was given during gestation including organogenesis, revealed that NaF affected development, but was not teratogenic.

7. Formic acid (FA)

FA is a colorless gas with a pungent and penetrating odor [70]. Exposure to FA can occur through inhalation, ingestion, and contact via the eyes or skin. The primary physiological characteristic of FA is an irritating action on the mucous membrane, eyes and skin [71].

A TLV-TWA of 5 ppm, TLV-STEL of 10 ppm [71], IDLH of 30 ppm [70] and OEL of 5 ppm [23] are recommended for FA (Table 1).

FA was dysmorphogenic and developmentally toxic to rat and mouse embryos in a whole embryo culture system [72]. No report is available on the reproductive and developmental toxicity of FA in vivo.

8. Discussion and conclusions

In this paper, we summarized the reproductive and developmental toxicity of degradation products of refrigerants, including TFA, CO_2, CO, CF, HF and FA, in experimental animals. The risk assessment of chemicals is difficult because there are many variables in the manifestation of reproductive and developmental toxicity. However, confirmation of adverse effects on reproduction and development in animals exposed by the anticipated route of human exposure would be important for risk assessment in humans.

Excessive exposure to CO_2 in humans is well reported, but there is a lack of information on reproductive and developmental toxicity [73]. In experimental animals, excessive CO_2 in the atmosphere is testicular and reproductive toxic, embryolethal, developmentally neurotoxic and teratogenic. The data for these animal studies suggest the adverse effects of CO_2 on reproduction and development to be due to a secondary effect such as acidosis, increased blood flow or increased oxygen tension.

Although human studies on CO are limited, developmental toxicity has mainly been confined to the central nervous system [74]. The inhalation of CO has not proved to be consistently teratogenic in animals, and only very early studies in rats and guinea pigs reported congenital malformations: more recent studies have not [74,75]. Studies in several species of animals showed that maternal exposure to CO cause prenatal and postnatal lethality and growth retardation, increased incidence of skeletal variations, cardiomegaly, blood biochemical changes, immunological changes, postnatal behavioral changes, and neurological impairment in offspring of exposed dams. Some of these changes in fetuses and pups were detected at levels which did not cause maternal toxic effects. Even at levels as low as 60–65 ppm, maternal exposure to CO can cause cardiomegaly and delayed reflex ontogeny in rat offspring. COHb levels in fetuses and pups increase to above the levels in the maternal circulation after maternal CO exposure. A further decrease in oxygen tension due to the presence of COHb could have potentially serious effects in fetuses with a lower oxygen tension, and increased COHb levels could have hypoxic effects in newborn pups with a high rate of oxygen consumption and lower oxygen transport capacity for hemoglobin [31]. These phenomena indicate that fetuses and pups are susceptible to CO exposure. Further studies are therefore required to evaluate the adverse effects of chronic exposure to low and near ambient levels of CO on the development of fetuses and newborn pups.

Little information is available on the toxicity of CF and HF. However, the results of toxicological studies on NaF have been used to obtain insight into the toxicity of CF and HF, because CF is rapidly hydrolyzed in contact with water and yields CO_2 and HF [48,49] and NaF is similar in kinetics and dynamics to HF. Developmental

toxicity studies, in which NaF was administered to rats and rabbits during gestation including organogenesis, revealed an increased incidence of fetal skeletal variations, but not fetal malformations. Rat multiple-generation toxicity studies revealed that NaF retarded ossification in F_2 fetuses, caused degenerative changes in the lung and kidney of F_2 male offspring, but had no adverse effects on parameters for reproductive toxicity including testicular or sperm toxicity. There is a discrepancy in testicular and sperm toxicity between regimens of NaF. Testicular and sperm toxicity was noted when NaF was directly given to young or adult rats. The efficiency of sperm production and epididymal sperm reserves of humans are considerably lower than those of experimental animal models [76]. It is also noted that human males have relatively low fertility and thus may be at greater risk from reproductive toxicants than experimental animals [77]. Furthermore, male rodents produce sperm in numbers that greatly exceed the minimum requirement for fertility while sperm production in human males appears to be closer to the infertility threshold, therefore a less severe reduction in sperm counts may affect male fertility [78]. These considerations suggest that definitive animal studies of chemicals suspected of having testicular and sperm toxicity are needed to assess the risk to reproduction in human males. Further histopathological studies of reproductive organs given NaF could help us to understand the reproductive toxicity of NaF, because histopathology is acknowledged as the most sensitive endpoint for detecting testicular toxicity [79].

There is a lack of information on the toxicity of TFA and FA.

Evidence from human studies is preferred for risk assessment as long as it is obtained humanely. It is sometimes claimed that the use of animal data for estimating human risk does not provide strong scientific support. However, a continuance of studies in experimental animals is required for risk assessment of chemicals because it is difficult to find alternative methods to test the direct toxic effects of chemicals.

Conflict of interest

None.

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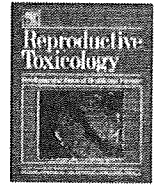
Fetal malformations and early embryonic gene expression response in cynomolgus monkeys maternally exposed to thalidomide☆

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ABSTRACT

The present study was performed to determine experimental conditions for thalidomide induction of fetal malformations and to understand the molecular mechanisms underlying thalidomide teratogenicity in cynomolgus monkeys. Cynomolgus monkeys were orally administered thalidomide at 15 or 20 mg/kg-d on days 26–28 of gestation, and fetuses were examined on day 100–102 of gestation. Limb defects such as micromelia/amelia, paw/foot hyperflexion, polydactyly, syndactyly, and brachydactyly were observed in seven of eight fetuses. Cynomolgus monkeys were orally administered thalidomide at 20 mg/kg on day 26 of gestation, and whole embryos were removed from the dams 6 h after administration. Three embryos each were obtained from the thalidomide-treated and control groups. Total RNA was isolated from individual embryos, amplified to biotinylated cRNA and hybridized to a custom Non-Human Primate (NHP) GeneChip® Array. Altered genes were clustered into genes that were up-regulated (1281 genes) and down-regulated (1081 genes) in thalidomide-exposed embryos. Functional annotation by Gene Ontology (GO) categories revealed up-regulation of actin cytoskeletal remodeling and insulin signaling, and down-regulation of pathways for vasculature development and the inflammatory response. These findings show that thalidomide exposure perturbs a general program of morphoregulatory processes in the monkey embryo. Bioinformatics analysis of the embryonic transcriptome following maternal thalidomide exposure has now identified many key pathways implicated in thalidomide embryopathy, and has also revealed some novel processes that can help unravel the mechanism of this important developmental phenotype.

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1. Introduction

Thalidomide (α-phthalimidoglutarimide) was synthesized in West Germany in 1953 by the Chemie Grünenthal pharmaceutical firm, and was marketed from October 1957 into the early 1960s. It was used for treating nausea and vomiting late during pregnancy and was also said to be effective against influenza. The first case of the phocomelia defect, although not recognized at the time as drug-related, was presented by a German scientist in 1959; subsequently, malformed children were reported in 31 countries [1]. A pattern of defects of limbs as well as the ocular, respiratory, gastrointestinal, urogenital, cardiovascular and nervous systems caused by maternal thalidomide exposure during early pregnancy was observed. Limb defects such as phocomelia, amelia, micromelia, oligodactyly, and syndactyly were the most common malformations [2]. After removal from the global market in 1962, thalidomide was reintroduced in 1998 by the biotechnology firm Celgene as an immunomodulator for the treatment of erythema nodosum leprosum, a serious inflammatory condition of Hansen's disease, and in orphan status for treating Crohn's disease and several other diseases [1].

Animal species are not equally susceptible or sensitive to the teratogenicity of chemical agents, and some species respond more readily than others [3]. For thalidomide, a variety of developmental toxic effects were reported in 18 animal species, but the responses have been highly variable across species. Limb defects that mimic

human thalidomide embryopathy have only been observed and replicated in a few strains of rabbits and in primates [1,3,4]. Eight of nine subhuman primates treated with thalidomide showed characteristic limb reduction malformations ranging from amelia to varying degrees of phocomelia at a dosage and timing comparable to those observed in human thalidomide embryopathy [3,5]. Since the first report of thalidomide embryopathy appeared 50 years ago, considerable information regarding the therapeutic applications of this drug has accumulated, but the mechanisms by which thalidomide produce congenital malformations are still not well understood [2,3,5].

The non-human primate *Macaca fascicularis* (cynomolgus monkey) is widely used in prenatal developmental studies because of year-round rather than seasonal breeding behavior [6]. Kalter [5] noted that non-human primates, especially macaques and baboons, are favorable for mechanistic studies; however, only two full reports of the teratogenicity of thalidomide in cynomolgus monkeys are available [7,8]. In those studies, cynomolgus monkeys were given thalidomide by gavage at doses of 5–30 mg/kg-d during gestation days 20–30, and fetuses were examined morphologically. The findings of these studies determined the critical period and doses of thalidomide required for the production of fetal malformations in this macaque species. Although amounts taken were not always accurately recorded in humans, available documents show that typical malformations resulted from the ingestion of as little as 25 mg three times a day or 100 mg/day for 3 days during the sensitive period, equivalent to an astonishingly small dosage of about 1 mg/kg-d [5]. In teratology studies using cynomolgus monkeys, the timing of dosing was comparable to the human one and the doses were estimated to be 5–30 times higher than those which produced typical malformations in humans [5,7,8].

Knowledge of the patterns of altered gene expression in embryonic target organs on a global scale is an important consideration for understanding the mechanisms of teratogenesis [9–13]. The application of cDNA microarray technology, a genome-wide analysis technique, to cynomolgus monkeys facilitates the rapid monitoring of a large number of gene alterations in this species [14]. In order to obtain information about the molecular mechanisms underlying the detrimental effects of thalidomide teratogenicity, the present study has determined the experimental conditions required to produce thalidomide-induced fetal defects that mimicked human abnormalities in cynomolgus monkeys and then profiled altered patterns of gene expression in these embryos during the critical period. The dosing used in the present study was 15 or 20 mg/kg-d thalidomide given by gavage to pregnant dams at days 26–28 of gestation for teratological evaluation, and 20 mg/kg given on day 26 for gene expression profiling 6 h post-treatment.

