perhomocysteinemia in the general population (1). Neugebauer et al. (2) reported a significantly higher prevalence of the mutant allele in diabetic patients with retinopathy. However, in their study, patients with retinopathy showed severe renal failure with higher levels of serum creatinine compared with those without retinopathy. Considering that renal failure accelerates atherosclerosis, we investigated the relationship between the MTHFR genotype and DR in 156 type 2 diabetic patients with <133 μmol/l serum creatinine level. According to international standards, patients with retinopathy in each genotype were classified into three groups: no DR (NDR), nonproliferative DR (NPDR), and proliferative DR (PDR). Their genotypes were analyzed using the PCR-restriction fragment–length polymorphism method (3).

The allelic frequency of the C677T mutation was 0.40. Genotype frequencies were in accordance with the Hardy-Weinberg equation (677C/677C, 35.3%, $n = 55$; 677C/677T, 50.0%, $n = 78$; 677T/677T, 14.7%, $n = 23$; χ^2 test, $P = 0.9995$). Statistical analyses showed no association of genotypes with clinical parameters such as sex, age, known diabetes duration, BMI, HbA_{1c}, fasting blood glucose, total cholesterol, triglyceride, HDL cholesterol, LDL cholesterol, and creatinine (data not shown). The frequency of 677T/677T homozygous patients with retinopathy was the highest among the three genotypes (677T/677T, 60.9%; 677C/677T, 25.6%; 677C/677C, 32.7%; χ^2 test, $P = 0.007$). The frequency of 677T/677T homozygous patients with NPDR was the highest among the three genotypes (677T/677T, 39.2%; 677C/677T, 17.9%; 677C/677C, 18.2%; χ^2 test, $P = 0.03$). This implies that the first signs of DR could appear earlier in 677T/677T homozygous patients than in those with the other two genotypes. These data indicate that the 677T/677T mutation in the MTHFR gene could be an independent risk factor for retinopathy. Based on the previous reports that hyperhomocysteinemia induces endothelial dysfunction, causing angiopathy (4), it is possible that the decrease in serum homocysteine level, such as with a high-folate diet, prevents the onset of DR, especially in diabetic patients with the C677T mutation. In summary, presence of the MTHFR genotype in diabetic patients can be a predictive marker of the progression of DR. Additionally, it is proposed that treatment for diabetes based on the MTHFR gene polymorphism could delay the progression of DR.

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Diabetic Retinopathy Possibly Results From Poor Blood Sugar Control Associated With MTHFR Gene Polymorphism in Type 2 Diabetic Patients

Response to Yoshioka et al.

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We appreciate the comments of Dr. Yoshioka et al. (1). As described previously (2), we excluded the patients with >133 μmol/l serum creatinine level. In addition, the patients with >300 mg/dl urinary protein levels did not participate in our study. We considered that these exclusions must elucidate the effects of the methylenetetrahydrofolate reductase (MTHFR) gene polymorphism, not the effects of nephropathy, on the progression of diabetic retinopathy (DR) in type 2 diabetic patients. We agree with their comment that we analyzed the correlation with a smaller number of subjects. However, we cannot help referring to the difference in the backgrounds of the subjects between the two studies. In our study, the subjects had a mean age of 59.4 years, a mean diabetes duration of 10.8 years, a mean HbA_{1c} of 8.1%, and a mean serum creatinine of 0.76 mg/dl. The noteworthy difference between the two studies is the mean HbA_{1c} level (8.1 vs. 7.3%). The discrepancy may be attributable to this difference.

To support this hypothesis, the subjects with >9.8% HbA_{1c} level were excluded from the previous analysis (2) to get the mean HbA_{1c} level down to 7.3%, and then the data were analyzed again. As a result, there was no significant difference in the relationship between the MTHFR gene polymorphism and DR (n = 124, χ^2 test, P = 0.08). Fong et al. (3) described that epidemiological analysis of the U.K. Prospective Diabetes Study data showed a continuous relationship between the risk of microvascular complications and glycemia, such that for every percentage point decrease in HbA_{1c} (e.g., from 8 to 7%), there was a 35% reduction in the risk of microvascular complications. Based on this description, improved control of blood glucose may mask the retinopathic background derived from the MTHFR gene polymorphism. Thus, in this letter, we propose that the MTHFR gene polymorphism contributes to the progression of DR synergistically with impaired blood glucose control. In other words, blood glucose control could override the effects of the MTHFR gene polymorphism in type 2 diabetic patients.

Prospective cohort studies are required to understand the influence of the MTHFR gene polymorphism on the progression of DR. We thank Yoshioka et al. again for their comment, which has illuminated that blood glucose control may be associated with the effect of the MTHFR gene polymorphism on DR.

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Homocysteine induces vascular endothelial growth factor expression in differentiated THP-1 macrophages

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Abstract

Hyperhomocysteinemia has been reported to be an independent risk factor for atherosclerosis and atherothrombosis. However, the molecular mechanism by which hyperhomocysteinemia can lead to atherosclerosis and atherothrombosis has not been completely described. Vascular endothelial growth factor (VEGF) has been proposed to play an important role in the progression of atherosclerosis. In the present study, we hypothesized that hyperhomocysteinemia might be associated with VEGF expression in atherosclerotic lesions. We investigated VEGF mRNA expression and VEGF secretion by homocysteine (Hcy) in differentiated THP-1 macrophages. As a result, it has been revealed that VEGF mRNA was upregulated by Hcy in a dose- and time-dependent manner in THP-1 macrophages with the increase in VEGF secretion. Importantly, other sulfur compounds, such as methionine and cysteine, showed no effect on VEGF expression, indicating that homocysteine specifically induced VEGF. Our findings suggest that hyperhomocysteinemia could promote the development of atherosclerotic lesions through VEGF induction in macrophages.

Keywords: Homocysteine; Vascular endothelial growth factor; THP-1; mRNA; Macrophage

1. Introduction

Hyperhomocysteinemia, elevated plasma homocysteine, was initially thought to be associated with advanced atherosclerosis in patients with homozygous defects in the enzymes responsible for homocysteine (Hcy) metabolism, such as cystathionine β-synthase [1,2]. The clinical observations from a patient not only with genetic defects, but also with abnormal cobalamine metabolism, a nongenetic factor, revealed that hyperhomocysteinemia is associated with vascular disease [3]. So far, accumulating evidence has confirmed that hyperhomocysteinemia can cause atherosclerotic vascular disease as an independent risk factor for atherosclerosis and atherothrombosis [4–6]. Hyperhomocysteinemia is associated with vascular endothelial cell injury [7], proliferation of vascular smooth muscle cells [8] and activation of the coagulation cascade [9]. Ross [10] proposed that dysfunction of endothelial cells, with subsequent infiltration of circulating monocytes/lymphocytes, is the primary event for atherogenesis. Thus, endothelial cell injury by hyperhomocysteinemia and consequent cellular responses to this injury can lead to the formation of atherosclerotic and atherothrombotic lesions.

Vascular endothelial growth factor (VEGF) has been known to induce migration and proliferation of endothelial cells, enhance vascular permeability, stimulate angiogenesis [11] and modulate thrombogenicity [12]. It has been demonstrated that VEGF activates monocytes and promotes their migration [13,14]. Importantly, VEGF was remarkably expressed in activated macrophages, endothelial cells, and smooth muscle cells in human coronary atherosclerotic lesions, but not in normal artery [15]. Moreover, Celletti et al. [16] proposed that VEGF caused an increase in atherosclerotic plaque size as well as the number of vascular cells associated with plaque. These data strongly suggest that VEGF plays a role in the chemotaxis of monocytes/macrophages in the process of inflammatory reactions of atherosclerosis.

In these contexts, we hypothesized that hyperhomocysteinemia might be associated with expression of VEGF in the accumulated macrophages of inflammatory lesions where atherogenesis occurs, resulting in the promotion of

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atherosclerosis and thrombosis. In the present study, to reveal the involvement of Hcy in vascular inflammation, we investigated the effects of Hcy on VEGF expression in macrophages.

