dietary patterns as well as dietary metal intake vary among countries and regions. For example, like many Asians, Japanese eat rice as a staple food and consume a lot of seafood, and this diet differs from that in other parts of the world. When reviewing or establishing local drinking water quality standards, it is important to take into account these local dietary patterns. Recently, an issue about risk assessment of essential yet toxic metals has been posed (Aggett 2008; WHO 2008). For example, copper plays an essential role as a central component of many redox active enzymes (Stern 2008), and manganese is essential for skeletal development, immune system function, and energy metabolism (Santamaria 2008). Deficiencies in such essential metals linked to enzyme activities contribute to the progression of disease (Gambling 2008). Although these elements are essential to biological processes in humans, they can also be toxic when consumed in excessive amounts (Fraga 2005). Therefore, both deficient and excessive intake of certain essential elements poses health risks.

In the present study, we investigated daily metal intake (DMI) of 17 metals (toxic, essential yet toxic, and essential metals) from total diet and drinking water in six cities in Japan. We estimated the contribution to TDI of toxic elements as proposed by the WHO or Joint FAO/WHO Expert Committee on Food Additives (JECFA). We also estimated the contribution to recommended dietary allowances (RDAs) proposed by the Japanese Ministry of Health, Labour and Welfare (JMHLW). The RDA represents the dietary intake level that is sufficient to meet the nutrient requirements for 97–98% of a Japanese population of a certain age and gender (JMHLW 2005). We focused on the contribution of metal intake via drinking water to TDI and/or RDA. For toxic metals, the margins of TDI that could be allocated to drinking water for establishment or review of drinking water quality standards are discussed. The contribution of drinking water to daily essential metal intake is also discussed.

METHODS

Sample collection

Samples were collected by the market-basket method from six cities across Japan. At each sampling location, about 150 kinds of food were purchased from grocery stores according to the methods used by the National Nutrition Survey (JMHLW 2004). Food items that are generally cooked before consumption were cooked by the usual methods such as boiling or baking. The items were cooked without addition of any oil or seasoning, because these additives belonged to different categories. Then, all food items were categorized into 13 groups in accordance with the classification of the National Nutrition Survey (Figure 1). Finally, food samples of each group were homogenized to make a composite sample. Drinking water samples were collected from the tap where the preparation of the food was performed. In all six cities, drinking water was processed in municipal drinking water treatment plants that use surface water as the source. The drinking water samples were assigned to the 14th group. The food composite samples were stored at −30°C in a deep freezer and were defrosted just before analysis. Drinking water samples were stored at 4°C in a refrigerator.

Analytical methods

The composite food samples were digested in a microwave digestion system (ETHOS TC, Milestone S.r.l., Bergamo, Italy) by the following procedure. A portion of 0.5–1.0 g (wet weight) was weighed into a PTFE vessel, and 4 mL of nitric acid and 1 mL of hydrogen peroxide (Ultra Pure Grade; Kanto Chemical Co., Inc., Tokyo, Japan) were added. The basic program of the microwave digester was as follows: increase the temperature from room temperature

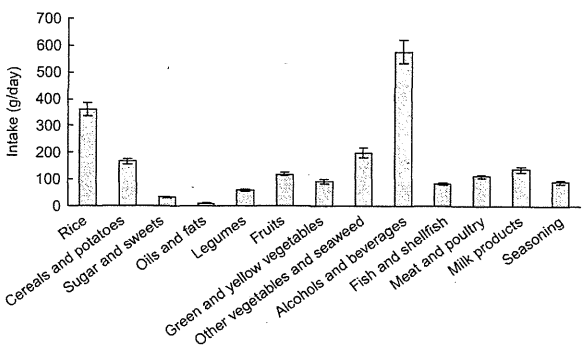


Figure 1 | Thirteen food sample groups and intake of every group, as determined by methods used by the National Nutrition Survey (JMHLW 2004). Columns represent the average of six cities, and error bars are one standard deviation.

to 210°C over 30 min, maintain that temperature for 15 min, and then cool to room temperature over 10 min; the maximum power was 1,000 W (Dolan & Caper 2002). Digested solution was brought up to 50 mL with Milli-Q water (Milli-Q Advantage, Millipore, Billerica, MA, USA).

The concentrations of 14 metals (boron, aluminium, chromium, manganese, nickel, copper, zinc, arsenic, selenium, molybdenum, cadmium, antimony, lead, and uranium) in drinking water and in the digested solutions of the food composite samples were determined using an inductively coupled plasma-mass spectrometer (ICP-MS; HP-4500, Agilent Technologies, Inc., Palo Alto, CA, USA). The instrumental parameters were as follows: RF power, 1,200 W; RF matching, 1.8 V; sample skimmer cone in Ni; plasma flow rate, 16 L/min; auxiliary flow rate, 1.1 L/min; nebulizer flow rate, 1.2 L/min. Gallium (m/z = 69) and yttrium (m/z = 89) were used as the internal standards. The concentrations of magnesium, calcium, and iron in drinking water and in the digested solutions were determined by ICP-atomic emission spectrometry (ICPS-7510, Shimadzu Corp., Kyoto, Japan).

Validation of measurement method

To check the validity of the analysis, seven standard reference material (rice flour [SRM1568a], spinach leaves [SRM1570a], bovine muscle powder [SRM8414], oyster tissue [SRM1566b], nonfat milk powder [SRM1549], wheat flour [SRM1549a], and "typical diet" [SRM1548a]) were purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA). These standard reference materials were analyzed in the same manner as the food composite samples in this study and compared with the certified values of the standard reference materials.

The standard reference material–certified or reference values and our analyzed values were compared by the recovery ratio and z-score (Dolan & Caper 2002). For almost all metals, the recovery ratio was between 75 and 125% and/or the absolute value of the z-score was less than 2.5, which showed good agreement between the certified or reference values and our analyzed values. However, our analyzed values were outside of these ranges for selenium in the spinach leaves and the nonfat milk powder and for arsenic and molybdenum in the bovine muscle powder. The main cause of this disagreement may be that the certified or reference values are very low and near the detection limits of the present study. Because these values are very small, the effect of the disagreement was estimated to be negligible in the calculation of DMI in this study.

Detection limits were estimated to three times the standard deviation (SD) of the metal concentration derived from 10 measurements of the method blank. The method blank was a blank sample pretreated by the same procedure as for food composite samples.

Estimation of DMI

The National Nutrition Survey provides daily intake data for each food sample group (Figure 1). In the case of drinking water (14th group), the daily drinking water intake was assumed to be 2 L/day. To calculate DMI, the metal concentration in a food composite sample or drinking water was multiplied by the daily intake of each group, and the values were summed, as follows:

$$\sum_{i=1}^{14} a_i b_i$$

a_i = metal concentration in the food composite sample or drinking water of ith group
b_i = daily intake (consumption rate) of ith group

Calculation of TDI and RDA values and their assumptions

In this study, the values of TDI and RDA were calculated as for an average Japanese adult. Basically, the values of TDI (mg/kg/day) were derived from either the TDI reported in the *Guidelines for Drinking-water Quality* (WHO 2008) or from that proposed by JECFA (1982, 1983, 1989, 1993). TDI is not necessarily expressed as TDI but other similar terms, such as PTWI (provisional tolerable weekly intake) and PMTDI (provisional maximum tolerable daily intake). In this study, for simplification, the term "TDI" is used for all of these similar terms after weekly intakes were converted to daily intakes. Exceptions were the TDI values of manganese, molybdenum, and selenium. The original

TDI for manganese in the WHO guidelines is considered to apply only to drinking water, because it includes an uncertainty factor (UF) of 3 to take into consideration the possible increased bioavailability of manganese from water (WHO 2008). Therefore, the TDI of manganese was set to be 11 mg as daily intake per capita by excluding the UF of 3. This value was also applied as the upper limit for dietary reference intakes for Japanese (JMHLW 2005). For molybdenum, we applied the reference dose set for chronic oral exposure (0.005 mg/kg/day) by the U.S. Environmental Protection Agency (USEPA 1993). This value was also applied as the upper limit for Japanese (JMHLW 2005). For selenium, the no observed adverse effect level (NOAEL) of 4 μg/kg/day in humans was used directly for the derivation of the WHO guideline value (WHO 2003, 2008); this approach is the same as the TDI approach with a UF of 1. Therefore, we assume the NOAEL should be equivalent to TDI in the case of selenium. This value is more conservative than the upper limit for Japanese of 6.7 μg/kg/day (JMHLW 2005), which is based on a NOAEL of 13.3 μg/kg/day as determined by the study of Yang & Zhou (1994) and a UF of 2. To convert these TDI values to the unit of weight per capita, the average weight per capita was assumed to be 50 kg, which is the assumption used in establishing the drinking water quality standards in Japan.