2. Materials and methods

2.1. Teratological evaluation

The teratology study was performed at SNBL USA, Ltd. (Everett, WA, USA) in compliance with the Animal Welfare Act and recommendations set forth in The Guide for the Care and Use of Laboratory Animals [15]. Only females showing 25–32-day menstrual cycles were used in these experiments. Each female monkey was paired with a male of proven fertility for 3 days between days 11 and 15 of the menstrual cycle. When copulation was confirmed, the median day of the mating period was regarded as day 0 of gestation. Pregnancy was confirmed on day 20 or day 25 by ultrasound (SSD-4000, Aloka Co., Mitaka, Japan) under sedation induced by intramuscular injection of 5% ketamine hydrochloride (Sigma Chemical Co., St Louis, MO, USA). The monkeys were given (±)-thalidomide (Lot no. SEH7050, Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 15 or 20 mg/kg-d by oral administration using gelatin capsules (Japanese Pharmacopiae grade) on days 26–28 of gestation. The dosage was adjusted to the body weight on day 25 of gestation. Cesarean section was performed on day 100–102 of gestation under deep anesthesia induced by intramuscular injection of 5% ketamine hydrochloride (0.1–0.2 ml/kg) and inhalation of isoflurane (0.5–2.0%, Baxter, Liberty Corner, NJ, USA). Salivation was inhibited by atropine (0.01 mg/kg, Phenix Pharmaceutical, St. Joseph, MO, USA). Fetal viability was recorded, and the fetuses were euthanized by intraperitoneal injection

of pentobarbital and phenytoin solution (Euthasol®, Virbac Corp., Fort Worth, TX, USA). Fetuses were sexed and examined for external anomalies after confirmation of the arrested heartbeat. After the completion of external examinations, fetuses were examined for internal abnormalities.

2.2. Microarray experiments

The animal experiments were performed at Shin Nippon Biomedical Laboratories (SNBL), Ltd. (Kagoshima, Japan) in compliance with the Guideline for Animal Experimentation (1987), and in accordance with the Law Concerning the Protection and Control of Animals (1973) and the Standards Relating to the Care and Management of Experimental Animals (1980). This study was approved by the Institutional Animal Care and Use Committee of SNBL and performed in accordance with the ethics criteria contained in the bylaws of the SNBL committee.

Each female monkey was paired with a male of proven fertility for 1 day between day 11 and day 15 of the menstrual cycle. Pregnant females, aged 5–8 years and weighing 2.84–3.76 kg on day 22 of gestation, were allocated randomly to two groups, each with three monkeys, and housed individually. The monkeys were orally dosed with (±)-thalidomide (Lot no. SDH7273/SDJ3347, Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 0 or 20 mg/kg by oral administration of a gelatin capsule on day 26 of gestation, which was during the critical period for thalidomide-induced teratogenesis [7,8]. Dosage was adjusted to the body weight on day 22 of gestation. Control monkeys received the capsule only.

2.3. RNA sample collection

Hysterectomy was performed under terminal anesthesia at 6 h after the administration of thalidomide on day 26 of gestation. Whole embryos were rapidly removed from the uterus using a stereomicroscope and immersed in sterilized physiological saline. Three embryos each in the thalidomide-treated and control groups were obtained for RNA analysis and stored at −70 °C until further processing. General factors of maternal age, weight and date of processing these samples are shown in Table 1. Embryos were processed simultaneously, and aside from the blocking factors in Table 1, all six samples were handled concurrently through RNA isolation and hybridization.

2.4. RNA preparation and labeling

Total RNA was isolated from each day-26 embryo, amplified to cRNA, and biotin-labeled for analysis on the Affymetrix NHP GeneChip® Array at Gene Logic Inc. (Gaithersburg, MD, USA) using the TRIzol method and RNeasy columns according to protocols from Affymetrix (Santa Clara, CA, USA). The 28S/18S rRNA ratio of isolated RNA was assessed using a Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and found to be of sufficiently high quality. Biotinylated cRNA was finally cleaned up and fragmented by limited hydrolysis to a distribution of cRNA fragment sizes below 200 bases.

2.5. Affymetrix NHP GeneChip® Array and hybridization

Biotinylated cRNA samples from control and exposed embryos (n = 3 each) were hybridized using Biogen Idec's (NASDAQ: BIIB) proprietary Affymetrix NHP GeneChip® Array platform. This microarray chip contains a comprehensive representation of the Cynomolgus genome derived from Biogen Idec's proprietary sequencing efforts, from which Gene Logic (www.genelogic.com/) subsequently obtained the exclusive rights to provide as a service (personal communication, Jun Mano, Gene Logic). The steps for hybridization followed a protocol described in the Gene Logic GeneChip® Analysis Manual (Gaithersburg, MD, USA). Probe-sets for this analysis consisted of cynomolgus expressed sequence tags (ESTs), published rhesus monkey ESTs, predictive coding sequences from the rhesus genome, and human genes not represented by monkey sequences. Because of the incomplete state of annotation for the cynomolgus genome at the time this study was undertaken, we used human, mouse and rat gene annotations to characterize monkey genes on the NHP GeneChip® Array. This reasonably assumes that most cynomolgus sequences are well-annotated by human ortholog information. After hybridization the GeneChip® Arrays were scanned and raw signal values were subjected to subsequent normalization and processing.

2.6. Microarray data processing and analysis

Probe-level data normalization from the six *.cel files used the robust multichip average (RMA) method with perfect-match (PM) but not mismatch (MM) data from the microarrays. RMA returns a single file containing the 51,886 probes in six columns of normalized data, representing the log 2-intensity of each probe. To query differential transcript abundance between sample groups, the log 2 ratio of treated (Q) to reference (R) was computed for all six samples, with R being the average of the three controls. The six columns were centered to MEDIAN = 0.00 and scaled to STDEV = 0.50 [10,12]. These data were loaded to GeneSpring GX7.3 software (Agilent Technologies, Redwood City, CA, USA) for one-way analysis of variance (ANOVA) by treatment group. Due to the small sample size (n = 3) and limited annotation of the cynomolgus genome for this preliminary analysis we relaxed the selection criterion

Table 1

Procurement of cynomolgus embryos at SNBL for microarray study.

Group	Embryo	Maternal age in years	Maternal bw in kg (day 22)	Date of embryo collection (day 26)	*.cel filename (NIHS)
Control	001	6	3.76	November 2, 2006	137255bpcyna11.cel
	002	7	2.84	December 2, 2006	137256bpcyna11.cel
	003	8	3.68	December 2, 2006	137257bpcyna11.cel
Thalidomide	101	5	2.97	October 30, 2006	137258bpcyna11.cel
	102	6	3.01	November 6, 2006	137259bpcyna11.cel
	103	8	3.14	November 24, 2006	137260bpcyna11.cel

by not applying a false-discovery rate filter. Genes or probes passing the statistical (ANOVA) filter at a P value of 0.05 were subjected to K-means clustering, with cluster Set 1 and Set 2 that were up-regulated and down-regulated, respectively, in the thalidomide-exposed versus control embryos. Entrez gene identifiers were used for bioinformatics evaluation (http://www.ncbi.nlm.nih.gov/).

3. Results

3.1. Teratological evaluation

To confirm thalidomide embryopathy in the cynomolgus colony under the conditions used for this study, pregnant dams were given thalidomide at 15 and 20 mg/kg on days 26–28 of gestation. Four fetuses were obtained at each dose for teratological evaluation (Table 2). Although we did not observe a clear dose–response in this limited number of fetuses, we did observe a number of cases with limb defects consistent with human thalidomide embryopathy. Fig. 1 shows external appearance of fetuses of dams exposed to thalidomide on days 26–28 of gestation. Bilateral amelia in the fore-/hindlimbs was noted in one female fetus at 20 mg/kg, and bilateral

micromelia in the hindlimbs was observed in four fetuses at 15 mg/kg. Deformities of the paw and/or foot including hyperflexion, ectrodactyly, polydactyly, syndactyly, brachydactyly, and/or malpositioned digits, were observed in all fetuses at 15 mg/kg and in two fetuses at 20 mg/kg. Tail anomalies were found in one fetus at 15 mg/kg and three fetuses at 20 mg/kg. Small penis was noted in one fetus each in both thalidomide-treated groups. No internal abnormalities were noted in any of the thalidomide-treated fetuses examined here. This confirmed the relevant sensitivity of cynomolgus embryos to thalidomide, based on a maternally administered dose of 15–20 mg/kg during days 26–28 of gestation.

3.2. Genes altered by thalidomide

The embryonic transcriptome was evaluated at 6 h after 20 mg/kg maternal thalidomide exposure on day 26. For this analysis, we used a proprietary Non-Human Primate (NHP) microarray having representation of the cynomolgus genome (see Section 2 for details). The NHP array includes 18,293

Table 2

Morphological findings in fetuses of cynomolgus monkeys given thalidomide on days 26–28 of gestation.

Target Findings	Dose Fetus no. Gender	15 mg/kg				20 mg/kg			
		1 Female	2 Male	3 Female	4 Female	5 Male	6 Male	7 Male	8 Female
Forelimb									
Amelia		−	−	−	−	−	−	−	B
Paw									
Hyperflexion		B	−	−	−	−	−	−	−
Ectrodactyly		L	−	−	−	−	−	−	−
	Accessory digit(s)[a]	L	−	−	−	−	−	−	−
Polydactyly[a]		−	R	−	−	−	−	−	−
	Brachydactyly	−	−	−	B	−	−	−	−
Hindlimb									
Micromelia		B	B	B	B	−	−	−	−
Amelia		−	−	−	−	−	−	−	B
Foot									
Hyperflexion		−	B	B	B	−	−	−	−
Ectrodactyly		−	B	R	R	−	−	−	−
Polydactyly		−	−	−	−	B	B	−	−
Syndactyly		R	−	B	−	−	−	−	−
	Brachydactyly	−	−	−	L	−	−	−	−
	Malpositioned digit(s)	−	−	L	−	−	−	−	−
Craniofacial		−	−	−	−	−	−	−	−
Trunk		−	−	−	−	−	−	−	−
Tail									
Bent or curled tail		−	−	−	+	−	+	+	+
Short tail		−	−	−	−	−	−	+	−
External genital organs									
Small penis		−	+	−	−	+	−	−	−

−: No anomaly was observed.

+: Anomaly was observed.

B: Bilateral anomaly was observed.

R: Unilateral (right side) anomaly was observed.

L: Unilateral (left side) anomaly was observed.