2. Materials and methods

2.1. Cell culture

THP-1 cells, a human monocytic cell line, were provided from RIKEN CELL BANK (Cell No. RCB1189). Cells were cultured in RPMI 1640 medium (ICN Biomedicals, Inc. Aurora, OH 44202) containing 10% fetal bovine serum (ICN Biomedicals, Inc.). With HPLC method, we could not detect Hcy in the culture medium. THP-1 cells were differentiated into macrophages with phorbol 12-myristate 13-acetate (PMA). Briefly, equal quantities of cells (approximately 1×10^6 cells/ml) were suspended into 6- or 12-well dishes. After suspension, cells were treated with 50 nM concentration of PMA (Sigma-Aldrich Japan K.K., Tokyo, Japan) at 37 °C for 48 h.

2.2. Purification of total RNA from THP-1 cells

Differentiated THP-1 cells were stimulated with the indicated concentrations of D,L-homocysteine (Sigma-Aldrich), D,L-homocystine (Wako, Japan), L-methionine (Nacalai Tesque, Japan), L-cysteine (Katayama Chemical, Japan) for indicated time. After treatments, total RNA was purified by the acid guanidium thiocyanate–phenol–chloroform (AGPC) method.

2.3. Determination of VEGF mRNA level by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

One-step RT-PCR assays for VEGF and β-actin, an internal control, mRNA were performed using the PLATINUM® Quantitative RT-PCR THERMOSCRIPT™ One-Step System (Invitrogen™, Life Technologies) according to the manufacturer's instruction. Oligonucleotide primers used for PCR were designed according to published sources to flank target sequences shown in Table 1. To detect all isoforms of VEGF, the primers were designed in exons 1 and 8 to flank all known human VEGF isoforms. cDNA was synthesized form 1 μg of total RNA and amplified by PCR according to the protocol as follows: denaturing at 95 °C for 15 s, annealing at 59 °C for 30 s and extension at 68 °C for 1 min. Reaction was repeated 34 cycles for VEGF and 18 cycles for β-actin. PCR products were confirmed by sequencing. For semiquantitative analysis of PCR products, PCR products were separated by electrophoresis in 3% agarose gels (ultra pure grade, Bio-Rad) and were stained with ethidium bromide. DNA bands were visualized with UV light (302 nm) and band intensities were measured by NIH image computerized densitometry program. Band intensities of the PCR products for VEGF were normalized with those for β-actin.

2.4. Quantification of VEGF mRNA by real-time RT-PCR analysis

One set of primers and the fluorescent TaqMan® probe for VEGF mRNA were designed using Primer Expression® version 1.0 (PE Applied Biosystems Inc.) based on the sequence from the GenBank database (Accession no. NM_003376). The oligonucleotide sequences of the primers and the probe for real-time RT-PCR analyses are shown in Table 1.

There are several isoforms for VEGF; however, exon 1 to exon 5 is common to all isoforms. In this study, forward primer was selected in exon 3, and the reverse primer was in exon 4. The probe spanned exon 3 to exon 4 in order to detect all isoforms. After amplification using these primers, the coding for VEGF (988–1062) was confirmed by sequencing.

VEGF mRNA and β-actin mRNA levels were determined by quantitative real-time RT-PCR using the ABI-PRISM® 7700 sequence detection system (PE Applied Biosystems Inc.). Relative quantification was performed using the standard curve method according to User Bulletin #2 for this detection system.

Five hundred nanograms of total RNA was used in this assay. Each reaction was performed in triplicate wells, using the following conditions: 30 min at 60 °C, followed by a total of 40 cycles of two temperature cycles 95 °C for 15 s and 60 °C for 30 s.

Table 1
Oligonucleotide sequence of VEGF mRNA and β-actin mRNA specific primers

Target gene		Sequence	Amplicon position
VEGF	Forward primer	5′-TCGGGCCTCCGAAACCATGA-3′	686–705
	Reverse primer	5′-CCTGGTGAGAGATCTGGTTC-3′	1315–1334
β-actin	Forward primer	5′-CAAGAGATGGCCACGGCTGCT-3′	746–760
	Reverse primer	5′-TCCTTCTGCATCCTGTCGGCA-3′	1000–1020
VEGF for TaqMan® analysis	Forward primer	5′-CCACTGACGAGTCCAACATCAC-3′	988–1009
	Reverse primer	5′-CATCTCTCCTATGTGCTGGCCT-3′	1040–1062
	TaqMan® probe	5′-(FAM)-TGCAGATTATGCGGATCAAACCTCACC-(TAMRA)-3′	1011–1038

Accession number for VEGF mRNA: NM_003376. Accession number for β-actin mRNA: NM_001101.

2.5. VEGF protein quantification

After incubation of the THP-1 macrophages under the indicated conditions, the conditioned medium was collected and centrifuged at 100 × *g* for 5 min to remove the cells and cell debris. The supernatant was then used for the measurement of VEGF protein with a QuantiGlo® Human VEGF ELISA Kit (R&D Systems, Inc., MN, USA). All samples were measured in duplicate.

2.6. Statistical analysis

All results were expressed as mean ± SE. Statistical analyses of the data were performed by one-way analysis of variance and Fisher's Protected Least Significant Difference (PLSD) test. A value of $P<0.05$ was considered significant. The experiments presented are representative of at least three separate experiments.

3. Results

3.1. The effects of Hcy on VEGF mRNA levels in THP-1 macrophages

Three splicing variants of VEGF mRNA were observed in THP-1 macrophages. Splicing variants of VEGF mRNA, expressed in THP-1 macrophages, are controversial. To identify the splicing variants in THP-macrophages, we performed sequencing, and identified PCR products of

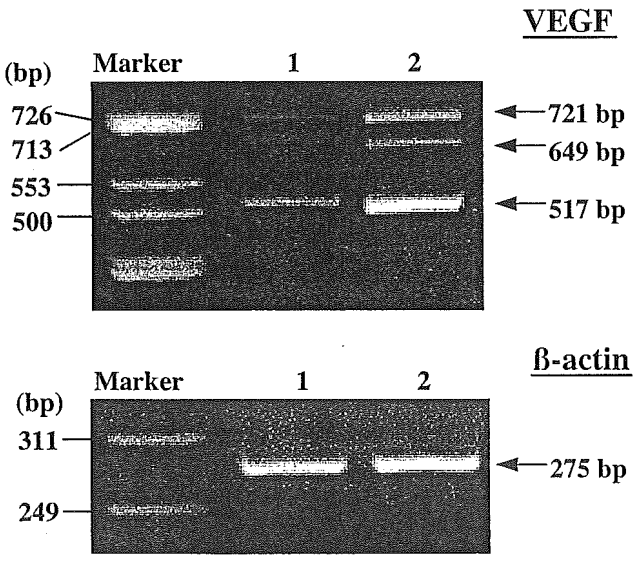

Fig. 1. Hcy upregulates $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ in THP-1 macrophages. THP-1 macrophages were incubated in the presence (lane 2) or absence (lane 1) of 200 μM Hcy for 24 h. After total RNA were prepared from THP-1 macrophages, RT-PCR was performed for VEGF (upper) and β-actin (lower). PCR products for VEGF (upper) showed 517, 649 and 721 bp, corresponding to $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$, respectively. All isoforms of the mRNA levels for VEGF were enhanced by Hcy treatment. PCR products of 275 bp corresponded to β-actin (lower).

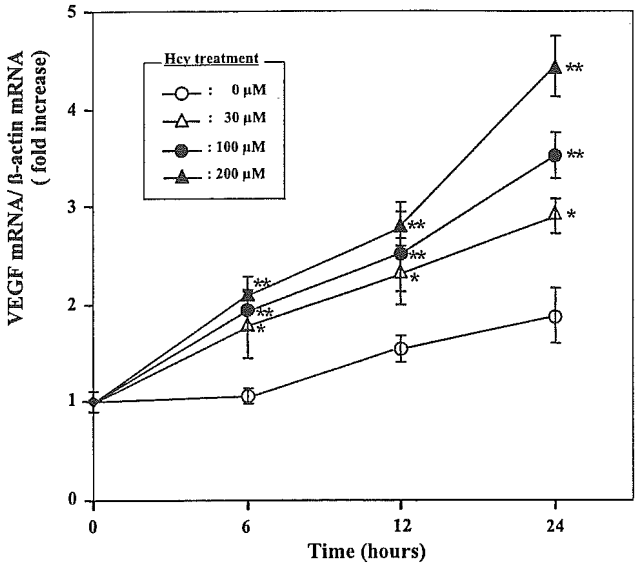

Fig. 2. Hcy induces VEGF mRNA expression in THP-1 macrophages in a dose- and time-dependent manner. THP-1 macrophages were incubated with various concentrations of Hcy for indicated time periods. VEGF mRNA expression was determined by an ABI PRISM® 7700 detection system using the relative standard curve method. Each of the VEGF mRNA levels were represented as fold induction relative to the VEGF mRNA level at time 0. The experiments were performed in triplicate. Results are expressed as mean ± S.E. of values from three independent experiments. $*P<0.05$ and $**P<0.01$—significantly different compared with nontreatment of each group (ANOVA, Fisher's PLSD).

