testosterone levels were 0, 5·2±0·15, and 1·5±0·15 ng/ml in castrated, castrated plus testosterone injected, and intact groups respectively. Serum testosterone levels reached 38 ng/ml, 1 h after a testosterone injection. The mRNA levels of identified secreted proteins decreased 1 week after castration, although the extent of the decrease differed among protein species. For instance, SPI-KT3 mRNA in the VP was greatly decreased in castrated animals to only 1/769 of the intact control level, while castration reduced SBP expression to 1/62 of the control. The extent of change in mRNA expression also varied between lobes. GRP78 mRNA in the DLP, for instance, was decreased to 1/11 of the control by castration but only to about 1/3 of the control in the VP. The mRNA levels were normalized by β-actin levels, which were not affected by castration and testosterone treatment. The β-actin levels in the VP were 3·5±0·36, 3·8±0·30, and 3·5±0·34 fg/ng total RNA in the castrated, the castrated plus testosterone and intact groups respectively. The values were 4·8±0·74, 5·3±0·99, and 4·3±0·40 for the DLP, and 3·9±0·24, 4·2±0·60, and 4·7±0·26 for the AP.

Ontogeny in mRNA expression of identified secreted proteins

The expression of identified secreted protein mRNAs was examined in each lobe of the prostate at ages 1, 2, 4, 6, and 11 weeks (Table 5). Low levels of mRNA expression were noted at 1 week. Significant increases of SBP and EAPA2 mRNAs began at 2 weeks and continued thereafter. Increases in other secretory protein mRNAs, including 91 kDa protein, PSP94 and IgGBPLP mRNAs, were apparent at 4 weeks.

Discussion

In the present study, the major secretory proteins of the mouse VP, DLP, and AP were identified by mass spectrometric analysis after 2D-gel electrophoresis (Table 6). IgBPLP and EAPA2 were major proteins in the DLP/AP. A 91 kDa protein predicted from a mouse urinary bladder cDNA (AK035662), Prdx6 and PLCα were also found in the prostatic secretion for the first time, in addition to previously reported prostatic proteins, including SBP (Mills *et al.* 1987*b*), SPI-KT3 (Mills *et al.* 1987*a*), PSP94 (Xuan *et al.* 1999), and probasin (Johnson *et al.* 2000). The mRNAs for these proteins were expressed in a lobe-specific manner and were regulated by androgen. Our study has delineated the main mouse prostatic secretion pattern for the first time. The data will be useful for studying androgen-dependent gene regulation in the prostate, and may also provide markers for studying functional differentiation of prostate tissue.

Production and secretion of prostatic proteins are the main physiological functions of the prostate gland. Prostatic secretory proteins have been studied in rats as well as in humans, especially from the viewpoint of androgen-dependent regulation of expression and to identify possible markers of prostate cancer. The major human prostatic-secreted proteins are PSA (prostate-specific antigen), PSP94, and prostatic acid phosphatase (Lee *et al.* 1986). In rat, the composition of prostatic proteins is different; only PSP94 is common with the human case, and the production of each protein varies among lobes. In the VP, prostatic-binding protein or prostatein is the major secreted protein, while cystatin-related protein and kallikreins are also produced abundantly (Heyns 1990). The LP and DP secrete probasin,

Table 5 Ontogeny of mRNA levels of identified proteins in the prostate

	SBP	91 K	PSP94	EAPA2	IgBPLP
VP					
1W	0·1±0·04	0·1±0·02	0·1±0·05	–	–
2W	17·9±3·8*	0·1±0·01	0·2±0·08	–	–
4W	78·5±15·3*	0·8±0·07†	2·6±0·23†	–	–
6W	503·0±106*	3·6±0·23†	16·0±3·9†	–	–
11W	474·9±77·3*	4·4±0·50†	4·2±0·6†	–	–
DLP					
1W	–	–	0·0±0·03	0·18±0·06	0·05±0·02
2W	–	–	0·0±0·01	0·42±0·06*	0·09±0·02
4W	–	–	1·1±0·32*	0·88±0·27	0·68±0·21
6W	–	–	12·7±1·5†	3·95±0·79*	7·43±1·5*
11W	–	–	4·0±0·2†	15·3±2·36†	102±22·0†
AP					
1W	–	–	–	0·18±0·04	0·04±0·01
2W	–	–	–	0·74±0·02*	0·1±0·02
4W	–	–	–	1·10±0·4	1·9±0·26†
6W	–	–	–	3·55±0·53†	30·1±3·6†
11W	–	–	–	5·50±0·80†	53·2±8·1†

Means±S.E.M. (n=4). Values are mRNA levels divided by β-actin mRNA levels (mol/mol β-actin) 1, 2, 4, 6 and 11-week-old (W) male C57BL mice were killed. Total RNA was isolated from each prostate lobe and amounts of mRNA were measured by real-time RT-PCR. *P<0·05 and †P<0·01 vs. 1W.

Table 6 Summary: identified mouse prostatic secretory proteins

Abbreviation	Lobe specificity	mRNA decrease by castration	Description
SBP	VP	++	Known prostatic protein (Mills *et al.* 1987*b*)
SPI-KT3	VP, (SV)	+++	Known prostatic protein (Mills *et al.* 1987*a*)
91 kDa	VP	++	Scavenger receptor cys-rich (SRCR) domains
PSP94	VP, DLP	+++	Known prostatic protein (Xuan *et al.* 1999)
ZnG	DLP > VP, AP	+	Ribonuclease activity?
GRP78	VP, DLP, AP	+	Heat-shock protein 70 family
AGR2	VP, DLP, AP	+	Human homolog expressed in prostatic cancer cell lines
PLCa	VP, DLP, AP	+	Enzyme involved in phosphatidylinositol metabolism
Calr	VP, DLP, AP	+	Calcium-binding protein
PDI	VP, DLP, AP	+	Enzyme involved in protein folding
Prdx6	DLP, AP > VP	++	Antioxidant protein
Probasin	DLP, AP	+++	Known prostatic protein (Johnson *et al.* 2002)
EAPA2	DLP, AP	+++	No homology with any known protein
IgBPLP	DLP, AP	+++	IgG binding? Willebrand factor D domains, trypsin inhibitor like

PSP94, and SVS2 (Imasato *et al.* 2001). A kinesin heavy chain-like protein and an IgG-binding protein were recently reported in the secretion of the AP (Esposito *et al.* 2001, Wilhelm *et al.* 2002).

In spite of the morphological similarity of the prostate in mouse and rat, previous studies have suggested a substantial difference in prostatic secretion between the two species (Donjacour *et al.* 1990). Since mouse prostatic proteins are known to be highly glycosylated, we first examined the effects of glycosidase digestion on prostatic proteins. Endo H glycosidase, which cleaves mainly within the chitobiose core of high mannose, did not change the SDS-PAGE pattern (data not shown). On the other hand, PNGase F, which removes all types of N-linked glycosylation, changed the pattern. A broad band of SBP in the VP was converted to a sharp band with smaller molecular weight, and smear-like bands between 40 and 100 kDa in the DLP/AP were also converted to sharper bands, indicating that proteins were de-glycosylated by the enzyme. In spite of highly glycosylated characteristics of mouse prostate proteins, the biological role of glycosylation is not yet understood.

In the mouse prostate, only VP-secreted proteins have been investigated, and two major proteins, SBP and SPI-KT3, were identified (Mills *et al.* 1987*a*,*b*). The present study confirmed the secretion of these two proteins and also revealed the presence of other proteins, including 91 kDa protein, Prdx6 and GRP78. The 91 kDa protein is expected to consist of 841 aa with two predicted extracellular (CUB) domains and three scavenger receptor cystine-rich (SRCR) domains, and is expressed preferentially in the VP. The size of the protein, however, seems to be less than 91 kDa in the gel. Since the sequence coverage of peptide mass fingerprinting is only 13%, the actual reading frame may be shorter than the predicted one. Prdx6 is another new component of the prostatic secretion found in the present study. Since it is an antioxidant enzyme that reduces peroxide and alkyl hydroperoxide to water and alcohol respectively (Wang *et al.* 2003), it may provide seminal plasma antioxidant capability. GRP78 belongs to the heat-shock protein 70 family, which had been considered as intercellular proteins. However, a recent proteomic analysis of human prostasomes revealed the presence of heat shock proteins in prostatic secretion (Utleg *et al.* 2003). In addition, heat-shock protein 70 has been reported to be secreted from a variety of prostatic cell lines, and to show growth-inhibitory activity (Jones *et al.* 2004, Wang *et al.* 2004). Secreted mouse GRP78 may have a similar activity.

Although mouse DLP proteins had not been biochemically identified, Cunha's group has recognized 110 and 55 kDa bands in SDS-PAGE as major DLP/AP proteins (Donjacour *et al.* 1990). They reported that DLP/AP proteins are highly glycosylated, which was confirmed by the present study. The predicted IgGBPLP sequence derived from the cDNA sequence (XM_620455), however, is calculated to contain 1866 aa with a molecular mass of 201 kDa. Because peptide sequencing by the peptide-mass fingerprinting method covered the whole predicted sequence (27% coverage), the 100 kDa spot probably contains a mixture of cleaved fragments derived from the 201 kDa protein, although this remains to be confirmed. Recently, an IgG-binding protein of 115 kDa was reported to be secreted also from the rat AP, suggesting that a rat homolog exists (Wilhelm *et al.* 2002). The predicted cDNA sequence corresponding to this rat protein (XM_620455), which became available more recently, encodes 206 kDa protein (1914 aa) instead of 115 kDa. There is 84% similarity between the mouse and the rat sequences. Secretion of EAPA2, which is one of the antigens found in experimental autoimmune prostatitis, is also a noteworthy finding in the present study. This protein of 914 aa contains no known domain structure and has no homology with any known functional protein. Secretion of both PSP94 and probasin was detected in the DLP, as expected, since both proteins are well characterized in rats and have been reported in mice (Xuan

et al. 1999, Johnson *et al.* 2000). The other identified DLP proteins include GRP78, Prdx6, ZnG, AGR2, Calr, and protein disulfide isomerase (PDI). Prostatic secretion of ZnG has been reported in humans, and ZnG is widely distributed in body fluids and in various epithelia (Lei *et al.* 1998, Hale *et al.* 2001). AGR2 is a mammalian homolog of *Xenopus* AGR2, which was recently reported to be secreted from human prostate under androgen regulation (Zhang *et al.* 2005). It is overexpressed in prostate cancer and the expression level is correlated with pathological grade. Calr is a highly conserved calcium-binding protein involved in a wide variety of cellular processes (Krause & Michalak 1997). Interestingly, the Calr gene was identified as an androgen-inducible gene in the rat VP (Zhu *et al.* 1998). PDI is involved in the maintenance of folding of synthesized proteins. Specific expression of PDI in the prostate was recently reported in humans (Lexander *et al.* 2005). Since both Calr and PDI are considered to be localized in the lumen of endoplasmic reticulum, they may represent contaminants introduced during preparation of the secretion sample. Secretion from the AP is similar to that from the DLP, i.e. the major secretory proteins are IgGBPLP and EAPA2, but little ZnG and no PSP94 are found in the secretion. The results of mass spectrometric identification of SV proteins were generally in agreement with previous reports, i.e. SVS2, 4, 5, and 6, as well as SPI-KT3 (Lai *et al.* 1991, Lundwall *et al.* 1997). Except SPI-KT3, SV proteins are specifically expressed in the SV and not in the prostate gland, which differs from the rat case, where SVS2, for instance, is highly expressed in DLP/AP.

