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# Area under the Plasma Concentration–Time Curve of Inorganic Fluoride following Sevoflurane Anesthesia Correlates with CYP2E1 mRNA Level in Mononuclear Cells

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***Background:*** **Because the amount of inorganic fluoride released after anesthesia with sevoflurane depends on the dose of administered sevoflurane and cytochrome P450 (CYP) 2E1 activity in the liver, a reliable and noninvasive probe for CYP2E1 would be useful for predicting plasma inorganic fluoride levels after anesthesia. In this study, the authors evaluated the relation between plasma concentration of inorganic fluoride after sevoflurane anesthesia and CYP2E1 mRNA level in mononuclear cells.**

***Methods:*** **Twenty patients (American Society of Anesthesiologists physical status I), aged 20–68 yr undergoing body surface surgery with general anesthesia with sevoflurane were enrolled. One milliliter of blood was obtained before administration of sevoflurane and mononuclear cells were obtained. Levels of CYP2E1 mRNA in mononuclear cells were measured by competitive reverse transcription polymerase chain reaction with a specific primer and competitor for CYP2E1 mRNA.**

***Results:*** **There was a significant correlation between level of CYP2E1 mRNA in mononuclear cells and the area under the plasma concentration–time curve of plasma inorganic fluoride from the beginning of sevoflurane administration to infinity in uninduced and uninhibited patients ($r^2 = 0.56$; $P < 0.01$).**

***Conclusions:*** **Area under the plasma concentration–time curve of inorganic fluoride after sevoflurane anesthesia correlates with CYP2E1 mRNA in mononuclear cells in peripheral blood. (Key words: Competitive RT-PCR; CYP2E1; mononuclear cells; sevoflurane.)**

INORGANIC fluoride is a common metabolite of fluorinated volatile anesthetics. Sevoflurane is a fluorinated volatile anesthetic that undergoes biotransformation, producing inorganic fluoride.[1-4] Because sevoflurane is selectively metabolized by cytochrome P450 (CYP) 2E1 in liver and the area under the plasma concentration–time curve (AUC) of inorganic fluoride after anesthesia with sevoflurane depends on chlozoxazone metabolic activity, a probe for hepatic CYP2E1 activity,[2,3] a noninvasive and reliable probe for hepatic CYP2E1 would be useful for predicting plasma inorganic fluoride level after sevoflurane anesthesia. Several methods have been suggested for evaluating CYP2E1 activity in the liver.[5] However, these methods necessitate liver biopsy or administration of test drugs. Recently, mRNA of CYP isoforms has been detected in mononuclear cells in peripheral blood, and the levels of these mRNAs are correlated with those of the corresponding CYP isoforms in the liver.[6-9] In this study, we evaluated the reliability of measurement of CYP2E1 mRNA in mononuclear cells as a specific method for determining CYP2E1-mediated inorganic fluoride production after sevoflurane anesthesia. For quantitative measurement of CYP2E1 mRNA levels in mononuclear cells, we developed a competitive reverse transcription polymerase chain reaction (RT-PCR), an

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improved method of conventional RT-PCR, which has been widely used to detect mRNA.[10]

## Materials and Methods

*Anesthetic Procedure*

The entire study protocol was approved by the Institutional Human Investigational Committee of Osaka City University. We obtained written informed consent from 20 patients before the study. The patients were classified as American Society of Anesthesiologists physical status I, aged 20–68 yr, and undergoing body-surface surgery. Exclusion criteria included abuse of ethanol, hepatic or renal dysfunction, heart disease, diabetes mellitus, treatment with any known inducer or inhibitor of CYP2E1 enzymes, and morbid obesity. All patients were premedicated with 100 mg secobarbital and 0.5 mg intramuscular atropine, administered 60 and 30 min before induction of anesthesia, respectively. Anesthesia was induced with intravenous 200 μg fentanyl, followed by 2 mg/kg propofol and 0.1 mg/kg vecuronium. After tracheal intubation, the lungs were ventilated mechanically with a tidal volume of 10–12 ml/kg to maintain the end-tidal carbon dioxide tension between 35 and 40 mmHg. End-tidal carbon dioxide tensions and concentrations of sevoflurane were measured by a gas analyzer (Capnomac Ultima; Datex Instrumentarium Corp., Helsinki, Finland). Anesthesia was maintained with sevoflurane (1–3 vol%) and 66% nitrous oxide in oxygen, with a fresh gas flow of 6 l/min. Inhaled sevoflurane concentrations were adjusted by the attending anesthesiologists to keep the systolic blood pressure and heart rate within 80–120% of the baseline values before anesthesia. The minimum alveolar anesthetic concentration (MAC)–hours exposure was calculated from the percent anesthetic concentration recorded every 5 min and the duration of exposure. MAC values of 1.71% were used for sevoflurane.[11] Administration of sevoflurane was discontinued when skin suturing was finished. Body temperature was monitored by a sensor placed in the urinary bladder and was maintained between 35.3–37.3°C. The trachea was extubated postoperatively and patients were then transferred to the recovery room.