The JMHLW (2005) provides separate RDA values for each gender and age group. In this study, the average of RDA values of both males and females from 18 to 69 years old was used as the RDA for an average Japanese person, except in the case of calcium and manganese. For these elements, JMHLW does not set a RDA but an adequate intake (AI) value. AI is defined as the amount of daily intake sufficient to maintain a stable nutritional state (JMHLW 2005). AI is set only when an RDA cannot be set because of the lack of experimental and epidemiological data. The values of TDI and RDA calculated in this study for an average Japanese person are shown in Table 1 with the results of DMI.

RESULTS AND DISCUSSION

Margin to TDI values for toxic metals

The mean and SD of DMI for the 17 elements, as well as the DMI of each food group, are presented in Table 1. The percentage of DMI to TDI was calculated for each of the 13 toxic metals (Figure 2). The mean DMIs of 10 toxic metals (excluding selenium, molybdenum, and arsenic) were less than 50% of TDI. A simple interpretation of this result indicates that there may be a substantial margin between TDI and mean DMI. Accordingly, for these 10 metals the total daily intake percentages allocated to drinking water could possibly be increased from the normal allocation percentage (i.e. 10–20% of TDI) in reviewing or establishing drinking water quality standards. In this case, however, only the mean intakes were considered. Variation of the intakes among individuals and areas should be further investigated and considered. Exposure from other possible sources such as air and soil should be considered as well. Furthermore, it may not be necessary to increase the allocation ratio if drinking water can meet the current standards with commonly-used drinking water treatment processes.

The six-city average of daily arsenic intake exceeded TDI by a considerable degree (280% of TDI); the highest intake was 359% of the TDI. The toxicity of arsenic varies greatly according to its chemical form (i.e. inorganic or organic). The toxicity of organic arsenic is much lower than that of inorganic arsenic. In this study, the chemical form was not considered, and the DMI of arsenic was calculated on the basis of the total arsenic. According to the analysis of daily arsenic intake (Table 1), among the 13 food groups two groups ("Other vegetables and seaweeds" and "Fish and shellfish") accounted for high ratios. The sum of arsenic intake from the other 11 food groups was lower than the TDI; therefore, the chemical forms of arsenic in these two groups should be further investigated. The mean molybdenum intake was 99% of the TDI, and maximum ratio was 134%. The excess intake of molybdenum does not pose an immediate health hazard to humans, because the TDI represents a tolerable intake for a lifetime, and short-term exposure to levels exceeding the TDI is not a cause for concern (WHO 2008). Uncertainties exist in risk assessment of long-term exposure, however, including large UFs generally involved in establishing TDIs (WHO 2008) and the lack of quantitative risk information on intakes exceeding the TDI. These uncertainties are limitations in the current risk assessment approaches and should be improved in the future.

Table 1 | Daily metal intake of each food group, average of six cities

	B mg/day	Mg mg/day	Al mg/day	Ca mg/day	Cr μg/day	Mn mg/day	Fe mg/day	Ni μg/day	Cu mg/day
TDI × 50-kg body weight	8[*]	–	–	–	–	11[†,◦]	40[‡]	600[◦]	25[§]
RAD[†]	–	318	–	694 (Al)	34	3.75 (Al)	9	–	0.75
Food group	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD
Rice	0.1 ± 0.04	14 ± 4.1	0.07 ± 0.01	8.8 ± 2.5	197 ± 148	1.0 ± 0.2	0.04 ± 0.03	24 ± 8.7	0.3 ± 0.1
Cereals and potatoes	0.09 ± 0.02	30 ± 7.0	0.4 ± 0.1	41 ± 10	22 ± 11	0.5 ± 0.2	0.9 ± 0.2	13 ± 8.9	0.2 ± 0.03
Sugar and sweets	0.04 ± 0.01	7.2 ± 1.6	0.4 ± 0.3	17 ± 4.9	21 ± 24	0.1 ± 0.04	0.2 ± 0.1	4.0 ± 2.2	0.04 ± 0.01
Oils and fats	0.003 ± 0.0001	0.02 ± 0.02	0.004 ± 0.01	0.3 ± 0.3	29 ± 7.9	0.0001 ± 0.0001	0.004 ± 0.002	0.1 ± 0.1	0.001 ± 0.0004
Legumes	0.2 ± 0.05	47 ± 12	0.1 ± 0.04	52 ± 15	21 ± 7.3	0.4 ± 0.06	0.7 ± 0.2	38 ± 9.3	0.1 ± 0.01
Fruits	0.3 ± 0.03	13 ± 0.8	0.03 ± 0.02	14 ± 1.7	10 ± 7.9	0.2 ± 0.09	0.1 ± 0.03	6.2 ± 2.8	0.05 ± 0.01
Green and yellow vegetables	0.2 ± 0.02	23 ± 7.6	0.2 ± 0.2	34 ± 14	11 ± 10	0.2 ± 0.06	0.3 ± 0.04	5.8 ± 5.1	0.04 ± 0.01
Other vegetables and seaweed	0.4 ± 0.2	30 ± 10	0.6 ± 0.5	65 ± 21	23 ± 12	0.4 ± 0.1	0.6 ± 0.3	10 ± 1.2	0.09 ± 0.03
Alcohols and beverages	0.2 ± 0.1	20 ± 13	1.1 ± 1.1	18 ± 23	34 ± 19	0.8 ± 0.6	0.2 ± 0.1	21 ± 18	0.08 ± 0.06
Fish and shellfish	0.06 ± 0.02	36 ± 13	0.2 ± 0.1	109 ± 48	44 ± 19	0.05 ± 0.02	0.6 ± 0.2	2.9 ± 1.2	0.1 ± 0.06
Meat and poultry	0.05 ± 0.04	26 ± 8.2	0.1 ± 0.1	33 ± 20	64 ± 25	0.03 ± 0.005	1.7 ± 0.6	3.0 ± 4.7	0.08 ± 0.02
Milk products	0.06 ± 0.02	17 ± 5.2	0.02 ± 0.02	188 ± 90	39 ± 11	0.01 ± 0.01	0.1 ± 0.07	2.0 ± 1.5	0.01 ± 0.01
Seasoning	0.2 ± 0.05	27 ± 3.2	0.2 ± 0.1	22 ± 7.7	51 ± 10	0.4 ± 0.1	0.5 ± 0.2	25 ± 2.8	0.05 ± 0.02
Drinking water	0.07 ± 0.03	6.1 ± 4.1	0.08 ± 0.07	31 ± 17	0.2 ± 0.1	0.004 ± 0.005	0.14 ± 0.30	0.7 ± 0.6	0.005 ± 0.003
DMI	1.93 ± 0.22	295 ± 38.1	3.60 ± 1.37	631 ± 126	568 ± 194	4.15 ± 0.66	6.13 ± 1.11	156 ± 0.03	1.20 ± 0.17

	Zn mg/day	As μg/day	Se μg/day	Mo μg/day	Cd μg/day	Sb μg/day	Pb μg/day	U μg/day
TDI × 50-kg body weight	50[§]	107[‖]	200[*]	250[†,¶]	50[*]	300[*]	179[*,**]	30[*]
RAD[†]	8	–	28	23	–	–	–	–
Food group	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD	Intake SD
Rice	2.4 ± 0.4	15 ± 4.2	6.7 ± 2.2	122 ± 38	4.3 ± 2.6	0.4 ± 0.3	9.1 ± 5.9	0.09 ± 0.08
Cereals and potatoes	0.7 ± 0.1	1.5 ± 0.4	23 ± 5.5	12 ± 4.9	2.6 ± 0.5	0.2 ± 0.1	4.1 ± 2.9	0.0003 ± 0.001
Sugar and sweets	0.1 ± 0.02	0.8 ± 0.4	1.1 ± 0.4	3.6 ± 0.3	0.5 ± 0.3	0.04 ± 0.02	0.7 ± 0.5	0.05 ± 0.02
Oils and fats	0.003 ± 0.005	0.1 ± 0.06	0.4 ± 0.02	0.2 ± 0.2	0.08 ± 0.004	0.01 ± 0.001	0.5 ± 0.3	0.004 ± 0.01
Legumes	0.6 ± 0.07	0.5 ± 0.3	3.4 ± 1.7	40 ± 15	1.0 ± 0.3	0.07 ± 0.06	2.1 ± 1.2	0.03 ± 0.03
Fruits	0.07 ± 0.01	0.6 ± 0.3	2.5 ± 0.1	2.5 ± 0.4	0.4 ± 0.0	0.2 ± 0.09	2.3 ± 2.0	0.07 ± 0.08
Green and yellow vegetables	0.3 ± 0.04	0.4 ± 0.2	1.9 ± 0.1	4.0 ± 2.0	1.8 ± 0.2	0.08 ± 0.04	2.5 ± 2.1	0.06 ± 0.07
Other vegetables and seaweed	0.4 ± 0.04	103 ± 64	7.3 ± 4.6	11 ± 5.3	3.7 ± 1.2	0.2 ± 0.1	4.3 ± 2.9	0.7 ± 0.3
Alcohols and beverages	0.2 ± 0.2	2.1 ± 1.4	10 ± 4.0	13 ± 8.5	2.0 ± 0.8	0.7 ± 0.4	8.7 ± 1.1	0.4 ± 0.4

