curve (Fig. 4C). In Figure 4C, H0 through H5 indicate p53 heterozygous-deficient mice exposed to graded increases in dose of radiation (0–5 Gy), which show more steeper and shortened lifespans. Similarly, benzene-induced leukemias make the lifespan curve in the Gompertzean expression also steeper with benzene exposure (Fig. 4D). Although these slopes seem to be based on the incidence of various types of tumorigenesis, the slopes may be modified by other chronic factors, such as nutrition related to cardio- and/or renal-vascular diseases. Two prevention studies provide interesting prevention curves in Gompertzean expression; one on caloric restriction in radiation-induced leukemogenesis [9] and the other on thioredoxin overexpression against benzene toxicity [31], which shows potentially equivalent antioxidative functions (Fig. 4E and F). Gompertzean expression curves for toxic compounds and for inhibitory compounds shown in Figure 4C to F imply that the slopes for Gompertzean expression may be based on the model shown in Figure 4B (Fig. 4E corresponds to "T+P (Tox & Prevention)" in Fig. 4B; Fig. 4F corresponds to ideal prevention, "Id 2" in Fig. 4B.). The significance of differences in slopes in Gompertzean expression among the non-treated animals, animals treated with toxic compounds, and animals treated with inhibitory compounds, and possible deterministic factors for genomic stabilization, cell-cycle regulators and active caloric metabolic enzymes, among others, are not clearly understood. However, this relevance would be an important factor for elucidating the mechanism underlying toxicities vs prevention of lifespan.

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Membrane Channel Connexin 32 Maintains Lin$^-$/c-kit$^+$ Hematopoietic Progenitor Cell Compartment: Analysis of the Cell Cycle

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Abstract Membrane channel connexin (Cx) forms gap junctions that are implicated in the homeostatic regulation of multicellular systems; thus, hematopoietic cells were assumed not to express Cxs. However, hematopoietic progenitors organize a multicellular system during the primitive stage; thus, the aim of the present study was to determine whether Cx32, a member of the Cx family, may function during the primitive steady-state hematopoiesis in the bone marrow (BM). First, the numbers of mononuclear cells in the peripheral blood and various hematopoietic progenitor compartments in the BM decreased in Cx32-knockout (KO) mice. Second, on the contrary, the number of primitive hematopoietic progenitor cells, specifically the Lin$^-$/c-kit$^+$/Scal$^+$ fraction, the KSL progenitor cell compartment, also increased in Cx32-KO mice. Third, expression of Cx32 was detected in Lin$^-$/c-kit$^+$ hematopoietic progenitor cells of wild-type mice (0.27% in the BM), whereas it was not detected in unfractionated wild-type BM cells. Furthermore, cell-cycle analysis of the fractionated KSL compartment from Cx32-KO BM showed a higher ratio in the G_2/M fraction. Taken together, all these results imply that Cx32 is expressed solely in the primitive stem cell compartment, which maintains the stemness of the cells, i.e., being quiescent and noncycling; and once Cx32 is knocked out, these progenitor cells are expected to enter the cell cycle, followed by proliferation and differentiation for maintaining the number of peripheral blood cells.

Keywords Connexin 32 · Hematopoiesis · Hematopoietic stem cell · Cx32-knockout mouse

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Introduction

Connexin (Cx) functions in the organization of cell-cell communication via gap junctions in multicellular organisms. Gap junctions have been implicated in the homeostatic regulation of various cellular functions, including growth control and differentiation (Loewenstein, 1979), apoptosis (Wilson, Close & Trosko, 2000) and the synchronization of electrotonic and metabolic functions (Bruzzone, White & Paul, 1996).