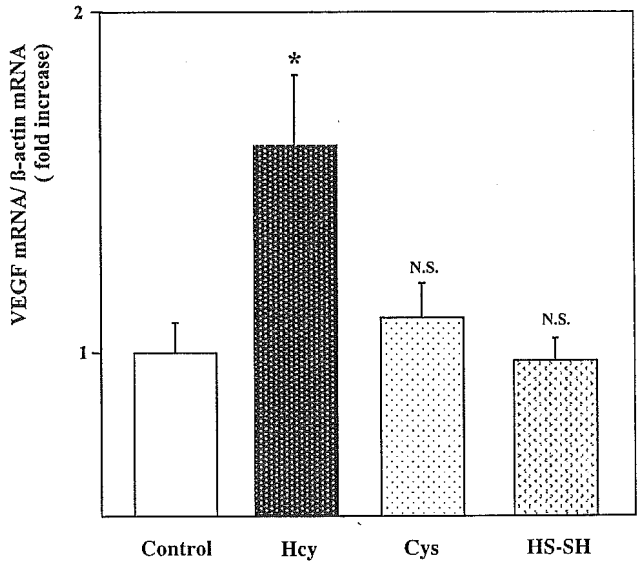

Fig. 3. Hcy specifically upregulates VEGF mRNA. To investigate the possibility of a thiol effect on the induction of VEGF mRNA, L-cysteine (Cys), L-homocysteine (Hcy) and L-homocystine (HS-SH) were tested for their ability to induce the VEGF expression. All compounds were studied at 100 μM. After 24 h of treatment, there was no increase in mRNA levels of VEGF with L-cysteine or L-homocystine. $*P<0.01$, significantly different compared with nontreatment. N.S., not significantly different (ANOVA, Fisher's PLSD).

517, 649 and 721 bp corresponded to splicing variants $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$, respectively. We could also confirm the PCR product of β-actin, which had 275 bp (Fig. 1).

We then analyzed if VEGF mRNA level in THP-1 macrophages might be altered by Hcy (Fig. 1). As analyzed by semiquantitative RT-PCR, $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{185}$ splicing variants are constitutively expressed in THP-1 macrophages, and the intensity for $VEGF_{121}$ was the highest. All of the mRNA levels for $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ were enhanced by 200 μM of 24-h Hcy treatments.

Next we examined the effects of Hcy on VEGF mRNA expression from THP-1 macrophages by real-time RT-PCR. THP-1 macrophages were incubated with various concentrations (0, 30, 100 and 200 μM) of Hcy for 6, 12 and 24 h ($n=3$ for each set of conditions). Each of the VEGF mRNA levels was represented as value of fold induction relative to the VEGF mRNA level at time 0 h when Hcy treatments were started (Fig. 2). Hcy increased VEGF mRNA levels both in a dose- and time-dependent manner. VEGF mRNA increased significantly 6 h after Hcy stimulation.

3.2. Effects of other sulfur compounds on VEGF expression

It was reported that homocysteine is the only plasma thiol compound that is considered as a risk factor for preclinical cardiovascular disease [17]. Therefore, we evaluated the possible effects of plasma thiol compounds such as homocysteine, cysteine and homocystine on the induction of VEGF mRNA. D,L-homocysteine, D,L-homocystine and L-cysteine were tested at 100 μM for this study. After 24 h of treatment, there was no significant increase in mRNA levels of VEGF with cysteine or homocystine (Fig. 3).

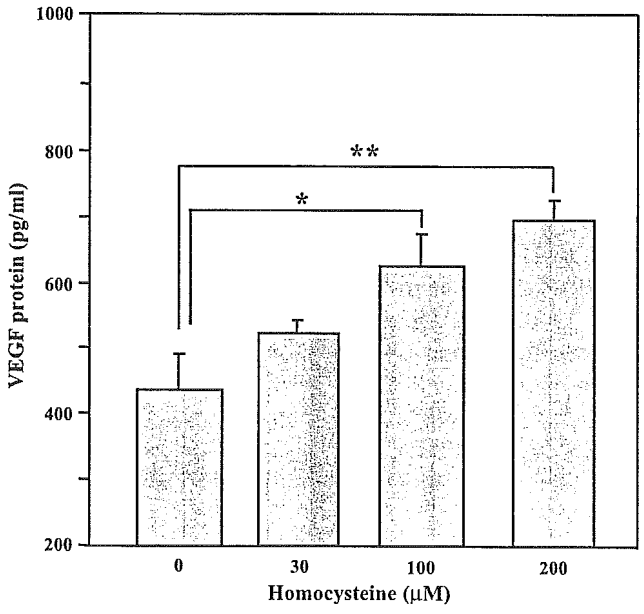

Fig. 4. Hcy upregulates VEGF protein from THP-1 macrophages. THP-1 macrophages were incubated with Hcy (0, 30, 100 and 200 μM) for 24 h ($n=3$ for each set of conditions). After the incubation of the THP-1 macrophages, the conditioned medium was collected and used for the measurement of VEGF protein with ELISA. The experiments were performed in triplicate. Results are expressed as mean ± S.E. of values from three independent experiments. *$P<0.01$ and **$P<0.001$, significantly different compared with nontreatment (ANOVA, Fisher's PLSD).

3.3. Effects of Hcy on the secretion of VEGF protein

VEGF levels were determined in culture medium by ELISA. VEGF concentration was 0 in medium prepared for this study (data not shown). As shown in Fig. 4, Hcy significantly increased VEGF secretion in a dose-dependent manner after treatment for 24 h. This study demonstrated that pathological concentrations of Hcy not only upregulated VEGF mRNA but also triggered the secretion of VEGF protein from THP-1 macrophages.

4. Discussion

In this study, we demonstrated that Hcy increased both the expression of VEGF mRNA and protein in THP-1 macrophages in a dose-dependent and a time-dependent manner. We also confirmed that among plasma thiol compounds, only Hcy exclusively induced VEGF mRNA in THP-1 macrophages. Our results suggest that hyperhomocysteinemia induces VEGF expression in macrophages in the process of arteriosclerosis. Many prospective epidemiological studies have indicated that hyperhomocysteinemia is involved in atherogenesis. Because VEGF has been reported to be abundant in atherosclerotic lesions [15] and involved in the progression of atherosclerosis [16], it is supposed that hyperhomocysteinemia may be associated with the induction of VEGF expression, which contributes to atherosclerotic plaque progression.

Monocytes/macrophages play a central role in atherogenesis and the development of atherosclerotic plaque progression [18]. In the present study, we used THP-1 cells as a macrophage model because more than 90% of THP-1 cells become terminally differentiated with PMA activation and demonstrate macrophage-like characteristics [19–23]. We then showed here that the stimulation of THP-1 macrophages with Hcy, but not with other sulfur compounds, such as homocystine, methionine and cysteine, leads to VEGF induction. Taken together with the previous report that macrophages are one of the major sources for producing VEGF in atherosclerotic plaque [24], our present data proposed that macrophages could be major target of Hcy, in atherosclerosis, leading to VEGF expression, as well as oxidized low-density lipoprotein [25] and prostaglandin E_2 [26]. The previous study demonstrated that Hcy activates NF-κB in THP-1 cells in vitro [27]. Furthermore, recent data have provided the evidence for the in vivo activation of NF-κB and downstream inflammatory marker expression in

atherosclerotic lesion in hyperhomocysteinemic apolipoprotein E (apoE)-deficient mice [28], indicating the pathological significance of Hcy in NF-κB activation. Notably, in human macrophages, VEGF expression is regulated by NF-κB, a proinflammatory transcriptional factor [29]. Collectively, these contexts could support our hypothesis that Hcy is involved in vascular inflammation with VEGF expression in macrophages.

