Application of Toxicogenomic Analysis to Risk Assessment of Delayed Long-Term Effects of Multiple Chemicals, Including Endocrine Disruptors in Human Fetuses

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Our previous studies analyzing umbilical cords show that human fetuses in Japan are exposed to multiple chemicals. Because of these findings, we believe it is necessary to establish a new strategy for examining the possible delayed long-term effects caused by prenatal exposure to multiple chemical combinations and evaluating the health risk to human fetuses. In this commentary we describe our attempts to apply toxicogenomic analysis of umbilical cords, using DNA microarray for future risk assessment. Because the umbilical cord is part of the fetal tissue, it is possible to estimate the effects of chemicals on the fetus by analyzing alteration of the gene expression. This type of toxicogenomic analysis could be a powerful and effective tool for developing a new risk assessment strategy to help investigators understand and possibly prevent long-term effects caused by fetal exposure to multiple chemicals. Worldwide cooperation is needed to establish a new stragegy for risk assessment using toxicogenomic analysis that focuses on the human fetus. *Key words:* **delayed long-term effects, DNA microarray, human fetus, multiple chemicals, risk assessment, toxicogenomics, umbilical cord.** *Environ Health Perspect* **111:803–809 (2003). doi:10.1289/txg.5958 available via** *http://dx.doi.org/* **[Online 12 November 2002]**

Exposure to multiple chemical combinations occurs throughout our lives from air, water, soil, food, and household products. Environmental contamination by multiple chemicals, including endocrine disruptors (EDs), over the last 50 years has been suggested as a cause of human health disorders (Andersson et al. 2001; Colborn et al. 1996; Perera et al. 2002; Safe 2000; Sharpe and Skakkebaek 1993; Toppari et al. 1996). EDs are chemicals that disturb the function of natural hormones in humans and wildlife (Andersson et al. 2001; Colborn et al. 1996; Safe 2000). In animal experiments, potential EDs have adverse effects on the development and/or function of the reproductive and nervous systems, particularly when exposure occurs during fetal or neonatal periods (Colborn et al. 1996; Newbold 2001; Newbold et al. 1984; Williams et al. 2001). Similarly, human fetuses and infants are significantly more sensitive to a variety of environmental toxicants than adults (Charnley and Putzrath 2001; Needam and Sexton 2000; Perera et al. 2002). Several investigators have shown that fetuses and young children are especially vulnerable to the toxic effects of environmental tobacco smoke, pesticides, polychlorinated biphenyls (PCBs), and metals (Calabrese 1986; Jacobson and Jacobson 1996; Moore and Persaud 1998; Needleman 1979; Perera 1996; Whyatt and Perera 1995; WHO 1986).

The current risk assessment strategy, established in 1983 by the U.S. National Research Council (NRC), is based on the risk of exposure to only single chemicals and focuses on the adverse health effects on adults not children or fetuses (NRC 1983). It does not even suppose the risk of complex mixtures of chemicals to human fetuses. Several investigations have shown that combined effects of multiple chemicals enhance the proliferation of human breast cancer cells (Payne et al. 2001) and induce congenital anomalies in rats (Gray et al. 2001; Price et al. 2000). Our recent studies in Japan using human umbilical cords have shown that human fetuses are exposed to multiple chemicals (Mori 2001; Mori et al. 2001; Todaka and Mori 2002). There is genuine concern that these multiple chemical exposures in humans may cause delayed long-term adverse health effects. Therefore, in addition to the current risk assessment, a new method of health risk assessment of fetal exposure to multiple chemicals must be developed.

Toxicogenomics is now developing. It is defined as the study of the genes and their products, which are important in adaptive responses to toxic exposures (Iannaccone 2001). One applicable and efficient method for developing this assessment of fetal exposure to multiple chemicals is the toxicogenomic analysis of umbilical cords, using DNA microarray.

Certain adverse health effects can be prevented if symptoms are observed during the postnatal period. Newborn diagnosis is very important from the viewpoint of preventive medicine. Using toxicogenomic analysis, if the new risk assessment of effects of exposure to multiple chemicals is applied to preventive medicine, it can possibly secure the health of future generations.