The role of Cxs in hematopoietic organs is poorly understood, except that the expression of Cx43 between hematopoietic progenitor cells and bone marrow (BM) stromal cells sustains hematopoiesis (Rosendaal, Gregan & Green, 1991; Ploemacher et al., 2000; Cancelas et al.,

2000; Montecino-Rodriguez, Leathers & Dorshkind, 2000). As Cxs are essential molecules for multicellular organisms, Cxs that organize cell-cell communication within the hematopoietic progenitor cell compartment are surmised to be present in BM tissue. If Cxs are present among hematopoietic progenitor cells, what would be their functions?

Krenacs & Rosendaal (1998) previously reported that Cx32 is not expressed in the BM. Therefore, if Cx32 is expressed in the blood cells, such Cx32-expressing cells would likely be, e.g., solely hematopoietic stem/progenitor cells. Such a specific study was supposed to be supported by the use of knockout (KO) mice for specific Cx molecules. Consequently, we found a functional impairment of the BM in Cx32-KO mice in our benzene exposure experiment (Yoon et al., 2004).

Cx32-KO mice were first established in 1996 by Willecke (Nelles et al., 1996). Using these Cx32-KO mice, an analysis of the possible functions of Cx32 in hematopoietic stem/progenitor cells was conducted using a reverse biological approach. Cx32-KO mice showed decreased numbers of peripheral mononuclear cells, various progenitor cell compartments and an increased primitive stem cell fraction, such as the lineage marker-negative (Lin^-)/c-kit-positive ($c\text{-}kit^+$)/stem cell antigen-1-positive ($Sca1^+$) (=KSL) fraction. On the contrary, in wild-type mice, expression of Cx32 was detected by immunocytochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR), although it was not detected in unfractionated wild-type BM cells. Subsequent cell-cycle analyses, one for colony-forming progenitors using the method for evaluation of cycling progenitor cells with incorporation of bromodeoxyuridine (BrdUrd) followed by exposure to ultraviolet A (UVA) (see, BUUV Assay in Materials and Methods) and the other using a cell sorter with Hoechst 33342 for the KSL fraction, showed a significant increase in the ratio of the cell-cycle fraction in both compartments in the BM of Cx32-KO mice. The functions of Cx32, which is expressed in primitive hematopoietic stem/progenitor cells, are likely restoration of stem/progenitor cell quiescence and maintenance of primitive stem cells to prevent exhaustion.

Materials and Methods

Experimental Animals

Cx32-KO mice ($Cx32^{-/-}$ or $Cx32^{-/Y}$) were genetically modified from the F_1 embryonic cell line 129/J and the C57BL/6 strain developed by Willecke (Nelles et al., 1996). Heterozygous mice ($Cx32^{-/+}$) backcrossed with the C57BL/6 strain and maintained at the animal facility of the National Institute of Health Sciences (NIHS), Tokyo, Japan, were used. The pups were genotyped by PCR for screening of DNA from their tails.

Eight-week-old C57BL/6 male mice from Japan SLC (Hamamatsu, Japan) were used for the colonization assay. All experimental protocols involving laboratory mice in this study were reviewed by a peer review panel, the Interdisciplinary Monitoring Committee for the Right Use and Welfare of Experimental Animals, established at the NIHS, and approved by the Committee for Animal Care and Use at the NIHS with the experimental code 224-37009639415-2002.

Blood and BM Separation

The numbers of peripheral white blood cells, platelets and red blood cells were measured using a Coulter counter (Sysmex K-4500; Sysmex, Kobe, Japan). BM cells were harvested from the femur of each mouse (Yoon et al., 2001) after the animals were killed by cervical dislocation under deep anesthesia with ethyl ether. A 26-gauge needle was inserted into the femoral bone cavity through the proximal and distal ends of the bone shafts, and BM cells were flushed out under pressure by injecting 2 ml of α-minimum essential medium (α-MEM) with ribonucleosides and deoxyribonucleosides (Invitrogen, Carlsbad, CA).