Hyperhomocysteinemia is a condition where total plasma Hcy levels in the fasting state are higher than normal (5–15 μM) [30,31]. Plasma Hcy level is affected not only by genetic defects of Hcy metabolism but also by various nongenetic factors such as nutritional deficiency of cobalamin, folate, pyridoxine or choline as well as other disease and some medications [31–34]. Fifteen to thirty micromolars of plasma Hcy is described as moderate hyperhomocysteinemia, 30–100 μM as intermediate, and more than 100 μM as severe hyperhomocysteinemia [30,31,35]. Importantly, our results showed that VEGF was upregulated in THP-1 macrophages by concentrations as low as 30 μM of Hcy, that is the same concentrations of patients with moderate hyperhomocysteine. Consistently, accumulating epidemiological evidence has demonstrated that moderate hyperhomocysteinemia is an independent risk factor for atheromatous vascular disease [32,33,36]. Therefore, our data biologically support the possibility that macrophage-derived VEGF by Hcy stimulation promotes atherosclerosis in patients with moderate to intermediate hyperhomocysteinemia.

In summary, we have demonstrated for the first time that Hcy induces VEGF expression in macrophages. VEGF then may contribute to drive plaque formation by its various effects on the vascular endothelium in atherosclerotic lesion. Our findings suggest the possible involvement of hyperhomocysteinemia in the development of atherosclerosis lesions through VEGF induction in macrophages. It is proposed that pharmacological intervention in Hcy/VEGF signaling pathway is promising therapeutics against the progression of atherosclerotic lesion.

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In vitro inhibitory effects of Kampo medicines on metabolic reactions catalyzed by human liver microsomes

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SUMMARY

Background: **Although it is well known that drug–drug interactions may lead to toxicity and therapeutic failure, little is known about the incidence and consequences of herb–drug interactions in patients receiving Kampo medicines.**
Methods: **We evaluated the frequency of the combined use of Kampo medicines and Western drugs at Osaka University Hospital, and investigated the effects of these formulae on the metabolic activity of different cytochrome P450 (CYP) isoforms using pooled microsomes obtained from human liver.**
Results: **Twenty-two Kampo formulae were used together with 40 Western drugs catalyzed by the CYP isoforms CYP3A4, CYP2C9, CYP2D6 and CYP1A2. Among the Kampo medicines, HOCHUEKKI-TO, SHOSAIKO-TO, NINJINYOUEI-TO, SAIREI-TO and KAKKON-TO were most frequently used during the study period (1996–2000). These were co-administered with 11 categories of drugs, which are substrates for CYP3A4. HOCHUEKKI-TO and SAIREI-TO were competitive inhibitors of CYP3A4 with *Ki* values of 0·65 and 0·1 mg/mL, respectively. HOCHUEKKI-TO, SHOSAIKO-TO and SAIREI-TO inhibited the metabolic activities of CYP2C9, but had no effect on CYP2D6. HOCHUEKKI-TO and SAIREI-TO exhibited non-competitive inhibition of the metabolic activity of CYP2C9 with a similar *Ki* value (0·7–0·8 mg/mL). SAIRE-TO (0·25 mg/mL) was a potent inhibitor of CYP1A2 (inhibition > 68%).**
Conclusions: **Frequently used Kampo medicines may interact with Western drugs, which are substrates for CYP3A4, CYP2C9 and CYP1A2. Their co-administration should be undertaken with care.**

Keywords: **CYP450 activity, drug interaction, human liver microsomes, Kampo medicine**

INTRODUCTION

In 1976, Kampo medicine was officially integrated into the Japanese health care system. According to 1983 statistics, many Japanese medical doctors utilized Kampo-formulas in their daily practice either as the sole source of therapy or in combination with Western drugs. Different Kampo formulae have been prescribed for diseases such as hypertension, arthritis, neuralgia, bronchial asthma, collagenous diseases, chronic hepatitis, diabetes mellitus, as well as common cold, headache, and others (1). Although toxicity and therapeutic failure have long been recognized as possible consequences of drug–drug interactions, little is known about the incidence and consequences of herb–drug interactions in patients receiving Kampo medicines. As the pharmacokinetics of a drug may be affected by the induction or inhibition of specific drug-metabolizing enzymes such as cytochrome P450 (CYP) (2, 3), it seems interesting to investigate the effects of herbal drugs co-administered with Western medicines known to be metabolized by CYP isoforms. This study estimated the frequency of the combined use of Kampo medicines and Western drugs at Osaka University Hospital. The effects of co-administered Kampo medicines on the metabolic activity of each

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CYP isoform responsible for the metabolism of possible interacting Western drugs were evaluated.

MATERIALS AND METHODS

Drug profile generation

This study was conducted at Osaka University Hospital on 200 patients who received at least one Kampo formula during a 1-month period (April–May) in 1996, 1998 or 2000. The frequency of the combined usage of Kampo medicines and Western drugs was recorded. Next we examined whether the Kampo formulae used affected the isoform of human CYP principally responsible for the metabolism of identified co-administerd Western drugs. Information on the human CYP isoforms was obtained from the CYP Drug Interaction Table listed on the Georgetown University Medical Center homepage (http://www.dml.georgetown.edu/depts./pharmacology/davetab.html).

Materials

The Kampo formulae were kindly donated by Tsumura Co., (Tokyo, Japan). Human liver microsomes (pool: H161) were purchased from GENTEST (Wobun, MA, USA). NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were from Oriental Yeast Co., Ltd. (Tokyo, Japan). Nifedipine, ketoconazole and quinidine were purchased from Nakarai tesque (Kyoto, Japan), while oxidized nifedipine and methylhydroxytolbutamide were from Funakoshi (Tokyo, Japan). Phenacetin, tolubutamide, acetaminophen, sulfaphenazole, and 3-amidophenol were purchased from Sigma Chemical Co. (St Louis, MO, USA). Bufuralol and 1-hydroxybufuralol were from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Fluvoxamine malate was a gift kindly donated by Solbei Meiji Co. (Tokyo, Japan). Methanol and ethyl acetate of high performance liquid chromatography (HPLC) grade were purchased from Katayama Chemicals (Osaka, Japan) and ULTRAFINE Chemicals (Manchester, UK).

Metabolic assays

Microsomal incubations were carried out in a total volume of 0·5 mL and in the presence of freshly prepared NADPH generating system. The reaction mixture composed of 200 mM potassium phosphate (250 μL, pH 7·4), 50 mM $MgCl_2$ (50 μL), NADPH-generating system (20 mM NADP, 100 mM glucose 6-phosphate and 10 unit of glucose 6-phosphate dehydrogenase in 50 μL), microsomal protein (25 μL) and substrates (5 μL) prepared in water with minimal use of methanol or acetone [nifedipine (CYP3A4: methanol), tolubutamide (CYP2C9: acetone), bufralol (CYP2D6: water) and phenacetin (CYP1A2: methanol)]. A well-pulverized Kampo formula was extracted with distilled water by sonication for 15 min. The extract was centrifuged at 12 000 × *g* for 10 min at 4 °C and the supernatant was collected for examination of its effect on microsomal drug metabolism. The reaction mixture was preincubated at 37 °C for 2 min. Reaction was initiated by the addition of the NADPH-generating system. The concentration of the substrate, the incubation time and the protein concentration used in each assay were specified in Table 1. The reaction was terminated by the addition of 3 mL of EtOAc (for CYP3A4 and CYP1A2) or 200 μL of 1 M HCl following either 3 mL of EtOAc (for CYP2C9) or 75 μL of 60% $KClO_4$ (for CYP2D6). Except for CYP2D6 assay, propylparaben (4-hydroxybenzoic acid propyl ester) was used as an internal standard for the determination of CYP3A4, sodium phenobarbital for CYP2C9 and 3-amidophenol for CYP1A2. Incubation mixtures were centrifuged for 5 min (1500 *g*). The supernatant fraction from each mixture was transferred to a 10-mL test tube and evaporated to dryness under a mild stream of N_2 gas. The residue was suspended in 200 μL of the HPLC mobile phase and filtered (0·45 μm), and 80 μL of the filtrate was used for analysis. In the case of CYP2D6 assay, 50 μL of water was added and the mixture was centrifuged at 7000 *g* for 5 min. The supernatant was filtered through a 0·45 μm Millipore filter and 100 μL of the filtrate were analyzed by HPLC. All microsomal incubations were duplicated.