*Blood Sampling and Determination of Plasma Concentration of Inorganic Fluoride*

To determine the plasma concentrations of inorganic fluoride, blood samples were drawn from an indwelling arterial catheter before anesthesia, at 1-h intervals during anesthesia, and 0, 1, 1.5, 2, 4, 8, 12, 15, 18, 21, and 24 h after discontinuation of sevoflurane. Plasma was separated within 10 min and stored at −40°C until analysis. The amount of blood collected for the study was less than 30 ml/patient, an amount insignificant compared with surgical loss. Plasma inorganic fluoride concentrations were measured using an ion-selective electrode (Ionmerter model 920A; Orion Research Inc., MA) and extrapolated for an infinite amount of time using the terminal elimination rate constant.[4] The coefficients of intra- and interassay variation were less than 4% and 3%, respectively. The area under the plasma concentration of inorganic fluoride–time curve (AUC) was calculated from the beginning of sevoflurane administration to infinity.

*Isolation of Total RNA and Competitive Reverse Transcription Polymerase Chain Reaction*

Mononuclear cells were isolated from 1 ml blood obtained before administration of sevoflurane by density-gradient centrifugation using a cell-preparation tube (Vacutainer CPT; Becton Dickinson Co., Franklin Lakes, NJ). Total RNA from mononuclear cells was isolated with the RNA isolation solution Isogen (Nippon Gene, Toyama, Japan), and the resulting RNA was treated with deoxyribonuclease (Nippon Gene) to remove contaminating genomic DNA. Total RNA was dissolved in nuclease-free water, quantified using spectrophotometry, and converted to cDNA with reverse transcriptase (RNA PCR kit AMV version 2.1; Takara Corp., Shiga, Japan). Polymerase chain reaction was performed using a method described previously.[12] Briefly, 2 μg total RNA was used in a total volume of 20 μl and heated at 30°C for 10 min, at 55°C for 60 min, and at 99°C for 10 min to synthesize cDNA. One microliter of each reverse transcription reaction mixture was used for PCR reaction containing 1 × PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each deoxynucleotide 5′-triphosphatase, 10 pmol of each of a pair of specific primers for CYP2E1, 1.5 U recombinant AmpliTaq DNA polymerase (AmpliTaq Gold; Perkin Elmer Corp., Branchburg, NJ), and a dilution series of DNA competitor (0, $10^2$, $10^3$, $10^4$, $10^5$ copies, number of molecules).

The nucleic acid sequences of the primers for CYP2E1 were sense 5′-CTG CAA CGT CAT AGC CGA CA-3′ (nucleotide positions 540–559 in the CYP2E1 sequence) and antisense 5′-TCC ATT TCC ACG AGC AGG CA-3′ (nucleotide positions 814–833).[13] The predicted size of the fragment amplified from cDNA is 294 base pairs (bp).[13] DNA competitor for CYP2E1 was synthesized from each of a pair of specific primers for CYP2E1 using

a competitive DNA construction kit (Competitive DNA Construction Kit No. RR017; Takara Corp.) modeled after the method described in the instruction manual. The size of the competitor DNA for CYP2E1 cDNA is 340 bp. PCR amplification was performed, after preheating at 94°C for 10 min, with 35 rounds of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 2.5 min. We used β-actin cDNA as an internal standard. DNA competitor for β-actin cDNA was designed using each of a pair of specific primers for β-actin (sense 5′-CAA GAG ATG GCC ACG GCT GCT-3′, nucleotide positions 714–734 in the CYP2E1 sequence,[14] antisense 5′-TCC TTC TGC ATC CTG TCG GCA-3′, nucleotide positions 968–988). Predicted size of fragment amplified from cDNA is 275 bp, as previously described.[14] The size of competitor DNA for β-actin cDNA is 342 bp, which is amplified by PCR using template DNA.