[a] Polydactyly means (almost) complete extra digits existed, and accessory digit incomplete "digit like tissue" attached to a normal digit.
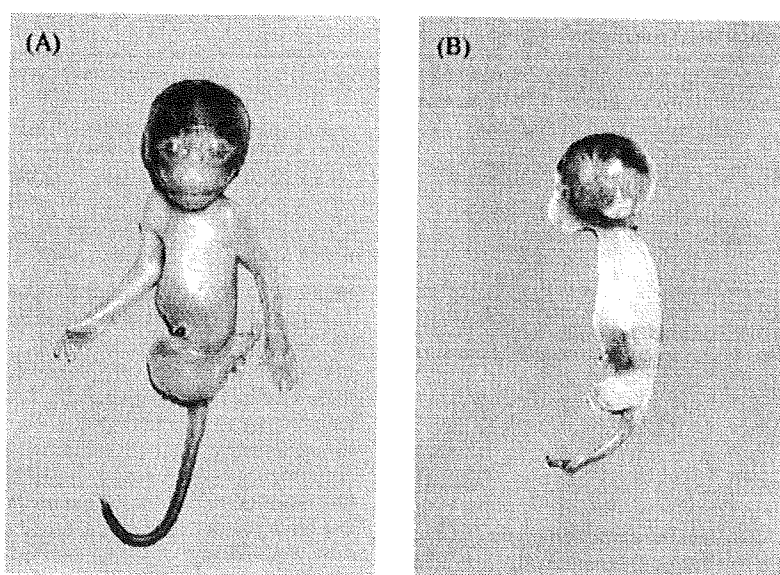
Fig. 1. Malformed fetuses of cynomolgus monkeys exposed to thalidomide on days 26–28 of gestation. (A) The fetus of maternal monkey given thalidomide at 15 mg/kg-d exhibiting brachydactyly in the paw, micromelia in the hindlimb, hyperflexion, ectrodactyly and brachydactyly in the foot and curled tail. (B) The fetus of maternal monkey given thalidomide at 20 mg/kg-d exhibiting amelia in the fore- and hindlimb and bent tail.

cynomolgus genes and 8411 Rhesus genes as well as genes from several other species. The six-array dataset conforming to MIAME standards resides in the Gene Expression Omnibus repository (www.ncbi.nlm.nih.gov/geo/) under platform accession number GPL8393 (series GSM389350–GSM389355). A thalidomide-sensitive subset of genes in the embryonic transcriptome was reflected in the high-percentage of present calls for genes whose expression levels showed ≥ 1.5-fold difference between thalidomide-treated and control embryos.

Statistical (ANOVA) analysis identified 2362 genes that differed significantly between control and thalidomide groups ($P \leq 0.05$). The heat map for these genes showed a clear pattern (Fig. 2). K-means clustering partitioned them into primary sets of up-regulated (1281) genes and down-regulated (1081) genes for thalidomide relative to control embryos.

3.3. Annotation systems

Ranking functional categories of genes in an expression cluster is an important step to unravel the cellular functions and pathways represented in the differentially expressed gene list. To derive the highest ranking biological themes across the up-/down-regulated gene lists, Entrez gene IDs were annotated by Gene Ontology (GO) category using the Database for Annotation, Visualization, and Integrated Discovery (http://apps1.niaid.nih.gov/david/). Table 3 lists the significantly over-represented themes when the 1281 up-regulated genes (Table 3A) and 1081 down-regulated genes (Table 3B) were mapped by GO category. We used level-4 annotation for Biological Processes, Cellular component and Molecular Function as well as curated pathways from the KEGG (Kyoto Encyclopedia of Genes and Genomes) open source pathway resource to obtain categories passing by Fisher exact test ($P \leq 0.05$). For clarity and greater specificity we limited the categories in Table 3 to those having at least 10 hits for sensitivity and no more than 50 hits to improve specificity.

Integrated biological processes evident across the up-regulated categories addressed the regulation of cellular growth, including cell cycle progression, DNA repair and nucleic acid transport. Other up-regulated biological processes addressed the regulation of metabolism, the cytoskeletal cycle, heart development

and vesicle transport. Many of these processes were logically reflected in the ontologies for cellular components addressing the nucleo-ribosomal system, the microtubule network, and molecular functions for GTPase activity and actin binding. Up-regulated signaling pathways (KEGG) included several oncogenic growth pathways as well as the TGF-beta, GnRH and insulin signaling pathways.

Integrated biological processes evident across the down-regulated categories addressed ion homeostasis and cellular secretion. These processes were logically reflected in the ontologies for cellular components addressing the endoplasmic reticulum, GTPase activity and transferases. Other down-regulated biological processes addressed cell growth, muscle and vasculature development, and the inflammatory response—consistent with KEGG pathways for hematopoietic cells and antigen processing.

4. Discussion

The results from this study show that a teratogenic dose of thalidomide (20 mg/kg) significantly alters global gene expression profiles in the cynomolgus monkey embryo within 6 h of exposure on day 26 of gestation. Bioinformatics analysis of the embryonic transcriptome following maternal thalidomide exposure revealed up-regulation in several signaling pathways with roles in morphogenesis and oncogenesis (e.g., TGF-beta, insulin signaling), and down-regulation of the endoplasmic reticulum and inflammatory response. As might be anticipated, this implies a broad reaction of the embryo to the mechanism of thalidomide and a generalized reprogramming of pathways known to be important in development and teratogenesis.

The dosing scenario used in the present study was 15 or 20 mg/kg-d thalidomide given by gavage to pregnant dams on days 26–28 of gestation for teratological evaluation, and 20 mg/kg given on day 26 for gene expression profiling 6 h post-treatment. The teratological exposure induced limb malformations consistent with earlier studies with thalidomide in pregnant macaques. For example, it was previously reported that two fetuses with amelia were obtained from two of four cynomolgus monkeys given thalidomide by gavage at 10 mg/kg-d on days 32–42 after commencement of menses (approximately equivalent to days 20–30 of gestation)
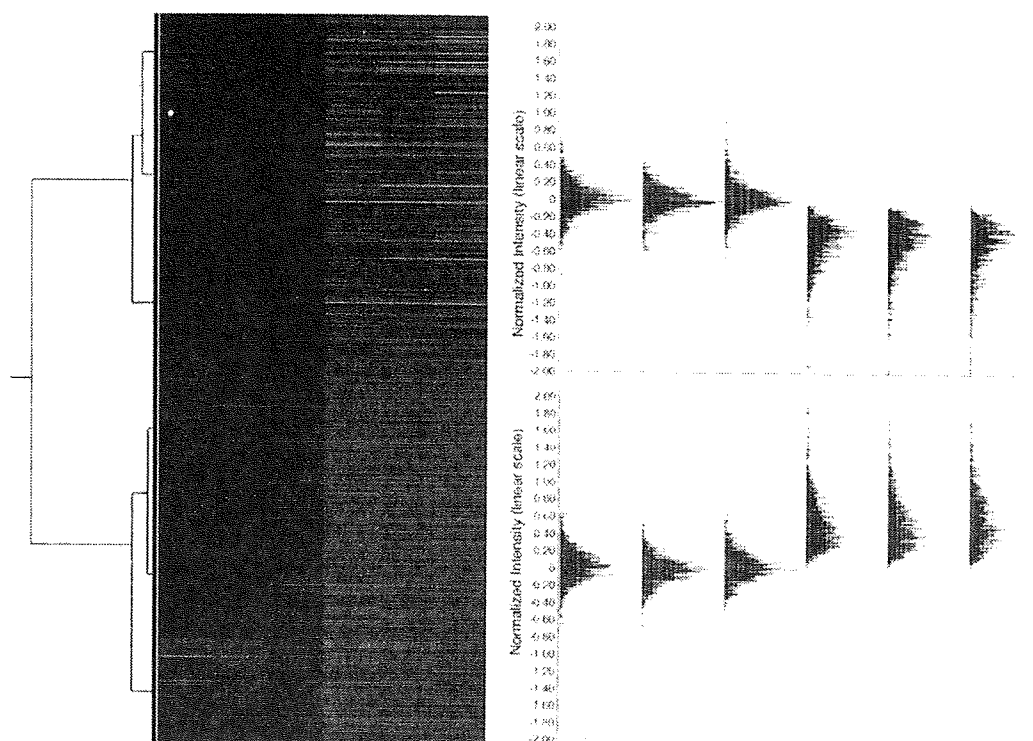
Fig. 2. Molecular abundance profiles of the thalidomide-sensitive genes in the cynomolgus embryonic transcriptome on day 26 of gestation. RNA was isolated from day 26 embryos 6 h after maternal exposure to 20 mg/kg thalidomide or vehicle control. Values represent log 2 ratios of treated/reference, where the reference is an average of all three controls for each gene. ANOVA returned 2362 genes that were significantly different between the groups ($n = 3$, $P \leq 0.05$). The heat map visualizes the genes in rows and the embryos in columns, and the histogram shows the distribution of genes in each cluster. Columns left to right: 1–3 from control embryos (#001, #002, #003) and 4–6 from thalidomide embryos (#101, #102, #103). Genes were partitioned by K-means clustering into two primary expression clusters with 1281 up-regulated genes (red) and 1081 down-regulated genes (green).

and that the fetal malformations were similar to malformations reported in children whose mothers had taken thalidomide during pregnancy [7]. Forelimb malformations in the cynomolgus fetus were noted following a single oral administration of thalidomide on days 25, 26 or 27 of gestation at 10 and 30 mg/kg and daily administration on days 25–27 of gestation at 5 mg/kg, and both fore- and hindlimb malformations were observed following a single oral administration on day 25 or 28 of gestation at 30 mg/kg [8]. The present study, taken together with the previous studies [7,8], indicate that orally administered thalidomide induces fetal malformations in cynomolgus monkeys similar to human pregnancies and furthermore localizes the vulnerable period to days 25–28 of gestation and the effective doses to 5–30 mg/kg-d.

Given the limitations of working with this species the preliminary application of a custom NHP microarray, the analysis at one dose and time point, and the incomplete state of annotation of the macaque genome, the current study design focused on RNA collected from individual embryos rather than the specific target organ system (forelimb, hindlimb). Ideally a follow-up study on focused gene expression analysis should be performed for specific embryonic limbs in which malformations have been induced with thalidomide; however, the present study is among the first to provide genomic information on the initial changes in gene expression occurring in macaque embryos during the critical events following a teratogenic dose of thalidomide. A total of 43 and 26 functional categories of redundant genes were up- and down-regulated, respectively, based on the GO annotation system for human Locus Link identifiers.

Statistically, the top-ranked 20 up-regulated genes included 4 hits to cell shape and polarity genes: KIAA0992 (twice), FNML2,

FMNL3. Palladin, encoded by the KIAA0992 gene, plays a role in cytoskeletal organization, embryonic development, cell motility, and neurogenesis [16]. Formin-related proteins play a role in Rho GTPase-dependent regulation of the actin cytoskeletal cycle and have been implicated in morphogenesis, cell movement and cell polarity [17]. Several genes in the focal adhesion/actin cytoskeleton pathway were up-regulated. Guanine nucleotide exchange factors (GEFs) DOCK1, which forms a complex with RhoG, and VAV2 and ARHGEF7 that act on Rho family GTPases, play a fundamental role in small G-protein signaling pathways that regulate numerous cellular processes including actin cytoskeletal organization [18–22]. To further understand the mechanisms of thalidomide-induced teratogenicity the regional and developmental stage of expression for these genes and corresponding proteins should be determined; however, these preliminary findings suggest that thalidomide perturbs a general program involving the up-regulation of Rho family GTPases and their GEFs.