Quantitative determination of mRNA expression revealed a clear transcriptional differentiation of secreted proteins among the lobes. The levels of secretory protein mRNAs were very high, ranging from 1 to 500 times that of the housekeeping gene β-actin, used as an internal control in the present study. The mRNA levels overall correlated with the intensity of protein staining in the gel, although spots of protein with larger molecular weight, such as EAPA2 and IgBPLP show lesser intensity in the 2D-gel, since the 1D-gel used in the present study is only able to hold proteins with molecular weights less than 80–100 kDa. Since all the identified secreted proteins decreased significantly a week after castration of the animal, these protein transcripts are androgen-dependent directly or indirectly through involution of the gland. In rats, various studies have shown a faster response of the VP to androgen action, as compared with other lobes. For instance, castration decreased probasin mRNA expression to 1% of the control level after a week, while the decrease in the DLP was only 50% (Imasato *et al.* 2001). In the mouse, however, large decreases in mRNAs were evident in all the lobes. We examined the effect of a single injection of testosterone on the mRNAs in castrated animals to confirm the androgen dependency of transcription. The serum testosterone level well exceeded the control level within 1 h and was still high 24 h after an injection. Although most mRNA levels increased significantly after an injection, which clearly demonstrates their androgen inducibility, most of them were not restored to the intact control level. It may suggest that the full activity of androgen-dependent genes in the prostate is involved in both short- and long-term transcriptional regulation mechanisms by androgen.

Although rodent prostate models have been used for investigating the mechanism of prostate carcinogenesis, anatomical differences between rodent and human prostate have led to concerns about the validity of rodents as suitable models for human prostate cancer. Besides, mice are resistant to induction of prostate tumors by chemical carcinogens. However, a number of transgenic or knockout mouse lines have become available in which prostate carcinomas preferentially occur. For instance, the TRAMP transgenic line expresses the SV40 antigen under the control of the rat probasin promoter. The TRAMP mice develop high-grade prostatic intraepithelial neoplasia and prostate cancer within 12 weeks of birth, and ultimately develop metastases to the regional lymph nodes and lung by 30 weeks. In addition, androgen depletion by castration results in decreased tumor incidence. Their futures are similar to the human case, although metastasis to bone, a characteristic feature of human prostate cancer, is rare. The expression pattern of secretion proteins may be related to development of prostate carcinogenesis. Since the present study has revealed mouse prostate secretion, these can now be examined in relation to the development of prostate carcinogenesis as well as androgen-dependent differentiation of the gland. The ontogeny of mRNA expression of secreted proteins indicated that significant expression started 2 weeks after birth, which is consistent with the fact that branching morphogenesis of the mouse prostate is completed in the first 15 days of birth.

The present study has provided an understanding of the major secretory function of the mouse prostate, and identified common aspects of secretory functionality between mouse and human, e.g. for heat-shock proteins, ZnG and peroxiredoxin. The identified secretory proteins should be available as models of androgen-dependent gene regulation and are candidates as markers for prostatic differentiation. Like human PSA or PSP94, some of the identified proteins may be useful as pathological markers associated with prostate disorders; this would facilitate prostate research in mouse models.

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A POSSIBLE MECHANISM FOR THE DECREASE IN SERUM THYROXINE LEVEL BY POLYCHLORINATED BIPHENYLS IN WISTAR AND GUNN RATS

Yoshihisa Kato [1], Shin-ichi Ikushiro [2], Rie Takiguchi [1], Sekihiro Tamaki [1], Koichi Haraguchi [3], Toshiyuki Sakaki [2], Shizuo Yamada [1], Jun Kanno [4] and Masakuni Degawa [1]

[1] *School of Pharmaceutical Sciences, University of Shizuoka, 52-1, Yada, Suruga-ku, Shizuoka 422-8526, Japan*
[2] *Biotechnology Research Center, Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama, 939-0398, Japan*
[3] *Daiichi College of Pharmaceutical Sciences, 22-1, Tamagawa-cho, Minami-ku, Fukuoka 815-8511, Japan*
[4] *Division of Cellular & Molecular Toxicology, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*

Introduction

Most polychlorinated biphenyl (PCB) congeners are known to decrease the levels of serum thyroid hormone and to increase the activities of hepatic drug-metabolizing enzymes in rats [1,2]. As possible mechanisms for the PCB-mediated decrease in level of serum thyroid hormone, enhancement of thyroid hormone metabolism by PCBs and displacement of the hormone from serum transport proteins (transthyretin (TTR)) are considered [3-5]. Especially, the decrease in the level of serum thyroxine (T_4) by 3,3',4,4',5-pentachlorobiphenyl, Aroclor 1254, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rats is thought to occur mainly through the induction of the UDP-glucuronosyltransferase (T_4-UDP-GT) responsible for glucuronidation of T_4 [2,4]. However, the magnitude of decrease in level of serum total T_4 is not necessarily correlated with that of increase in T_4-UDP-GT activity [1,6]. Recently, we suggested that the decrease in serum total T_4 level by a single administration of either Kanechlor-500 (KC500) or 2,2',4,5,5'-pentachlorobiphenyl in UGT1A-deficient Wistar rats (Gunn rats) was not dependent on the increase in hepatic T_4-UDP-GT activity, and further suggested that even in Wistar rats, the PCB-mediated decrease in serum T_4 level might occur not only through the increase in hepatic T_4-UDP-GT [7]. In the environment, humans and animals are exposed to PCB of very low level extent over a long period of time.

In the present study, therefore, to clarify possible mechanisms for the PCB-mediated decrease in level of serum thyroid hormone, we examined a relationship between the decrease in serum total T_4 level and the increase in the hepatic T_4-UDP-GT (UGT1A1 and UGT1A6) by the consecutive treatment of Wistar and Gunn rats with PCB and demonstrated that the PCB-mediated decrease in serum total T_4 level in rats was not necessarily dependent on the increase in hepatic T_4-glucuronidation, but the decrease occurs through the increased transport of T_4 to the liver.

Materials and Methods

Animal treatments. Male Wistar rats (160-200 g) and Gunn rats, (180-210 g) were obtained from Japan SLC., Inc. (Shizuoka, Japan). Male Wistar and Gunn rats were housed in three or four per cage with free access to commercial chow and tap water, and were maintained on a 12-h dark/light cycle (8:00 a.m.-8:00 p.m. light) in an air-controlled room (temperature: 24.5 ± 1℃, humidity: 55 ± 5%), and were handled with humane care under the guidelines of the University of Shizuoka (Shizuoka, Japan). The rats were treated with ip injection of KC500 (10 mg/kg) dissolved in Panacete 810 (5 ml/kg) at 24 h-intervals for 10 days. Control animals were treated with vehicle alone (5 ml/kg).

A) *In vivo* study. Rats were killed by decapitation on day 4 after the final dosing, and the liver was removed, and hepatic microsomes were prepared according to the method of Kato *et al.*[8] and stored at –85℃ until used. Blood was collected from each animal between 10:30 and 11:30 a.m. After clotting at room

temperature, serum was separated by centrifugation and stored at –50℃ until used.

Analysis of serum hormones. The levels of total T_4, free T_4, and thyroid-stimulating hormone (TSH) were measured by radioimmunoassay using the T4-RIABEAD (DAINABOT Co., Ltd, Tokyo, Japan), free T_4 (Diagnostic Products Corporation; Los Angels, CA), and Biotrak rTSH [^{125}I] assay systems (Amersham Life Science Ltd.; Little Chalfont, UK), respectivery.

Hepatic microsomal UDP-GT assay. The amount of protein was determined by the method of Lowry *et al.*[9] with bovine serum albumin as a standard. The activity of microsomal UDP-GT toward T_4 was determined by the method of Barter and Klaassen [3]. The UDP-GT activity was measured after activation of the UDP-GTs by 0.05% Brij 58.

Western blot analysis. Polyclonal anti-peptide antibodies against the common region of UGT1A isoforms and specific antibodies against UGT1A1, UGT1A6, and UGT2B1 were used [10]. Western analyses for microsomal UGT isoforms were performed by the method of Luquita *et al.*[11]

B) *Ex vivo* study. At 4 day after last treatment with KC500, animals were anesthetized with 50 mg/ml sodium pentobarbital combined 1:1 with 1 mg/ml potassium iodide at 2 mg/ml. The femoral artery was cannulated and primed with heparinized saline. Fifteen minutes later, animals were given [^{125}I]T_4 i.v. at 15 μCi/ml in 10 mM NaOH saline including 1 % normal each animal serum.

Clearance of [^{125}I]T_4 from serum. Five minutes following i.v. administration of [^{125}I]T_4 and five more times at the indicated time, a portion of blood was sampled from the artery, and serum was collected and stored at -50℃ for assay. Two aliquots were taken from serum samples for γ-counting.

Analysis of [^{125}I]T_4 binding to serum proteins. The levels of serum [^{125}I]T_4-albumin, [^{125}I]T_4-thyroxine binding protein, and [^{125}I]T_4-TTR complexes were determined by the method of Kato *et al.*[12]

Tissue distribution of [^{125}I]T_4. At 60 min after the administration of [^{125}I]T_4, blood was sampled from abdominal aorta, and tissues were removed and weighted. Radioactivities in serum and tissues were determined by γ-counting, and concentration ratios of the tissue to serum were determined.

Results and Discussion

The serum total T_4 and free T_4 levels were markedly decreased not only in the Wistar rats but also in the Gunn rats 4 days after final treatment with KC500 (10 mg/kg, i.p., once daily for 10 days), and there was no significant difference in magnitude of the decrease between Wistar and Gunn rats (Fig. 1). At the same time, the level and activity of T_4-UDP-GT (UGT1A1 and UGT1A6) were significantly increased by treatment with KC500 in Wistar rats but not in Gunn rats (Fig. 2). In contrast, the level of UGT2B1 was increased by KC500 in both Wistar and Gunn rats. Hepatic microsomal enzyme activities (benzyloxyresorufin *O*-dealkylase activity, 48- and 35-fold; pentoxyresorufin *O*-dealkylase activity (CYP2B1/2), 17- and 10-fold; ethoxyresorufin *O*-dealkylase activity (CYP1A1/2), 64- and 10-fold in Wistar and Gunn rats, respectively) were significantly increased by KC500 treatment. In addition, no significant change in the level of serum TSH by the KC500 treatment was observed in either Wistar or Gunn rats.