For precise measurement of the levels of CYP2E1 mRNA in mononuclear cells, we performed competitive RT-PCR with various doses of competitors in a more narrow range than the first dilution series of DNA competitors. After a dilution series of DNA competitor (0, $3 \times 10^4$, $10^5$, $3 \times 10^5$, $10^6$ copies) was added to cDNA, 0.1 μl each reverse transcription reaction product was used for PCR. PCR for β-actin was performed according to the methods described previously herein, except that PCR amplification was performed with 30 rounds. These PCR cycles are suitable for the current experiments and were previously determined after testing of various numbers of PCR cycles about samples and standards.[6]

**Table 1. Patient Demographic Data**

| Variable | Value |
|---|---|
| Sex (male/female) | 12/8 |
| Age (yr) | 43.1 (16.9) |
| Weight (kg) | 59.0 (11.0) |
| Height (cm) | 163.7 (8.4) |
| MAC hours of sevoflurane | 4.00 (1.55) |
| Duration of sevoflurane administration (min) | 210.6 (66.0) |

Values are mean (SD) in 20 patients.
MAC = minimum aveolar concentration.

### *Analysis of Polymerase Chain Reaction Products*

The competitive RT-PCR products were subjected to electrophoresis in a 2% (wt/vol) agarose gel, containing ethidium bromide, and cDNA bands were visualized with ultraviolet light. Nucleotide sequences of amplified fragments were confirmed by auto-DNA sequencer (Prism 310; Perkin Elmer Corp.) After being photographed, the image was exported in a file and the den-

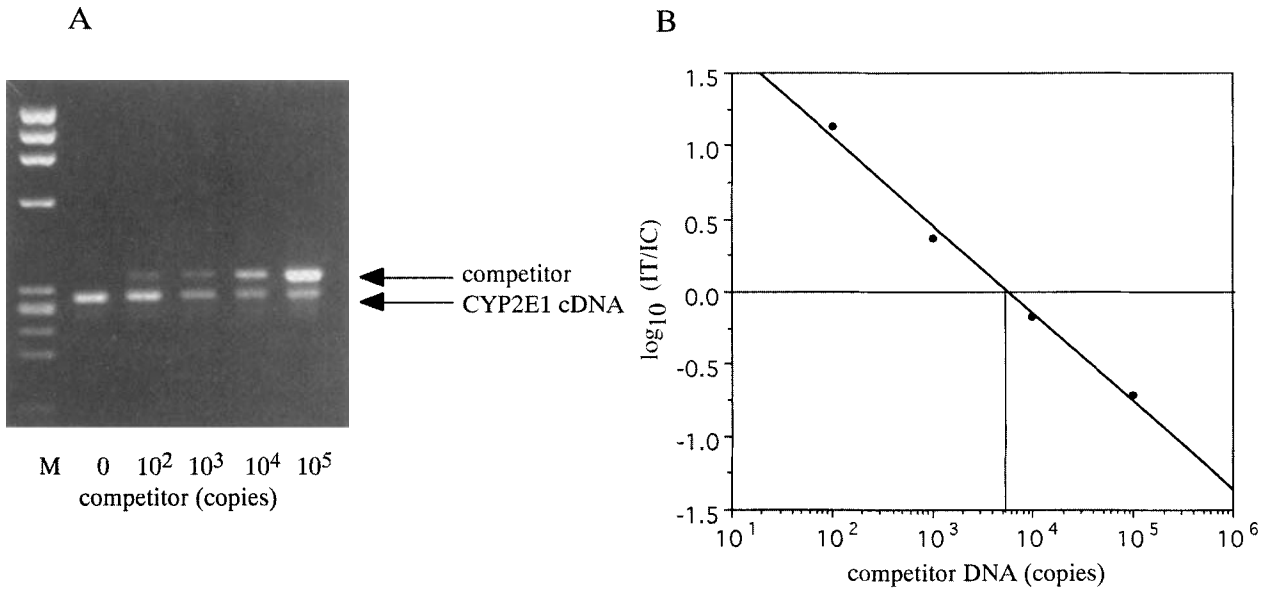


**Fig. 1. Measurement of CYP2E1 mRNA in human mononuclear cells by competitive RT-PCR. (*A*) Ethidium bromide-stained PCR products separated on agarose gel using a dilution series of DNA competitor (0, $10^2$, $10^3$, $10^4$, $10^5$ copies). The predicted PCR products are 275 bp for CYP2E1 cDNA and 340 bp for DNA competitor. Marker of molecular size (M). (*B*) Logarithm of the intensity of CYP2E1 cDNA band (IT) divided by that of competitor band (IC) plotted as a function of the logarithmic number of the competitor. The resulting regression equation was solved for $x$ when $y = 0$ to estimate the number of copies of CYP2E1 mRNA.**