In this commentary we introduce *a*) a summary of our analysis of human fetal exposure to multiple chemicals, *b*) our preliminary toxicogenomic analysis of animals, *c*) our toxicogenomic analysis of human umbilical cords, *d*) technical problems and socioethical issues to be resolved to make this method more practical, and *e*) the necessity of a worldwide effort to establish a new risk assessment using toxicogenomic analysis that focuses on the human fetus.

Analysis of Human Fetal Exposure to Multiple Chemicals

Our group has investigated human fetal exposure to multiple chemicals in Japan by analyzing umbilical cords and cord blood (Mori 2001; Mori et al. 2001; Todaka and Mori 2002). Human umbilical cords, part of the fetal tissue, were collected from normal newborns. This study has been approved by the Congress of Medical Bioethics of Chiba University, Yamanashi Medical College, and Kyoto University. Informed consents of the mothers were also obtained.

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A summary of our previous studies of human fetal exposure assessment is as follows:

a) Multiple chemicals and toxicants were detected in human umbilical cords or cord serum. The detected chemicals and toxicants were dioxins (polychlorinated dibenzo-*p*-dioxins + polychlorinated dibenzofurans + coplanar-PCBs), PCBs, dichlorodiphenyltrichloroethane (DDT), dichlorodiphenylchloroethane (DDE), aldrin, chlordane, hexachlorobenzene (HCB), hexachlorocyclohexane (BHC), heavy metals (cadmium and lead), bisphenol A, and phytoestrogens (Mori 2001; Mori et al. 2001; Todaka and Mori 2002). All these chemicals and toxicants were detected in more than 50% of the umbilical cords investigated in our studies. Some umbilical cord data showed large amounts of several chemicals (Todaka and Mori 2002). As a result of these findings, it is now clear that fetuses in Japan are exposed to multiple chemicals.

b) Concentrations of persistent chemicals such as dioxins, PCBs, and DDT and its metabolites in human umbilical cords of first-, second-, and thirdborn babies were examined and compared. Concentrations of dioxins, PCBs, and DDT, and its metabolites seem to be higher in first born babies than in second- or thirdborn babies (Mori et al. 2001).

There are correlations between the concentrations of PCBs and other persistent chemicals such as *p,p´*-DDE, HCB, and BHC in human umbilical cords (Figure 1). Individuals who accumulated PCBs at higher levels also accumulated other persistent chemicals at higher levels. As shown in Figure 1, three umbilical cords that had accumulated levels of PCBs greater than 200 pg/g-wet weight also showed high levels of *p,p´*-DDE, HCB, and BHC. There are two possible reasons for this. One is that these people might have been highly exposed because of their eating habits or because they are living (or used to live) in an area polluted by these chemicals. Another possible reason is that these people may have lower abilities to exclude the chemicals because of specific genetic backgrounds. This analysis suggests that there are fetuses highly exposed to multiple persistent chemicals in Japan.

From the above, it is clear that at least 20 chemicals and toxicants have been transplacentally transferred from mothers to their fetuses. Moreover, some fetuses are more highly exposed to multiple chemicals (Mori 2001; Mori et al. 2001; Todaka and Mori 2002).

There is no evidence these situations will cause any adverse health effects in children in the future. However, reports from animal experiments and wildlife animal disorders give us genuine concern about possible delayed long-term adverse health effects on humans (Colborn et al. 1996). Diethylstilbestrol (DES) is a synthetic estrogen commonly used for pregnant women until the1970s to prevent miscarriage. Delayed long-term effects, typically reported as DES syndrome, are phenomena caused by fetal exposure to DES that do not emerge until a child reaches puberty, or sometime later in life (Colborn et al. 1996; Herbst et al. 1971, 1974; McLachlan et al. 2001). Because the human fetus is toxicologically much more sensitive to chemicals than adults (Calabrese 1986; Jacobson and Jacobson 1996; Moore and Persaud 1998; Needleman 1979; Perera 1996; Perera et al. 2002; WHO 1986; Whyatt and Perera 1995), it is important that the scientific community establish a new method of risk assessment that considers human fetal exposure to multiple chemicals.