Table 1 | *(continued)*

	Zn mg/day	As μg/day	Se μg/day	Mo μg/day	Cd μg/day	Sb μg/day	Pb μg/day	U μg/day
TDI × 50-kg body weight	50[§]	107[‖]	200[*]	250[†,¶]	50[*]	300[*]	179[*,**,‡‡]	30[*]
RAD[†]	8	–	28	23	–	–	–	–
Food group	**Intake SD**	**Intake SD**	**Intake SD**	**Intake SD**	**Intake SD**	**Intake SD**	**Intake SD**	**Intake SD**
Fish and shellfish	0.9 ± 0.2	159 ± 44	42 ± 5.4	2.3 ± 1.6	3.8 ± 3.2	0.2 ± 0.2	2.6 ± 3.0	0.4 ± 0.1
Meat and poultry	2.2 ± 0.5	1.9 ± 0.3	36 ± 6.9	7.5 ± 2.0	0.6 ± 0.2	0.3 ± 0.2	2.5 ± 1.1	0.2 ± 0.06
Milk products	0.5 ± 0.06	0.8 ± 0.2	8.0 ± 2.2	6.9 ± 0.9	0.6 ± 0.1	0.1 ± 0.08	2.0 ± 0.5	0.1 ± 0.04
Seasoning	0.3 ± 0.04	13 ± 3.2	12 ± 2.8	21 ± 5.1	1.4 ± 0.8	0.2 ± 0.2	2.4 ± 1.2	0.1 ± 0.03
Drinking water	0.06 ± 0.1	0.7 ± 0.3	1.3 ± 0.0	1.3 ± 0.7	0.07 ± 0.06	0.2 ± 0.1	0.7 ± 0.3	0.04 ± 0.01
DMI	8.65 ± 0.74	300 ± 58.2	156 ± 16.1	247 ± 46.4	22.8 ± 3.08	2.90 ± 1.25	44.4 ± 18.1	2.38 ± 0.64

*WHO (2008).
†JMHLW (2005).
‡JECFA (1983).
§JECFA (1982).
‖JECFA (1989).
¶USEPA (1993).
**, ‡‡ JECFA (1993).
TDI: tolerable daily intake; RDA: recommended dietary allowances; DMI: daily metal intake; SD: standard deviation; AI: adequate intake.

Daily essential metal intakes relative to RDA

The percentages of DMI to RDA or AI were calculated for nine essential metals (Figure 3). The mean dietary intakes of magnesium, calcium, and iron were less than the RDA or AI. In terms of minimum daily intake among the six cities, the intakes of five metals (magnesium, calcium, manganese, iron, and zinc) were lower than the RDA or AI. Magnesium and calcium intakes were lower than the RDA or AI in five of the six cities. The differences between the RDA or AI and the intakes, however, were not very large, and the variation of the intake among cities and among years should be further investigated. As discussed for TDI, RDA and AI values also include many uncertainties that should be improved in the future, which limit our ability to discuss risks associated with deficiency of essential metals.

Iron intake was lower than the RDA in all six cities, and manganese and zinc intakes were lower in one city. Iron, manganese, and zinc are considered to be both essential and toxic metals. In terms of toxicity, the DMI values of these three metals were less than 50% of the TDI. Therefore, Japanese may consume more of these metals to satisfy the RDA or AI value.

Daily intakes of chromium, selenium, and molybdenum were far greater than the RDA values. In terms of toxicological aspects, the DMI values of selenium and molybdenum were near, or a slightly higher than, the TDI (Figure 2). Thus, it is not recommended that Japanese consume more selenium or molybdenum. For chromium, JMHLW (2005) does not provide a tolerable upper intake level, and the WHO does not describe the quantitative risk of chromium intake, although it has established a provisional drinking-water quality guideline value 0.05 mg/L for total chromium (WHO 2006).

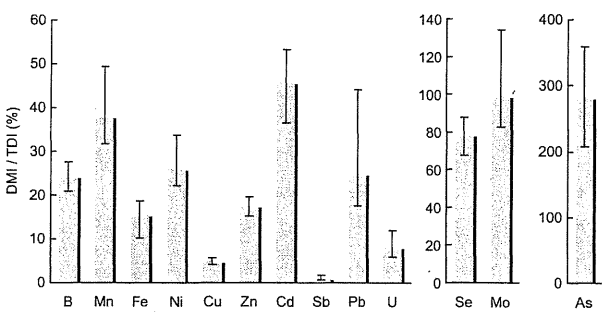


Figure 2 | Daily toxic metal intake as a percentage of TDI. Columns represent the average of six cities; bars represent minimum and maximum values.

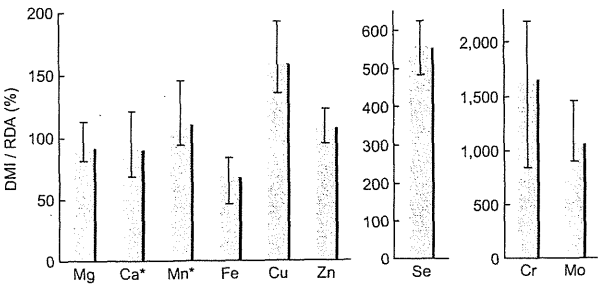


Figure 3 | Daily essential metal intake as a percentage of RDA. Columns represent the averages of six cities; bars represent minimum and maximum values. Asterisk indicates AI instead of RDA.

Contribution of drinking water to TDI and/or RDA

For the 13 toxic metals analyzed, the contribution of drinking water to TDI was 2% or less in all six cities (Figure 4). Basically, in establishing the Japanese water quality standards, 10 of the TDI is allocated to intake via drinking water for most metals. The actual contribution is much less than 10% of the TDI, because the metal contents are controlled to be about 20–30% of the Japanese drinking water quality standard values in most water purification plants in Japan (Japan Water Works Association 2009).

The drinking water contribution to the RDA or AI of essential metals is less than 10% in all cities. On average, drinking water contributed only about 5% of the AI of calcium. Hardness in water is derived from calcium and magnesium. According to the WHO *Guidelines for Drinking-water Quality*, very soft waters may have an adverse effect on mineral balance and cardiovascular health (WHO 2008). The calcium concentration in drinking water is low in many cities in Japan (Japan Water Works Association 2009), and calcium compounds are added at some drinking water treatment plants where the hardness of the water is very low. This calcium addition was originally designed to prevent the corrosion of water pipes, but it may also help to reduce the health risks posed by very soft water. In general, the process increases the calcium concentration by ~8 mg/L (Japan Water Works Association 2009), which is equivalent to a daily intake of 16 mg/day or 2.3% of the AI of calcium. Therefore, this addition of calcium to drinking water does not help with calcium intake deficiency; it is better to consume calcium in foods such as milk.

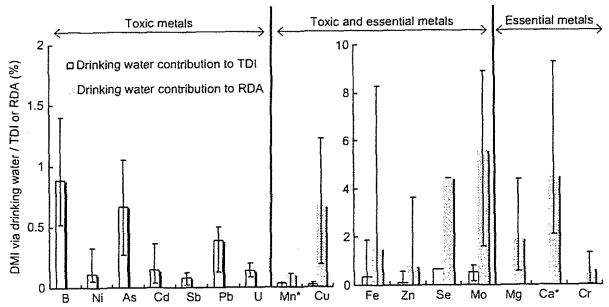


Figure 4 | Drinking water contributions to TDI or RDA. Columns represent the averages of six cities; bars represent minimum and maximum values. Asterisk indicates AI instead of RDA.

CONCLUSION

The daily intakes of 17 metals in six cities in Japan were estimated by analyzing the concentrations of metals in locally obtained food composite samples and drinking water samples. The mean daily intake of 10 of the 13 toxic metals was less than 50% of TDI, except in the cases of arsenic, selenium, and molybdenum. The allocation ratio of intake to drinking water in establishing drinking water quality standards could possibly be increased from the normal allocation of 10–20% of TDI for these 10 metals. However, not only the mean intakes but also variation of the intakes among individuals and areas should be further investigated and considered. For the 13 toxic metals analyzed in this study, the contribution of drinking water to TDI was 2% or less in all six cities. For essential metals, the DMI values of magnesium, calcium, iron, manganese, and zinc were lower than RDA or AI in at least one city. The drinking water contribution to RDA or AI was less than 10% for all essential metals in all the six cities, indicating that drinking water did not contribute very much to essential metal intake.

