducible heme oxygenase-2 mRNA was detected as two bands of 1.4 and 2.4 kb in these brain tumors and the brain tissue. The 1.4-kb heme oxygenase-2 mRNA is more abundant than the 2.4-kb species, as observed in various regions of the human brain (35). The expression of iNOS mRNA was also determined using the same RNA blot (Fig. 1). The iNOS mRNA of about 4.5 kb was expressed in the six brain tumors, with the highest contents of iNOS mRNA found in a choroid plexus carcinoma (CPC) (lane 2). In contrast, iNOS mRNA was

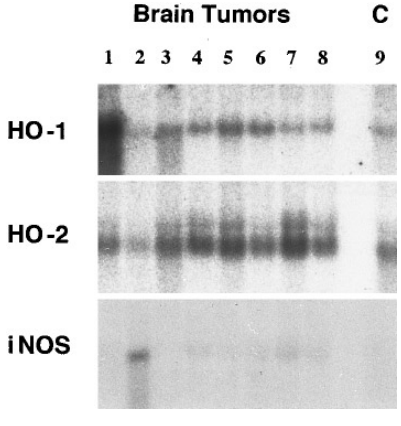

FIG. 1. Northern blot analysis of heme oxygenase isozyme and iNOS mRNA in human brain tumors. Shown is the autoradiogram of the RNA blot hybridized with each of ^{32}P-labeled cDNA probes, heme oxygenase-1, heme oxygenase-2, and iNOS. The numbers for brain tumors (lanes 1–8) represent the patient numbers shown in Table 1. Lane 9, designated with C, contained RNA derived from control brain tissue.

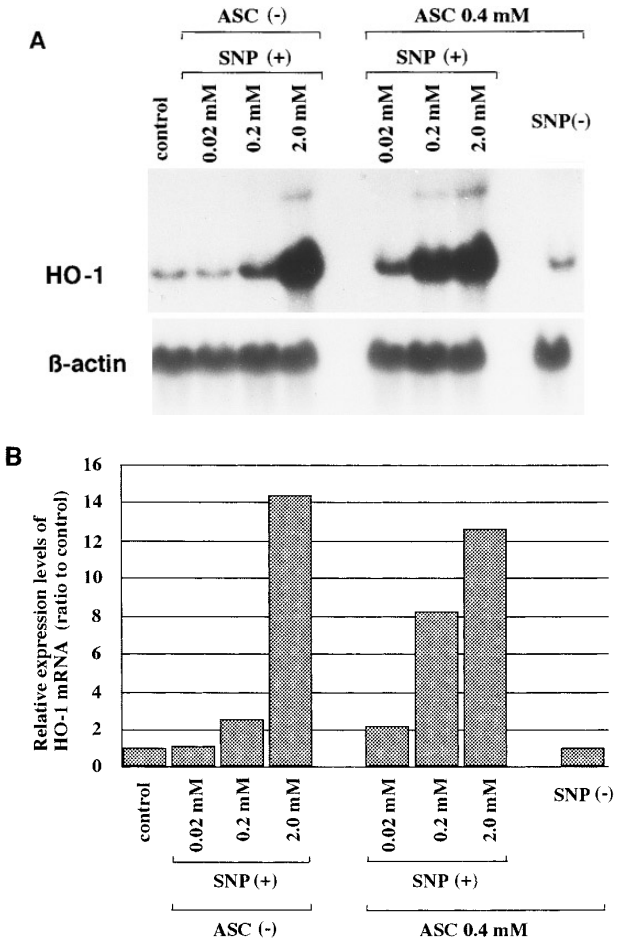

FIG. 2. Induction of heme oxygenase-1 mRNA in A172 human glioblastoma cells by an NO donor. A. Northern blot analysis. A172 cells were treated for 5 h with the indicated concentrations of SNP in the presence or absence of 0.4 mM ascorbate. Shown is the autoradiogram of the RNA blot hybridized with ^{32}P-labeled heme oxygenase-1 or β-actin cDNA probe. B. Relative expression levels of heme oxygenase-1 mRNA. The intensity of hybridization signals in A was quantified with a Bioimage Analyzer, and the intensity representing heme oxygenase-1 mRNA in each lane was normalized with the intensity for β-actin mRNA. The data are shown as the ratio of each normalized with the intensity for β-actin mRNA. The data are shown as the ratio of each normalized value to that of the control.