One candidate pathway for the control of cytoskeletal remodeling evident in studies of early induction of the Fetal Alcohol Syndrome (FAS) in mouse embryos is the receptor tyrosine kinase (RTK) signaling pathway, mediating insulin-like growth factors [12]. Genes in the RTK insulin signaling pathway were significantly up-regulated by thalidomide treatment as in FAS. AKT1 and GSK3β, which were up-regulated by thalidomide, are key genes in this pathway. AKT1, a serine–threonine protein kinase, is regulated by PDGF and insulin through PI-3 kinase signaling [23–25]. GSK3β, a substrate of AKT, is a proline-directed serine–threonine kinase that was initially identified as a phosphorylating and inactivating glycogen synthase [26]. IGF-I and IGF-II are expressed in the anterior and posterior mesodermal cells of the developing limbs [27–29]. IGF-I can influence chick limb outgrowth [29–31] and regulate mus-

Table 3A

GO-annotated biological categories for genes up-regulated in the embryo following maternal thalidomide exposure.

Category	Term	Count	P value	List Total	Pop Hits	Pop Total	Log 2 Fold Change
GOTERM_BP_4	Biological Process (level 4)						
GO:0015931	Nucleobase, nucleoside, nucleotide and nucleic acid transport	15	0.001	694	100	13,532	+2.92
GO:0050658	RNA transport	13	0.002	694	87	13,532	+2.91
GO:0050657	Nucleic acid transport	13	0.002	694	87	13,532	+2.91
GO:0051236	Establishment of RNA localization	13	0.002	694	87	13,532	+2.91
GO:0051028	mRNA transport	11	0.007	694	79	13,532	+2.71
GO:0045941	Positive regulation of transcription	40	0.000	694	326	13,532	+2.39
GO:0007507	Heart development	15	0.006	694	128	13,532	+2.28
GO:0051276	Chromosome organization and biogenesis	45	0.000	694	394	13,532	+2.23
GO:0006281	DNA repair	28	0.001	694	267	13,532	+2.04
GO:0022618	Protein–RNA complex assembly	12	0.035	694	116	13,532	+2.02
GO:0031325	Positive regulation of cellular metabolic process	42	0.000	694	416	13,532	+1.97
GO:0009893	Positive regulation of metabolic process	44	0.000	694	445	13,532	+1.93
GO:0051169	Nuclear transport	14	0.035	694	145	13,532	+1.88
GO:0016481	Negative regulation of transcription	28	0.003	694	300	13,532	+1.82
GO:0006461	Protein complex assembly	27	0.005	694	295	13,532	+1.78
GO:0045786	Negative regulation of progression through cell cycle	19	0.022	694	209	13,532	+1.77
GO:0009892	Negative regulation of metabolic process	38	0.002	694	436	13,532	+1.70
GO:0031324	Negative regulation of cellular metabolic process	32	0.009	694	387	13,532	+1.61
GO:0000074	Regulation of progression through cell cycle	42	0.005	694	526	13,532	+1.56
GO:0051726	Regulation of cell cycle	42	0.005	694	529	13,532	+1.55
GO:0007010	Cytoskeleton organization and biogenesis	41	0.008	694	526	13,532	+1.52
GO:0016192	Vesicle-mediated transport	39	0.013	694	509	13,532	+1.49
GOTERM_CC_4	Cellular component (level 4)						
GO:0005830	Cytosolic ribosome (sensu Eukaryota)	10	0.017	743	76	14,201	+2.51
GO:0005681	Spliceosome	16	0.004	743	134	14,201	+2.28
GO:0000785	Chromatin	22	0.001	743	194	14,201	+2.17
G O:0031965	Nuclear membrane	15	0.012	743	136	14,201	+2.11
GO:0012506	Vesicle membrane	13	0.030	743	125	14,201	+1.99
GO:0005874	Microtubule	23	0.005	743	233	14,201	+1.89
GO:0005635	Nuclear envelope	18	0.015	743	182	14,201	+1.89
GO:0005768	Endosome	18	0.028	743	196	14,201	+1.76
GO:0005694	Chromosome	32	0.011	743	385	14,201	+1.59
GO:0030529	Ribonucleoprotein complex	41	0.047	743	584	14,201	+1.34
GOTERM_MF_4	Molecular Function (level 4)						
GO:0051427	Hormone receptor binding	10	0.001	578	57	12,599	+3.82
GO:0051020	GTPase binding	11	0.003	578	78	12,599	+3.07
GO:0003712	Transcription cofactor activity	41	0.000	578	311	12,599	+2.87
GO:0003779	Actin binding	27	0.002	578	302	12,599	+1.95
GO:0008234	Cysteine-type peptidase activity	15	0.027	578	172	12,599	+1.90
KEGG_PATHWAY							
hsa05220	Chronic myeloid leukemia	10	0.016	225	74	4,214	+2.53
hsa05222	Small cell lung cancer	11	0.016	225	87	4,214	+2.37
hsa05215	Prostate cancer	11	0.016	225	87	4,214	+2.37
hsa04350	TGF-beta signaling pathway	11	0.020	225	90	4,214	+2.29
hsa04912	GnRH signaling pathway	11	0.026	225	94	4,214	+2.19
hsa04910	Insulin signaling pathway	14	0.025	225	134	4,214	+1.96

cle mass during early limb myogenesis [32]. Although these facts may implicate IGF signals as a potential mediator of thalidomide embryopathy, the present study did not find significant expression or thalidomide-induced alteration in the global pattern of several key transcripts in this signaling pathway, including IGFBPs 13, 5, 6 and 7, IGF1, IGF1R, and IRS14 (data not shown). It is certainly plausible that thalidomide exposure may locally alter upstream events in IGF-1 signaling without necessarily altering the molecular abundance profiles of the pathway in the developing limb of monkey embryos. On the other hand, our preliminary microarray analysis does find evidence for the up-regulation of GSK3β and AKT1 transcripts that are downstream in the insulin signaling pathway. Effects on TGF-beta and WNT signaling may be critical here. Thalidomide-induced oxidative stress in chick embryos can enhance signaling through BMPs (bone morphogenetic proteins), leading to up-regulation of the WNT antagonist Dickkopf1 (Dkk1) and subsequent cell death [33]. We note here a significant up-regulation of genes in the TGF-beta pathway and similarities with genes in the cytoskeletal cycle and WNT pathways for the murine FAS [12].

Some of the responsive genes found in this study are known to play roles in vascular development pathways. For example, vascular endothelial growth factor (VEGF) was down-regulated and platelet-derived growth factor receptor β (PDGFRβ) was up-regulated during early stages in thalidomide embryopathy. VEGF is a key stimulator of vascular cell migration and proliferation and acts directly on endothelial cells, whereas PDGF attracts connective tissue cells that can also stimulate angiogenesis. The reciprocal effect on these transcript profiles, potentially leading to an overall decrease in VEGF/PDGFRβ activities, might be predicted to interfere with vascular cell recruitment and proliferation in the developing embryo or limb. It is well known that thalidomide reduces the activity or production of VEGF and TNF-α, leading to inhibition of angiogenesis [34]. The present microarray data are consistent with this effect. Furthermore, VEGF stimulates PDGFRβ and induces tyrosine phosphorylation [35]. The reciprocal effect that maternal thalidomide exposure had on these transcripts may suggest a key event in the programming or induction of vascular cells or their progenitors has been disrupted within 6 h after exposure. This notion is supported by the study of D'Amato et al. [36] that

Table 3B
GO-annotated biological categories for genes down-regulated in the embryo following maternal thalidomide exposure.

Category	Term	Count	P value	List Total	Pop Hits	Pop Total	Log2Fold Change
GOTERM_BP_4	Biological Process (level 4)						
GO:0008284	Positive regulation of cell proliferation	24	0.000	556	240	13,532	−2.43
GO:0007517	Muscle development	16	0.006	556	177	13,532	−2.20
GO:0009889	Regulation of biosynthetic process	18	0.005	556	207	13,532	−2.12
GO:0006417	Regulation of translation	14	0.027	556	174	13,532	−1.96
GO:0032940	Secretion by cell	23	0.004	556	287	13,532	−1.95
GO:0001944	Vasculature development	15	0.026	556	191	13,532	−1.91
GO:0045045	Secretory pathway	18	0.020	556	239	13,532	−1.83
GO:0051246	Regulation of protein metabolic process	23	0.008	556	307	13,532	−1.82
GO:0006873	Cellular ion homeostasis	16	0.031	556	214	13,532	−1.82
GO:0006954	Inflammatory response	22	0.012	556	301	13,532	−1.78
GO:0016192	Vesicle-mediated transport	35	0.004	556	509	13,532	−1.67
GO:0042127	Regulation of cell proliferation	34	0.005	556	499	13,532	−1.66
GO:0019752	Carboxylic acid metabolic process	36	0.012	556	572	13,532	−1.53
GO:0046907	Intracellular transport	40	0.043	556	714	13,532	−1.36
GOTERM_CC_4	Cellular component (level 4)						
GO:0005625	Soluble fraction	21	0.004	602	244	14,201	−2.03
GO:0005768	Endosome	15	0.039	602	196	14,201	−1.81
GO:0005789	Endoplasmic reticulum membrane	28	0.031	602	435	14,201	−1.52
GO:0044432	Endoplasmic reticulum part	30	0.047	602	494	14,201	−1.43
GO:0005624	Membrane fraction	44	0.026	602	749	14,201	−1.39
GO:0005783	Endoplasmic reticulum	46	0.049	602	827	14,201	−1.31
GOTERM_MF_4	Molecular Function (level 4)						
GO:0030594	Neurotransmitter receptor activity	14	0.000	531	99	12,599	−3.36
GO:0051020	GTPase binding	11	0.002	531	78	12,599	−3.35
GO:0016747	Transferase activity, transferring other than amino-acyl groups	15	0.028	531	188	12,599	−1.89
GO:0004175	Endopeptidase activity	31	0.012	531	463	12,599	−1.59
KEGG_PATHWAY							
hsa04640	Hematopoietic cell lineage	12	0.005	223	85	4,214	−2.67
hsa04612	Antigen processing and presentation	10	0.024	223	80	4,214	−2.36

Results for the embryo 6 h after a teratogenic dose of thalidomide (20 mg/kg) on day 26 of gestation for 1281 significantly up-regulated genes (Table 3A) and 1081 significantly down-regulated genes (Table 3B) based on the population of arrayed genes. The annotated system used the NIH/NIAID Database for Annotation, Visualization, and Integrated Discovery (DAVID) at level 4. Count refers to the number of altered genes in the ontology (min = 10 and max = 50). P value refers to results from Fisher exact test ($P \leq 0.05$); List Total refers to the number of annotated genes on the array; Pop Hits and Pop Total refers to the number of annotated genes in the database for the category and overall; Log 2 Fold Change is computed as the mean Log2 (treated/control) for genes in the category.

suggested limb defects caused by thalidomide were secondary to inhibition of blood vessel growth in the developing limb bud. Down-regulation of the vascular development program is consistent with this notion and with the supposition that correct limb bud formation requires a complex interaction of both vasculogenesis and angiogenesis during development [37]. Perhaps these genes might be considered as potential biomarkers of thalidomide-induced teratogenesis in cynomolgus monkeys. A recent study with the teratogenic thalidomide analogue, CPS49, has shown direct evidence for the suppression of endothelial angiogenetic sprouting and failure to establish a normal vascular network as a key event in thalidomide embryopathy [38]. CPS49 mimics the antiangiogenic properties, but not anti-inflammatory properties, of thalidomide.