Analysis of metabolites produced from model substrates in CYP-mediated drug metabolism by HPLC

Isocratic HPLC was sufficient for the separations described using a SHISEIDO NANOSPACE SI-1 (Shiseido, Tokyo, Japan) connected to 2001

Table 1. *In vitro* assay conditions of CYP-mediated drug-metabolism in human liver microsomes enzymes

(A) CYP model substrates and CYP-mediated reactions in human liver microsomes

CYP isoforms	Substrates (μM)	Reactions	HLMs content (mg protein/mL)	Reaction time (min)	Inhibitor (μM)
3A4	Nifedippine (20)	Oxidation (1)	0·2	5	Ketoconazole (1)
2C9	Tolubutamide (50)	4-Methylhydroxylation (1)	0·5	30	Sulfaphenazole (1)
1A2	Phenacetin (5)	*O*-deethylation	0·2	20	Fluvoxamine malate (10)
2D6	Bufralol (20)	1-hydroxybufralol (1)	0·25	10	Quinidine (1)

(B) HPLC conditions for analysis of metabolites produced via CYPs

Conditions	CYP3A4	CYP2C9	CYP1A2	CYP2D6
Column		Inertsil ODS-3 (5 mm, 4·6 × 150 mm i.d. GL Science Inc.)		
Column temperature	40 °C	40 °C	40 °C	Room temperature
Detection	UV 254 nm	UV 240 nm	UV 254 nm	EX.: 252 nm EM.: 302 nm
Mobile phase	A: H_2O B: CH_3OH	A: 5 mM KH_2PO_4 B: CH_3OH	A: 5 mM KH_2PO_4 B: CH_3OH	CH_3OH/20 mM $NaClO_4$-$HClO_4$ (pH 2·5) = 40/60
Gradient profile	0–12 min: A/B = 55 : 45 12–19 min A/B = 20 : 80 19–34 min: A/B = 55 : 45	0–15 min: A/B = 40 : 60 15–20 min: A/B = 80 : 20 20–30 min: A/B = 40 : 60	0–15 min: A/B = 12 : 88 15–20 min: A/B = 80 : 20 20–35 min: A/B = 12 : 88	
Flow rate	1 mL/min	1 mL/min	1 mL/min	0·5 mL/min
Retention time (min)	Oxidized nifedipine: 7·6 I.S.(4-hydroxybenzoic acid isopropyl ester): 9·3	Hydrxytolbutamide: 6·6 I.S. (Phenobarbital sodium salt): 8·4	Acetaminophen: 7·8 I.S. (3-acetamido-phenol): 12·5	1-Hydroxybufralol: 17·7

CYP, cytochrome P450; HLMs, human liver microsomes; HPLC, high performance liquid chromatography.

semi-micropump with a 2002 ultraviolet (UV) detector, a 2003 autosampler and a 2004 column oven, or SHIMADZU 10A connected to LC-10AT pump with CTO-10A column oven, SIL-10A auto-injector. The HPLC column [Inertsil ODS-3 (5 mm, 4·6 × 150 mm i.d., GL Science Inc., Tokyo, Japan)] was used with a 0·2 mm precolumn filter for all the assays described in Table 1. The mobile phase used for each assay is shown in Table 1. The influences of Kampo formulae on microsomal drug metabolism were estimated as the alteration in the production of CYP-mediated metabolites from model substrates using human liver microsomes. The inhibitory effects of Kampo formulae is given as the ratio (%) of the level of CYP-mediated metabolites produced from model substrates with and without Kampo formulae.

RESULTS

Classification of medication profiles

The following 22 Kampo formulae were used: HOCHUEKKI-TO, SHOSAIKO-TO, HACHIMIJIO-GAN, NINJINYOEI-TO, SAIREI-TO, KAKKON-TO, CHOREI-TO, SAIKOKARYUKOTSUBOREI-TO, KAMISHOYO-SAN, TOKISHAKUYAKU-SAN, SHAKUYAKUKANZO-TO, GOSHAJINKI-GAN, KEISHIBUKURYO-GAN, GOREI-SAN, BAKUMON-TO, ORENGEDOKU-TO, DAIKEN-CHU-TO, DAISAIKO-TO, KAMIKIHI-TO, JUZENTAIHO-TO and SHOSEIRYU-TO. HO-CHUEKKI-TO, SHOSAIKO-TO, HACHIMIJIO-GAN, NINJINYOUEI-TO, SAIREI-TO and KAK-KON-TO were frequently used during 1996–2000. SHOSAIKO-TO the most frequently used product appeared in 30% of all prescriptions in 1996. However the number has decreased to 17% in 1998 and 9% in 2000. In 1998 and 2000, the most frequently used Kampo formula was HOCHUEKKI-TO.

Table 2 summarizes the 34 Western drugs co-administered with Kampo formulae and the four gene families of human CYPs, i.e.CYP3A4, CYP2C9, CYP2D6 and CYP1A2. There were 28 drugs classifiable into 12 categories of medicines, which were considered to be substrates for CYP3A4 (Table 2). The co-administration frequency with CYP3A4-metabolized drugs was 41·6% (Table 2). Diclofenac, an NSAID, and phenytoin, an

Table 2. The list of Western drugs co-administered with 22 Kampo medicines and co-administration rate

CYP isoforms	Drugs		Co-administration rate (%)
3A4	Antianxiety agents	Alprazolam, diazepam, triazolam, brotizolam	12·2
	Calcium channel blockers	Amlodopine, diltiazem, nasodipine, nitrendipin, nifedipine, nilvadipine, benidipine, manidipine	8·0
	Hormones	Ethinylestradiol, predonisolone, mestranol	9·4
	5-HT agonists	Cisapride	2·3
	Anticonvulsants	Carbamazepine	2·3
	HMG-CoA reductase inhibitors	Simvastatin	1·6
	Antihistamines	Terfenadine, astemizole	0·7
	Antineoplastic agents	Tamoxifen	0·5
	Immunosuppressants	Cyclosporine	0·2
	Antibiotics	Clarithromycin	0·2
	Ergot alkaloids	Ergotamine tatrate	0·2
	Tricyclic antidepressants	Amitriptyline, clomipramine, imipramine	6·1
	Major tranqulizers	Chlorpromazine	0·7
2C9	NSAIDs	Diclofenae	1·9
	Anticonvulsants	Phenytoin	1·2
2D6	Tricyclic antidepressants	Amitriptyline, imipramine, clomipramine	6·1
1A2	Bronchodilators	Theophylline	0·7

Data were analyzed from the prescriptions in 1998.

Table 3. Rates of co-administration of Western drugs and Kampo medicines in patients treated with Kampo medicines

	Co-administration rate (% of total patients medicated with Kampo-medicines)					
CYP3A4	SHOSAIKO	HOCHUEKKI	SAIREI	NINJINYOEI	KAKKON	DAISAIKO
Antianxiety agents	4/62 (6·5)	14/68 (21)	4/28 (14)	5/32 (16)	2/27 (7)	1/5 (20)
Calcium channel blockers	14/62 (23)	3/68 (4)	–	3/32 (9)	5/27 (19)	–
Hormones	1/62 (1·6)	2/68 (3)	8/28 (29)	7/32 (22)	2/27 (7)	–
5-HT agonists	3/62 (4·8)	1/68 (1)	–	2/32 (6)	1/27 (4)	1/5 (20)
Anticonvulsants	1/62 (1·6)	1/68 (1)	1/28 (4)	–	1/27 (4)	1/5 (20)
HMG-CoA reductase inhibitors	–	2/68 (3)	1/28 (4)	–	2/27 (7)	–
Antihistamines	–	2/68 (3)	–	–	–	–
Antineoplastic agents	–	–	1/28 (4)	1/32 (3)	–	–
Antibiotics	1/62 (1·6)	–	–	–	–	–
Ergot alkaloids	–	1/68 (1)	–	–	–	–
Tricyclic antidepressants	–	5/68 (7)	3/28 (11)	–	–	1/5 (20)
Total patients/month	62	68	28	32	27	5
Prescribed number/month	98	110	34	42	32	5

Data were analyzed from the prescriptions in 1998.

anticonvulsant drug, are metabolized by CYP2C9 and their frequency of use with Kampo products were 1·9% and 1·2%, respectively (Table 2). The tricyclic antidepressants, amitriptyline, imipramine and clomipramine, are substrate for CYP2D6, and the total frequency of Kampo co-administration was 6·1% (Table 2). The co-administration rate of theophylline, a substrate for CYP1A2 was 0·7% in 1998, but the drug was not used in 1996.