not detected in the glioblastoma multiforme expressing the highest contents of heme oxygenase-1 mRNA (lane 1) and in an astrocytoma (lane 3). Furthermore, iNOS mRNA expression was not detected in the frontal cortex obtained from the control subject (lane 9) and in other regions of the control brain, such as cerebellum, pons, and hypothalamus (data not shown), whereas expression of heme oxygenase isozyme mRNA was expressed in every region of the brain examined (35). The amounts of the RNA loaded were similar among the lanes assessed by staining the RNA gel (data not shown). Thus, iNOS mRNA appeared to be expressed at higher levels in brain tumors compared to the brain tissue, but no obvious relationship was noted between the expression levels of iNOS and heme oxygenase-1 mRNA.

Highest expression levels of heme oxygenase-1 mRNA in a glioblastoma multiforme prompted us to use A172 human glioblastoma cells, which were reported to express iNOS mRNA (40). Thus, this cell line is suitable to study the regulation of expression of both heme

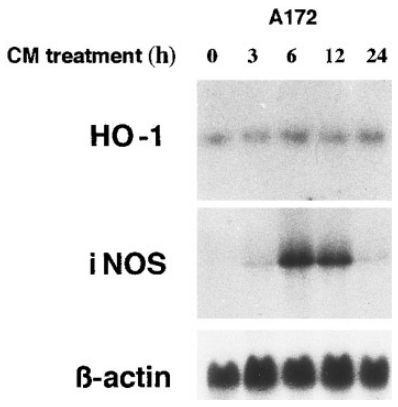

FIG. 3. Expression of heme oxygenase-1 and iNOS mRNA in A172 human glioblastoma cells. Shown is the autoradiogram of the RNA blot hybridized with ^{32}P-labeled heme oxygenase-1 or iNOS cDNA probe. Each lane contained RNA prepared from A172 cells untreated (0 h) or treated with the cytokine mixture (CM) for the indicated hours. A bottom panel shows the expression of β-actin mRNA as an internal control.

oxygenase-1 and iNOS mRNA. Northern blot analysis showed that heme oxygenase-1 mRNA expression was increased by the treatment with SNP in a dose-dependent manner (Fig. 2). We also examined the effects of ascorbate on the induction of heme oxygenase-1 mRNA expression, because ascorbate could increase the formation of NO from SNP (41, 42). The presence of ascorbate with the submaximal concentrations of SNP (0.02 and 0.2 mM) caused greater induction, suggesting that NO is responsible for the increase in the expression levels of heme oxygenase-1 mRNA. However, expression of iNOS mRNA was not detectable under the conditions used (data not shown). We are currently investigating the molecular mechanism by which heme oxygenase-1 mRNA expression is increased by SNP.

We then examined the effects of cytokines on the expression of heme oxygenase-1 and iNOS mRNA in A172 cells. Expression levels of iNOS mRNA were remarkably increased by cytokine stimuli from the undetectable levels in untreated cells, whereas the expression of heme oxygenase-1 mRNA was not noticeably affected (Fig. 3). This observation is consistent in part with a recent report of transient transfection assays, showing that the human iNOS gene promoter is able to confer the induction of a reporter gene in response to the mixture of IFN-γ, IL-1β and TNF-α (43). On the other hand, the lack of induction of heme oxygenase-1 mRNA in cytokine-stimulated cells suggested that the amount of endogenously produced NO is not sufficient to induce heme oxygenase-1 mRNA. We have also obtained the similar results using a cell line, established from the CPC tissue (44) which expressed the highest levels of iNOS mRNA as shown in Fig. 1. Treatment of CPC cells with cytokines increased the expression of iNOS mRNA but not heme oxygenase-1 mRNA (data not shown).

In summary, by using brain tumors, we provide the first evidence that expression of both inducible genes is regulated in a different manner in humans. These findings will facilitate the research on the possible coordinated regulation at the post-transcriptional levels of expression of heme oxygenase-1 and iNOS.

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