Our Preliminary Toxicogenomic Analysis of Animals

In the past 5 years, toxicogenomics has become a remarkable scientific field that combines studies of genetics, genomewide mRNA expression, cellwide and tissuewide protein expression, bioinformatics, and toxicology to understand the roles of gene–environment interactions in diseases (Bartosiewicz et al. 2000; Iannaccone 2001; Lobenhofer et al. 2001; Medlin 1999; Nuwaysir et al. 1999; Rockett and Dix 1999; Tennant 2002). Toxicogenomic analysis using DNA microarray is a powerful and high-throughput method for monitoring the expression of thousands of genes simultaneously (Adachi et al. 2002; Duggan et al. 1999; Hossain et al. 2000; Komiyama et al. 2002; Rockett and Dix 1999). If gene expression profiles can be used as a predictive parameter, toxicogenomic analysis using microarrays would be an ideal tool for developing risk assessment of chemicals (Iannaccone 2001).

Our group conducted animal experiments to investigate the long-term alteration of gene expression affected by neonatal exposure to estrogenic compounds in mouse testis (Adachi et al. 2002; Komiyama et al. 2002; Shibayama et al. 2001). Male and female reproductive organs including testis and uterus are vulnerable to estrogenic compounds. The estrogenic compounds used in the experiments were DES and genistein (a phytoestrogen produced by plants).

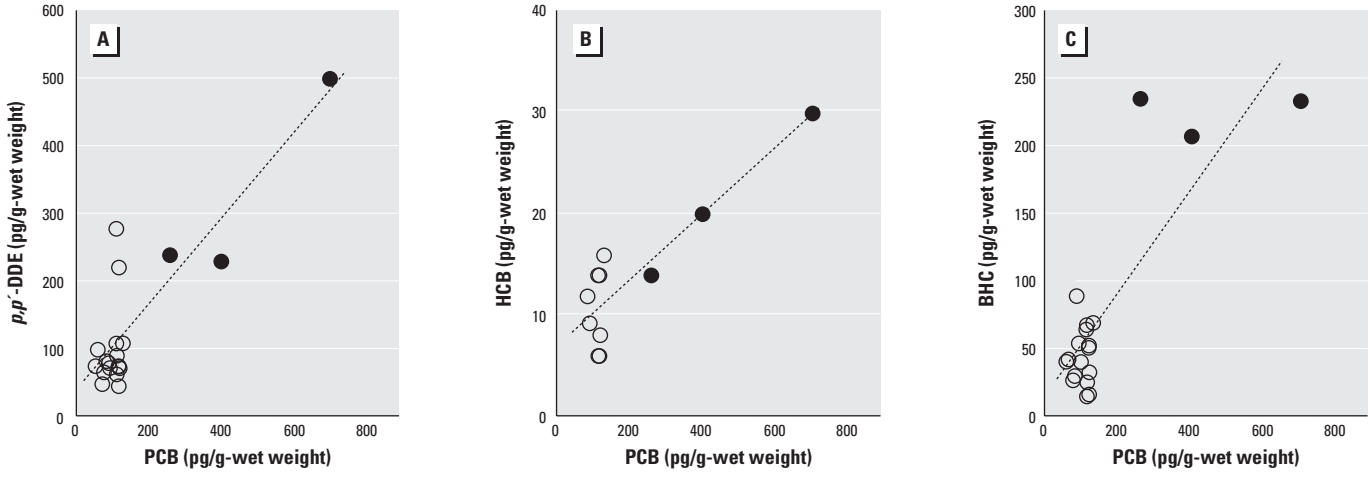

Figure 1. Correlation analysis between the people who accumulated high amounts of PCBs and the people who accumulated high amounts of *p,p´*-DDE (*A*), HCB (*B*), and BHC (*C*). The number of samples in each analysis is 19, 11, and 19, respectively. The amount of each chemical is expressed as picograms per gram tissue wet weight. The group including fetuses highly exposed to multiple chemicals is shown as filled circles. In these data, three samples are included in the multiple highly exposed group.

Table 1. Evaluation of effects of neonatal exposure to DES or genistein at three different end points in mice.