Furthermore, significant increases in the disappearance of [^{125}I]T_4 from the serum and in the distribution volume of [^{125}I]T_4 by KC500 treatment were observed in both Wistar and Gunn rats. A concentration ratio of the liver to serum was approximately one in either Wistar or Gunn rats, and KC500-treatment increased the ratio by 4 times. The concentration of [^{125}I]T_4 appeared to be the highest in the liver in both Wistar and Gunn rats. The hepatic levels of [^{125}I]T_4 in both rats were further increased by KC500 treatment. More than 40% of [^{125}I]T_4 dosed was transported to the liver of both rats (Fig. 3). In contrast, a significant increase in liver weight was observed in KC500-treated Wistar rats but not in the Gunn rats. In addition, significant decrease in the binding of [^{125}I]T_4 to serum TTR and significant increase in the binding to serum albumin by KC500 treatment were observed in either Wistar or Gunn rats.

In conclusion, the present findings demonstrate that the PCB-mediated decrease in serum total T_4 level in Gunn rats occurs without an increase in hepatic T_4-UDP-GT activity; they further suggest that in both strain rats, the PCB-mediated decrease occurs through the increased transportation of T_4 to the liver. Furthermore, the decrease in the binding of T_4 to serum TTR and hepatic hyperplasia might be attributed to the increase in the level of T_4 in the liver. In Wistar rats, however, the PCB-mediated induction of T_4-UDP-GT might, at least in part, contribute to the decrease. Further studies are necessary for understanding the susceptibility toward a PCB-mediated decrease in serum T_4 level in animals including humans.

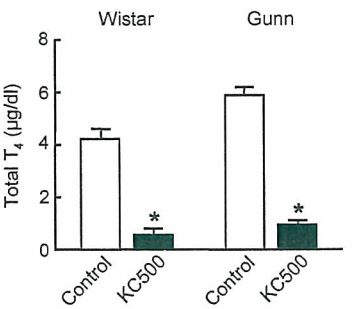

Fig. 1. Effect of KC500 on the level of serum total thyroxine in Wistar and Gunn rats

Animals were killed 4 days after the final administration of KC500 (10 mg/kg, i.p., once daily for 10 days). Each column represents the mean ± S.E. (vertical bars) for five to six animals. *P<0.01, significantly different from each control.

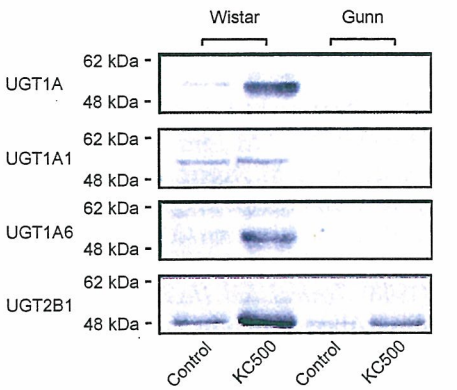

Fig. 2. Representive Western blot patterns for hepatic microsomal UGT isoforms in KC500-treated Wistar and Gunn rats

Animals were killed 4 days after the final administration of KC500 (10 mg/kg, i.p., once daily for 10 days).

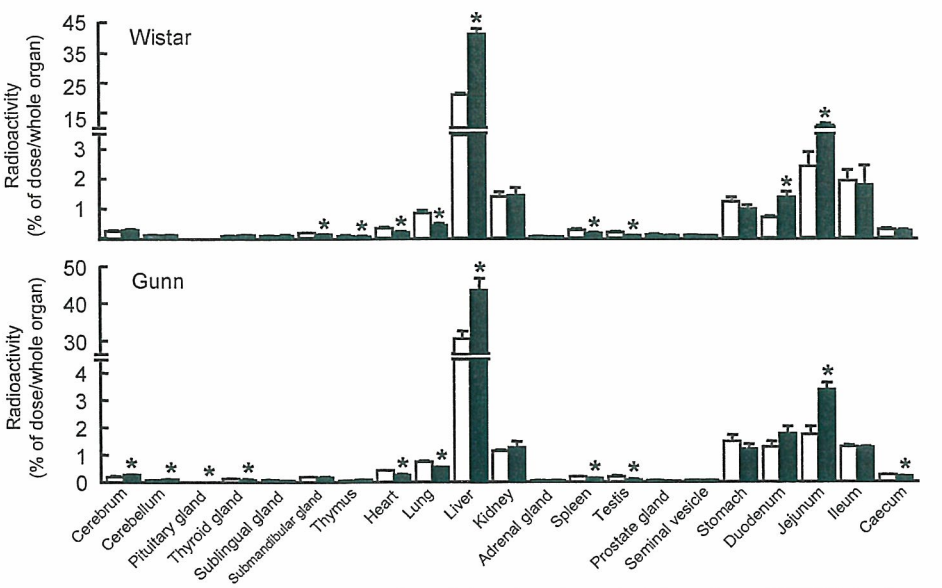

Fig. 3. Tissue distribution of total radioactivity after the administration of [^{125}I]T_4 to the KC500-treated Wistar and Gunn rats

KC500 (10 mg/kg) was given i.p. to animals at 24 hr-intervals for 10 days. The radioactivity of each tissue was measured at 60 min after the i.v. administration of [^{125}I]T_4. Each column represents the mean ± S.E. (vertical bars) for three to six animals. *P<0.05, significantly different from each control. ☐, control; ■, KC500.

Acknowledgements

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Drug Metab. Pharmacokinet. 21 (1): 70–74 (2006).

Regular Article

Monospecific Antipeptide Antibodies Against Human Hepatic UDP-Glucuronosyltransferase 1A Subfamily (UGT1A) Isoforms

Shin-ichi IKUSHIRO[1], Yoshikazu EMI[2], Yoshihisa KATO[3], Shizuo YAMADA[3] and Toshiyuki SAKAKI[1]

[1]*Biotechnology Research Center, Faculty of Engineering, Toyama Prefectural University, Toyama, Japan*
[2]*Graduate School of Life Science, University of Hyogo, Hyogo, Japan*
[3]*School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka, Japan*

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Summary: Expression of UDP-glucuronosyltransferases (UGT) in mammals is thought to be regulated in both a tissue- and developmental-specific manner. Furthermore, induction of genes encoding UGT occurs after exposure to xenobiotics including drugs, environmental pollutants and dietary compounds. In human, isoforms of UGT 1A subfamily catalyze the glucuronidation of a greater proportion of drugs, suggesting that the expression of UGT1A isoforms is responsible for the clearance of a diverse range of drugs. To analyze the expression of human UGT1A isoforms, we have developed polyclonal antibodies against specific peptide regions within the isoforms (UGT1A1, 1A3, 1A4, 1A6 and 1A9). The prepared antipeptide antibodies were found to be highly monospecific for each UGT1A isoform and no cross-reactivity with UGT2B isoforms was detected. Analysis of UGT1A protein levels in hepatic microsomes using these antibodies demonstrated interindividual differential expression of each isoform. These highly specific antipeptide antibodies provide an important tool to analyze tissue distribution and interindividual expression levels of human UGT1As.

Key words: antipeptide antibody; glucuronidation; humanUGT1A isoform; liver microsomes

Introduction

UDP-glucuronosyltransferases (UGTs, EC 2.4.1.17), which are located mainly in the endoplasmic reticulum, play a major role in the detoxification of drugs and other xenobiotics including environmental pollutants and dietary compounds in all vertebrates.[1)] UGTs catalyze the transfer of glucuronic acid to the hydroxyl, carboxyl, sulfhydryl or amine groups of structurally unrelated compounds, resulting in the formation of water-soluble glucuronides for excretion in the bile or urine. Mammalian *UGTs* can be classified, according to their substrate specificity, sequence similarity and gene structure, into three subfamilies: *UGT1*, *UGT2* and *UGT3*.[2)] Many of the UGT isoforms have been identified and characterized in terms of their substrate specificity after expression of the corresponding cDNA in heterologous cells.[3)]

In human liver, five UGT1A cDNAs have been identified and characterized: UGT1A1, 1A3, 1A4, 1A6 and 1A9.[4)] Although the expression of different UGT isoforms at the mRNA level in hepatic and extrahepatic tissues has been quantified, there is a paucity of protein data for each isoform due to the lack of specific antibodies. Without a detailed knowledge of the relevant protein levels it is difficult to accurately determine the contribution of specific UGT isoforms to the metabolism of the liver and other tissues. Therefore, the development of isoform-specific antibodies is needed to fully characterize the protein level of each UGT in major tissues and to define the substrate specificity for each UGT isoform. We have developed isoform-specific antipeptide antibodies to rat UGT isoforms and characterized inducer-dependent induction and tissue specific expression.[5-9)] In this report, we developed human UGT1A isoform-specific antipeptide antibodies to analyze the expression of the isoforms in hepatic microsomes. These highly specific antibodies provide an important tool to study tissue distribution and cellular expression levels of UGT isoforms.

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To whom correspondence should be addressed: Shin-ichi IKUSHIRO, *Tel. +81-766-56-7500 (ext 566), Fax. +81-766-56-2498, E-mail: ikushiro@pu-toyama.ac.jp*

Table 1. Amino acid sequences of antigen peptides for human UGT1A isoforms

Isoforms	Positions	Sequences
UGT1A	520–533	KKGRVKKAHKSKTH
UGT1A1	108–121	RVIKTYKKIKKDSA
UGT1A3	82–100	ISWTQDEFDRHVLGHTQLY
UGT1A4	107–120	LKRYSRSMAIMNNV
UGT1A6	82–95	YDQEELKNRYQSFG
UGT1A9	102–115	VRSIYSLLMGSYND

Methods

Materials: Human hepatic microsomes from 5 individual livers (HH13, HH18, HK25, HG43 and HG89) were purchased from BD Biosiences (Woburn, MA). Informed consents for the preparation of the microsomes were obtained from all donors. Recombinant human UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15 and UGT2B17 expressed in baculovirus-infected insect cells (Supersomes) were also purchased from BD Biosciences. All other chemicals were of the best commercially available grade.