The CYP3A has a central role in drug metabolism as it is the most abundant form of CYPs (20–60%) in human liver (4). CYP3A4 is expressed in human liver and about 50% of currently used drugs are substrates for it (5). Of the most popular Kampo formulae, HOCHUEKKI-TO, SHOSAIKO-TO, NINJINYOUEI-TO, SAIREI-TO and KAKKON-TO were co-administered with 11 categories of Western drugs which are CYP3A4 substrates (Table 3). Fourteen patients or 21% of patients given HOCHUEKKI-TO (68 patients) were also receiving antianxiety agents. In the case of SHOSAIKO-TO, calcium channel blockers were co-administered to most patients (14 of 62: 23%).

Influences of Kampo formulae on the metabolic activity of CYPs

HOCHUEKKI-TO, SHOSAIKO-TO, NINJINYOUEI-TO, SAIREI-TO, KAKKON-TO and DAISAIKO-TO were the most frequently used in Osaka University Hospital (Table 3). These formulae were selected for an *in vitro* study on CYP-mediated drug metabolism in human liver microsomes. The herbal compositions of these formulas are shown in Table 4. As the usual daily dose of Kampo medicines is 6–9 g, the experiment was performed in the drug concentration range of 0·25–2·0 mg/mL. Table 5 shows the influence of six Kampo medicines on the CYP-mediated metabolism of several substrates: phenacetin O-deethylation for CYP1A2, tolbutamide hydroxylation for CYP2C9, bufuralol 1-hydroxylation for CYP2D6, and nifedipine oxidation for CYP3A4. HOCHUEKKI-TO, SHOSAIKO-TO, and SAIREI-TO inhibited the metabolic activities of CYP3A4 and CYP2C9 in a dose-dependent manner but did not affect that of CYP2D6. The inhibition of the metabolic activity of CYP3A4 by SHOSAIKO-TO was mild and inhibition at even a highest dose (2 mg/mL) did not reach 50%. However, 1 mg/mL of HOCHUEKKI-TO produced 36–72% decrease in the metabolic activities of CYP1A2, 2C9 and 3A4 and the Kampo formula was a potent inhibitor of CYP2C9, which was similarly inhibited by SHOSAIKO-TO. One milligram per milliliter of SHOSAIKO-TO inhibited the metabolic activities of CYP3A4, CYP2C9 and CYP1A2 by 38, 54 and 51%, respectively. At a concentration of 0·25 mg/mL, SAIRE-TO was a

Table 4. Herbal compositions of Kampo medicines

	[Composition (%, w/w)]					
Herb	SHOSAIKO	HOCHUEKKI	SAIREI	NINJINYOEI	KAKKON	DAISAIKO
SAIKO = Bupleuri radix	29·2	8·3	17·5	–	–	26·1
HANGE = Pinelliae tuber	20·8	–	12·5	–	–	17·4
OGON = Scutellariae radix	12·5	–	7·5	–	–	13·0
NINJIN = Ginseng radix	12·5	16·7	7·5	9·7	–	–
KANZO = Glycyrrhiza radix	8·3	6·3	5·0	3·2	11·1	–
SHOKYO = Zingberis rhizoma	4·2	2·1	2·5	–	11·1	4·3
TAISO = Zizyphi fructus	12·5	8·3	7·5	–	16·7	13·0
OGI = Astragali radix	–	16·7	–	4·8	–	–
SOJUTSU = Atractylodis lanceae rhizoma	–	16·7	7·5	–	–	–
BYAKUJUTSU = Atractylodis rhizoma	–	–	–	12·9	–	–
TOKI = Angelicae radix	–	12·5	–	12·9	–	–
CHINPI = Autanti nobilis Pericarpium	–	8·3	–	6·5	–	–
SHOMA = Cimicifugae rhizoma	–	4·2	–	–	–	–
TAKUSHA = Alismatis rhizoma	–	–	12·5	–	–	–
CHOREI = Polyporus	–	–	7·5	–	–	–
KEIHI = Cinnamomi cortex	–	–	5·0	8·1	11·1	–
SHAKUYAKU = Paeoniae radix	–	–	–	6·5	11·1	–
KIJUTSU = Aurantii fructus immaturus	–	–	–	–	–	13·0
DAIO = Rhei rhizoma	–	–	–	–	–	8·7
KAKKON = Puerariae radix	–	–	–	–	–	4·3
MAO = Ephedrae herba	–	–	–	–	22·2	–
BUKURYO = Hoelen	–	–	–	12·9	16·7	–
JIO = Rehmanniae radix	–	–	7·5	12·9	–	–
ONJI = Polygalae radix	–	–	–	6·5	–	–
GOMISHI = Schisandrae fructus	–	–	–	3·2	–	–

potent inhibitor of CYP1A2 (inhibition > 68%). Treatment with 0·5 mg/mL of DAISAIKO-TO decreased the metabolic activity of CYP1A2 by 18%. NINJINYOEI-TO and KAKKON-TO inhibited the metabolic activities of CYP3A4, CYP2C9 and CYP1A2 in the following order: CYP2C9 > CYP1A2 > CYP3A4.

When the inhibitory effect of HOCHUEKKI-TO, one of the most frequently used and co-administered Kampo medicines in 1998 and 2000, on microsomal CYP-mediated drug metabolism was estimated by Dixon plot, this formula was found to be a competitive inhibitor of CYP3A4 and its *Ki* value was 0·65 mg/mL (Fig. 1). In the 1998 sample, 31 or 46% of patients given HOCHUEKKI-TO also took drugs metabolized byCYP3A4 (Table 3). SAIREI-TO was also a competitive inhibitor of CYP3A4 with a *Ki* value of 0·1 mg/mL. HOCHUEKKI-TO and SAIREI-TO exhibited a non-competitive inhibition of CYP2C9 with *Ki* values of 0·76 and 0·74 mg/mL, respectively (Fig. 1).

DISCUSSION

Extensive studies of human CYP over recent years, have yielded much information on the role of its individual isoforms in the metabolism of therapeutic agents (6, 7). In 1996, SHOSAIKO-TO the most frequently used Kampo medicine in Osaka university Hospital accounted for 30% of all prescriptions. However, as the Japanese news media reported 10 cases of death because of interstitial pneumonia among about 1–2 million patients who had received SHOSAIKO-TO, its use has decreased sharply (8). The information was initially released by the Japanese Ministry of Health and Welfare as part of its Information on Drug Adverse Reactions (IDAR) programme.

Table 5. Influence of Kampo medicines on the metabolic activity of CYP

	Activity (% of control)			
	3A4	2C9	2D6	1A2
HOCHUEKKI (mg/mL)				
0·25	87	102	107	64
0·5	82	57	82	59
1·0	55	28	88	64
2·0	37	17	67	64
SHOSAIKO (mg/mL)				
0·25	84	58	–	55
0·5	77	–	116	58
1·0	62	46	84	49
2·0	55	22	70	67
SAIREI (mg/mL)				
0·25	76	82	98	32
0·5	49	52	102	34
1·0	33	29	96	28
2·0	–	–	89	38
DAISAIKO (mg/mL)				
0·25	47	67	111	71
0·5	64	19	92	18
1·0	41	29	58	–
2·0	0	20	39	–
NINJINYOEI (mg/mL)				
0·25	69	73	–	44
0·5	68	81	130	46
1·0	43	16	103	4
2·0	34	18	89	8
KAKKON (mg/mL)				
0·25	80	49	–	61
0·5	76	27	–	60
1·0	62	32	–	24
2·0	43	–	–	30
Ketoconazole 1 μM	12	–	–	–
Sulfaphenazole 1 μM	–	6	–	–
Quinidine 1 μM	–	–	30	–
Fluvoxamine 10 μM	–	–	–	4

The effects of Kampo medicines were estimated as the alteration of CYP-mediated metabolism of model substrates in human liver microsomes. Substrates; CYP3A4: Nifedipine (20 μM), CYP2C9: Tolubutamide (50 μM), CYP2D6: Bufuralol (20 μM), CYP1A2: Phenacetin (5 μM).
Data were shown as the mean of at least duplicated determinations.