	End points		
	Gene expression level (4–12 weeks)[a]	Reproductive disorder level (12 weeks)[a]	Cancer level (18 months)[a]
Evaluation methods	Toxicogenomic analysis	Conventional toxicologic assessment	Delayed long-term effect
DES	Alteration of gene expression	Adverse effects	Adenocarcinoma[b]
Genistein	Alteration of gene expression	No adverse effects	Adenocarcinoma[b]

[a]Term after neonatal exposure to chemicals. [b]Data from Newbold (2001) and Newbold et al. (2002).

The goal of our investigation was to determine if toxicogenomic analysis at the molecular level could detect a delayed long-term effect that cannot be detected by conventional toxicological assessment. Table 1 is a summary of the data from our experiments and others (Adachi et al. 2002; Komiyama et al. 2002; McLachlan et al. 2001; Newbold 2001; Newbold et al. 1984, 2002; Shibayama et al. 2001). The table shows the effects of neonatal exposure to DES or genistein in experimental animals at three different end points (gene expression level, reproductive disorder level, and cancer level). The gene expression level was examined by toxicogenomic analysis during weeks 4–12 after neonatal exposure. The reproductive disorder level was examined by conventional toxicologic assessment at week 12 after neonatal exposure. The cancer level shows the delayed long-term effect. The delayed long-term effect was detected as the form of adenocarcinoma in the uterus at month 18 after neonatal exposure (Newbold 2001; Newbold et al. 2002).

Details are as follows: *a*) Neonatal exposure to DES in animal experiments can cause marked atrophy of the testes, testicular carcinoma, uterine adenocarcinomas, and carcinoma of cervicovaginal region (Arai et al. 1983; McLachlan et al. 2001; Newbold 2001; Newbold et al. 1984; Visser et al. 1998). In our experiments, DES caused several adverse effects as reported before by conventional experimental methods. Furthermore, using molecular level analysis, we were able to detect long-term alteration of gene expression (Shibayama et al. 2001).

b) Neonatal exposure to genistein in animal experiments has been reported to cause uterine adenocarcinomas (Newbold et al. 2002), but other adverse effects have not been reported. Using the conventional method in our experiment, we did not detect any other effects. However, long-term altered gene expressions at mRNA and protein levels were found (Shibayama et al. 2001). The evalution presented in Table 1 shows that delayed long-term effects not detectable with the conventional method can be detected at the molecular level with toxicogenomic analysis.

Table 2. Transition of gene expression change in testes of neonatally DES-treated mice during 4–12 weeks of age using microarray analysis.

Transition pattern	4 weeks	8 weeks	12 weeks	Number of genes
A	Up	Up	Up	1
B	Up	Up	–	3
C	Up	–	–	21
D	–	Up	Up	6
E	–	Up	–	27
F	–	–	Up	7
G	Down	Up	Up	1
H	Down	Up	–	1
I	Down	–	Up	2
J	Down	–	–	12
K	–	Down	–	8
L	–	–	Down	3
M	Down	Down	Down	0

Abbreviations: Down, downregulated more than 2-fold; Up, upregulated more than 2-fold; –, same as control.

c) Next, using DNA microarray, our group conducted experiments to examine global gene expression that was altered in the long term. The effect of neonatal exposure to DES (50 µg/mouse/day) on mouse testicular gene expression was examined using in-house cDNA microarray (Adachi et al. 2002; Komiyama et al. 2002). The in-house cDNA microarray contained 2,304 cDNA probes prepared from mouse fetuses (day 14.5). As a result, 11 genes were upregulated (more than 1.5-fold) at expression levels in DES-treated mice 4 weeks of age. Real-time reverse transcription–polymerase chain reaction (RT–PCR) analysis also revealed that expression levels of 8 of 11 genes were still higher in DES-treated testes at 8 and 12 weeks of age (Adachi et al. 2002). Moreover, transitions of gene expression profiles in testes of DES-treated mice were also investigated during 4–12 weeks of age using the in-house cDNA microarray (Table 2). Many of the genes affected by DES were upregulated or downregulated at restricted periods (Table 2, patterns B–F, J–L), but some genes were continuously upregulated (Table 2, pattern A) or shifted from a downregulated state to an upregulated state (Table 2, patterns G–I) (Komiyama et al. 2002).