Preparation of anti-peptide antibodies: The amino-terminal half of each human UGT1A, deduced from the corresponding cDNA sequence, was selected for the preparation of human UGT1A isoform-specific antipeptide antibodies (**Table 1**). The carboxyl-terminal region (14 aa) of UGT1A, which is common to all UGT1A isoforms, was selected for preparing anti-UGT1A antibodies. An additional cysteine residue was included at the amino-terminus of each peptide sequence to facilitate conjugation to a carrier protein, Keyhole limpet hemocyanin (KLH). The synthetic peptides (14–19 aa) were conjugated with KLH *via* the amino-terminal cysteine using maleimidobenzoyl-N-hydroxysuccinimide.[5] Rabbits were immunized with the KLH-peptide conjugates emulsified in Freund's complete adjuvant. Subcutaneous immunization (250 μg KLH-peptides per rabbit) was performed three times, at 2 week intervals, and the rabbits were bled two weeks after the third injection. The antisera titers were monitored by ELISA assay using synthetic peptides conjugated with bovine serum albumin as a standard. The antipeptide antibodies were purified from antisera by using the corresponding peptides coupled to a BrCN-activated Sepharose 4B column. Six monospecific antipeptide antibodies against human UGT1A isoforms were generated corresponding to the sequence of UGT1AC, UGT1A1, 1A3, 1A4, 1A6 and 1A9, which were designated h1AC, h1A1, h1A3, h1A4, h1A6 and h1A9, respectively.

Immunoblot analysis: SDS-polyacrylamide gel electrophoresis (PAGE) was performed using a 4% (w/v) stacking and a 10% (w/v) separating gel.[5] The proteins in the gel were blotted to nitrocellulose membranes using a semi-dry blotting method. The membranes were washed in distilled water and blocked at 25°C for 1 hour in blocking buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1.5% (w/v) bovine serum albumin), followed by incubation with diluted antibodies (1:1000–1:6000 dilution) at 25°C for 15 hours. The membranes were washed in PBS containing 0.05% (v/v) Tween 20 and incubated with a 1:5000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (CTS. Inc., MA, USA) at 25°C for 2 hours. Immunodetection was developed by adding 1.7 mg/mL bromochloroindolyl phosphate and 0.7 mg/mL nitro blue tetrazolium. The band intensity of the blot sheet was quantitated using densitometric scanner with high resolution and NIH image software (Version 1.61). Endoglycosidase H (EndoH) treatment under denaturing conditions was performed as follows. A 20 μL aliquot of sample used for the SDS-PAGE analysis was directly subjected to EndoH treatment (200 units) for 60 min at 37°C. The reaction was terminated by heating at 98°C for 5 min.

Results and Discussion

The human *UGT1A* gene cluster spans more than 200 kb of chromosome 2 and is organized in a unique arrangement.[2] A series of divergent first exons are located consecutively over 150 kb, each encoding approximately 280 aa. The 3′ region of the locus comprises exons 2–5, which encode the conserved 245 aa carboxyl terminal region of UGT1A. The amino- and carboxyl-terminal halves of UGT are thought to be involved in the recognition/binding of substrate and UDP-glucuronic acid, respectively.[5] Because the UGT1A isoforms share a very high level of overall sequence identitiy (66–93%), it has proved difficult to prepare isoform-specific antibodies. Previously, we have successfully developed specific antipeptide antibodies against rat UGT1A isoforms and demonstrated that the isoforms are independently induced by various xenobiotics.[5-9] In this work, a peptide-specific antibody strategy was chosen to raise specific antibodies against human UGT isoforms.

A comparative analysis of the amino acid sequence of human UGT isoforms identified unique regions for designing peptides for isoform-specific antibody preparation (**Table 1**). UGT1A peptide is located at the carboxyl-terminal end of all UGT1A isoforms, but is distinct from the sequence found in UGT2B isoforms. The isoform-specific peptides were unique to each isoform and corresponded to the hypervariable region I (residues 60–150) of UGT1, which is thought to be involved in aglycon recognition.[10] Affinity-purified antibodies were tested for specificity by immunoblot

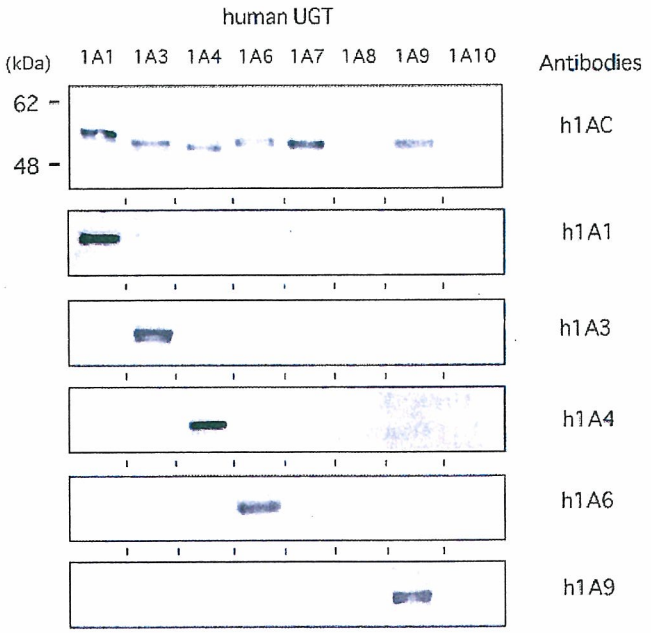

Fig. 1. Immunospecificity of anti-UGT1A isoform-specific peptide antibodies. Approximately 1 μg of microsomes containing recombinant human UGT1A isoforms was used for the analysis of h1AC and 5 μg for each UGT1A isoform-specific antibody preparation.

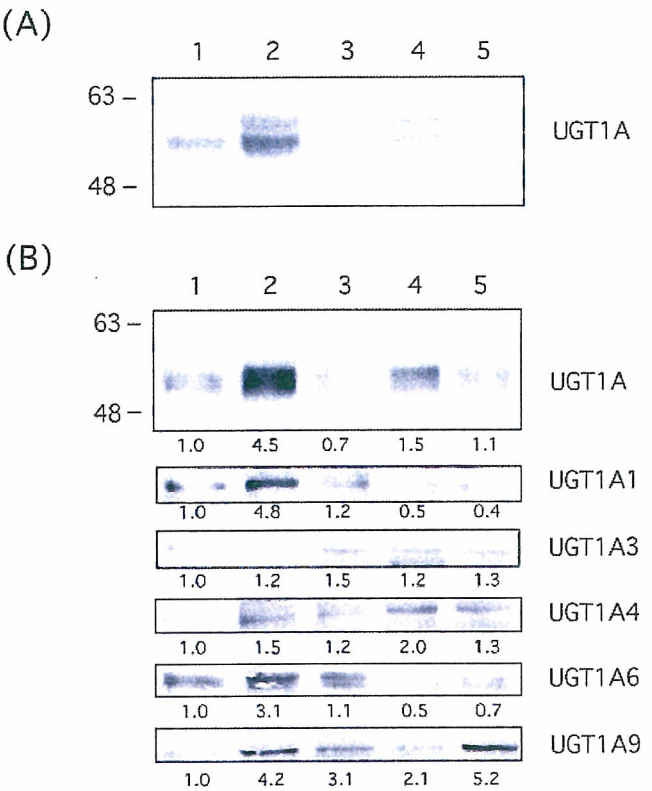

Fig. 2. Analysis of interindividual expression of human UGT1A isoforms in hepatic microsomes using specific antibodies. Commercially available human hepatic microsomes from 5 individuals were used: lane 1; HH13, lane 2; HH18, lane 3; HK25, lane 4; HG43, lane 5; HG89. Hepatic microsomes were untreated (A) or treated (B) with endoglycosidase H. Approximately 20 and 40 μg of microsomes was used for the analysis of h1AC and isoform-specific antibodies, respectively. Values of each panel represent the relative expression level of UGT isoforms in hepatic microsomes.

analysis of commercially available recombinant human UGTs. The antipeptide antibodies against each UGT1A isoform displayed highly specific immunoreactivity (**Fig. 1**). Furthermore, these antibodies did not recognize human UGT2B isoforms UGT2B4, 7, 15 and 17 (data not shown).

Despite the high level of sequence identity between UGT1A3 and 1A4 (93%), h1A3 and h1A4 only reacted with the corresponding isoform. Recently, Iwai *et al.*[11] used an antipeptide antibody preparation against UGT1A3 to detect the recombinant enzyme in an expression system, but did not verify the antibody specificity. Girard *et al.*[12] and Miles *et al.*[13] developed an antibody against the N-terminus of recombinant UGT1A9 protein to determine the protein content of UGT1A9 in human liver microsomes. However, the antibodies recognized closely related UGT1A isoforms, UGT1A7, 1A8 and 1A10, in addition to UGT1A9. Our antipeptide antibody, h1A9, did not recognize UGT1A7, 1A8 and 1A10, indicating that the expression level of hepatic UGT1A9 protein could be determined without cross-reactivity against related UGT1A isoforms.

Recently, several lines of evidence indicate significant interindividual variability in *UGT1A* expression and the associated glucuronidating activity. Genetic variations in the promoter region of each isoform affect the expression of the corresponding protein. Polymorphism of the UGT1A1 promoter region is known to be associated with disorders of bilirubin metabolism and an adverse effect to SN-38.[14] In addition to genetic variation, regulation of the UGT1 locus is under selective transcriptional control with a number of xenobiotic and steroid receptors.[15–18] Antipeptide antibodies were used to determine the UGT protein levels in a panel of human hepatic microsomes. A considerable degree of heterogeneity in UGT1A expression in individuals is evident from the cross-reactivity of h1AC with several bands (**Fig. 2, panel A**). Treatment of microsomes with endoglycosidase H resulted in a shift of these bands, indicating the modification of UGT1A isoforms with high mannose-type sugar chains (**Fig. 2, panel B**). Furthermore, different patterns of expression of UGT1A isoforms between individuals was detected using the isoform-specific antibodies. Because the levels of NADH-cytochrome b_5 reductase and NADPH-cytochrome P450 reductase do not vary between individuals, these microsomal proteins were used as a convenient control for our experiments (data not

shown). The UGT1A1 protein level in hepatic microsomes, which is associated with bilirubin glucuronidating activity, showed a variation factor of approximately 10. Although the expression levels of UGT1A3 and UGT1A4 did not vary between the individuals examined in this study, recent findings have demonstrated that human PXR variants is implicated in the expression of UGT1A3 and UGT1A4.[19] The UGT1A9 protein level in hepatic microsomes, associated with propofol glucuronidating activity, showed a variation factor of approximately 4. Yamanaka *et al.*[20] recently identified sequence variations in the UGT1A9 promoter that may affect the level of enzyme expression.

In human, UGT1A isoform-dependent glucuronidation plays an important role in the metabolism of drugs as xenobiotic compounds. The expression of UGT1A isoforms varies during development and with different dietary intake. Polymorphism in the flanking promoter regions of the *UGT1A* gene cluster may affect the transcriptional efficiency. It is therefore important to define the level of hepatic UGT protein for each individual to estimate the glucuronidating efficiency *in vivo*. The antipeptide antibodies developed in this study allow us to analyze the expression of the human UGT1A isoforms and assess the relative contribution of each isoform for the glucuronidation of various drugs.