Antibodies and Immunomagnetic Bead Separation

For the depletion of differentiated (lineage marker-positive) cells from BM cells, immunomagnetic bead separation (BD IMag Mouse Hematopoietic Progenitor Cell Enrichment™ set; BD Biosciences, San Jose, CA) or immunobead density gradient separation (SpinSep™; StemCell Technologies, Vancouver, Canada) was performed. As for lineage (Lin) markers, a biotinylated antibody cocktail (BD Biosciences) containing anti-mouse CD3e (145-2C11), CD11b (M1/70), CD45R/B220 (RA3-6B2), Ly-6G and Ly-6C/Gr-1 (RB6-8C5) and TER-119/erythroid cell (TER-119) antibodies and a monoclonal antibody cocktail (SpinSep) containing anti-CD5/Ly-1, CD45R, CD11b/Mac-1, Ly-6G/Gr-1, TER119 and 7/4/neutrophil antibodies were used. As a secondary antibody for the former biotinylated antibody cocktail, streptavidin (StAv)-coated beads (BD Biosciences) for depletion and StAv-peridinin chlorophyll-a protein (PerCP, BD Biosciences) for visualization were used. For the latter cocktail (SpinSep), an optimized combination antibody cocktail against it that had been coated on dense microparticles, i.e., SpinSep Mouse Dense Particles (StemCell Technologies), was used for immunoprecipitation.

For enrichment of the $c\text{-}kit^+$ fraction by immunomagnetic bead separation, CD117/c-kit-conjugated phycoerythrin (PE, StemCell Technologies) was used as a progenitor

marker and, as a secondary antibody, an anti-PE tetrameric antibody complex (StemCell Technologies) was used.

For detection of Cx32-positive cells by flow cytometry, a mouse anti-Cx32 monoclonal antibody from two sources (Chemicon International, Temecula, CA; Santa Cruz Technology, Santa Cruz, CA) as a primary antibody and an anti-mouse immunoglobulin (Ig) conjugated with fluorescein isothiocyanate (FITC) as a secondary antibody (BD Biosciences) were used.

For cell-cycle analysis by flow cytometry, as lineage markers, the same antibody cocktails from BD Biosciences were used. In addition, CD117/c-kit conjugated with allophycocyanin (APC), stem cell antigen (Sca1) antibody conjugated with PE and an AT-rich DNA-binding dye, Hoechst 33342 (Sigma, St. Louis, MO), were used.

Immunohistochemical Analysis

The same anti-Cx32 antibody (Chemicon International) was used as the primary antibody. As for the secondary antibody, a biotinylated horse anti-mouse Ig G (Vector Laboratories, Burlingame, CA) was used, and streptavidin labeled with peroxidase and 3,3′-diaminobenzidine was used to detect immunoreactivity (Vector Laboratories).

Enrichment of BM Cells in Lin^-/c-kit^+ Fraction

The Lin^-/c-kit^+ fraction is rich in hematopoietic stem cells (HSCs). To obtain a large number of Lin^-/c-kit^+ progenitor cell-enriched fraction in BM cells, a combination of immunobead density gradient and immunomagnetic bead separation techniques was carried out. First, for the depletion of lineage-positive BM cells, harvested BM cells were processed through an immunobead density gradient using a density-matched medium and dense microparticles coated with a cocktail of an optimized combination of antibodies called SpinSep (StemCell Technologies). Second, for the selection of the c-kit^+ fraction, immunomagnetic bead separation using magnetic nanoparticles with a magnetic holder was carried out according to the manufacturer's instruction (StemCell Technologies). For each procedure, the antibodies used are described under Antibodies and Immunomagnetic Bead Separation, above.

Flow-Cytometric Analysis using Anti-Cx32 Antibody

BM cells with or without fractionation for Lin^-/c-kit^+ HSC enrichment were stained with the biotinylated antibody cocktail of StAv-PerCP, c-kit-PE, the anti-Cx32 antibody and anti-mouse IgG conjugated with FITC. Flow-cytometric analysis was carried out using FACSVantage and FACSAria (both from BD Biosciences).