Finally, the inflammatory response pathway was found to be significantly down-regulated in the early thalidomide embryome. Although down-regulation of the inflammatory response might be anticipated to protect the embryo, studies in laboratory animals have implicated a role for reactive oxygen species (ROS) in thalidomide embryopathy [39]. In that study, thalidomide was found to preferentially increase ROS in embryonic limb cells from a sensitive species (rabbit) but not the insensitive species (rat). Down-regulation of the inflammatory pathways in thalidomide-exposed monkey embryos reinforces this notion.

In conclusion, these findings show that thalidomide exposure perturbs a general program of morphoregulatory processes in the cynomolgus monkey embryo. Bioinformatics analysis has now identified many key pathways implicated in thalidomide embryopathy in cynomolgus monkeys, and has also revealed some novel processes that can help unravel the mechanism of this important developmental phenotype. Several pathways, including actin cytoskeleton remodeling and downstream insulin signaling-related genes, in addition to vascular development pathways may provide candidate biomarkers for key events underlying the teratogenicity of thalidomide in primates. To clarify the molecular mechanisms further studies must examine protein expression, phosphorylation, and other modifications in the precursor target organ system.

Conflict of interest statement

None.

Acknowledgements

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産科医療の現場からみる今日の妊娠・出産

晩婚化・晩産化の進展が招く様々な問題

政府が多くの出産・子育て支援策を打ち出している背景には、わが国が直面する少子高齢化という深刻な問題があります。じりじりと低下する出生率を早急に回復していかなければ経済活動が停滞し、増大する高齢人口を支えることが困難となっていくおそれも増大します。

第1部では、政府が少子化支援策を緊急課題とする背景について、出産を取り巻く環境変化、産科医療の現場にスポットを当てて考えます。

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産む側に起こった変化

■ 妊娠・出産を少し遅らせても大丈夫という認識の広がり

わが国で1年間に生まれてくる子どもの数は、1970年代前半では約200万人だったものが、現在は約110万人。合計特殊出生率（1人の女性が生涯に産む子どもの数を示す指標）をみても、1975年頃に2を割り込んだ数字は低下の一途をたどり、2005年に1.26と過去最低を記録したのはまだ記憶に新しいところです。ここ数年では、団塊ジュニア世代が出産年齢を迎えたことで、2007年が1.34、2008年は1.37とやや持ち直してはいますが、この世代が出産時期から離れていく今後の推移がどうなるか、まだよく見えてはいないようです（図表1-1参照）。

近年少子化が急速に進んだ理由にはいろいろな要因が考えられますが、千葉大学大学院医学研究院の生水真紀夫教授（専門は生殖機能病態学）は、「最大の原因は晩婚化だと考えられます」と言います。

「厚生労働省の調査でも、産みたい子どもの数は2人程度と以前からほとんど変化はありません。また30代前半の独身男性の9割以上は『結婚したい』と考えており、決して『子どもがほしくない』、『結婚したくない』という人が増えているわけではない。むしろ様々な要因で、結婚したくてもできない人が増え、結果的に未婚率の上昇や晩婚化・晩産化が進んでいるものと思われます」（図表1-2・1-3参照）。

要因の1つとして生水教授が挙げるのが"女性の意識変化"です。職業を持った女性が"ワーク・ライフ・バランス"の比重を"ワーク"へシフトさせる傾向が強まり、晩婚化を進めているのだろうという見方です。「そうした女性たちが仕事のほうにウエートを置く背景には、『少しくらい妊娠・出産を遅らせても大丈夫』という意識があるものと思われます」と生水教授。

女性の生殖能力は30代前半から徐々に衰え始めると考えられていますが、社会で活躍する女性にとって、出産に適している年齢は職業人として大きな飛躍が期待できる時期とも重なります。NPO法人ファイン（現在・過去・未来の不妊体験者を支援する会）とNPO法人日本不妊予防協会が実施した「不妊に関する意識調査」（2007年）によると、女性の生殖能力が衰え始める時期を知っていた人は約1割（不妊女性18％、一般女性11％）。女性が"出産適齢期"を十分に理解しないまま、ワークへシフトしている様子が見えてきます。

現在、高齢出産は「35歳以上の初産」と定義されていますが、これは1993年以降のことで、それまでは30歳以上の初産をそう呼んでいました。しかし30歳以上の初産婦の増加と、医療技術におけるサポート体制の進歩などを背景に、WHOなど先進各国の定義に合わせて年齢が引き上げられました。

しかし人間の身体そのものが、35歳で子どもを産んでも大丈夫なように変

図表1-1 ■ 出生数および合計特殊出生率の年次推移
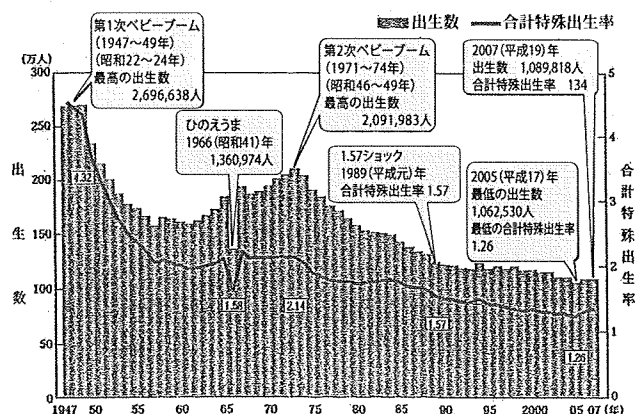
出所：厚生労働省「人口動態統計」

化したわけではなく、30歳を過ぎる頃から妊娠・出産時のリスクが高まるのは今も昔も変わりはありません。高齢出産に伴うリスクには医療現場でもかなり対応できるようになりましたが、どのようなリスクにも必ず対応できるわけではありません。生水教授も、「医療側から言わせていただくと、高齢出産でも安全と思い込むことにはいささか問題があります」と釘を刺します。

■ 高齢出産に伴うリスク

高齢出産に伴うリスクにはどのようなものがあるのでしょうか。生水教授によると、最も如実に現れてくるのが"妊娠高血圧症候群"（旧称：妊娠中毒症）の発症率の増大です。妊娠後期に高血圧や蛋白尿などの症状が出るもので、ひどいむくみが現れたり、重症になると分娩時に母子ともに危険にさらされたりするリスクも高まり、帝王切開を余儀なくされるケースが増えます。

また高齢での妊娠は、糖尿病や心臓疾患、脳血管疾患など、生活習慣病のような年齢とともに発症率の高まる疾患を誘発しやすくなることもわかっています。先頃も、脳出血を起こして救急車で運ばれ、あちこちの病院を回っても受け入れてもらえずに亡くなってしまった妊婦さんをめぐる対応が問題になりましたが、こうした病気を伴うケースも高齢出産の増大とともに目立ってきました。

高齢での妊娠・出産は、母体に大きな負担を与えるだけでなく、子どものほうにも先天異常の発症リスクを高めます。特に40歳を過ぎた出産では、染色体異常によってもたらされる"ダウン症候群"の発生率が明らかに増大し、早産や流産のリスクも高まります。このように、母子を様々な危険にさらす出産のことを"ハイリスク出産"と呼んでいます。厳密には、妊娠・出産に際しての何らかのリスク因子を持った人々のことを指す言葉で、初産かどうかに関係なく、出産年齢が上がるほどハイリスク出産となる可能性も高まります。

またリスクではないものの、30代半ばを過ぎると妊娠そのものが成立しにくくなっていきます。流産率も高まるため、女性の年齢が上がるほど相対的に子どもは生まれにくくなるわけです。「初産年齢が上がると、こうした理由でなかなか第二子を授かることができず、結果的に子どもは1人だけというケースが増えるのです」（生水教授）。さらには長引く不況による経済的な事情も加わって、「子どもは2人ほしい」と思う人が多いにもかかわらず、現実には少子化に歯止めがかからない……。これが妊娠や出産をめぐる現在の日本の状況です。結婚し、これから子育ての時期を迎えていく世代のプランニングには、こうした実情を理解しておくことが望ましいでしょう。

ちなみに、たとえハイリスク出産となっても、たいていは健康保険でカバーされますから、妊婦側に経済的な負担が増大されるわけではありません。しかし社会全体からみると、ハイリスク出産が増えればそれだけ社会保障費用の肥大化にもつながり、社会そのものの疲弊を招きます。その意味では、高齢出産の増加に伴ってハイリスク出産が増えることには、個人の健康や人生設計の問題だけでなく、大きな社会的課題があるといえそうです。

医療機関側で進む変化

■「ハイリスク出産」増加の裏で 産婦人科医が減っていく──

一方、ハイリスク出産の増大は、医療機関側にも深刻な影響を及ぼしています。

そもそも、ハイリスクとローリスクの妊娠・出産を区別する理由は、医療機関側の限られた資源を最大限かつ効率的に活用した医療を実現することにあります。一定数の妊産婦の母集団には、必ず一定比率でハイリスクの因子を持った人々が含まれます。こうした人々を少しでも早い段階で見つけ、ハイリスク出産に対応できる設備を持った医療機関（例えば大学病院）で集中して対応すれば、医療機関にとっても効率よく資源の投入が図れるだけでなく、リスク因子を持った妊産婦も安心して出産に臨むことができます。

図表1-2 ■ 生涯未婚率の年次推移

2005（平成17）年
男性：15.96

1975（昭和50）年
女性：4.32

2005（平成17）年
女性：7.25

1975（昭和50）年
男性：2.12
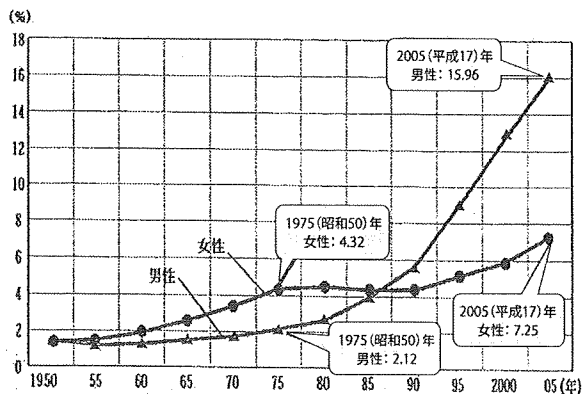
出所：国立社会保障・人口問題研究所「人口統計資料集（2009年版）」