As above, with toxicogenomic analysis using cDNA microarray, we proved that neonatal exposure to chemicals induces long-term effects on testicular gene expression in adult mice. Moreover, global analysis using DNA microarray can detect some genes that are upregulated or downregulated continuously by chemical exposure. Such genes can be a biomarker to predict adverse effects caused by chemicals. Bartosiewicz et al. (2000) also recently reported that microarray is a powerful tool to evaluate the effects of multiple chemical exposure. Several studies by our lab and others, using animals, have shown that application of toxicogenomic analysis to human risk assessment of multiple chemical exposure is possible.

Our Toxicogenomic Analysis of Human Umbilical Cords

Microarray technology has been used in many human studies such as cancer research. Some of the main objectives of using microarray technology in human cancer research are to improve differential diagnosis of malignant neoplasia and to increase the effectiveness of therapeutics (Lobenhofer et al. 2001). Microarray technology has been applied to a field of toxicology using animal experiments and will be applied to the field of risk assessment of human exposure to several environmental toxicants. A recent study using cDNA microarray by Hossain et al. (2000) reported the identification of lead-sensitive genes in immortalized human fetal astrocytes. The study showed the potential of DNA microarray in the discovery of novel toxicant-induced gene expression alterations and in the understanding of the mechanisms underlying lead neurotoxicity.

As mentioned above, we are newly applying toxicogenomic analysis using

DNA microarray to develop a new risk assessment method to evaluate the effects of chemicals. Figure 2 shows our challenging framework to establish toxicogenomic analysis using human umbilical cords for this purpose. Because the umbilical cord is part of the fetal tissue, it is possible to estimate the effects of chemicals on the fetus by analyzing alteration of the gene expression. In addition, it is easy to collect the umbilical cords technically and socially. Therefore, the use of umbilical cords for risk assessment of chemical effects on the fetus seems to be an ideal method.

We are developing this new risk assesment method using four steps (Figure 2). Step 1 analysis is the global gene expression analysis of the umbilical cords by DNA microarray. Step 2 analysis is a combined analysis of the data from Step 1 and exposure assessment data in each umbilical cord. In this analysis a relationship between a global gene expression profile and chemical exposure levels will be clarified. Step 3 analysis using an *in vitro* experiment is DNA microarray analysis of the alteration of gene expression in human umbilical cord–derived cells (HUCCs) after exposure to chemicals. For this purpose, human umbilical vein endothelial cells (HUVEC) is one of the candidates, as we found that HUVEC genes changed their expression after chemical exposure. Although HUVEC can be applied in this step, other cells from umbilical cords also may be applicable. Step 4 analysis is an integrated analysis of a comparison between Step 2 and Step 3. In this integrated analysis, biologic reactions at the molecular level caused by exposure to chemicals in fetuses can be detected. To extend the toxicogenomic analysis method to develop a new risk assessment strategy, comprehensive studies are required to clarify the correlation between data from toxicogenomic analysis, data from animal experiments observing the adverse effects of chemical exposure, and data from human prospective studies. By establishing the toxicogenomic analysis with the *in silico* integrated analysis of human umbilical cords, the new risk assessment for multiple chemical exposures will be practical.

However, in establishing the procedure for toxicogenomic analysis, some doubt the possibility of detecting alterations of gene expressions because the umbilical cord is not the target organ affected by the chemicals. Others doubt if toxicogenomic analysis using microarray is possible with umbilical cords. To answer these questions and to prove the importance of toxicogenomic analysis using umbilical cords, we conducted the following two preliminary experiments.

First, the total RNA was purified from umbilical cords (n = 5), and RT–PCR analysis was conducted. As a result, mRNAs of cytochrome P450 (CYP) 1A1, CYP1A2, CYP1B1, and sex hormone receptors were detected in human umbilical cords. We also found the difference of expression levels of mRNA of CYP1A1 in each umbilical cord (unpublished data). These results show that umbilical cords can be targets for toxicogenomic analysis.