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Meeting Report: Validation of Toxicogenomics-Based Test Systems: ECVAM–ICCVAM/NICEATM Considerations for Regulatory Use

Raffaella Corvi,[1] Hans-Jürgen Ahr,[2] Silvio Albertini,[3] David H. Blakey,[4] Libero Clerici,[5] Sandra Coecke,[1] George R. Douglas,[4] Laura Gribaldo,[1] John P. Groten,[6] Bernd Haase,[7] Karen Hamernik,[8] Thomas Hartung,[1] Tohru Inoue,[9] Ian Indans,[10] Daniela Maurici,[1] George Orphanides,[11] Diana Rembges,[5] Susanna-Assunta Sansone,[12] Jason R. Snape,[13] Eisaku Toda,[14] Weida Tong,[15] Joost H. van Delft,[16] Brenda Weis,[17] and Leonard M. Schechtman[18,19]

[1]European Centre for the Validation of Alternative Methods (ECVAM), Institute for Health and Consumer Protection (IHCP), Joint Research Centre of the European Commission (JRC), Ispra, Italy; [2]Bayer HealthCare AG, Wuppertal, Germany; [3]Hoffmann-La Roche, Basel, Switzerland; [4]Environmental Health Centre, Health Canada, Ottawa, Ontario, Canada; [5]Physico-Chemical Exposure, IHCP, JRC, Ispra, Italy; [6]TNO, Utrecht, the Netherlands; [7]QIAGEN, Hilden, Germany; [8]U.S. Environmental Protection Agency, Washington, DC, USA; [9]National Institute of Health Sciences, Tokyo, Japan; [10]Health Safety Executive, London, United Kingdom; [11]Syngenta, Macclesfield, United Kingdom; [12]European Molecular Biology Laboratory, European Bioinformatics Institute, Hinxton, Cambridge, United Kingdom; [13]AstraZeneca, Brixham, United Kingdom; [14]Organisation for Economic Co-operation and Development, Paris, France; [15]Food and Drug Administration, National Center for Toxicological Research, Jefferson, Arkansas, USA; [16]University of Maastricht, Maastricht, the Netherlands; [17]National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services, Research Triangle Park, North Carolina, USA; [18]U.S. Interagency Coordinating Committee on the Validation of Alternative Methods, Research Triangle Park, NC, USA; [19]U.S. Food and Drug Administration, National Center for Toxicological Research, Rockville, Maryland, USA

This is the report of the first workshop "Validation of Toxicogenomics-Based Test Systems" held 11–12 December 2003 in Ispra, Italy. The workshop was hosted by the European Centre for the Validation of Alternative Methods (ECVAM) and organized jointly by ECVAM, the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). The primary aim of the workshop was for participants to discuss and define principles applicable to the validation of toxicogenomics platforms as well as validation of specific toxicologic test methods that incorporate toxicogenomics technologies. The workshop was viewed as an opportunity for initiating a dialogue between technologic experts, regulators, and the principal validation bodies and for identifying those factors to which the validation process would be applicable. It was felt that to do so now, as the technology is evolving and associated challenges are identified, would be a basis for the future validation of the technology when it reaches the appropriate stage. Because of the complexity of the issue, different aspects of the validation of toxicogenomics-based test methods were covered. The three focus areas include *a)* biologic validation of toxicogenomics-based test methods for regulatory decision making, *b)* technical and bioinformatics aspects related to validation, and *c)* validation issues as they relate to regulatory acceptance and use of toxicogenomics-based test methods. In this report we summarize the discussions and describe in detail the recommendations for future direction and priorities. *Key words:* acceptance, alternatives, biomarker, predictive test, regulatory use, standardization, toxicogenomics, toxicology, validation. *Environ Health Perspect* 114:420–429 (2006). doi:10.1289/ehp.8247 available via *http://dx.doi.org/* [Online 17 August 2005]

Toxicogenomics, an emerging field in molecular toxicology, offers the promise of new approaches to identify and characterize such factors as the biologic activity of new and existing chemicals and drugs and could play an important role in hazard assessment for human health. This revolutionary field can potentially affect many scientific and medical areas, including the development of a new generation of alternative predictive testing and screening methods that could lend themselves to the reduction, refinement, and replacement of animals used for such purposes.

The European Centre for the Validation of Alternative Methods (ECVAM), the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) are currently investigating the specific considerations necessary for adequate validation of toxicogenomics-based test methods. The primary objective of ECVAM and ICCVAM/NICEATM is to facilitate development, validation, and regulatory acceptance of new, revised, and alternative test methods that reduce, refine, and replace the use of animals (referred to as the three Rs; Russell and Burch 1959) in testing while maintaining and promoting scientific quality and the protection of human health, animal health, and the environment. The efforts of such organizations as ICCVAM/NICEATM and ECVAM have helped foster the principles of the three R's and have contributed to progress in the use of alternative methods for regulatory, research, and educational purposes.

Experience in the validation of conventional alternative test methods has led to an understanding that new and innovative approaches likely will be necessary to standardize test methods based on toxicogenomics and to evaluate the scientific validity and regulatory applicability of such test methods. It is envisioned that the entire validation process will be more complex and challenging than that typically encountered thus far for other alternative test methods. This is because not only will the technology itself need to be standardized and validated, but the methods that are based upon the technology and their predictive aspects will also need to undergo validation if they are to be employed in regulatory decision-making processes. In addition the validation process must be able to accommodate the anticipated rapid changes in technology that could affect the performance of the test method and its reliability for a specific purpose.

Toxicogenomics-based methods are being widely applied in toxicology and biomedical research. Because data are already being generated using these technologies, it is both timely and important to address the subject of validation now with the aim of establishing a foundation that will facilitate future regulatory acceptance of scientifically validated

Address correspondence to R. Corvi, European Centre for the Validation of Alternative Methods, IHCP, Joint Research Centre of the European Commission, Via E. Fermi 1, 21120 Ispra, Italy. Telephone: 39-0332-785266. Fax: 39-0332-785845. E-mail: raffaella.corvi@jrc.it

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toxicogenomics-based test methods. By addressing the critical validation issues early, and in parallel with the evolutionary and maturation phases of the technologic development of toxicogenomics-based methods, it should be possible to preempt many potential pitfalls and data gaps encountered with retrospective method evaluations that could impede validation of this promising research and regulatory tool. Such a strategy will also facilitate early buy-in and confidence in the technologies by the regulatory arena in its quest for new, improved, and relevant methods by which to help ensure human health, protect the environment, and demonstrate responsiveness to animal welfare issues.

In consideration of all these related issues, ECVAM and ICCVAM/NICEATM held the first of a planned series of workshops to address the validation principles that lend themselves to toxicogenomics-based test methods, for example, gene expression technologies and associated bioinformatics. Given the complexity of the rapidly evolving toxicogenomics field, a variety of issues were addressed. These included but were not limited to *a*) differences in and evolution of technology platforms including changes in genome coverage for model species; *b*) quality assurance (QA) and Good Laboratory Practice (GLP) compliance; *c*) technology standardization, transferability, and reproducibility; *d*) relevance to *in vivo* biological responses; *e*) yardsticks against which toxicogenomics responses should be measured; *f*) data evaluation, statistical approaches, and databases; *g*) validation approaches; and *h*) regulatory acceptability.

To begin to examine these complex issues, three breakout groups were formed. Each group concentrated on different aspects of the validation of toxicogenomics-based test methods, and the discussions were shared with the other participants in plenary sessions. The three focus areas were *a*) biological validation of toxicogenomics-based test methods for regulatory decision making, *b*) technical and bioinformatics aspects related to validation, and *c*) validation issues as they relate to regulatory acceptance and use of toxicogenomics-based test methods.

Validation of Toxicogenomics: Focus on the Biological Systems

The biological issues related to the validation of toxicogenomics-based test methods involved two strategies proposed for developing and validating such methods so that they can be employed to support regulatory decision making. One strategy involves phenotypic anchoring of gene expression changes to identify molecular mechanisms and candidate biomarkers of toxicity (i.e., single genes, proteins, or biological pathways). A second strategy involves the identification and validation of predictive gene expression signatures of toxicity. Validation considerations specific to data quality and cross-platform and interlaboratory variability that are common to both strategies were identified. It is acknowledged that any new toxicogenomics-based methods will need to address established validation criteria for determination of reliability and relevance (Balls et al. 1995; ICCVAM 1997, 2003) as well as articulate the advantages and limitations of a given toxicogenomics-based test method. In addition biological validation of such a test method, that is, assessment of the concordance of gene changes with biological events, is essential but is contingent upon validation of the technology itself, which is addressed elsewhere in this article.

Strategy 1: use of toxicogenomics data to define mechanism and identify biomarkers. Toxicogenomics offers the opportunity to enhance existing toxicity prediction strategies through elucidation of biological mechanisms around critical events. This sentiment is captured in the recent U.S. Environment Protection Agency (EPA) and U.S. Food and Drug Administration (FDA) strategies regarding the inclusion of genomics data in submissions of regulated substances (U.S. EPA 2002; U.S. FDA 2005). Although these agencies currently preclude basing regulatory decision making on genomics data alone, they do encourage the voluntary submission of well-documented, quality genomics data. Both agencies are considering the use of submitted data on a case-by-case basis for assessment purposes (e.g., to help elucidate mechanism of action or contribute to a weight-of-evidence approach) or for populating relevant comparative databases by encouraging parallel submissions of genomics data and traditional toxicologic test results. This approach is appropriate given the state of scientific knowledge of toxicogenomics and the requisite need for a clear understanding of the toxicologic relevance of the gene expression signals detected by this technology. There is a small but rapidly increasing number of published reports demonstrating a linkage between gene expression changes and adverse phenotypic changes (Huang et al. 2003; Orphanides 2003). These reports provide qualitative evidence of the power of genomics to link phenotype with gene expression, thereby contributing to an understanding of mechanism of action. Some such reports demonstrate the predictive power of these data to classify compounds. However, they fail to address adequately quantitative dose- and time-dependent (e.g., threshold) responses that are the hallmark of toxicologic evaluation, making their immediate acceptance in regulatory arenas circumspect.

Nonetheless, toxicogenomics data may eventually be useful in hazard and risk assessment if data quality and validity can be adequately substantiated. Some regulators are finding that these data have the potential to add to the body of knowledge about compound mechanism of action. With appropriate dose- and time-dependent measurements, gene and protein changes can be used to mark the molecular events that occur as an organism moves through the continuum from exposure to response. The obvious benefit is the identification of early markers of response, including responses that mark the point of departure from adaptation to toxicity. In addition, it may be possible to detect unforeseen effects at very low doses or in unexpected tissues (Brown et al. 2002). This is important because changes in gene or protein expression alone are not sufficient to differentiate toxicity from biologic adaptation after exposure to an exogenous compound. The challenge for predictive toxicology is to link changes in gene and protein expression to sequential changes in phenotype, both adaptive and adverse, in a manner that is consistent with the underlying biologic mechanisms. For example, gene expression profiling has been used to classify hepatotoxins based on mechanism of action and to differentiate early, presumably adaptive, responses from later responses that are reflective of toxicity (Hamadeh et al. 2002a, 2002b; Waring et al. 2001, 2003). The gene expression changes correlated well with changes in histopathology and clinical chemistry, supporting the liver as target organ for the test compounds.