図表1-3 ■ 婚姻件数および婚姻率の年次推移

最高の婚姻率
1947（昭和22）年
婚姻率：12.0

最高の婚姻件数
1972（昭和47）年
1,099,984組
婚姻率：10.4

2007（平成19）年
婚姻件数：719,822組
婚姻率：5.7

最低の婚姻率
2004、2005
（平成16、17）年
婚姻率：5.7
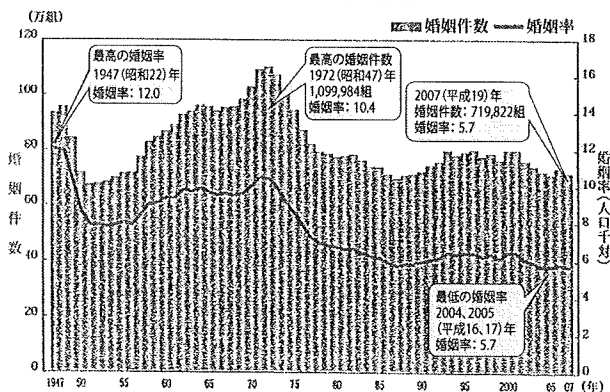
出所：厚生労働省「人口動態統計」

しかし、このために大学病院のように高度な医療を提供できる医療機関にはハイリスクの妊産婦が集中。ローリスクだった人が突然何らかの問題を発症し、ハイリスク患者となって搬送されてくるケースもあり、病院の負担はただでさえ増大しがちです。それに加えて「一般の産科医院より信頼できる」といった理由で、ローリスクの妊産婦も集まってきます。

こうして、大学病院のような医療機関の負担が大きくなり、産婦人科医師の減少といった深刻な問題を招いているのです。

生水教授によると、医師の総数はこの10年間で15%増加しているのに対し、産婦人科の医師は20年もの期間にわたって減少傾向が続いており、10年前に比べて12%も少なくなっています（図表1-4参照）。

理由の1つは、産婦人科を選択する新人医師そのものの減少です。産婦人科では深夜の救急対応なども多く、分娩に伴う長時間拘束などの労働環境の悪さから元々新人医師に忌避される傾向がありました。

そこへ大きな打撃を与えたのが、2004年に導入された「新臨床研修制度」です。同制度は新人医師に2年間のアルバイトを禁止しており、これにより医療現場は当直を引き受けてくれる主要な"働き手"の供給を失い、研修期間を過ぎた中堅医師にその負担が回ってきます。こうして中堅医師たちの疲弊ぶりを見た若手医師が、なおさら産婦人科を選択しなくなるという負のスパイラルが発生しているのです。

もう1つが、ベテランの域に達しようという医師たちの"病院"離れです。背景にあるのは、医療訴訟リスクの高まりです。ハイリスクの妊娠・出産はトラブル発生の確率もそれだけ高くなりますが、望まぬ結果がもたらされた妊産婦側は、「大きな病院に来ている以上は無事に産まれて当然」と医療機関側の責任追及や訴訟に動きがちです。

「新生児関係の損害賠償請求は、予想された生存期間をもとに逸失利益が算出されるため、請求額は一般にきわめて高額となり、それが訴訟件数を高める要因ともなっています」と生水教授。このため、責任を問われる医長や準医長などの立場となった医師たちにとって、病院に留まることへのモチベーションが急激に下がり、病院を離れ、リスクの低い（かつ報酬のよい）一般の診療所に移るという現象が起こっているのです。

加えて、長らく現場を牽引してきたベテラン医師たちが定年退職時期を迎え、産婦人科医師の減少がさらに加速化。こうして、わが国において長期にわたって維持されてきた質の高い医療の提供はいよいよ困難になり、「まさに"医療崩壊"ともいうべき深刻な状況に直面しているのです」（生水教授）。

打開策は女性医師が活躍できる環境整備と助産師との連携に

では、何らかの解決策はあるのでしょうか。「女性医師の働き方の見直しに1つの活路がある」と生水教授は語ります。

ここ10年ほどで産婦人科の医療現場では女性医師が増えており、生水教授によると産婦人科入局者の8割近くは女性です。しかし彼女たちが結婚し、家事や出産・子育てで現場を離れる期間を換算すると、その労働力は男性医師の7割程度とみなさざるを得ないのが現実。女性医師に代わって当直勤務負担が増える男性医師には不公平感が募り、女性医師数が増加していても現場の労働環境改善には必ずしもプラスにならないという認識が男性医師の間に広がってしまいがちです。

「しかし、男性医師たちも自分の子育ては妻任せにしてきた人が多いはずですから、この問題には長い目で取り組み、構造変革を進める必要があります」と語る生水教授が提唱するのは、女性医師が女性医師を支えていく仕組みづくりです。
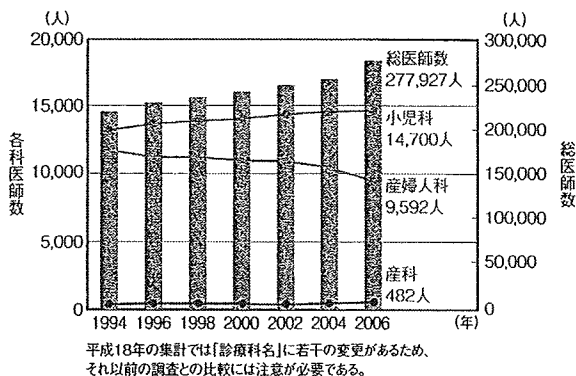
従来は、女性医師が家事や子育てに手を取られる部分を男性医師が支えるという構造がありました。これを、女性医師が自らのキャリアアップのために医療現場に戻ることを前提とした制度に変え、子育てを終えた女性たちが若い女性医師を支えていく仕組みを作っていけば、男女間の問題解消につながる可能性があります。

もちろん個々に対応すべき問題があり、過渡期においては男性医師の負担はまだまだ続きます。「過渡期として、もうしばらく男性医師に頑張ってもらう必要はあります。しかし質の高い医療の継続的な提供という観点から、従来の医局システムの殻を破ってでも、女性医師たち自らが現場を支えていく仕組みを早急に作っていく必要があります」と生水教授は強調します。

もう1つの打開策として生水教授が唱えるのが、助産師との連携の見直しです。

ハイリスク出産に対応するのが病院の役割だとすれば、ローリスク出産を地域で支える存在が助産師です。助産師の教育に長年携わってきた生水教授も、「助産師は正常分娩のスペシャリスト。ローリスクなら医師の介在なしで子どもを取り上げることができる助産師は、われわれにとっても重要なパートナーです。助産師には産後のきめ

図表1-4 産婦人科医師数の減少

平成18年の集計では「診療科名」に若干の変更があるため、それ以前の調査との比較には注意が必要である。

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細かい経過管理、授乳や赤ちゃんの扱いといった母親へのサポートなど、医師ではかなわないスキルがあり、もっと活躍してもらえるはずだと考えます」と語ります。

助産師と病院医師との連携とは、ローリスク分娩であったものに突然異常が発生したときなどに活かされる仕組みをいいます。例えば、ローリスクの妊産婦については病院に常駐する助産師が主体的に管理し、医師も健診などには部分的に関与します。その代わり、異常が発生した際にはすぐ医師に引き継ぎ、助産師は補助者となって分娩に関わります。すべてが病院内で受け継がれるため、医師も直ちに出動することができ、迅速な治療にあたることができます。

「助産師にはハイリスク出産を扱う資格はなく、異常発生に際しては直ちに医師の応援を仰ぐことが義務づけられています。しかし突然ハイリスク化した分娩では、設備の整った医療施設で多くのスタッフが集中して処置にあたらなければ救えないケースも多く、医師が助産所に駆けつければいいというものではないのです」と生水教授。

医師の負担も軽減され、妊産婦にとってもメリットの多いこうした仕組みが各地で本格的に導入できれば、医師の減少という深刻な問題に直面する産科医療の現場環境も改善に向かっていくものと期待されます。

国の少子化対策の視点

■ いかに少子化の進展と医療崩壊を食い止めるか

ここまでの話で、晩婚化・晩産化の進展とハイリスク出産の増加が大学病院など高度医療に携わる医療機関を疲弊させていることがご理解いただけたことでしょう。

ハイリスク出産の増大は、最終的に社会保障費用の増大となって国民一人ひとりにも跳ね返ってくる問題です。政府も少子化対策に乗り出していますが、解決に向けて明確な道筋が見えているわけではありません。

日本の医療の水準は国際的にもきわめて高く、分娩時の医療事故の発生率は世界でも最も低い部類に属しています。健康保険への加入も行き届いています。しかし国が医療の振興にかけている費用はOECD（経済協力開発機構）加盟国のなかでは下位クラスと、政策的にも十分な予算が投じられてはきませんでした。生水教授は、「医療機関側からの主張や情報発信が十分ではなかったことにも一因がある」と言いますが、同じように少子化に悩む欧州諸国では、国の制度によって出生率が上昇した事例もあります。

軽々に論じることはできませんが、わが国でも女性が子どもを産み育てやすくするための公的助成制度と、医療崩壊を食い止めるための施策が進められることによって、いま私たちが直面する事態の改善を図っていくことができるかもしれません。

■ 明確な決定打に欠ける政府の少子化対策

政府も妊娠・出産に関する様々な助成制度を打ち出していますが、生水教授は「意義はあるが、まだまだ十分ではない」と語ります。

例えば、妊婦健診の無料化について考えてみましょう。これは従来5回分まで無料になっていた妊婦健診が14回分まで無料になるというもので、新たに無料となる9回分の費用は、国と市町村が折半して負担します（図表1-5参照）。

「妊婦健診費用を14回すべて無料にします！」と政府首脳が語っていましたが、現状、妊婦全員が恩恵を受けているわけではありません。奈良県では、ほかの都道府県に先がけて「妊婦健診費用」の全額公費負担を公表しましたが、厚生労働省の調査では、財政状況の悪い自治体などで助成をしない、国庫補助を他の支出に振り分けるケースも散見され、運用にばらつきがあります。

また特定不妊治療費の助成についても、「子どもを増やすという意味からも、金額はもっと上げたほうがよい」（生水教授）。出産手当金の増額も、当然の措置とおっしゃっています（第2部参照）。

政府は医療機関側に対し、産科医の数を増やすことを目的に、「産科医等育成・確保支援事業」と銘打った財政支援措置をとったり、ハイリスク出産を多く扱う病院には「ハイリスク分娩管理料」の加算措置を行っていますが、要件が厳しいなどの問題があり、生水教授はその実効性はまだまだ不透明だと指摘します。

今年1月には「産科医療補償制度」がスタートしました。この制度は通常の分娩にもかかわらず、分娩に際し重度脳性まひとなった赤ちゃんがすみやかに補償を受けることができる制度で、