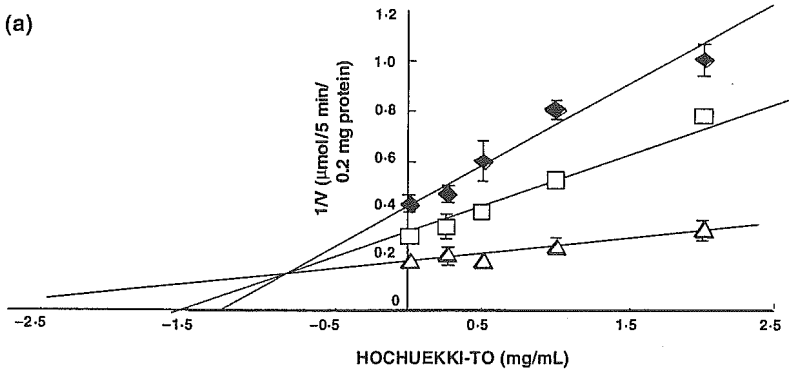
Interstitial pneumonia was also highlighted as a serious side-effect of SHOSAIKO-TO (8) and since then the number of SHOSAIKO-TO prescriptions in our hospital also decreased between 1998 and 2000.

A well-known example of the influence of a natural product on drug metabolism, is that of grapefruit juice's effect on the bioavailabilities of many clinically important drugs through its inhibition of CYP3A4 in humans (9). A traditional Japanese plant 'KIJITSU' bears fruit similar to a grapefruit. DAISAIKO-TO, which contains KIJITSU (unripe fruits of a trifoliate orange), inhibited the metabolic activity of all CYP isoforms tested, but it was not used frequently in Osaka University Hospital (Tables 3 and 5). Treatment with 0·5 mg/mL of DAISAIKO-TO decreased the metabolic activity of CYP1A2 by 18%. However, it was reported that KIJITSU showed no inhibitory effect on the metabolic activity of human CYP3A4, based on microsomal testosterone 6β-hydroxylation (10). NINJINYOEI-TO and KAKKON-TO also inhibited the metabolic activities of CYP3A4, CYP2C9 and CYP1A2.

CYP3A4 is known to metabolize many clinically important drugs, such as rifampicin, cyclosporine and ritonavir (11–14) as well as endogenous compounds such as testosterone (15). CYP3A4 is known to be a major form of CYPs expressed in both the small intestine and the liver (16). Therefore, Western drugs with low oral bioavailability may be affected by co-administration of Kampo medicines.

Little experimental data exist on herb–drug interactions. Even case-reports are scarce and case-series rarer still. Among the few studies, SHOSAIKO-TO (Xiao Chai Hu Tang) was reported to decrease the area under the plasma concentration–time curve (AUC) of predonisolone (17), whereas SAIBOKU-TO increased it and SAIREI-TO did not influence it (18). Both SHOSAIKO-TO and RIKKUNSHI-TO were found to not affect the pharmacokinetics of a single oral dose of ofloxacin (18). However, in many cases there is no plausible mechanism to explain the interaction and causality is uncertain (19). Herbal medicines tend to be complex mixtures of more than one active ingredient. For example, HOCHUEKKI-TO, SHOSAIKO-TO and SAIREI-TO consist of eight, seven and 12 herbs, respectively (Table 4). It is still unclear which constituents of Kampo medicines are pharmacologically important The multitude of active ingredients in herbal medicines may increase interactions between herbal medicines and conventional Western drugs. Moreover, users of

(a)



(b)

		Kampo medicines		
		HOCHUEKKI-TO	SHOSAIKO-TO	SAIREI-TO
3A4	Inhibition type K_i (mg/mL)	Competitive 0·65	No inhibition	Competitive 0·1
2D6	Inhibition type	No inhibition	No inhibition	No inhibition
2C9	Inhibition type K_i (mg/mL)	Non-competitive 0·76	-	Non-competitive 0·74

Fig. 1. Inhibitory effects of Kampo medicines on the metabolic activity of CYPs in human liver microsomes. The s of the Kampo medicines were estimated as the alteration of CYP-mediated metabolism of model substrates (nifidipine oxidation for CYP3A4; tolbutamide hydroxylation for CYP2C9; bufuralol for CYP2D6) in human liver microsomes. See Materials and methods for experimental details. (a) A representative Dixon plot obtained from results at 5-min incubation with 10 μM (closed diamond), 20 μM (open square), and 100 μM (open triangle) of nifedipine in the absence or presence of HOCHUEKKI-TO (0·25–2·0 mg/mL). Each data point represents the mean ± SEM of triplicate determinations. (b) The *Ki* values and inhibition types of three Kampo medicines on CYP-mediated drug metabolism in human liver microsomes. Data are shown as the mean of at least duplicated determinations with variation <10%.

medicinal herbs tend to be individuals suffering chronic diseases who usually take prescribed Western drugs concomitantly (20).

A recent review (21) summarized the potential of herbal medicines to cause interactions with Western drugs. Such interactions are usually derived from our understanding of the mode of action of herbal medicines (22, 23). This information can point us in the right direction but is certainly not conclusive. Evidence on herb–drug interactions is still sparse systematically investigated and the results made known widely and swiftly.

The results of the present study suggest that co-administration of Kampo medicines may cause herb–drug interactions through inhibition of CYP-catalyzed drug metabolism. Therefore care must be exercised with such co-administraions.

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今、医学研究を支える人体由来のモノと情報

個人の気づかないところで進んでいるカラダの情報を利用したゲノム研究。医学研究は人間の尊厳とどのように折り合うのか？　法学に期待されるものは何か。

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1　はじめに

人間を生物（いきもの）として、対象として、まるのままで研究することは、医療の場における初歩的観察にはじまり、人体実験までを含む。そして現在、まるのままの人間に介入することなく、例えばその個人に由来する「一滴の血」を利用して、ゲノム研究の成果を物差しとして、その個人を研究対象とできる道具立てがそろってきた。このような流れから考えると、二〇〇〇年一〇月のヘルシンキ宣言の改訂において、「人体由来のモノと情報」を利用する研究を「医学研究」と位置付けたことは、象徴的な出来事であった[1]。

医学研究の枠がこのように広げられ、研究対象となっている個人が、痛くも痒くも、また、知覚もできない、そして、その個人の生が終わったずっと後に、「生物としての個人」を研究できる現代の医学研究のあり方は、人体実験ほどのインパクトはないのだが、しかし、それだけに、個人を対象とした研究活動として厄介な問題を提起している[2]〜[9]。

本稿では、広範な市民の研究参加なしには成り立たない、ゲノム研究の新展開を例にとって解説し、未来的、予測的、想像的な問題としての医学研究と、今、此処で護られるべき人間の尊厳との折り合いについて、素描を試みる。果たして法律、あるいは法学と言うものがどのような役割を果たすのかについて、考えていただく縁（よすが）となればと考えている[10]。

2　病歴情報の二次利用

具合が悪いと私たちは病院へ行く。そして、医師による問診から始まり、身長、体重、血圧、血液検査、レントゲン撮影のような検査から、診断、薬や処置に対する指示が出る。「お大事に」ということで経過観察に入る場合が多い。「薬をX日分出しますが、それでも症状が思わしくなければまた診せてください」ということになる。この間の記録は、患者の氏名、年齢、生年月日に始まり詳細な連絡先と共にカルテとして医師の元に保存される。

このカルテ情報、病歴と言われるものは、例えば家庭医がいれば、そこを拠点にして、専門医、専門病院への紹介、その紹介を受けた専門医・専門病院からの家庭医への返事、また、行った専門病院内のカルテとして保管される。

普段私たちはこのことを意識しないが、自分の体の情報は、病歴という形で、他人の手が届くところに保管されているのである。私たちが現在安心してそのことを忘れていられるのは、それが「自分の医療のためにのみ」用いられていて、医師は守秘義務を負っていると考えているからである。

そして、今、この個人の病歴情報が、現在のゲノム研究を支える必須の研究資源として注目されている[11]〜[16]。ゲノム研究の成果を市民へ還元するために大規模な研究支援体制を構想している英国での状況について解説し、日本の状況について考察する。