Although good technical progress has been made in recent years, additional proof-of-principle studies are needed for the regulatory community to become more accepting of the use of toxicogenomics data as part of the regulatory decision-making process. It would be important to demonstrate, for instance, that toxicogenomics not only can confirm what is already known about specific compounds and toxic end points (i.e., phenotypic anchoring) but also can accurately predict toxicity for unknown compounds. The task is to present regulatory scientists with new knowledge gained from toxicogenomics approaches in a familiar context. Ideally, at least in the short term, the focus will be the identification of single, or small sets of, genes or proteins that serve as biomarkers of response, as opposed to signatures of response that are the typical output of microarray experiments. Simple biomarkers of response are favored over complex expression signatures because they are familiar in toxicology assessment, are easy to maintain over time (e.g., are independent of the microarray platform), and can be readily validated. Validation strategies for toxicogenomics-based markers can be modeled after protocols for existing biomarkers. Thus, global gene expression technologies such as microarrays can be used to identify a specific gene marker,

or a suite of markers, that can then be validated by conventional methods such as Northern blot analysis, *in situ* hybridization, and quantitative polymerase chain reaction. This approach has advantages because regulatory agencies such as the U.S. FDA have proposed procedures to address gene and protein biomarkers, and other organizations, such as the Organisation for Economic Co-operation and Development (OECD 2005), are embarking on establishing similar guidance (Supplemental Material, Section 1; http://ehp.niehs.nih.gov/members/2005/8247/suppl.pdf).

Proof-of-principle studies could be conducted concurrently with existing regulatory test methods using similar samples of test compounds. In such situations, it may be appropriate to use *in vivo* systems, which are widely accepted by the regulatory community. Parallel *in vitro* studies could be conducted in situations where an appropriate test system is available. It may be wise to focus initial efforts on defining relationships between gene expression changes and toxicity for individual compounds or compound classes with well-defined end points. The experimental design should address conventional aspects of dose and time (dose response), species and strain susceptibility, group size and sex, and selection of end points for study (e.g., histopathology, clinical chemistry). Numerous commercial microarray platforms offer genomewide coverage for model systems such as rat, mouse, *Caenorhabditis elegans*, and humans. Commercial microarrays are also available for genes that are highly expressed in specific tissues (e.g., liver, breast) and during specific biological processes such as metabolism (e.g., P450 enzymes). Both genomewide and dedicated arrays can be used with RNA samples from *in vivo* and *in vitro* (tissue and cell culture) systems, enabling parallel studies to be conducted with a single microarray platform. This is important because the results of microarray experiments can vary depending on the array design and the selection and performance of gene probes on the array. Encouraging results on cross-platform comparisons and between-laboratory reproducibility are now emerging (Bammler et al. 2005; Chu et al. 2004; Irizarry et al. 2005; Larkin et al. 2005; Yauk et al. 2004). Toxicogenomics studies conducted in parallel and comparative systems can demonstrate the biologic relevance of *in vitro* models as surrogates for *in vivo* models without the need to address cross-platform (technologic) issues (Boess et al. 2003; Huang et al. 2003). Although initial efforts should focus on defining simple gene and protein biomarkers for specific compound classes, end points, and model systems, the end goal is to establish a compendium of compound-specific knowledge that transcends technology platform. Ideally, the markers should be robust enough to withstand technologic advances in toxicology that add to the existing knowledge about the compound. Once sufficient and adequately validated data are available, toxicogenomics can become part of a hierarchical approach to compound assessment.

The use of toxicogenomics to identify (screen) compounds with the potential to cause adverse effects may present opportunities to reduce the need for full animal tests, or perhaps refine animal use, and/or reduce the numbers of animals needed when *in vivo* tests are necessary. Of course, the statistical power of any test will influence the number of animals used in an *in vivo* test as well. Screening-type assessments may be appropriate for priority setting, dose setting, chemical ranking, and so forth. The extent of validation required for screening tests may be different than that required for full replacement tests because negative compounds might still undergo full animal testing. Establishing a compendium of compound-specific information will enable regulators and sponsors to access what is known about a compound across multiple test systems, species, and end points, thereby improving the biological relevance of regulatory decisions to safeguard human health and the environment.

Strategy 2: use of gene expression signatures to predict toxicity. Toxicogenomics holds great promise for improving predictive toxicologic assessments. Gene expression profiling has been used to classify compounds by chemical class and mechanism (Hughes et al. 2000; Scherf et al. 2000; Steiner et al 2004; Thomas et al. 2001), tumors by origin and type (Chung et al. 2002), and breast cancer patients for follow-up chemotherapy (van 't Veer et al. 2002). In all cases, classification was based on a set of discriminatory gene elements, between 10 and several hundred, identified from a larger pool of genes on a microarray. The pattern of gene expression, not the measurement of a single or a small set of genes, was the basis for classification. A variety of gene expression analysis algorithms were used to discriminate samples based on gene expression signature. In all cases, the compound class or tumor status was known *a priori*, and gene expression signatures for known samples were used to predict classification for other known but blinded samples (Blower et al. 2002; Brindle et al. 2002). Such models are currently being developed in the private sector (e.g., Gene Logic, Iconix) and are commercially available but cannot, as yet, be exploited by regulators and the scientific community because the underlying data sets and algorithms have not been made available outside the private sector.

Predictive model development will require an extensive "training" set of gene expression measurements for classes of model compounds in a variety of test systems, both *in vivo* and *in vitro*, at multiple doses and time points. Initial studies can be conducted concurrently with conventional testing systems as a way to confirm model predictions. In the short term, it is unlikely that sufficient data will be available for gene expression signatures to replace conventional approaches. Until then, such data can be used as part of a hierarchical approach to toxicity testing in conjunction with accepted methods routinely used for regulatory purposes. In the long-term, sufficient data should accumulate from well-designed validation studies such that gene expression signatures could be part of a battery of tests that reduce or replace animal procedures.

Model validation will necessitate multiple independent data sets and application of sophisticated statistical approaches. Acceptance of these models will require that research and regulatory communities have access to the data analysis tools used to build the models, and that they become familiar with the limitations and uncertainties of using these complex computational models. Confidence in and acceptance of these models will also require rigorous performance standards and appropriate controls to ensure reproducibility and stability over time (see below) and adequate sensitivity and specificity to discriminate toxic from nontoxic responses. Initial model development could easily be accelerated through coordinated sector-spanning efforts. Coordinated efforts across academia, government, and industry partnerships will accelerate progress in defining gene sets that are robust and discriminatory both within and across technology platforms. This is an ideal scenario given the rapidly advancing pace of technology development.

An important aspect of any toxicogenomics validation strategy is the need to measure the range of biological variability of gene responses for a given test system. Ideally, this should be accomplished by one species, tissue, and end point at a time, in order to adequately assess cross-species differences that often hamper risk assessments. Measurements of biologic variability under baseline and toxicant-challenged conditions will enable regulators to better discriminate biologically relevant responses from baseline homeostatic fluctuation. This is an important issue for toxicogenomics, as studies conducted on cell culture populations demonstrate a wide range of biological variability in gene expression measurements for individual cells under both baseline and challenged conditions (Kuang et al. 2004). Therefore, it is necessary to define criteria to adequately address biological variability in a data submission and to establish whether the burden of maintaining these data is that of the regulator or sponsor.

The recommendations related to the biological validation of toxicogenomics-based test methods are listed in Table 1.

Standardization and Validation of Toxicogenomics-Based Methods: Focus on the Technology

Considerations given to validation of the technology encompassed the technical and bioinformatics issues related to the validation of toxicogenomics-based test methods. The starting premise adopted was that with the availability of bioinformatics expertise, biological data generated from toxicogenomics studies could be interpreted with a high degree of confidence. The ultimate aim was to identify a strategic approach that would enable credible biological observations and consequential judicious regulatory decisions, and that this approach would be independent of the toxicogenomic platform used. Moreover, standardization and validation of toxicogenomic platforms were seen as essential for identifying and reducing technologic artifacts. Standardization would also be required to increase the certainty by which biological observations could be extrapolated across and between different microarray platforms. It is therefore important to build on the learning of previous and ongoing efforts in standardization of toxicogenomics (reviewed by Sansone et al. 2004).

Three distinct levels where validation is necessary were identified (see Figure 1 and discussion below). The first level of validation is the responsibility of the array manufacturer or provider and has to be performed only once. This can be seen as a "one-off validation" and relates to both the microarray quality and the instrumentation. The second level of validation is the responsibility of both the experimental toxicologist and the array manufacturer or provider. This can be seen as "routine validation" or best practice to allow data comparability. It encompasses quality control (QC) aspects of the critical experimental components and is a process that occurs on a regularly scheduled basis. The third level of validation, that is, determination of reliability and relevance, is needed every time a change is introduced into the test procedure. Performance standards developed based upon the original test method would serve as the criteria against which the revised method would be compared. Despite these multilevel validation needs, it was repeatedly emphasized that significant technologic development and progress in microarray platforms are still under way and that efforts to validate and standardize these technologic platforms must not be at the expense of innovation.

One-Off Validation

The one-off validation is the responsibility of the array manufacturer or array provider. This is required to ensure that the array platform being used is robust and that the inherent variability within the platform is transparent to the user and the regulator (Figure 1). The following were identified as being necessary for microarray-based toxicogenomics to be used in regulatory assessments:

- Microarrays should be fabricated in accordance with the principles of Good Manufacturing Practice (GMP).
- Specifications and performance criteria for all instrumentation and method components should be available.
- All quality assurance/quality control (QA/QC) procedures should be transparent, consistent, comparable, and reported.
- The array should have undergone sequence verification, and the sequences should be publicly available.
- All data should be exportable in a MAGE (MicroArray and Gene Expression)-compatible format.

Table 1. Recommendations: focus on biological systems.