図表1-5 ■ 妊婦健診の公的負担の拡充について
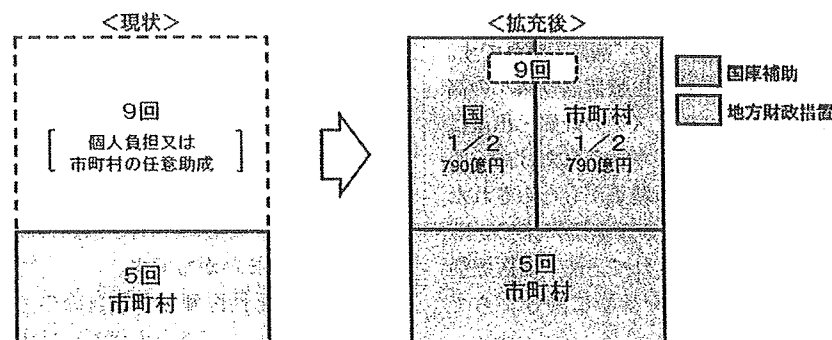
出所：厚生労働省

医療機関の抱える訴訟リスクの軽減が期待されています。

「脳性まひの8割は医療機関側が慎重に管理をしても防ぐことのできなかったもので、残る2割が分娩時の措置が適切であれば蘇生の可能性のあったものといわれています。この制度によって、今後こうしたトラブルの原因解析を個々に行うことになりましたし、医療機関側に責任のないトラブルについての訴訟が減少する可能性はあります」と生水教授。しかし補償額が少ないことと、訴訟自体を禁じているわけではないため、医師側にはまだ訴訟リスクの不安が残るとも言います。

ちなみに、この産科医療補償制度は分娩機関がお産1件ごとに約3万円の掛金を負担することで、掛金相当分の分娩費上昇が見込まれたため、加入機関（現時点で100%に近い医療機関が加入）で分娩した場合の出産育児一時金が3万円引き上げられることになりました（図表1-6参照）。

GDP（国内総生産）の2～3%を少子化対策に投じている欧州各国に比べ、日本の財政支出はわずか0.83%（2007年）。その意味では、わが国における少子化・晩婚化対策への取り組みはまだまだ十分なものではなく、政府と医療現場による抜本的な改革に期待が集まっているといえるでしょう。

図表1-6■産科医療補償制度加入機関のマーク

産科医療補償
制度加入機関

財団法人 日本医療機能評価機構／厚生労働省

出所：厚生労働省

■注目される
フランスとスウェーデンの少子化対策

先進各国では子育ての費用がかかることなどを背景に、総じて少子化の傾向にあります。しかし政策によって合計特殊出生率の引き上げに成功している国もあります。フランスは1980年代から出生率が下がり始め、95年には1.65にまで下がりましたが、女性が労働と育児を両立させやすくする環境整備や、子どもの数に応じた税率の低減措置、育児手当の20歳まで引き上げ、公共機関での家族割引などを導入し、2006年には2.01にまで回復させています。婚姻関係にない男女の間に生まれた子どもにも同じ権利を与えている点も大きな特徴です。

スウェーデンでも80年代に出席率が1.6にまで低下。働く女性や低所得者層に対する出産・育児支援策やフランスと同様に婚外子への権利の向上を図った結果、出席率は2を突破するまでに回復しました。しかし社会保障費の上昇で財難となったことで一部制度の廃止や減額を行ったところ、2000年には出生率は再び1.5に低下。そこで男女ともに就労者の育児支援につながる労働体系の見直しや公教育の低コスト化を図った結果、2005年には1.77、2006年には1.85に回復しています。子どものいる若い夫婦のニーズに合致した経済的支援や労働環境の改善、教育費の低廉化などは、少子化対策をもたらすことは間違いないようです。

■26.7%
数字に見る「できちゃった婚」

第一子出生数のうち、「できちゃった婚」（結婚期間が妊娠期間より短い出生）で生まれた子の推移をみると、1980年の12.6%が2000年には26.3%と、20年間で倍増（00～04年は26～27%台）。2009年もおそらく3割近くであろうと想像できます。

少子化の理由として晩婚化が注目されるなか、「できちゃった婚」は、10代後半～20代前半が多数。なお、人工妊娠中絶は少なくなってきています（国立社会保障・人口問題研究所統計）。"産む"ことを選べる社会になってきたということでしょうか。

FPとしては「できちゃった婚」に対する倫理観はさておき、そんな夫婦のフィナンシャル・プランが気になるところ。収入が少ないうちに無計画で結婚することで将来のリスクも発生するでしょう。

後に収入増を目指しキャリアアップを図りたくなっても、目の前の養育費や子育ての時間が優先され、キャリアアップの道が閉ざされる可能性も少なくありません。収入が少ないゆえの不仲、離婚、シングルマザー……最悪の事態を想定すると、できちゃった婚夫婦には、せめてその後FPのアドバイスに耳を傾けてほしいものです。

■ 結婚期間が妊娠期間より短い嫡出子が第一子出生に占める割合

厚生労働省 平成17年度「出生に関する統計の概況」
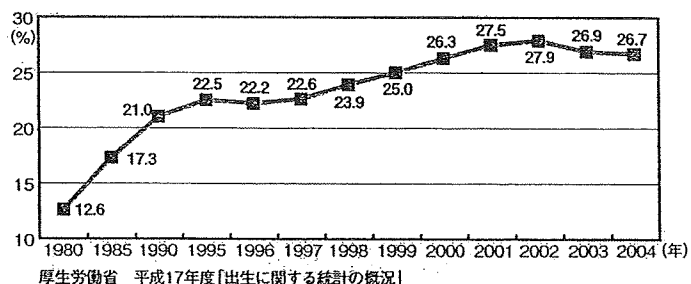
Relationship of Th1/Th2 Cell Balance With the Immune Response to Influenza Vaccine During Pregnancy

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To determine the optimal timing for influenza vaccination in pregnant women, we measured alterations in the types 1 and 2 T helper cell (Th1/Th2) balance during pregnancy, monitored specific immunity to inoculated antigens after vaccination with inactivated influenza vaccine, evaluated the relevance of the Th1/Th2 ratio and immune responses to the vaccination, monitored the maintenance of high antibody titers until delivery and measured the transplacental antibody transfer rate. No significant alterations of the Th1/Th2 balance were noted in the 65% of pregnant women among whom the Th1/Th2 ratio was lower than 9.9% in the first trimester. In those groups with a ratio higher than 10% in the first trimester, there was a tendency for the ratio to decrease as gestation advanced. The efficiency of immunization was not influenced by the Th1/Th2 status or by the stage of gestation. The antibody titer decreased steadily with time from 1 month after vaccination to the time of delivery. Conversely, the transfer rate of antibodies from maternal to fetal blood at the time of delivery increased with the duration of gestation after vaccination. Nevertheless, the antibody titers in both maternal and fetal blood were sufficient to afford protection against infection. Thus, efficient influenza vaccination can be undertaken at any stage of pregnancy. *J. Med. Virol. 81:1923–1928, 2009.* © 2009 Wiley-Liss, Inc.
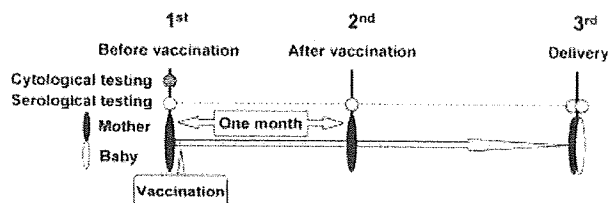
KEY WORDS: Th1/Th2 cell balance, pregnancy, influenza vaccination, passive immunity, transplacental transfer

INTRODUCTION

The fetus and placenta are perceived as "foreign" to the maternal immune system and facilitation of immune tolerance to the fetus in successive pregnancies is

important. One alteration of the immune response in pregnant patients is decreased cellular immunity, reflecting the relative predominance of type 2 T helper cells (Th2) in the Th1/Th2 ratio [Saito, 2000; Tsuda et al., 2002]. Although it is not clear whether the alteration arises locally or systemically, a decrease in cytotoxic lymphocyte activity associated with a predominance of Th2 is capable of attenuating the immune defense against viral infections. In addition, decreased maternal lung capacity caused by the enlarging gravid uterus and increases in plasma volume, heart rate, stroke volume, and oxygen consumption during pregnancy can impair the mother's cardiopulmonary functions. These physiological changes might explain the increased vulnerability of pregnant women to viral infections of the respiratory system.

Increased morbidity and mortality rates in pregnant women were reported following three major historical influenza pandemics: the Spanish flu (viral strain H1N1) in 1918, Asian flu (H2N2) in 1957, and Hong Kong flu (H3N2) in 1968. The mortality rate in pregnant women was 49% and pregnant women accounted for about half of the fatalities in women of reproductive age during the epidemics of 1918 and 1957 [Harris, 1919; Greenberg et al., 1958; Freeman and Barno, 1959; Widelock et al., 1963; Mullooly et al., 1986]. These reports were supported by the recent findings of increasing risk of respiratory hospitalization at later

suspension in PBS was added to each well and incubated at RT for 1 hr. The titers of the specific antibodies for the various strains were read by inverting the plates to produce a streak [Hist, 1942; Francis et al., 1944; Stephenson et al., 2003].

Statistical Analysis

All P values were two-sided and were not adjusted for multiple testing. Differences between groups in terms of maintenance of elevated antibody titers and transplacental transfer rate were assessed using the Mann–Whitney nonparametric U test. Analyses were performed using SPSS II software (SPSS, Inc., Chicago, IL).

RESULTS

Patients

There were 125 patients in this series: 71 in their second trimester at the time of vaccination and 54 in their third trimester (Table I). There was little opportunity for administering the vaccine during the first trimester as we usually schedule the vaccination at a follow-up appointment following the first visit, 1 month after the diagnosis of pregnancy. Although a few patients received the vaccination in the first trimester, we did not enter these data into this analysis. No significant differences in mean age, white blood cell (WBC) count, differential lymphocyte count or natural killer (NK) cell activities were observed in the patients between the second and third trimester. There were no adverse events in terms of pregnancy or fetal medical conditions.

The fetus is a source of "foreign" antigens for the maternal immune system during pregnancy and NK cell activities in maternal blood have relevance to the maternal–fetal interface [Boyson et al., 2008]. Stability of NK cell activities is important to maintain pregnancy and is also a parameter of smooth pregnancy. Given that activated NK cells have a potent influence on T-cells, we analyzed NK cell activities in this study.

Alteration of the Th1/Th2 Ratio During Pregnancy

In a preliminary investigation, the Th1/Th2 ratio was analyzed in healthy pregnant women. No significant alteration was observed when evaluated using mean data from subjects with a wide range of the ratio. Therefore, to evaluate any alterations to the Th1/Th2 balance during pregnancy we classified the subjects into three groups based on the ratio in the first trimester (Fig. 2). Those women with a low Th1/Th2 ratio ($\leq 9.9\%$) did not show any significant alterations during pregnancy, whereas women with a ratio in the mid-range (10.0–14.9%) showed a tendency toward decrease of the ratio. This tendency for a decrease was accentuated in the group with a high initial Th1/Th2 ratio ($\geq 15\%$).