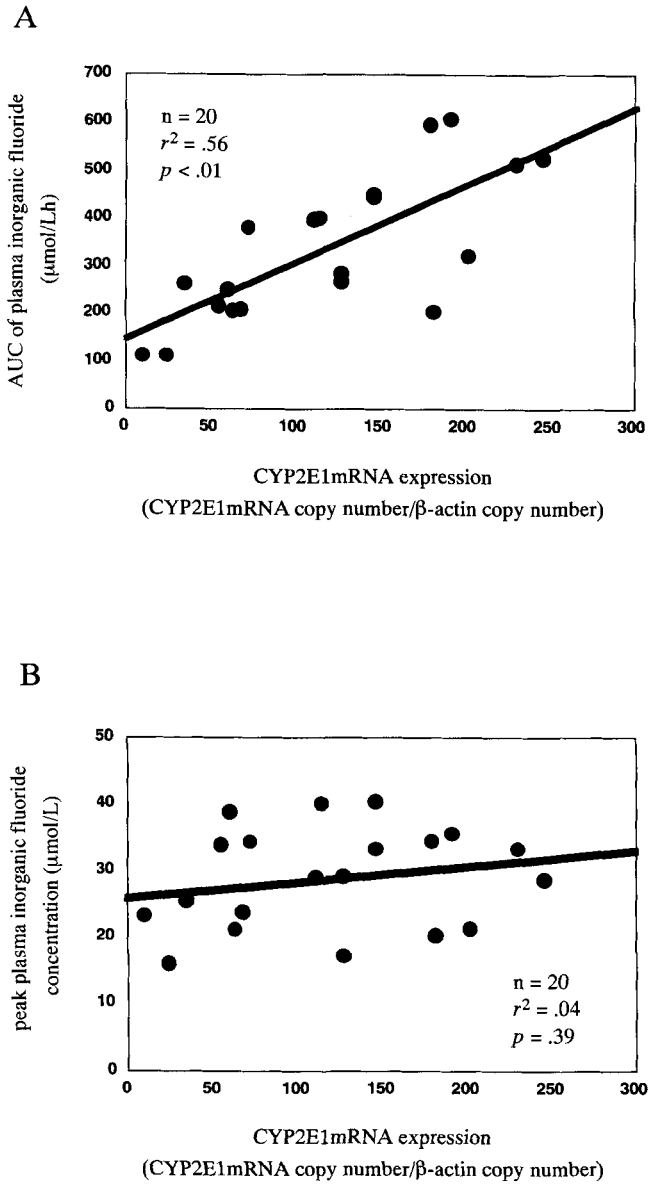


**Fig. 2. Correlation between CYP2E1 mRNA expression in mononuclear cells and the area under the curve of plasma inorganic fluoride ($r^2 = 0.56$; $P < 0.01$) and peak plasma inorganic fluoride ($r^2 = 0.04$; $P = 0.39$) in 20 patients.**

sities of cDNA bands were quantified using molecular analyzer (Bio-Rad Laboratories, Hercules, CA).

*Statistical Analysis*

Linear regression was used to evaluate the relations between the levels of CYP2E1 mRNA or MAC-hours of sevoflurane and the AUC or peak levels of plasma inorganic fluoride. Values are presented as the mean ± SD. Differences were considered to be significant at $P < 0.05$.

## Results

Patient demographic characteristics are shown in table 1. Arterial pressure and heart rates were almost unchanged during anesthesia, and no patients required blood transfusion. Concentrations of plasma inorganic fluoride were highest at 1 or 2 h after discontinuation of sevoflurane. Peak levels of plasma inorganic fluoride ranged from 14.6 to 66.2 μM, and the mean peak value was 31.4 ± 11.5 μM. Increase in serum creatinine or discharge of albumin in urine was not detected in any patients after anesthesia. The mean AUC of plasma inorganic fluoride from the beginning of sevoflurane administration to infinity was 344.0 ± 157.7 μM/h. These values are comparable with those reported previously.[3,4]

A typical gel image obtained by competitive RT-PCR is shown in figure 1A. When the dose of DNA competitor added to CYP2E1 cDNA was increased, the intensity in CYP2E1 cDNA bands on the gel was decreased. There were inverse liner correlations between the logarithm of competitor level and the logarithm of the ratio of intensity of CYP2E1 cDNA band to intensity of competitor band. The level of CYP2E1 cDNA was equal to the known amount of competitor when the logarithmic ratio of intensity of CYP2E1 cDNA band to intensity of competitor band was 0 (fig. 1B).[6] The levels of β-actin cDNA were also measured using this method. Levels of CYP2E1 cDNA normalized to those of β-actin cDNA are regarded as CYP2E1 mRNA levels.[12]

There was a significant linear correlation between CYP2E1 mRNA level and the AUC of plasma inorganic fluoride ($r^2 = 0.56$; $P < 0.01$; fig. 2A). There was no correlation between CYP2E1 mRNA level in mononuclear cells and peak concentration of plasma inorganic fluoride (fig. 2B). There were no correlations between MAC-hours of sevoflurane and the AUC or peak concentration of plasma inorganic fluoride either (data not shown).