Second, to conduct a toxicogenomic analysis of umbilical cords, alteration of the gene expression profile was analyzed in five umbilical cords using the human I cDNA microarray (human 15154 genes; Agilent Biotechnology, Japan, Tokyo, Japan). As a reference, we used the mRNA purified from HUVEC (Cell Applications, Inc., San Diego, CA, USA). Global analysis of gene expression profiles in five umbilical cords showed that more than 7,000 of 15,154 genes were expressed in umbilical cords, and expression levels of 265 of more than 7,000 genes were higher or lower than the expression levels of those

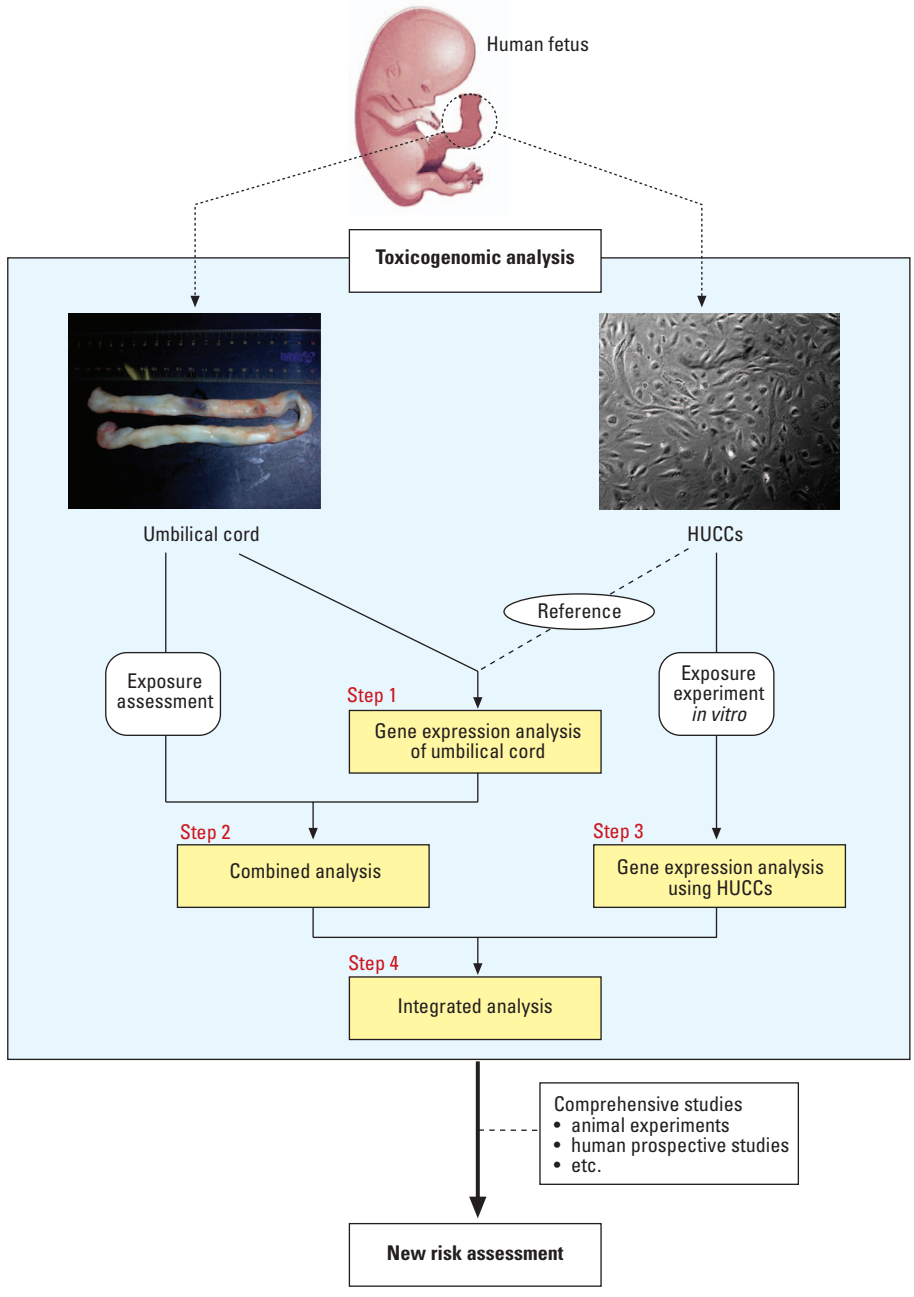

Figure 2. Challenging framework to establish toxicogenomic analysis of multiple chemical exposure using umbilical cords for new risk assessment to decrease the risks to future generations.