ACKNOWLEDGEMENTS

The authors are grateful to the following people for providing the prepared food composite samples: i) Ms. R. Matsuda and Mr. T. Watanabe (National Institute of Health Sciences, Tokyo, Japan); ii) Ms. Y. Tsuchida (Niigata Prefectural Institute of Public Health and Environmental Sciences, Niigata, Japan); iii) Mr. H. Terada (Nagoya City Public Health Research Institute, Nagoya, Japan); iv) Mr. H. Obana (Osaka Prefectural Institute of Public Health, Osaka, Japan);

v) Ms. C. Nishioka (Kagawa Prefectural Research Institute for Environmental Sciences and Public Health, Takamatsu, Japan); and vi) Mr. H. Tamashiro (Okinawa Prefectural Institute of Health and Environment, Nanjo, Japan). This research was supported by a Grant-in-Aid from the Ministry of Health, Labour and Welfare, Japan (2007–2009). This work has not been subject to the ministry's view and therefore, does not reflect the views of the ministry, and no official endorsement should be inferred.

REFERENCES

Aggett, P. 2008 Toxicology due to excess and deficiency. *Workshop Proceedings on Health Risk Assessment of Essential Metals Workshop*. University of Ottawa, Canada, pp. 48–55. http://www.mclaughlincentre.ca/events/metals/Final Essential Metals Report University of Ottawa May 6–7 2008.pdf (accessed 3 November 2009).

Biego, G. H., Joyeux, M., Hartemann, P. & Debry, G. 1998 Daily intake of essential minerals and metallic micropollutants from foods in France. *Sci. Total Environ.* **217**(1–2), 27–36.

Bordajandi, L. R., Gomez, G., Abad, E., Rivera, J., Fernandez-Baston, M. D., Blasco, J. & Gonzalez, M. J. 2004 Survey of persistent organochlorine contaminants (PCBs, PCDD/Fs, and PAHs), heavy metals (Cu, Cd, Zn, Pb, and Hg), and arsenic in food samples from Huelva (Spain): levels and health implications. *J. Agric. Food Chem.* **52**(4), 992–1001.

Dolan, S. P. & Caper, S. G. 2002 Multi-element analysis of food by microwave digestion and inductively coupled plasma-atomic emission spectrometry. *J. Food Compos. Anal.* **15**, 593–615.

Fraga, C. G. 2005 Relevance, essentiality and toxicity of trace elements in human health. *Mol. Asp. Med.* **26**, 235–244.

Gambling, L. 2008 Metal specific risk assessments: European perspective. *Workshop Proceedings on Health Risk Assessment of Essential Metals Workshop*. University of Ottawa, Canada, pp. 9–13. http://www.mclaughlincentre.ca/events/metals/Final Essential Metals Report University of Ottawa May 6–7 2008.pdf (accessed 3 November 2009).

Japanese Ministry of Health, Labour and Welfare (JMHLW) 2004 Heisei 16 Nen Kokumin Kenkou, Eiyou Tyousa Houkoku (*The National Health and Nutrition Survey in Japan, 2004*). Daiichi Shuppan Publishing, Tokyo, Japan.

Japan Water Works Association 2009 *Suidou Toukei (Statistics on Water Supply)*, (**Vol. 90**). Japan Water Works Association, Tokyo.

JECFA 1983 *Evaluation of Certain Food Additives and Contaminants: Twenty-seventh Report of the Joint FAO/WHO Expert Committee on Food Additives*, WHO Technical Report Series 696, WHO, Geneva, Switzerland.

JECFA 1989 *Evaluation of Certain Food Additives and Contaminants: Thirty-third Report of the Joint FAO/WHO Expert Committee on Food Additives*, WHO Technical Report Series 776, WHO, Geneva, Switzerland.

JECFA 1993 *Evaluation of Certain Food Additives and Contaminants: Forty-first Report of the Joint FAO/WHO Expert Committee on Food Additives*, WHO Technical Report Series 837, WHO, Geneva, Switzerland.

JMHLW 2005 Nihonjin no Syokuji Sessyu Kijun 2005 Nenban (*Dietary Reference Intakes for Japanese, 2005*). Daiichi Shuppan Publishing, Tokyo, Japan.

Joint FAO/WHO Expert Committee on Food Additives (JECFA) 1982 *Evaluation of Certain Food Additives and Contaminants: Twenty-sixth Report of the Joint FAO/WHO Expert Committee on Food Additives*, WHO Technical Report Series 683, WHO, Geneva, Switzerland.

Maitani, T. 2004 Evaluation of exposure to chemical substances through foods–exposure to pesticides, heavy metals, dioxins, acrylamide and food additives in Japan. *J. Health Sci.* **50**(3), 205–209.

Santamaria, A. 2008 Manganese: overview and update. *Workshop Proceedings on Health Risk Assessment of Essential Metals Workshop*. University of Ottawa, Canada, pp. 28–34. http://www.mclaughlincentre.ca/events/metals/Final Essential Metals Report University of Ottawa May 6–7 2008.pdf (accessed 3 November 2009).

Santos, E. E., Lauria, D. C. & da Silveira, C. L. P. 2004 Assessment of daily intake of trace elements due to consumption of foodstuffs by adult inhabitants of Rio de Janeiro city. *Sci. Total Environ.* **327**(1–3), 69–79.

Stern, B. R. 2008 Copper: overview and update. *Workshop Proceedings on Health Risk Assessment of Essential Metals Workshop*. University of Ottawa, Canada, pp. 21–26. http://www.mclaughlincentre.ca/events/metals/Final Essential Metals Report University of Ottawa May 6–7 2008.pdf (accessed 3 November 2009).

Turconi, G., Minoia, C., Ronchi, A. & Roggi, C. 2009 Dietary exposure estimates of twenty-one trace elements from a total diet study carried out in Pavia, northern Italy. *Br. J. Nutr.* **101**(8), 1200–1208.

United States Environmental Protection Agency (USEPA) 1993 *Molybdenum, Integrated Risk Information System*. http://www.epa.gov/ncea/iris/subst/0425.html (accessed 3 November 2009).

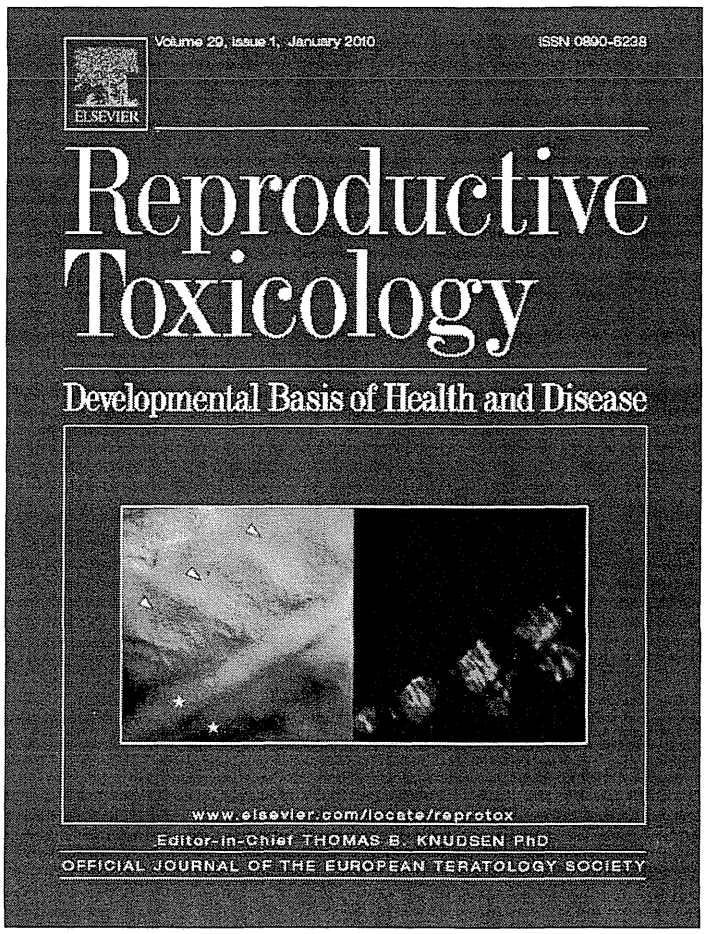
World Health Organization (WHO) 2003 *Selenium in Drinking-water, Background Document for Development of WHO Guidelines for Drinking-water Quality*, Report WHO/SDE/WSH/03.04/13, WHO, Geneva, Switzerland.

WHO 2004 *Guidelines for Drinking-Water Quality*, 3rd edition. WHO Publications, Geneva, Switzerland.

WHO 2006 *Guidelines for Drinking-Water Quality, First Addendum to Third Edition*. WHO Publications, Geneva, Switzerland.