- Encourage increased use of toxicogenomics-based approaches to define the mechanistic context of toxic responses to exogenous compounds
- Promote greater understanding of the relationships between gene expression responses and altered phenotype, considering the biological pathways affected, dose response, and the point of departure from adaptive to toxic response
- Favor the identification of biomarkers that are independent of technology platform but acknowledge the potential strengths of pathway analysis
- Characterize the range and extent of biological variability of responses for the test systems (e.g., diurnal effects, animal care and use, age-related context)
- Encourage the immediate use of toxicogenomics-based approaches in conjunction with conventional toxicity testing approaches
- Explore the extent to which toxicogenomics can address cross-species responses and specific disease states
- Promote the conduct of parallel and comparative *in vivo* and *in vitro* studies to identify *in vitro* systems that can serve as surrogates for *in vivo* systems
- Characterize predictive toxicology models with respect to parameters such as dose, time, study design, relevance; characterize the system to fulfill validation criteria
- Promote the identification of gene and protein biomarkers as early (prognostic) markers as a refinement to existing toxicity testing methods
- Establish a compendium of toxicant information based on gene expression responses for model compounds across multiple species, end points, and test systems
- Foster the development of effective partnerships between academic, government, and industry groups to promote collaborative efforts to validate toxicogenomics-based test methods and generate sufficient high-quality data to support regulatory decision making

Routine Validation

Routine validation is an ongoing process that is the responsibility of the experimental toxicologist and the array manufacturer or provider (Rockett and Hellmann 2004). Again, for microarray-based toxicogenomic assays to be used in regulatory decision making the following important factors were identified (Figure 1):

- Oligos, cDNAs, or clones that are arrayed should be randomly sequence-verified to ensure that no errors are introduced between batch syntheses. This verification process should be recorded and reported by the manufacturer
- All reagent components should be identified. Reagents should be prepared according to GMP and/or GLP as appropriate. Data regarding batch variability should also be recorded and reported
- Common reference RNA standards (housekeeping genes) should be adopted to facilitate comparison between array platforms. This may be achieved in collaboration with the international Microarray Gene Expression Data (MGED) Society and other related efforts (see below).

Biological standards. Performance standards, test component standards, and QC measures are key components of any validation strategy for a toxicologic test method. Establishing standards is particularly important for gene expression technologies due to the inherent technologic and biological "noise" in these systems. Commonly used biological standards are reference RNAs that are competitively hybridized with the sample of interest in two-channel array formats, and *in vitro* RNA transcripts that are "spiked into" RNA samples of interest in either one-channel or two-channel array formats. Establishing accepted RNA standards will address concerns of regulatory reviewers about data quality and variability within and between laboratories and across different technology platforms. The standards will also provide a common benchmark for regulators to assess platform performance over time. To achieve this goal, we must establish standards that maintain a defined level of accuracy, sensitivity, specificity, and reproducibility across platforms.

Reference RNAs can be derived from tissue extracts, cell lines, or both and serve a variety of purposes. Workshops sponsored by governments and industry have focused on defining the specifications for reference RNAs for clinical and regulatory applications (Joseph 2004). The consensus is a that multiple RNA standards are needed to measure the accuracy, dynamic range, sensitivity, and specificity of varied technology platforms under varied conditions. Important questions are whether regulatory agencies will define preferred sources of RNA standards, and, if so, who will generate and maintain baseline information about these

standards. Although the selection of a given RNA standard depends primarily on the purpose and application, all RNA standards should be tested for a clearly defined number of copies of a given sequence within an RNA preparation over some linear range (Cronin et al. 2004).

Some initiatives are raising awareness of the effects of variables that might hamper data comparability and are working toward developing best practice guidelines for microarray-based measurements (Hopkins et al. 2004). For example, recommendations for best practice in array normalization, together with performance characteristics in terms of sensitivity, accuracy, and comparability of different array platforms (cDNA and oligo, spotted and *in situ* synthesis), are beginning to emerge together with proposals for transparency and availability through publicly accessible databases (http://www.vam.org.uk). Other initiatives are considering the use of quality metrics for standardizing and validating array-based toxicogenomics measurements. The extent to which such efforts will be pursued and the impact they will have upon the standardization issues that are a necessary prerequisite to the validation exercises remain to be seen.

Quality assurance and Good Laboratory Practice. GLP is intended to promote proper documentation, quality, and authenticity of toxicity test data and is required for data acceptance by regulatory agencies (e.g., U.S. FDA, U.S. EPA). At the international level, GLP has been promulgated under the OECD guidelines program (OECD 1998). As part of the progression toward regulatory acceptance, toxicogenomics experiments should ideally be conducted in accordance with GLP. However, at present, most large-scale toxicogenomics efforts are not arising from GLP-compliant laboratories, and requiring compliance for data submission could greatly hamper the technical advancement of new technologies and retard their migration into the regulatory arena. To avoid discouraging technologic progress while maintaining a level of GLP conformity, it could be argued that for research and technical development and improvement purposes, it might be acceptable if array-based studies could at least measure up to the reporting standards required by GLP. However, with the adoption of the toxicogenomics-based technologies into regulatory decision-making practices, GLP compliance undoubtedly will be expected. Procedural aspects of GLP compliance not currently captured in MIAME-Tox (minimum information about a microarray experiment for toxicogenomics) will need to be identified but can be incorporated over time. Until then, it may be possible to allow for proof-of-principle and prevalidation studies to be conducted in accordance with the "intent" of GLP practices by requiring submitters to adequately document procedures and control measures and make experimental data open to regulatory review. "Best practices" for toxicogenomics can be established until formal procedures are adopted. This may be a more realistic solution that permits the advancement of science while addressing the need for QA and QC.

Validation as a Result of Procedural Changes

This third level of validation is necessary whenever a technical or methodologic change is introduced into the test. Such changes might, on one hand, be restricted to the microarray technology (e.g., modification or addition of sequences to a microarray, changes in data analysis procedures). Alternatively, they could involve the experimental design (e.g., dose, time, cell culture procedures). One consideration is that a distinction between minor and major procedural changes that might be incorporated into a test would help determine the extent of such validation necessary. Additionally, to facilitate the process, performance standards should be defined based upon the original validated test procedure. Minor changes would entail a demonstration of equivalence of results obtained with the modified test to that obtained from the validated test. Major changes would involve the need to define a new set of reference materials to be tested and a more extensive validation. Guidance on the use of performance standards and the elements comprising them have been published (ICCVAM 2003) and have been employed for *in vitro* dermal corrosion assessment methods (ICCVAM 2004). Such guidance can also help facilitate the establishment performance standards for toxicogenomics-based test methods in which procedural modifications have been introduced after an initial validation exercise, thereby providing a basis for the comparison of reliability and accuracy of the modified method relative to the validated and accepted reference test method.

The concept of performance standards was originally developed to evaluate the acceptability (accuracy and reliability) of proposed test methods that are based on similar scientific principles and that measure or predict the same biologic or toxic effect as an accepted (previously validated) test method. Because some regulatory authorities and international test guidelines programs (e.g., OECD) have restrictions regarding the use of proprietary test methods (methods that are copyrighted, trademarked, or patented), performance standards also allow for the development and validation of comparable nonproprietary methods based on performance standards derived from the corresponding proprietary antecedent method. Under these circumstances, performance standards allow the characteristics and functional attributes of a proprietary method or technique to be described and offer a procedure for evaluating the performance of methods claimed to be substantially similar. A method that meets the established performance standards is

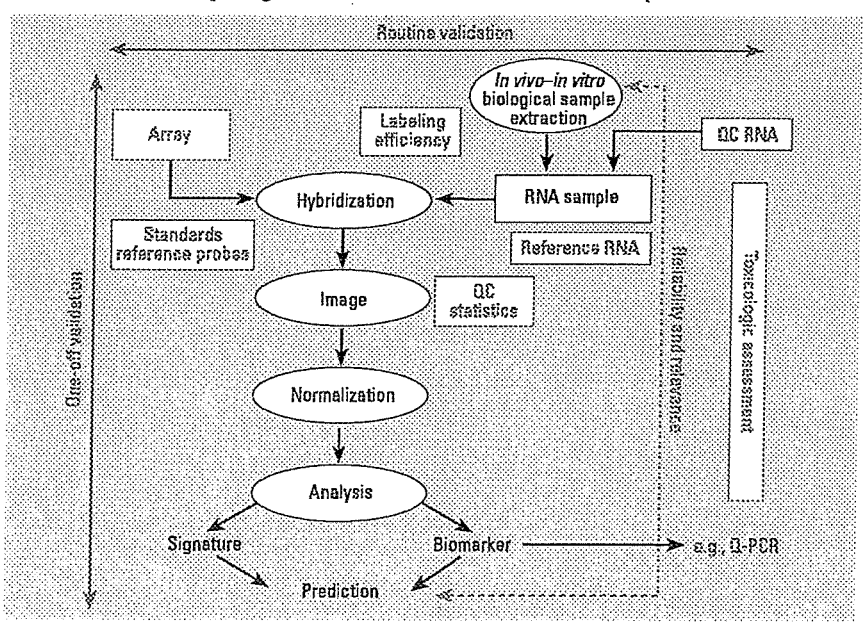

Figure 1. Scheme of the different steps in a toxicogenomics-based test. Three distinct levels were identified where validation is necessary: one-off validation (left), which should be performed once and is mainly related with the quality of the microarray and the instrumentation (blue); routine validation and QC (top), representing the ongoing requirements that are the responsibilities of the experimental toxicologist and the manufacturer (red); and the extent of validation necessary whenever a technical or methodologic change is introduced in the test (right): a method should meet the preestablished performance standards in order to be considered reliable and relevant as the original test method (green). Q-PCR, quantitative PCR.

considered sufficiently accurate and reliable for the specific testing purpose for which it is designed and is viewed as comparable with the original test method upon which it is based. If the correct performance standards have been developed, a method for which the results have the same accuracy and reliability as the original should by definition also be as relevant as the original method.

The conceptual framework and scope of performance standards could be expanded or adapted to include innovations or advancements in areas such as microarray or protein or metabolite separation and identification technology, where proposed improvements might or might not be generally or completely analogous to those in existing systems but would still enable similar applications. Performance standards could still provide a gauge for evaluating newer or revised technologies to ensure that their reliability and accuracy were at least comparable with that of existing acceptable techniques using similar chemicals even if essential test method components (i.e., structural, functional, and procedural elements of a validated test method to which a proposed, mechanistically and functionally similar test method should adhere) were not substantially similar.

This level of validation, which does not imply that a test needs to be completely revalidated, is of extreme importance for tests based on rapidly evolving technologies. It would be a mistake to immobilize these technologies by enforcement of a strict and inflexible validation approach that would hamper progress and test improvement. Finally, a periodic reassessment of a test method's performance (accuracy and reliability) employing established performance standards would help ensure adherence to essential test method components and the reliability and accuracy of the modified test method relative to the validated antecedent method (Hartung et al. 2004). Such assurance could be best established and reported by international validation bodies such as ECVAM and ICCVAM/NICEATM, which could track the history, performance, and validation status of a given test.