in the HUVEC. Approximately 6,800 genes did not show any alteration in expression levels between the umbilical cord and HUVEC. Among at least four umbilical cords, the number of genes commonly altered was only 27. Moreover, we found variations in gene expression patterns (Figure 3). Some genes, such as X53331 (matrix Gla protein) and AA314429 (expression sequence tag [EST], similar to ribosomal protein S12), were upregulated in some umbilical cords and downregulated in others. Conversely, some genes such as M60527 (deoxycytidine kinase) were downregulated among all five umbilical cords. (Gene accession numbers are from the GenBank database [*http://www.ncbi.nlm.nih.gov*].) These results indicate that DNA microarray analysis of each umbilical cord can detect individual differences of gene expression.

In summary, the analysis using DNA microarray could reveal global gene expression alterations and detect individual differences in gene expression in each umbilical cord. The next step would be to increase the number of cord samples and analyze *in silico* both the data from toxicogenomic analysis and exposure assessment.

As it is already used in the field of cancer research, if the approach shown in Figure 2 becomes practical, the toxicogenomic analysis can be used for the early diagnosis and possible prevention of adverse effects caused by multiple chemicals in humans.

Technical Problems and Socioethical Issues to Be Resolved

Although the toxicogenomic analysis using human umbilical cords can be an ideal method to predict possible future effects in humans, certain technical and socioethical points must be cleared to make it more practical.

First, a technical problem is the choice of reference. For this study, we chose HUVEC as a reference because we expected the gene expression pattern after exposure would be useful in HUCCs. However, as it is from human tissue, the genetic background differs from one HUVEC sample to another.

Second, investigators must be certain if an alteration in a microarray expression profile is a sign of a future adverse effect. At this stage an adverse effect is only suspected; however, if genes alter with chemical exposure, it could be a sign of some delayed long-term effect. It is therefore very important to accumulate information about the gene expression profile for the time when the meaning of the gene expression changes becomes clear. In addition, as mentioned above, comprehensive studies are required to clarify the correlation between the data from toxicogenomic analysis and data from chemical exposure experiments in animals. Moreover, if the toxicogenomic analysis data from each umbilical cord can be compared with data from prospective survey studies that follow up on the growth of the cord-related babies, the correlation between the two types of data from the toxicogenomic analysis and the human follow-up surveys could clarify the effectiveness of the newly developed risk assessment.

Third, as the toxicogenomic analysis method develops, socioethical issues surface. Naturally, researchers must receive informed consent from parents. In addition, information on gene expression with each umbilical cord should be managed properly and restricted to authorized use.

Fourth, because of the sensitive nature of the implications of the results, researchers must prepare for emotional care of the involved subjects and parents. With the rapid growth of toxicogenomics and environmental genome projects (Guengerich 1998; Shalat et al. 1998; Sharpe and Barrett 2000), people can now obtain an individual analysis of multiple chemical exposure addressing exposure levels, toxicogenomic analysis data, and genetic background. Susceptibilities of individuals to chemicals will differ, mainly because of the unique genetic background of each individual (Guengerich 1998; Shalat et al. 1998; Sharpe and Barrett 2000; Spearow et al. 1999). When a child's exposure level, toxicogenomic analysis data (gene expression level at present), and genetic background become clear, parents should be made clearly aware of this information, e.g., their children's exposure levels to which chemicals, how sensitive their children are to these chemicals, and how their children might be affected by these chemicals. How this information will be sensitively presented and how parents might emotionally respond need to be carefully planned. Researchers must develop a system that both accurately informs and sensitively cares for the involved parents. People who are informed of their own or their children's toxicogenomic analysis data should also be told of whatever methods are known and available to reduce the risk of possible adverse effects.