WHO 2008 *Guidelines for Drinking-Water Quality, Third Edition, Incorporating First and Second Addenda*. WHO Publications, Geneva, Switzerland.

Yang, G. & Zhou, R. 1994 Further observations on the human maximum safe dietary selenium intake in a seleniferous area of China. *J. Trace Elem. Electrolytes Health Dis.* **8**(3–4), 159–165.



Reproductive Toxicology 29 (2010) 49–56

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

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

Makoto Ema[a,*], Ryota Ise[b], Hirohito Kato[c], Satoru Oneda[d], Akihiko Hirose[a], Mutsuko Hirata-Koizumi[a], Amar V. Singh[e], Thomas B. Knudsen[f], Toshio Ihara[c]

[a] Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[b] Shin Nippon Biomedical Laboratories (SNBL), Ltd., Tokyo, Japan
[c] Shin Nippon Biomedical Laboratories (SNBL), Ltd., Kagoshima, Japan
[d] SNBL USA, Ltd., Everett, WA, USA
[e] Contractor to NCCT, Lockheed-Martin, Research Triangle Park, NC 27711, USA
[f] National Center for Computational Toxicology (NCCT), U.S. Environmental Protection Agency, Research Triangle Park, NC 27711, USA

ARTICLE INFO

Article history:
Received 2 June 2009
Received in revised form 3 September 2009
Accepted 4 September 2009
Available online 12 September 2009

Keywords:
Thalidomide
Teratogenicity
Fetal malformation
Gene expression profile
Embryo
Cynomolgus monkey

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.

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

☆ *Disclaimer*: The U.S. EPA, through its Office of Research and Development collaborated in the research described here. It has been subjected to agency review and approved for publication. The authors declare they have no competing financial interests.

* Corresponding author. Tel.: +81 3 3700 9878; fax: +81 3 3700 1408.
E-mail address: ema@nihs.go.jp (M. Ema).

0890-6238/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.reprotox.2009.09.003

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 multi-chip 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	Dose	15 mg/kg				20 mg/kg			
Findings	Fetus no.	1	2	3	4	5	6	7	8
	Gender	Female	Male	Female	Female	Male	Male	Male	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.

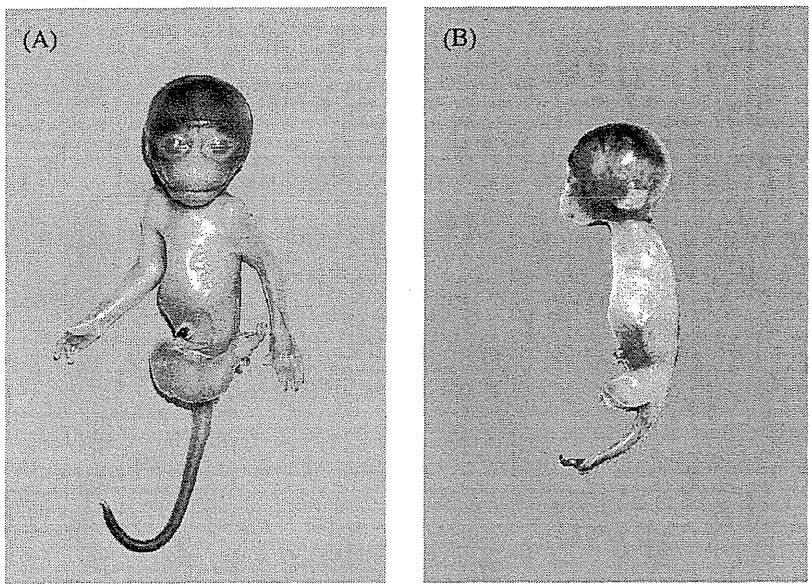


Fig. 1. Malformed fetuses of cynomolgus monkeys exposed to thalidomide on days 26–28 of gestation. (A) The fetus of maternal monkey given thalidomide at 15 mg/kg-d exhibiting brachydactyly in the paw, micromelia in the hindlimb, hyperflexion, ectrodactyly and brachydactyly in the foot and curled tail. (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)

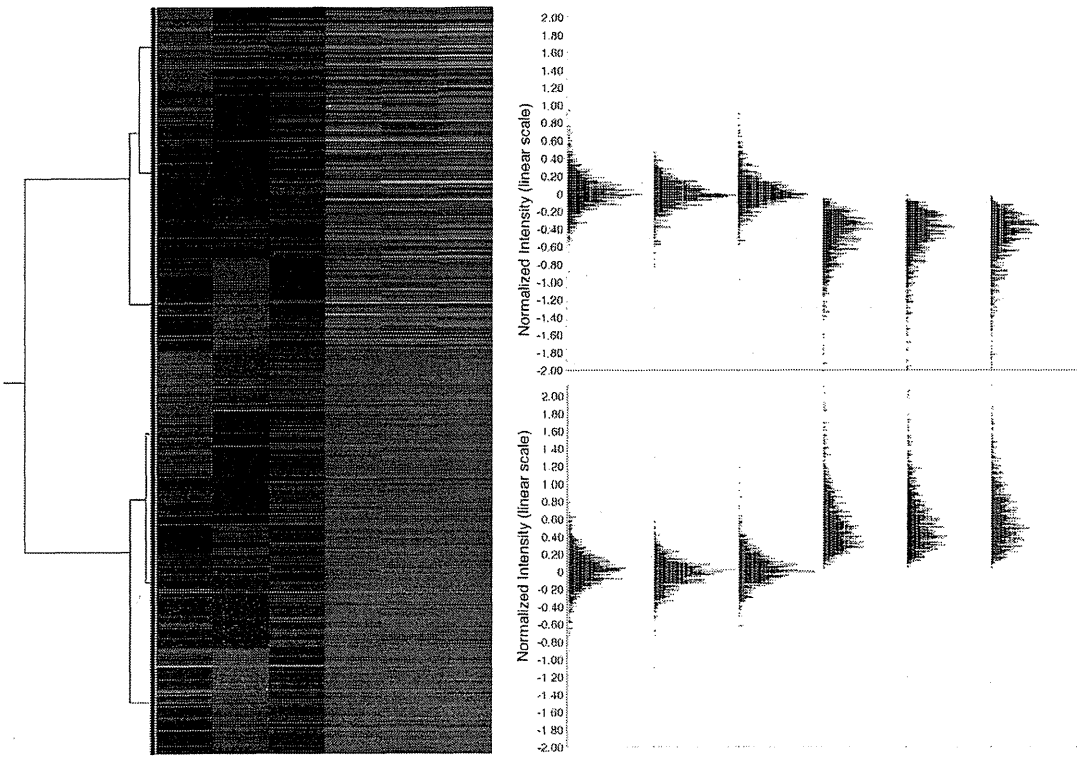


Fig. 2. Molecular abundance profiles of the thalidomide-sensitive genes in the cynomolgus embryonic transcriptome on day 26 of gestation. RNA was isolated from day 26 embryos 6 h after maternal exposure to 20 mg/kg thalidomide or vehicle control. Values represent log2 ratios of treated/reference, where 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	Log2 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 Log 2 (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

This work was partially supported by Health and Labour Sciences Research Grants (Research on Regulatory Science of Pharmaceuticals and Medical Devices: H16-Kenkou-066; Research on Risk of Chemical Substances: H17-Kagaku-001) from the Ministry of Health, Labour and Welfare of Japan. The bioinformatics analysis was performed at the National Center for Computational Toxicology, US EPA. Authors are grateful to Dr. Robert MacPhail of EPA's National Health and Environmental Effects Research Laboratory for helpful comments on the manuscript.

References

[1] Schardein JL, Macina OT. Thalidomide. In: Human developmental toxicants—aspects of toxicology and chemistry. Boca Raton: CRC Press, Taylor & Francis Group; 2007. p. 127–141.

[2] Hansen JM, Carney EW, Harris C. Differential alteration by thalidomide of the glutathione content of rat vs. rabbit conceptuses in vitro. Reprod Toxicol 1999;13:547–54.

[3] Schardein JL. Thalidomide: the prototype teratogen. In: Chemically induced birth defects. 3rd edition, revised and expanded New York: Marcel Dekker Inc.; 2000. p. 89–120.

[4] Teo SK, Denny KH, Stirling DI, Thomas SD, Morseth S, Hoberman AM. Effects of thalidomide on developmental, peri- and postnatal function in female New Zealand white rabbits and offspring. Toxicol Sci 2004;81:379–89.

[5] Kalter H. Thalidomide. In: Teratology in the twentieth century-congenital malformations in humans and how their environmental causes were established. Amsterdam: Elsevier Science; 2003. p. 167–175.

[6] Yoshida T. Introduction. In: Yoshida T, Fujimoto K, editors. The TPRC handbook on the care and management of the laboratory cynomolgus monkey. Tokyo: Springer Japan; 2006. p. 1–3.