Flow-Cytometric Analysis for Cell Cycle of KSL Fraction

Lineage-depleted BM cells were stained with the biotinylated antibody cocktail with StAv-PerCP, c-kit-APC, Sca1-PE and Hoechst 33342. Flow-cytometric analysis was carried out using FACSAria.

BUUV Assay

Hematopoietic progenitor cell-specific kinetic studies were evaluated by continuous labeling by an osmotic minipump (Alza, Palo Alto, CA) of BrdUrd for cycling cells, followed by UVA exposure and hematopoietic colonization assay (BUUV assay, details in Hirabayashi et al., 1998, 2002).

Irradiation

In the assay of hematopoietic progenitor cells, recipient mice were exposed to a lethal radiation dose of 915 cGy, at a dose rate of 124 cGy per minute, using a ^{137}Cs-gamma irradiator (Gammacell 40 Exactor; MDS Nordin, Ottawa, Canada) with a 0.5-mm aluminum-copper filter.

Assay for Colony-Forming Units in Spleen

The Till & McCulloch (1961) method was used to determine the number of hematopoietic spleen colonies, i.e., colony-forming units in spleen (CFU-S), formed by hematopoietic progenitor cells. Aliquots of a BM cell suspension were used for evaluating the numbers of CFU-S. Spleens were harvested 9 or 13 days after BM transplantation for determining the number of CFU-S-9 or CFU-S-13 and then fixed in Bouin's solution. Macroscopic spleen colonies were counted under an inverted microscope at ×5.6. It was previously shown, using the C57BL/6 strain, that all colonies visible on days 9 and 13 originate from transplanted BM cells under the condition that the recipient mice are exposed to a lethal radiation dose of 915 cGy (Hirabayashi et al., 2002).

Assay for Granulocyte-Macrophage Colony-Forming Units

Granulocyte-macrophage colony-forming units (CFU-GM) were assayed in semisolid methylcellulose culture (Yoon et al., 2001; Hirabayashi et al., 2002). Briefly, 8×10^4 BM cells suspended in 100 μl of α-MEM were added to 3.9 ml of culture medium containing 1% methylcellulose (Nakarai-Tesque, Kyoto, Japan), 30% fetal calf serum (HyClone Laboratories, Logan, UT), 1% bovine serum albumin (Sigma), 10^{-4} M mercaptoethanol (Sigma) and 10 ng/ml murine granulocyte-macrophage

colony-stimulating factor (GM-CSF; R&D Systems, Minneapolis, MN). One-milliliter aliquots containing 2 × 10^4 cells were placed in 35-mm tissue culture wells (Nalgen Nunc International, Rochester, NY) in triplicate and incubated for 6 days in a fully humidified incubator at 37°C with 5% CO_2 in air. Colonies were counted using an inverted microscope at ×40 (Olympus Optical, Tokyo, Japan).

PCR Analysis for Genotyping

To detect Cx32 wild-type and Cx32-KO alleles, PCR analysis was performed using genomic DNA from the tail of each mouse, and synthetic oligonucleotides were used as primers (Nelles et al., 1996). To detect the wild-type allele, a 5′ primer (ccataagtcaggtgtaaaggagc) and a 3′ primer (a-gataagctgcagggaccatagg) were used; to detect the Cx32-KO allele, a common 5′ primer and a *neo*-primer (at-catgcgaaacgatcctcatcc) were used.

Reverse Transcription and PCR Analysis of Cx32 Expression

Expression of the gene encoding Cx32 was analyzed by reverse transcription followed by PCR. The total RNA from BM cells and other tissues was isolated using a Qiagen RNAeasy kit (Qiagen, Valencia, CA).