Immunity to Vaccinated Antigens

The vaccine we used contains three strains of virus. The HI titers of each specific antibody were analyzed one month after vaccination to evaluate strain-specific immune responses. HI antibody titers $\geq 1:40$ are usually regarded as protective and are an objective for successful vaccination [Center for Biologics Evaluation and Research, 2007]. This is important for assessing clinical efficacy; however, in this study we concentrated on analyzing the immunological response to vaccination. The immune responses of all patients to the three viral strains included in the vaccine are shown in Figure 2B. These confirm that the antibody response rate tended to increase inversely with the titer at the time of the vaccination and that subjects with a prevaccination HI antibody titer of $\geq 1:80$ showed less response.

We applied the following criteria for classification of the immune responses. Subjects with a prevaccination HI antibody titer $<1:10$ and a postvaccination HI antibody titer $\geq 1:40$, or a prevaccination HI antibody titer $\geq 1:10$ and a minimum fourfold rise in postvaccination HI antibody titer, were classified as responsive. Those with only twice the increase, or with no increase in postvaccination HI antibody titer, were classed as poorly responsive and nonresponsive subjects, respectively. Subjects with HI antibody titers $\geq 1:80$ of prevaccination specific antibodies and with no significant response to vaccination were classed as being nonresponsive with a high titer (Table II). This group was no longer available because their antibodies might have interfered with the immune response to the vaccine, so the immunologically responsive group was our prime focus for evaluating immune responses.

TABLE I. Maternal Condition at the Time of Vaccination

Conditions, mean ± SD (range)	Trimester	
	Second (n = 71)	Third (n = 54)
Age (y.o.)	34 ± 3.8 (25–40)	34 ± 4 (26–41)
Weeks of gestation	22 ± 4.2 (15–28)	33 ± 2.5 (29–39)
WBC (ml)	9023.6 ± 1966.9 (3900–12700)	8500.0 ± 2260.7 (4100–16700)
Lymph (%)	21.2 ± 5.9 (11–38)	22.4 ± 7.7 (12–36)
CD4 (ml)	44.8 ± 6.9 (29.1–63.8)	45.7 ± 7.3 (31.2–60.1)
CD4/CD8 (ratio)	1.5 ± 0.5 (0.66–2.58)	1.5 ± 0.5 (0.65–2.47)
Th1/Th2 (ratio)	10.7 ± 7.4 (3.9–44.3)	10.2 ± 4.3 (4.3–20.7)
NK cells activities (%)	23.0 ± 11.1 (5–63)	24.5 ± 12.7 (6–56)

A

Trimester   Postpatum
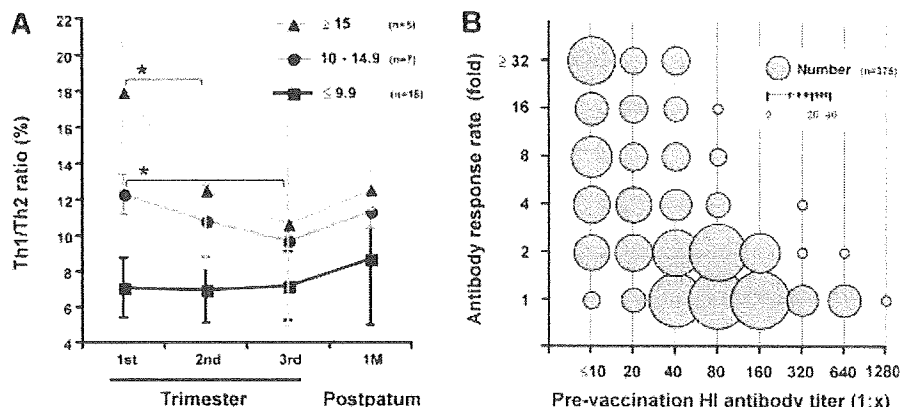
B

Pre-vaccination HI antibody titer (1:x)

Fig. 2. A: Alteration of the types 1 and 2 T helper cells (Th1/Th2) ratio during pregnancy. The three groups were classified based on the Th1/Th2 ratio during the first trimester. Data are shown as the mean ± SD from each group, *P < 0.05. B: Immune responses of all 125 patients to the three viral strains included in the vaccine. The HI antibody response rate tended to increase inversely with the HI titer at the time of vaccination, and subjects with a prevaccination HI antibody titer of ≥1:80 showed a lower response.

Th1/Th2 Ratio and the Immune Response to Vaccination

To analyze the association between the antibody immune response and the Th1/Th2 ratio at the time of vaccination, analysis was performed regardless of the stage of gestation. Although there was a wide range in the Th1/Th2 ratio in vaccinated patients, the numbers in the responder groups were generally inversely related to the ratio (Table III). Thus, most responders were observed in the group with a Th1/Th2 ratio of less than 9.9%, but no statistically significant differences were found across the range.

Serology

We compared the results of serological evaluation between the second and third trimesters (Table IV). The

TABLE II. Status of Immunity Against Antigens After Vaccination[a]

| Immunity (%) (n = 125) | Antigens[a] | | | |
	A	B	C	Whole[b]
Nonresponsive with a high titer[c]	34.4	43.2	32.8	36.0
Responsive[d]	51.2	28.0	40.0	57.6
Poorly responsive[e]	14.4	28.8	24.8	6.4
Nonresponsive	0.0	0.0	2.4	0.0

Percentage of immunity against each antigen at 1 month after the vaccination.
[a]A: A/Solomon/3/2006 (H1N1), B: A/Hiroshima/52/2005 (H3N2), C: B/Malaysia/2506/2004.
[b]Responded to either A or B or C.
[c]Subjects with HI antibody titers >1:80 to specific antibodies prevaccination and with no significant response to vaccination.
[d]Subjects with a prevaccination HI antibody titer <1:10 and a postvaccination HI antibody titer >1:40, or a prevaccination HI antibody titer >1:10 and a minimum fourfold rise in postvaccination HI antibody titer.
[e]Subjects with only twice the increase in postvaccination HI antibody titer.

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immunization rates in the vaccinated subjects without preexisting immunity were almost the same in the second and third trimesters. The rate of maintenance of an antibody level was calculated from the HI antibody titer in maternal serum at the time of delivery, relative to that measured at 1 month after vaccination. The transplacental transfer rate was calculated from the HI antibody titer in the serum of fetal umbilical cord blood, relative to that of maternal blood measured at the time of delivery. Maintenance of the antibody level was better in those subjects vaccinated in the third trimester than in those vaccinated in the second trimester. Although the transfer rate from the maternal to the fetal blood at delivery was inversely related to the duration of gestation after vaccination, higher titers were commonly observed in the fetal than in the maternal blood.

To clarify the effects of previous vaccination or infection, we also attempted to evaluate the results in relation to preexisting immunity and each patient's history of vaccination or infection. However, this was difficult to evaluate because not all the same antigens are used each past year to construct the vaccine and it is difficult to identify the infecting viral strains in each patient from a routine clinical examination.

DISCUSSION

Evaluating the clinical efficacy of vaccines is of prime importance for effective public health. However, the outcome of vaccination will depend on determining the appropriate vaccine for the strains anticipated to cause influenza in the following season and on many other factors such as each patient's history of influenza infection or vaccination and the clinical features of each particular infective strain.

Immunological evaluation of immune responses to influenza vaccination during pregnancy has not been

TABLE III. Th1/Th2 Cell Balance in the Responder Group

	Distribution			
Th1/Th2 ratio (%)	Total[a] (n = 80)	Responder (n = 72)	Percentage in each range (%)	Average Th1/Th2 ratio[b] (mean + SD)
≤9.9	52	48	92.3	7.2 ± 1.7
10–14.9	15	13	86.6	12.7 ± 1.5
15–19.9	9	8	88.8	17.0 ± 1.1
≥20	4	3	75.0	37.9 ± 5.5

[a]Vaccinated numbers without the number of preexisting immunity group.
[b]Average Th1/Th2 ratio in responders.

TABLE IV. Comparisons Between Second and Third Trimester

	Trimester		
Analysis	Second (n = 45)	Third (n = 27)	P
Immunized rate (%)[a] (n = 80) (responders/total subjects)	91.8 (45/49)	87.1 (27/31)	
Maintenance of elevated antibody level (%)[b]	66.4 ± 26.8	94.0 ± 30.9	<0.001
Transplacental transfer rate (%)[c]	161.1 ± 70.6	127.4 ± 84.8	0.020

[a]Percent of subjects showing antibody response among the vaccinated subjects without nonresponsive with a high titer immunity (n = 80).
[b]The rate of maintenance of an antibody level was calculated from the HI antibody titer in maternal serum at the time of delivery, relative to that measured at 1 month after vaccination.
[c]The transplacental transfer rate was calculated from the HI antibody titer in the serum of fetal umbilical cord blood, relative to that of maternal blood measured at the time of delivery.

conducted to date. Herein, we examined the effect of influenza vaccination in pregnancy on the Th1/Th2 ratio, on maintenance of the specific antibody response and the efficiency of transplacental transfer of the antibody to the fetus. Our aim was to determine the optimal timing of influenza vaccination in pregnant women. Therefore, our focus was not on evaluating the relationship between presence of the antibodies and the prevention of infection. We first clarified the alterations of the Th1/Th2 balance during pregnancy. Contrary to expectations, the balance was not significantly altered in the 65% of the pregnant women who had a Th1/Th2 ratio of lower than 9.9% in the first trimester. Subjects with a Th1/Th2 ratio of higher than 10% in the first trimester showed a tendency towards a decrease of the ratio, that is, a predominance of Th2 cells as gestation progressed. Although some systemic alteration of the balance was observed, this was not found in all pregnant women. These findings might be helpful to explain the alterations in diseases in which the pathogenesis, progression and improvement are affected by pregnancy, such as autoimmune and allergic diseases. The efficiency of the inactivated influenza vaccine was not influenced by the Th1/Th2 status. There were also no significant differences in the immunization rate between the second and third trimesters of pregnancy. It is conceivable that the same result would also be obtained for the first trimester on the basis of the Th1/Th2 status (Fig. 2). This outcome suggests that there might be no restriction related to the timing of influenza vaccination during pregnancy and also supports the recommendation of the ACOG or CDC in different ways.

The maintenance of an antibody level depends on the time elapsed after vaccination. Accordingly, the titers in these women decreased with time and were not related to the gestational stage. In contrast, the transfer rate from the maternal blood to the fetal blood at time of delivery tended to be inversely correlated with the duration of gestation after vaccination. The discrepancy might be accounted for by the time needed for receptor-mediated active transfer [Englund, 2007]. The presence of higher titers of antibodies in the fetal blood than in the maternal blood may also be related to the mechanism of active transfer.

In conclusion, a successful immunization rate of approximately 90% after vaccination was independent of both the Th1/Th2 balance and the stage of gestation. Although the antibody titers in maternal and fetal blood were affected by the timing of vaccination, vaccination at any time during the gestational period yielded sufficient antibody titers, in theory, to afford protection against infection. Therefore, we consider that efficient influenza vaccination can be undertaken at any stage of pregnancy.

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