[7] Delahunt CS, Lassen LJ. Thalidomide syndrome in monkeys. Science 1964;146:1300–5.

[8] Hendrickx AG. The sensitive period and malformation syndrome produced by thalidomide in the crab-eating monkey (*Macaca fascicularis*). J Med Prim 1973;2:267–76.

[9] Finnell RH, Gelineau-van Waes J, Eudy JD, Rosenquist TH. Molecular basis of environmentally induced birth defects. Ann Rev Pharmacol Toxicol 2002;42:181–208.

[10] Singh AV, Knudsen KB, Knudsen TB. Computational systems analysis of developmental toxicity: design, development and implementation of a birth defects systems manager (BDSM). Reprod Toxicol 2005;19:421–39.

[11] Daston GP. Genomics and developmental risk assessment. Birth Defects Res (Part A) 2007;79:1–7.

[12] Green ML, Singh AV, Zhang Y, Nemeth KA, Sulik KK, Knudsen TB. Reprogramming of genetic networks during initiation of the fetal alcohol syndrome. Dev Dyn 2007;236:613–31.

[13] Knudsen TB, Kavlock RJ. Comparative bioinformatics and computational toxicology. In: Abbott B, Hansen D, editors. Developmental toxicology. Target organ toxicology series, vol. 3. New York: Taylor and Francis; 2008. p. 311–60.

[14] Gene Logic. NHP GeneChip® Array Service http://www.genelogic.com/docs/pdfs/NHP_W.pdf [accessed September 14.09.07].

[15] Institute of Laboratory Animal Research, Commission of Life Sciences, National Research Council. Guide for the care and use of laboratory animals. Washington, DC: The National Academies Press; 1996.

[16] Otey CA, Rachlin A, Moza M, Arneman D, Carpen O. The palladin/myotilin/myopalladin family of actin-associated scaffolds. Int Rev Cytol 2005;246:31–58.

[17] Yayoshi-Yamamoto S, Taniuchi I, Watanabe T. FRL, a novel formin-related protein, binds to Rac and regulates cell motility and survival of macrophages. Mol Cell Biol 2000;20:6872–81.

[18] Marignani PA, Carpenter CL. Vav2 is required for cell spreading. J Cell Biol 2001;154:177–86.

[19] Brugneara E, Haney J, Grimsley C, Lu M, Walk SF, Tosello-Trampont AC, et al. Unconventional Rac-GEF activity is mediated through the Dock180–ELMO complex. Nat Cell Biol 2002;4:574–82.

[20] Katoh H, Negishi M. RhoG activates Rac1 by direct interaction with the Dock180-binding protein Elmo. Nature 2003;424:461–4.

[21] Rosenberger G, Jantke I, Gal A, Kutsche K. Interaction of alphaPIX (ARHGEF6) with beta-parvin (PARVB) suggests an involvement of alphaPIX in integrin-mediated signaling. Hum Mol Genet 2003;12:155–67.

[22] Shin EY, Woo KN, Lee CS, Koo SH, Kim YG, Kim WJ, et al. Basic fibroblast growth factor stimulates activation of Rac1 through a p85 PIX phosphorylation-dependent pathway. J Biol Chem 2004;279:1994–2004.

[23] Burgering BM, Coffer PJ. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature 1995;376:599–602.

[24] Franke TF, Yang SI, Chan TO, Datta K, Kazlauskas A, Morrison DK, et al. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell 1995;81:727–36.

[25] Kohn AD, Kovacina KS, Roth RA. Insulin stimulates the kinase activity of RAC-PK, a pleckstrin homology domain containing ser/thr kinase. EMBO J 1995;14:4288–95.

[26] Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 1995;378:785–9.

[27] Streck RD, Wood TL, Hsu MS, Pintar JE. Insulin-like growth factor I and II and insulin-like growth factor binding protein-2 RNAs are expressed in adjacent tissues within rat embryonic and fetal limbs. Dev Biol 1992;151:586–96.

[28] van Kleffens M, Groffen C, Rosato RR, van den Eijnde SM, van Neck JW, Lindenbergh-Kortleve DJ, et al. mRNA expression patterns of the IGF system during mouse limb bud development, determined by whole mount in situ hybridization. Mol Cell Endocrinol 1998;138:151–61.

[29] Stephens TD, Bunde CJ, Fillmore BJ. Mechanism of action in thalidomide teratogenesis. Biochem Pharmacol 2000;59:1489–99.

[30] Dealy CN, Kosher RA. Studies on insulin-like growth factor-I and insulin in chick limb morphogenesis. Dev Dyn 1995;202:67–79.

[31] Dealy CN, Kosher RA. IGF-I, insulin and FGFs induce outgrowth of the limb buds of amelic mutant chick embryos. Development 1996;122:1323–30.

[32] Mitchell PJ, Johnson SE, Hannon K. Insulin-like growth factor I stimulates myoblast expansion and myofiber development in the limb. Dev Dyn 2002;223:12–23.

[33] Knobloch J, Shaughnessy Jr JD, Rüther U. Thalidomide induces limb deformities by perturbing the Bmp/Dkk1/Wnt signaling pathway. FASEB J 2007;21:1410–21.

[34] Eisen T, Boshoff C, Mak I, Sapunar F, Vaughan MM, Pyle L, et al. Continuous low dose thalidomide: a phase II study in advanced melanoma, renal cell, ovarian and breast cancer. Br J Cancer 2000;82:812–7.

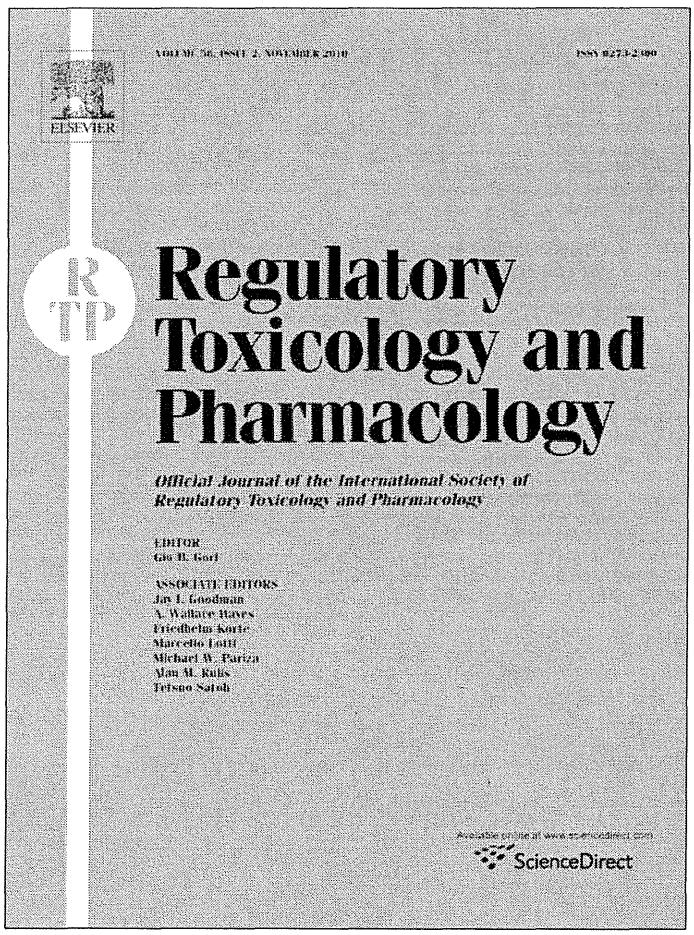
[35] Ball SG, Shuttleworth CA, Kielty CM. Vascular endothelial growth factor can signal through platelet-derived growth factor receptors. J Cell Biol 2007;177:489–90.

[36] D'Amato RJ, Loughnan MS, Flynn E, Folkman J. Thalidomide is an inhibitor of angiogenesis. Proc Natl Acad Sci USA 1994;91:4082–5.

[37] Seifert R, Zhao B, Christ B. Cytokinetic studies on the aortic endothelium and limb bud vascularization in avian embryos. Anat Embryol (Berl) 1992;186:601–10.

[38] Therapontos C, Erskine L, Gardner ER, Figg WD, Vargesson N. Thalidomide induces limb defects by preventing angiogenic outgrowth during early limb formation. PNAS Early Edition 2009, http://www.pnas.org/cgi/doi/10.1073/pnas.0901505106.

[39] Hansen JM, Harris KK, Philbert MA, Harris C. Thalidomide modulates nuclear redox status and preferentially depletes glutathione in rabbit limb versus rat limb. J Pharmacol Exp Ther 2002;300:768–76.