Last, perhaps of greatest concern are the attempts to decrease the presence of these unwanted chemicals in our environment and to develop methods to decrease the amount of accumulated chemicals already present in our bodies. We have

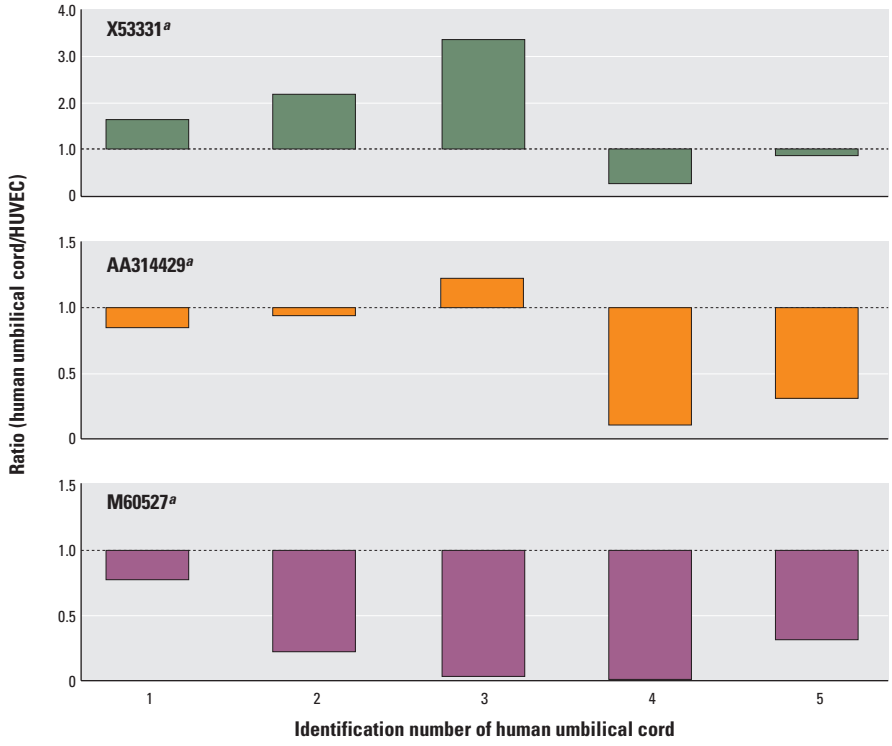

Figure 3. Individual differences of gene expression of some genes in each umbilical cord. [a]GenBank accession numbers are from the GenBank database (*http://ncbi.nlm.nih.gov*): X53331, matrix Gla protein; AA314429, EST, similar to ribosomal protein S12; M60527, deoxycytidine kinase.

established a framework to begin reducing levels of exposure through communication to the public of risk factors (Todaka and Mori 2002). Other groups are also trying to prevent chemical exposure to fetuses and infants in many ways. For example, Perera et al. (2002) have introduced a program of preventive methods against environmental toxicants in New York City. The U.S. National Institute of Environmental Health Sciences and U.S. Environmental Protection Agency have created 12 children's environmental health research centers in the United States and are trying to reduce the risk of exposure to chemicals and prevent damage to children (Wakefield 2002).

Conclusion

Increasing evidence clearly proves that human fetuses are contaminated by exposure to multiple chemicals. The currently accepted risk assessment focusing on the adverse effects of only single chemicals on only mature adults does not address this fact. Fetal exposure to multiple chemicals and the possible future adverse effects of these chemical combinations should be considered. Although the correlation between exposure to chemicals and adverse health effects such as congenital anomalies, disorders of the reproductive, immune, and nervous systems, etc., are not entirely clear. It is the responsibility of the people living in our modern society to decrease possible risks to future generations.

In this commentary we highlight the possible utility of developing a new risk assessment strategy using toxicogenomic analysis of umbilical cords by DNA microarray (Figure 2). As mentioned above, many steps are required to establish an accurate toxicogenomic analysis method using umbilical cords, and technical problems and socioethical issues still must be surmounted. However, when these problems are resolved, we are hopeful the new risk assessment strategy with toxicogenomic analysis can be applied to prevent the long-term effects of multiple chemical exposure. Furthermore, if the new risk assessment involves cooperation from current environmental genome projects, it could possibly lead to the development of new tailor-made preventive medicines.

The problems of multiple chemical exposure are not confined to Japan. People throughout the world are exposed to similar or even worse conditions of environmental contamination. Worldwide cooperation is necessary to establish a new risk assessment using toxicogenomic analysis focusing on the human fetus.

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