## Discussion

We found a significant linear correlation between CYP2E1 mRNA level in mononuclear cells and the AUC of plasma inorganic fluoride after sevoflurane anesthesia. Because sevoflurane is selectively metabolized by CYP2E1 to inorganic fluoride and the AUC of inorganic fluoride is correlated with chlorzoxazone metabolic ratio, a probe for CYP2E1 activity in the liver,[2,3] our findings suggest that the level of CYP2E1 mRNA in mononuclear cells would be correlated with CYP2E1 activity

in the liver. Several methods have been suggested for measuring CYP2E1 activity in the liver. CYP2E1 activity in the liver is directly measurable by liver biopsy, which is, however, invasive and can be performed in only a limited number of patients. Chlorzoxazone can be used as a molecular marker of CYP2E1 in the liver.[15] This method necessitates administration of chlorzoxazone and measurement of its metabolite. CYP2E1 and CYP2E1 mRNA are detected in mononuclear cells in peripheral blood, and, according to animal experiments, the level of CYP2E1 in mononuclear cells is also correlated with that of CYP2E1 in the liver, suggesting that CYP2E1 in mononuclear cells would be a marker of CYP2E1 in the liver.[8,9] However, direct measurement of CYP2E1 in mononuclear cells by immunoblot analysis necessitates large amounts of blood, limiting the use in clinical practice.[9] In contrast to these methods, only 1 ml blood is necessary to measure CYP2E1 mRNA in mononuclear cells.

We used competitive RT-PCR in this report of quantitative measurement of CYP2E1 mRNA in mononuclear cells. Conventional RT-PCR without competitors has been used to detect P450 mRNAs in human lymphocytes.[8,9] However, accurate quantitation with conventional RT-PCR is hampered by a number of variabilities that can occur in the course of reaction, and variations in reaction conditions are greatly magnified during the amplification process. Competitive RT-PCR is an improved and reliable method for measuring levels of mRNA, based on competitive coamplification of a specific target sequence, together with a known concentration of an internal standard in one reaction tube.[12]

In our experiments, levels of CYP2E1 mRNA in mononuclear cells were correlated with the AUC of plasma inorganic fluoride, but not with peak plasma inorganic fluoride level. Kharasch *et al.*[2] pointed out that peak plasma fluoride levels were correlated with rates of metabolism of anesthetics in the liver. They found a linear correlation between peak plasma inorganic fluoride levels and the microsomal defluorination rates of various anesthetics, including desflurane and methoxyflurane, respectively, an anesthetic with minimal rate of metabolism and an anesthetic with the most extensive metabolism.[2] In their study, there were more than 20-fold differences in the peak concentrations of plasma inorganic fluoride between patients after anesthesia with desflurane and sevoflurane and microsomal metabolic rates of these anesthetics. In our study, however, only sevoflurane was used, and interindividual variations in peak levels of plasma inorganic fluoride were smaller than those presented by Kharasch *et al.*,[2] accounting for the lack of correlation between CYP2E1 mRNA level and peak plasma inorganic fluoride level. In addition, there was no correlation between MAC-hours of sevoflurane and the AUC or peak concentration of plasma inorganic fluoride. Although the reason for this is not clear, Malan *et al.*[16] also found in animal experiments that rates of biotransformation of sevoflurane are only dependent on concentration of anesthetic up to 1.25%. In our clinical study, concentration of administered sevoflurane often exceeded 2%.

In our experiments, effect of inducers or inhibitors of CYP2E1 on CYP2E1 mRNA levels in mononuclear cells and on plasma inorganic fluoride was not evaluated. CYP2E1 activity in the liver is regulated at different cellular levels, and posttranslational regulations is an important level of regulation,[17] suggesting that CYP2E1 activity and CYP2E1 mRNA levels are not induced or inhibited in a comparable manner in response to inducers or inhibitors. Lack of inducers or inhibitors and small range of MAC-hour of administered sevoflurane may contribute to the significant correlation between CYP2E1 mRNA in mononuclear cells and the AUC of plasma inorganic fluoride in the current experiments.

In summary, plasma inorganic fluoride level after sevoflurane anesthesia was correlated with CYP2E1 mRNA level in mononuclear cells.

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