Regulatory Toxicology and Pharmacology 58 (2010) 237–242

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Proposal of new uncertainty factor application to derive tolerable daily intake

Ryuichi Hasegawa [a], Mutsuko Hirata-Koizumi [a], Michael L. Dourson [b], Ann Parker [b], Lisa M. Sweeney [b], Akiyoshi Nishikawa [a], Midori Yoshida [a], Atsushi Ono [a], Akihiko Hirose [a,*]

[a] National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[b] Toxicology Excellence for Risk Assessment, 2300 Montana Avenue, Suite 409, OH 45211, USA

ARTICLE INFO

Article history:
Received 2 February 2010
Available online 16 June 2010

Keywords:
Chemical risk assessment
Uncertainty factor
Probabilistic approach
Subdivision of UF

ABSTRACT

We propose new uncertainty factors (UFs) and a new subdivision of default factors in chemical risk assessment using a probabilistic approach based on the latest applicable information. Rounded values of 150 for mice, 100 for hamsters and rats, and 40 for rabbits, monkeys and dogs for inter- and intra-species differences (UF_{AH}) were derived from the probabilistic combination of two log-normal distributions. Further calculation of additional UFs when chronic data (UF_S) or NOAEL (UF_L) are lacking was conducted using available log-normal distribution information. The alternative UF_S and UF_L values of 4 are considered to be appropriate for both cases where data are lacking. The default contributions of inter-species difference (UF_A) and intra-species difference (UF_H) to the UF_{AH} of 100 for hamsters and rats as an example are considered to be 25 and 4, respectively. The UF_A of 25 was subdivided into $25^{0.6}$ (i.e., 7.0) for pharmacokinetics (PK) ($UF_{A,PK}$) and $25^{0.4}$ (i.e., 3.6) for pharmacodynamics (PD) ($UF_{A,PD}$), and the UF_H of 4 was evenly subdivided into $4^{0.5}$ (i.e., 2) ($UF_{H,PK}$ and $UF_{H,PD}$), to account for chemical-specific difference data between humans and laboratory animals for PK and/or PD. These default UFs, which come from actual experimental data, may be more appropriate than previous default UFs to derive tolerable daily intake values.

1. Introduction

Principle uncertainty factors (UFs) consisting of inter-species differences (or extrapolation from laboratory animals to humans, referred to as "UF_A") and intra-species differences (human variability, referred to as "UF_H") have commonly been used when extrapolating from animal experimental data to human risk values in chemical risk assessment. The current combined default UF of 100 ($10_A \times 10_H$) for extrapolation from animal data was introduced in the US in 1954 (Lehman and Fitzhugh, 1954) for food contaminants with a rationale for its suitability for environmental contaminants provided by Dourson and Stara (1983) years later. The physical size of laboratory animals is variable, with animals as small as mice to larger animals like dogs. In some cases the size difference results in more than a 500-fold difference in body weight indicating that some type of variable adjustment might be needed, rather than just a 10-fold factor.

Body surface area correction, (human body weight/animal body weight)$^{1/3}$ was the first data supported size adjustment (Freireich et al., 1966). It has been applied to cancer endpoints in US Environmental Protection Agency (US EPA) assessments and was also used in the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) residual solvent guidelines (Connelly et al., 1997). Recently, allometric scaling according to caloric demand or metabolic size, (human body weight/animal body weight)$^{1/4}$ was introduced as a more appropriate adjustment (Schneider et al., 2004) and is currently used by US EPA in cancer risk assessment (US EPA, 1992, 2005a). Size adjustment might be more appropriately based on allometric scaling as discussed by Falk-Filipsson et al. (2007). However, the use of allometric scaling in non-cancer endpoints remains untested by US EPA and other organizational assessments. The caloric demand adjustment factor for a mouse (0.030 kg) or a dog (16 kg) compared to a human (70 kg) based on body weight is 7 or 1.4, respectively, which is significantly lower than the default of 10. However, Schneider et al. (2004) demonstrated that caloric demand scaling was effective for predicting median differences between humans and animals on the basis of body weight in maximal tolerated dose (MTD) ratios of anti-cancer drugs, and also calculated the combined geometric standard deviation (GSD) of the empirical distribution.

Useful experimental data are quite limited for human intra-species differences, specifically variability between different ages (Dourson and Stara, 1983; Dourson et al., 1996, 2002). However, some insights can be gained from experimental animal work. For

* Corresponding author. Address: Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Fax: +81 3 3700 1408.
E-mail address: hirose@nihs.go.jp (A. Hirose).

0273-2300/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.yrtph.2010.06.006

example, a recent comparative investigation of no observed adverse effect levels (NOAELs) in repeat-dose studies of newborn and young rats for 18 chemicals was conducted (Hasegawa et al., 2007). The data provided the median and distribution of NOAEL ratios.

The default UF of 100 for inter- and intra-species differences is typically applied by multiplication of 10_A and 10_H. However, the default UF of 100 is not always appropriate to use. For example, multiplication of two log-normal distributions for inter- and intra-species differences also produces a log-normal distribution, and simple multiplication such as 10×10 causes overestimation if both individual values are in the 95th percentile. Kodell and Gaylor (1999) recommended standard statistical techniques that could be used to estimate the upper tolerance limits on the distribution of sums which can also be used for other UFs (e.g., the ratio of subchronic to chronic NOAELs). Swartout et al. (1998) also addressed this problem and gave hypothetical examples of UF combinations.

Another method for division of the default UF of 100 ($10_A \times 10_H$) for inter- and intra-species differences was proposed by Renwick (1993). He proposed a subdivision of these UFs into two parts, pharmacokinetics (PK) and pharmacodynamics (PD). Based on his analysis of experimental data and physiological parameters between animals and humans, the contribution ratios of PK and PD are 60:40 for inter-species differences and 50:50 for intra-species differences, leading to $10^{0.6}$ (4.0) $\times$ $10^{0.4}$ (2.5) and $10^{0.5}$ ($\sim$3.2) $\times$ $10^{0.5}$ ($\sim$3.2), respectively (IPCS, 1994). When chemical-specific data for the differences between animals and humans for PK and/or PD are available, the data should be used to develop chemical-specific adjustment factors instead of the default PK/PD factors (WHO, 2005). However, the default subdivision factors should be re-estimated if animal size-specific UFs are adopted as inter-species differences.

In this article, we propose new default UFs by a probabilistic approach using appropriate log-normal distribution data, taking animal size into consideration. We also propose development of new default values according to animal size for the subdivision of inter- and intra-species differences.

2. Data for each uncertainty

2.1. Inter-species difference data

Eight publications featuring chemical toxicity comparisons between humans and laboratory animals for anti-cancer drug toxicity were located. The first study by Freireich et al. (1966) showed MTD differences between humans and five animal species (mice, hamsters, rats, monkeys and dogs) in the analysis of 18 drugs. Recently, Schneider et al. (2004) extracted correlated human and animal data sets for 63 anti-cancer drugs from six additional publications (Goldsmith et al., 1975; Schein et al., 1979; Travis and White, 1988; Rozencweig et al., 1981; Grieshaber and Marsoni, 1986; Paxton et al., 1990) to demonstrate that caloric demand scaling was a suitable adjustment factor for the differences of inter-species median MTDs. Schneider et al. (2004) also derived a GSD of 3.23 from the combined distribution of all MTD ratios for humans versus the five animal species stated above.

Alternatively, inter-species differences in susceptibility could be derived based on the differences in NOAELs rather than MTDs. Schneider et al. (2004) also analyzed inter-species differences for pesticide NOAELs between mice/rats, rats/dogs and mice/dogs, providing further support to the caloric demand adjustment. Therefore, the median and GSD derived from MTD ratios of anti-cancer drugs might be equivalent to those based on NOAEL ratios between humans and animals. No other publications featuring an estimated direct comparison of chemical toxicity between humans and animals were identified.

2.2. Intra-species difference data

The NOAEL ratio of a sensitive subpopulation compared to that of the general population is a source of uncertainty for intra-species differences in risk assessment (Dourson et al., 2002). Occasionally the sensitive subpopulations are directly addressed in the risk assessment. For example, the Reference Dose (RfD) for nitrate on the Integrated Risk Information System (IRIS) used methemoglobinemia in children as the critical effect, therefore an intra-species UF may not be needed (US EPA, 2009). However, in most cases, the sensitive subpopulations are only considered protected with the use of an intra-species UF. Generally, infants, pregnant women, the elderly and other specified groups are considered high-susceptibility groups, although exceptions are not uncommon. For example, Tylenol overdose is more of a problem in adults than in children because the toxic metabolite is more readily formed in adults. Effects during pregnancy and gestation are considered to be adequately evaluated in the reproductive/developmental toxicity studies, while lifetime toxicity studies cover the potential for effects to the elderly. For a well-tested chemical, the only remaining sensitive subpopulation to be protected by an intra-species UF are infants. Currently, there are no experimental animal test guidelines intended for direct exposure of neonatal animals to chemicals. Other specified groups may include patients exhibiting hepatic or renal dysfunction and persons with a specific genetic background. These subpopulations need specific risk management and should not be the target population for a chemical risk assessment for public health because it is possible that their susceptibility to specific chemicals may be unexpectedly high owing to significantly reduced metabolism or excretion of toxic substances.