3　英国での動き

英国では、膨大な税金を投入して行

われたヒトゲノム研究の成果を最大限に国民に還元する目的でUK Biobankという四五歳から六九歳の英国国民男女五〇万人のゲノム試料（血液）と病歴と、生活習慣情報を含む環境情報を収集し、一〇年にわたって追跡するコホート研究計画が実施前の最終準備段階に入っている[17]-[20]。有名なアイスランドのほかに、エストニア、カナダ、トンガ、シンガポールが類似の計画を持っていると言われる。

英国では、一九九九年六月にWellcome財団とMRC（医学研究諮問委員会）が英国国民を対象としたコホート研究の計画作成のために科学的基礎と同時に、倫理的、法的、社会的検討を開始した。それら二機関にNHS（国民健康サービス）が加わったUK Biobank計画主体は二〇〇二年の四月二九日にこの計画を正式に実施すると宣言した。実際には二年間の更なる準備期間をおいて二〇〇四年に本格実施を目指している。

例えば、高血圧になりやすい体質の人が、高脂肪食、運動不足などという生活習慣をもち、ストレスの貯まる環境で生活すると、高血圧、動脈硬化、脳血管障害などの生活習慣病に罹ると考えられている。従来この「体質」は、客観的に書き表し、比較し、共有することができないと考えられていた。しかし、ゲノム研究は個人が持つゲノム差異によって、この体質を表すことができると言う仮説を導入し、ゲノム差異を書き表す基盤を作った。研究によって個人の病気に係るゲノム領域を調べ、病気を予防、診断、治療したりできるだろうと考えられている。このような医療の実現のためには、UK Biobankのような膨大な量のゲノム・生活習慣・病歴情報を収集し、構築されたデータベースを利用して研究を進める必要性が生じているのである。もちろん個人の体はゲノムだけで決定されているわけではない。また、体質は生活習慣や年齢によって変化することが知られている。この様な不確定要素があるために、意味のある情報をつかむためには広範な市民の長期にわたる協力が必要となるのである。実際にこの計画のためには、対象年齢の四%弱の参加が必要となる。

ヒトが生物として生きていくために必要な情報を含むヒトゲノムは、AGCTの四文字からなる三〇億文字の文字列として書き表わされる。個々人で約〇・一%異なり、その情報量は三〇〇万文字、普通の国語辞書一冊分に当たる。結論から言うと、このゲノム差異が意味するところも、UK Biobankのような研究によって明らかになるのである。このあたりの関係は相互依存的であり、やってみなければ判らないのだ。研究は、未来的、予測的、想像的であって、結果を確約しているわけではない。

以上述べてきた英国のゲノム研究の動きについて警告を発しているGEN-EWATCH UKは優れたパンフレットを公開している[21]。ゲノム研究反対派と言われる彼らとしても、理論的に考えて、ゲノム研究の成果を生かすために、人体由来のモノと情報を大量に収集する研究基盤の構築を否定することはできないのである。もちろん、彼らは彼らの立場から被験者保護の制度設計と科学的議論の上滑りについては、厳しく批判をしている。研究参加者（被験者）の個人情報は法律によって保護される。しかし、参加者のVolunteernessを尊重して、保護するためには、UK Biobank計画主体だけではなく研究者、医師、企業、社会や倫理審査委員会の活動整備を含めた広範な保護体制の構築が必要であるとする。

4 英国での病歴の研究利用について議論

UK Biobankが収集する情報のなかで、最も問題が大きいのが、病歴情報である。そこで、その問題についての検討が盛んに行われている。まず、実態の検証を踏まえて、倫理的・法的・社会的検討を行っているが、法律の側面でも一九九八年以降に成立・改定された個人情報保護法（Data Protection Act）、人権法（Human Right Act）、情報公開法（Freedom of Information Act）、健康社会福祉法（Health and Social Care Act）、内部告発者保護法（Public Interest Disclosure Act）などの法律が複雑に絡み合って、病歴の研究利用を可能にするとともに、研究参加者を保護するように働いているという。そして、現在これらの法律（特に情報公開法と個人情報保護法）と実態、情報に関する考え方、将来の構想に関しては、Information Commissionerを中心として、検討と調整が行われているようである。さらに、医師会も患者情報の取り扱いに関する検討や勧告を行っている。

去る二〇〇二年一一月二八日、Nuffield財団と王立医学会は「プライバシーと広い意味での医学研究での個人情報の二次利用」（Privacy and the Secondary Use of Data in Health Research）というシンポジウムを開催した。Nuffield財団は、健康政策の検討

を中心に活動する非営利団体である。一年半ほどの準備期間をかけたこのようなシンポジウムを年二回行っているという。

この会は「本来は研究のためと言うわけではなく収集された個人情報、特に医療の場で記録された病歴情報を、患者のプライバシーの侵害なしに診療以外の目的に再利用することはどのような場合に許されるのか」という素朴な問いかけから始まった(Under what conditions may data not collected specifically for research, such as primary medical data, be re-used for health research without compromising the privacy of the data-subjects ?)。

シンポジウムでは、この問題に対する異なった立場の人たちの講演、あるいはスコットランドやカナダの政策について講演があり、討議が行われた。大多数が英国からの参加者であり、その範囲は、研究者、医師、看護関係者、病院管理関係者、企業、報道、患者会関係者、一般市民と多様であり、参加登録は一九八名であった。会場の様子を見ると、各部門での中堅以上の責任を持つ立場の人たちが参加しているようであった。

この会の準備会の議事録を見ると、ゲノム研究と病歴利用という問題が大きく取扱われるかと期待していたのであるが、実際にはゲノム研究と病歴情報に関する発言は少なかった。それよりも、疾患登録や健康政策での病歴利用の話が中心であった。議論も白熱していて、講演や参加者のコメントに熱い拍手が巻き起こることがしばしばであった。

このシンポジウムではDiscussion Paperといわれる議論のための基礎論文が用意されている[21]。この論文は八〇ページ程度のものであるが、バランスよく英国の現状と、ガイドラインの示すところ、法律の問題がまとめられているが、将来に対する提言としてはかなりおとなしいものとなっている。英国での現状を理解する資料として、要領のよいものである。そのなかで、病歴等の個人情報の利用を以下の三つに分類している。①個人に戻ることのできない匿名化(連結不可能匿名化)の基に同意なしに利用する枠組み、②提供者の同意の許に個人に戻ることのできる匿名化(連結可能匿名化)の基に利用する枠組み、③法律によって規定された利用枠組み、である。

印象的であったのは、議論の上滑りを防ごうと言う発言が多かったことである。例えば、情報提供者個人に戻ることのできない匿名化は、その手軽さと、研究が個人情報保護から切り離され、いろいろな規制から自由になるという考え方から人気がある。しかし、今後の有効な個人情報利用の枠組み、特に多様なデータベースを組み合わせた立体的情報空間の構築を妨げると言う議論が研究者からなされた。複数のデータベースを比較する時に、共通して登録されている個人を特定して、データベース間のもつ情報を評価することが重要であると言われている。そのために連結可能匿名化でなければデータベース間の比較と評価は困難となる。この発言について、このような重要な問題に対し、研究者が責任を持って発言することの重要性が評価されていた。それは、発言する研究者側からも、自分たちの責務への言及があったからではないかと考えている。特に、共通の物差しとして個人情報保護法があり、それを基に、「個人に戻ることのできる形での情報の取り扱い」を可能にするための、研究者に要請される資金・労力・心遣いが共通認識されていたことが重要であったと考えている。

5 匿名化の問題

私たちに身近な匿名化は、新聞で見られる「少年A」というような方法である。実際に研究用のモノと情報は、個人名、生年月日、住所等連絡先など、個人を特定できる情報をはずして、研究用の整理番号をつけられて研究に供される。

この場合に、「何々さんはXXXXX番」という対照表を残し、しかし、厳重に管理する方法を「個人に戻ることのできる匿名化」と言う。それに対して、この対照表を廃棄する方法を、「個人に戻ることのできない匿名化」と言う。

英国においても、あるいは、日本の指針においても、あるいはヘルシンキ宣言においても、連結不可能匿名化されたモノと情報を研究に用いることは、かなり自由であると考えられている[1][22][23]。

しかし、日本と英国の議論では徹底的に異なる。英国では、まず最初に、完全に連結不可能匿名化することは「不可能」であるというところから始まることである。日本では「連結不可能匿名化」できるという仮定から出発する議論が大勢を占める。

このような例を考えていただきたい。皆様はどのように考えられるであろうか。

ケース一。ここで、連結不可能匿名化され整理番号とその整理番号に対応