Data Management

The lack of robust QC procedures and capture of adequate metadata has caused problems with the analysis and reproducibility of array-based transcriptomics investigations. Consequently, the international MGED Society proposed standards for publication (Nature 2002) that were designed to clarify the MIAME guidelines (Brazma et al. 2001). As a result, a number of journals now require that articles containing microarray experiments must be compliant with the MIAME standard; some also require that the data integral to the article's conclusions be submitted to the ArrayExpress database at the EBI (European Bioinformatics Institute) (Brazma et al. 2003), GEO (Gene Expression Omnibus) at NCBI (National Center for Biotechnology Information) (Edgar et al. 2002), and CIBEX (Center for Information Biology Gene Expression database) at DDBJ (DNA Databank of Japan) (Ikeo et al. 2003)—the European, American, and Japanese database counterparts, respectively.

There is a critical need for public toxicogenomics databases because of the significant volume of data associated with these experiments, the complexity of comparing different gene annotations and splice variants across platforms, and the need for a resource for complex informatics analyses of the traditional toxicology and microarray data in parallel. However, to fully achieve the potential of this emerging interdisciplinary field, it is necessary that we move toward the establishment of a common public infrastructure for exchanging toxicogenomics data (Mattes et al. 2004). The infrastructure should address *a*) the technical problems involved in data upload, *b*) the demand for standardizing data models and exchange formats, *c*) the requirement for identifying minimal descriptors to represent the experiment, *d*) the necessity of defining parameters that assess and record data quality, and *e*) the challenge of creating standardized nomenclature and ontologies to describe biological data. The goal is also to create an internationally compatible informatics platform integrating toxicology/pathology data with transcriptomics, providing the scientific community with easy access to integrated data in a structured standard format, facilitating data analysis and data comparison, and enhancing the impact of the individual data sets and the comprehension of the molecular basis of actions of drugs or toxicants. Ultimately, such a knowledge-base could be maintained (respecting confidentiality as appropriate) as a reference for regulatory organizations to evaluate toxicogenomics and pharmacogenomics data submitted by registrants to those organizations.

The potential exists for the international development of this public infrastructure. As part of the collaborative undertaking with the International Life Sciences Institute Health and Environmental Sciences Institute (ILSI–HESI) Technical Committee on the Application of Genomics to Mechanism Based Risk Assessment (http://www.hesiglobal.org/committees), the European Molecular Biology Laboratory of the European Bioinformatics Institute (EMBL–EBI; Brazma et al. 2003; http://www.ebi.ac.uk/microarray/Projects/toxnutri/index.html), the National Institutes of Health/National Institutes of Health National Institute of Environmental Health Sciences National Center for Toxicogenomics (NCT; Waters et al. 2003; http://www.niehs.nih.gov/nct/), and the U.S. FDA NCT (Tong et al. 2003; http://www.fda.gov/nctr/science/centers/toxicoinformatics/index.htm) have worked closely together. The respective databases are based on the international standards developed by the MGED Society (Brazma et al. 2001; Spellman et al. 2002). After the very favorable response that the MIAME received from the microarray community and key scientific journals (Ball et al. 2002, 2004; Nature 2002), the MIAME checklist was extended to describe array-based toxicogenomics experiments. The MIAME-Tox checklist (MGED 2004) is an attempt to define the minimum information required to interpret unambiguously and potentially reproduce and verify array-based toxicogenomics experiments. MIAME-Tox also supports a number of other objectives, for example, linking data from different experimental domains within a study and linking several studies from one institution and exchanging toxicogenomics data sets among public databases. The major objective of MIAME-Tox is to guide development of toxicogenomics databases and data management software. Without a sufficient depth of data in these resources, the scientific community's opportunity to develop consensus on analysis and application of these data for risk assessment or screening may be limited. The availability of this level of information regarding platform specification, appropriate common reference standards, and the toxicologic study alone will facilitate the predictive value of toxicogenomics across different array-based platforms. This, in turn, will result in a greater appreciation of and confidence in the value of toxicogenomics within a regulatory context, such that testing strategies can be optimized, predictive alternative models can be identified, and animal use can be reduced (Supplemental Material, Section 2; http://ehp.niehs.nih.gov/members/2005/8247/suppl.pdf).

Moreover, the long-term provision of a MIAME-Tox–compliant database with a MAGE-ML (Microarray Gene Expression Markup Language) export is required for the long-term storage of toxicogenomics data. This would directly support the role of ECVAM, ICCVAM/NICEATM, and other validation bodies in the validation of toxicogenomics-based test methods.

The recommendations related to the technical and bioinformatics aspects of validation are listed in Table 2.

Regulatory Acceptance of Validated Toxicogenomics-Based Methods

Regulatory scientists are increasingly being called upon to consider incorporation of toxicogenomics data in regulatory assessment processes that involve evaluation of potential human health or environmental hazard and risk. Those scientists will need to be able to

judge the level of confidence to place in both *in vivo* and *in vitro* toxicogenomics-based test methods and the resulting data that might be submitted in support of regulatory decision making. Whether a method has been determined to be valid for a specific purpose will be an important factor for the consideration of its use for regulatory purposes. Furthermore, the level of confidence held by regulators will influence regulatory acceptance of methods and data, and will affect both the further pursuit of toxicogenomics technologies and technologic improvements and the extent of industry application of these technologies.

Potential uses of toxicogenomics data in the regulatory area. The potential of toxicogenomics-based methods in contributing to regulatory assessment processes is broad. Examples might include, but would not be limited to, obtaining microarray data from individual *in vivo* bioassays or *in vitro* cell or tissue-based assays or from batteries of assays, using conventional or high-throughput approaches. In accordance with the current developing state of the science, realistic possibilities for initial uses of toxicogenomics data in regulatory settings might be first in the realm of hazard assessment, such as to support chemical mechanism of action arguments. Other early uses might include aiding individual chemical/chemical mixture screening or ranking exercises to set priorities for toxicity testing or to sort chemicals into batches. These types of applications might involve identification of individual genes or gene patterns associated with particular toxic effects or pathways, adaptive responses, or metabolic pathways. However, global pattern recognition–type techniques are, as yet, not considered to be ready to fully replace traditional bioanalytical methods for predicting toxicity or elucidating information on mechanism of action or biochemical pathway component identification.

Using only human or animal *in vitro* or *in vivo* data derived from toxicogenomics technology to estimate such parameters as adverse/no adverse effect levels or to determine dose–response relationships for conducting risk assessments is regarded as a much longer term goal. However, for hazard assessment purposes, the possibility of considering toxicogenomics data along with other types of toxicologic information and data [e.g., from *in vivo* and *in vitro* studies, determinations of quantitative structure–activity relationships (QSAR) or SAR] in a weight-of-evidence approach on a case-by-case basis was not discounted. Regulatory bodies have begun to craft preliminary proposals, policies, and guidance for the submission and use of omics-type data in regulatory deliberations and to provide encouragement for the use and further development of the technology (U.S. EPA 2002; U.S. FDA 2005). Additionally, organizations such as the OECD are actively working with member countries on approaches that seek to harmonize the use of omics-derived information for hazard assessment related to health and environmental effects.

Harmonization of toxicogenomics-based test methods will first necessitate the standardization and validation of the specific test protocol(s) developed for a specific purpose(s), as conducted by international validation bodies such as ECVAM and ICCVAM/NICEATM. It will then be important for such organizations to interface with the OECD to ensure the appropriate crafting of harmonized OECD toxicogenomics-based test guidelines that are based upon standardized, adequately validated procedures, that are considered practical, and that permit consistent regulatory judgments.

Case for a modular approach to validation. Because of the extraordinary rate at which toxicogenomics technologies are evolving, current validation processes might need to adapt so as to accommodate the rapidly developing changes and advancements while still observing the basic tried-and-true validation principles. To meet this anticipated need, a modular approach to validation (Hartung et al. 2004) was considered, not to abridge the process but to allow for more flexibility in data collection and evaluation throughout the progressive changes that the technology will undergo. Typically, in the conventional validation procedures for an alternative test method, a sequential approach to the process is taken. The test protocol is first optimized and its transferability is determined. The resulting standardized method is then evaluated for within-lab and between-lab reproducibility and for its accuracy. Thus, an optimized, standardized protocol linked to specific test method elements and a prediction of outcome for given classes of chemicals are evaluated together for performance characteristics and applicability. Such a linear validation model, although effectively employed for other test methods, might not be optimal for dynamic test methods in which changes are rapidly introduced that improve or alter the protocol or the technology incorporated in the protocol in any substantive way. The linear validation model might result in unnecessary delays in incorporating innovations into toxicogenomics-type test methods. In contrast, with a modular approach to validation, which capitalizes on the fundamental classic concepts of validation as defined by ECVAM and ICCVAM (Balls et al. 1995; ICCVAM 1997, 2003), the different steps in the validation process are subdivided into independent modules, each of which can be assessed individually so that those components that have been completed need not undergo repeated validation. Further validation activities would instead be directed to only that part of the process flow where needed. The proposed model would accommodate validation of innovation affecting only a particular part of the sequence such that incorporation of advancements in a particular sector into testing strategies would less likely be impeded. At the same time, a modular approach to validation could efficiently handle information/data gaps that could be filled over time without derailing the validation stages already achieved. The modular approach, complemented with the use of performance standards (see "Validation as a Result of Procedural Changes" above), is expected to facilitate and help expedite the validation of the toxicogenomics technology and test methods that are based on toxicogenomics.

The modular approach follows the fundamental classic concepts of validation as defined by ECVAM and ICCVAM. Validation is defined as the process by which the relevance and reliability of a test method for a specific purpose are determined (Balls et al. 1995; ICCVAM 1997, 2003). Adequate validation

Table 2. Recommendations: focus on technology.

- Validation and QA/QC should be mandatory during the manufacturing of the arrays
- The array should undergo sequence verification and sequences should be available in the public domain
- MIAME guidelines should be adhered to
- Initially, develop "best practices" for toxicogenomics, including the interpretation of data and how to manage uncertainties and limitations
- Subsequently develop guidance for and adherence to GLPs for toxicogenomics experiments
- Common reference standards should be considered
- A workshop should be convened to address the development of standards for RNA sample preparation (and other biologic aspects of microarray analyses)
- Develop a "common" RNA standard including developing consensus about sources and maintenance of baseline data for regulatory and research purposes
- Studies should be MIAME-Tox compliant
- Performance standards should be developed and implemented to evaluate reliability and accuracy of test methods incorporating procedural modifications
- An ongoing dialogue should be maintained between scientists in the various relevant disciplines, including bioinformaticians, through meetings, published papers, and advisory/discussion panels (e.g., ILSI-HESI committee, NCT consortium, OECD panel)
- Ensure that validation efforts and QA/QC criteria are not restrictive to the technology or its advancement
- Explore whether toxicogenomics measurements can define toxicologic effects quantitatively
- Develop prediction models (e.g., algorithms) for toxicogenomics-based test methods
- Develop a data infrastructure for capturing, storing, and reporting toxicogenomics data
- Ensure continuation of financial support for long-term public database maintenance