The comparative data between human adults and children/infants was assessed by many scientists. Glaubiger et al. (1981) compared MTDs in patients for 17 anti-cancer drugs demonstrating that children's MTDs were 50% higher than those of adults, indicating that children were less sensitive. Calabrese (1985) investigated the variation in physiological response to exogenous stress in humans, and judged that 80–95% of the variation in a human group for a given agent was less than 10-fold. Hattis et al. (1987) analyzed 101 PK parameter data sets for 49 substances (mostly medications) and showed that 96% of the human variation was also less than 10-fold. Ginsberg et al. (2002) compared PK in adults and children using a database of approximately 45 medications, and showed that the half-lives of medications for 1-week to 2-month old infants were twice as long as the half-lives in adults. Hattis et al. (2003) also showed significantly longer half-lives of medications in infants and children compared to adults.

Animal data has also been reviewed. Dourson and Stara (1983) analyzed acute rat toxicity data for 490 substances reported by Weil (1972). They concluded that the LD_{50}/non-lethal dose ratio for 92% of the chemical substances would be less than 10. In a meeting abstract, Sheehan and Gaylor (1990) stated that the LD_{50} of 238 substances in adult rats was about 2.6 times higher than the LD_{50} in newborn rat pups, and the LD_{50}/LD_{50} ratio for 86% of substances was less than 10. Calabrese (2001) showed that the LD_{50} in younger animals was within a 10-fold range of older animals for 86.3% of 313 substances. Charnley and Putzrath (2001) examined the influence of age on carcinogenesis caused by chemicals, but were unable to reach a clear conclusion. Similarly, the US EPA considered the effect of age in their most recent guidelines for carcinogen risk assessment. They estimated the geometric mean ratio of early-life to adult cancer potencies was 10.4 based on repeated and lifetime exposure data in the available scientific literature for six chemicals acting through a mutagenic mode of action

(US EPA, 2005b; Barton et al., 2005). As for chemicals causing cancer through other modes of action, the ratio was 3.4 for lifetime exposure (5 chemicals) and 2.2 for repeated exposure (6 chemicals).

The quantitative human and experimental animal data for severe endpoints and kinetic parameters are useful. However, a study design similar to the repeat-dose exposure studies used in risk analysis would be ideal to derive an intra-species UF. The UF is applied to the NOAEL derived from the results of repeated dose toxicity studies, therefore a comparative analysis of NOAELs from repeat-dose toxicity studies of newborn and young rats for 18 chemicals was considered more appropriate (Hasegawa et al., 2007). In this study, Hasegawa et al. (2007) strictly compared the NOAEL ratios for newborn and young rats in a repeat-dose study. The NOAEL ratios were log-normally distributed. The ratio median was 3, and 5 was equivalent to 94.4% of the whole data set, from which the GSD can be calculated (see below).

2.3. Data for supplemental uncertainty factors[1]

The appropriate adjustment from short-term NOAEL to lifetime NOAEL for risk assessment was evaluated using 33 data sets of subchronic (3 months)/chronic (2 years) NOAELs in rats and mice reported by Weil and McCollister (1963) and 68 additional data sets from analyses of published reports or papers that we previously summarized (Hasegawa, 1991). Comparison of NOAELs from published 3-month and 2-year repeated dose toxicity studies, unpublished data). The combined data sets yielded a median of 1.7 with a GSD of 3.30. If only a LOAEL was identified, the median LOAEL/NOAEL ratio of 3.5 with a GSD of 1.82 from Abdel-Rahman and Kadry (1995) from other chemicals can be adapted as an UF for this area, with the usual upper bound value of 10. However, it is recognized that the application of the benchmark dose approach is usually more appropriate in cases where only a LOAEL is available, and as such this UF is not used as frequently.

3. Calculation of new uncertainty factors based on experimental data by probabilistic approach, an example of rats

The distribution of both inter- and intra-species differences is log-normal because each component consists of the NOAEL ratios for two groups. If the default values of 10 are used, simple multiplication of 10 by 10, resulting in 100, leads to overestimation for the 95th percentile of the combined distribution, more appropriately it should be 51, as shown by Monte Carlo simulation (Swartout et al., 1998). Generically, the *N*th percentile of a log-normal distribution can be expressed as *N*th percentile = Exp [LN (median) + α_n × LN (GSD)]. For the 95th percentile, α_n = 1.645. The equation for the combination of two log-normal A and B distributions can be shown as follows: 95th percentile of (A × B) = Exp [LN ($median_A$) + LN ($median_B$) + 1.645 × $((LN(GSD_A))^2 + (LN(GSD_B))^2)^{0.5}$] (Kodell and Gaylor, 1999).

Inter-species differences were calculated using an analytical method presented by Schneider et al. (2004). A median of 4 was reported for the caloric demand adjustment, rounded from $3.76 = (70/0.35)^{1/4}$ (70 kg human body weight and 0.35 kg that of rats). A GSD of 3.23 was adopted from a combined distribution of MTD ratio for humans versus the 5 animal species previously described. For the 95th percentile, α_n = 1.645.

LN (95th percentile) = LN (4) + 1.645 × LN (3.23)

95th percentile = UF (95%) = Exp [1.39 + 1.645 × 1.17] = 27.5.

Intra-species differences were calculated using rat young/newborn NOAEL ratios in repeat-dose toxicity studies (Hasegawa et al., 2007). The median was 3 for 18 data sets and 5 was equivalent to 94.4% of all the data sets. For the 94.4th percentile, α_n = 1.590.

LN (5 as 94.4th percentile) = LN (3) + 1.590 × LN (GSD)
Rearranging,
LN (GSD) = (1.61 − 1.10)/1.590 = 0.321
Therefore,
GSD = Exp [0.321] = 1.38
95th percentile = UF (95%) = Exp [1.10 + 1.645 × 0.321] = 5.09.

From the above data for inter- and intra-species differences, the combined UF_{AH} was calculated as follows:

$LN(4) + LN(3) + 1.645 \times ((LN(3.23))^2 + (LN(1.38))^2)^{0.5} = 1.39 + 1.10 + 1.645 \times (1.17^2 + 0.321^2)^{0.5} = 4.48$
$Exp[4.48] = 88.7.$

For adjustment of short-term NOAEL to lifetime NOAEL, all 101 data sets of subchronic NOAEL/chronic NOAEL were used. The median was 1.7 with 10 equivalent to 93.1% of all the data sets. For the 93.1th percentile, α_n = 1.483.

LN (10 as 93.1th percentile) = LN (1.7) + 1.483 × LN (GSD)
Rearranging,
LN (GSD) = (2.30 − 0.531)/1.483 = 1.20
Therefore,
GSD = Exp [1.20] = 3.30
95th percentile = UF (95%) = Exp [0.531 + 1.645 × 1.20] = 12.1.

From the above UF calculations, the combined UF_{AHS} was calculated as follows:

$1.39 + 1.10 + 0.531 + 1.645 \times (1.17^2 + 0.321^2 + 1.20^2)^{0.5} = 5.82$
$Exp[5.82] = 337.$

If a benchmark dose approach cannot be applied, an additional UF should be applied when using LOAEL data. The LOAEL/NOAEL ratio for 24 chemicals was reported by Abdel-Rahman and Kadry (1995). The median was 3.5 and 10 was equivalent to 96% of the whole data. For the 96.0th percentile, α_n = 1.751.

LN (10 as 96.0th percentile) = LN (3.5) + 1.751 × LN (GSD)
Rearranging,
LN (GSD) = (2.30 − 1.25)/1.751 = 0.600
Therefore,
GSD = Exp [0.600] = 1.82
95th percentile = UF (95%) = Exp [1.25 + 1.645 × 0.600] = 9.39.

From the above UF calculations, the combined UF_{AHSL} was calculated as follows:

$1.39 + 1.10 + 0.531 + 1.25 + 1.645 \times (1.17^2 + 0.321^2 + 1.20^2 + 0.6^2)^{0.5} = 7.24$
$Exp[7.24] = 1400.$

4. Summary of combined uncertainty factors for six animal species by probabilistic approach

All fundamental values for the median, GSD and UF (95%) are shown in Table 1. The median for inter-species differences was derived using caloric demand adjustment from the standard human and animal body weights and rounded to a simple value. The

[1] The uncertainty factor used by several organizations for missing certain studies in the database (e.g., Dourson et al., 1992, 2002) was not considered here at this time, as it is being studied for applicability in Japan.