Statistical Analysis

The data obtained were stored in a computer and processed for statistical analysis using Student's *t*-test to evaluate the significance of differences in blood cell count, BM cellularity and the numbers of progenitor cells, CFU-GM, CFU-S-9 and CFU-S-13 between the wild-type group and the KO group. Differences with $p < 0.05$ were considered significant.

Results

Expression of Cx32 in Bone Marrow

Table 1 shows various blood cell parameters for the wild-type and Cx32-KO mice, with body weight and spleen weight as references. Although the total numbers of BM cells and red blood cells did not significantly differ between the wild-type mice and the Cx32-KO mice, the numbers of white blood cells and platelets from the peripheral blood, CFU-S-13 (primitive hematopoietic progenitor cells), CFU-S-9 (differentiated progenitor cells) and CFU-GM (progenitor cells cultured *in vitro*) were all significantly lower in the Cx32-KO mice than in the wild-type mice. These results suggest that the Cx32-KO mice have a potential disadvantage in hematopoiesis. However, when we studied the expression of Cx32 in BM cells by RT-PCR, as shown in Figure 1, neither the expression of Cx32 in the spleen (*not shown*) nor that in the BM was detected except in the positive known control, the hepatic tissue. Thus, the negative expression of Cx32 in BM cells is in good agreement with a previous observation (Krenacs & Rosendaal, 1998).

Table 1 Parameters associated with steady-state hematopoiesis

Parameter	Wild-type	Cx32-KO
Body weight (g)	22.6 ± 1.97	22.5 ± 1.77
Spleen weight (mg)	77.8 ± 17.7	88.3 ± 9.6
BM cellularity ($\times10^7$/femur)	2.28 ± 0.23	2.15 ± 0.08
Peripheral blood cells		
Red ($\times10^7$/ml)	960 ± 30.8	930 ± 50.4
White ($\times10^4$/ml)*	7,300 ± 283	5,633 ± 569
Platelets ($\times10^7$/ml)*	67.6 ± 0.14	48.7 ± 0.93
Hematopoietic progenitor cells		
CFU-GM ($\times10^2$/femur)*	387 ± 33.5	251 ± 27.4
CFU-S-9 ($\times10^2$/femur)*	45.8 ± 4.78	32.7 ± 5.23
CFU-S-13 ($\times10^2$/femur)*	27.7 ± 3.35	21.1 ± 2.85

Each value is expressed as average (n = 6 for each genotype) ± standard deviation except for the value of the hematopoietic progenitor cells. The numbers of hematopoietic progenitor cells in steady-state CFU-GM, day-9 spleen colonies (CFU-S-9) and day-13 spleen colonies (CFU-S-13) are expressed as average (three donor mice were used for each genotype, and six mice were used for each recipient group) ± standard deviation

* The difference calculated by *t*-test between wild-type and Cx32-KO is significant ($p < 0.05$)

We next studied Cx32 expression in colonies developed in the spleen in lethally irradiated wild-type recipient mice after injection of BM cells from wild-type mice or from Cx32-KO mice. Hematopoietic spleen colonies consist of a large number of immature cells rather than cells from the peripheral blood or unfractionated BM cells (Hirabayashi et al., 2002). Expression of Cx32 detected by RT-PCR analysis was only observed in the hematopoietic spleen colonies derived from wild-type BM cells (Fig. 1, lanes A1, A2). Expression of Cx32 was not detected in colonies derived from Cx32-KO BM cells, which are negative controls (Fig. 1, lanes B1, B2). Expression of Cx32 was also detected in spleen colonies from Cx32-KO recipient mice that had been repopulated with wild-type BM cells (Fig. 1, lanes C1, C2).

Immunohistochemical staining with the anti-Cx32 antibody was carried out to examine the hematopoietic spleen colonies originating from BM cells from wild-type mice and from Cx32-KO mice. A colony originating from a wild-type BM cell showed mild and mottled staining in the outer boundary of the spleen colonies, whereas a colony originating from Cx32-KO BM cells showed no staining

Template	None		Liv		BM		A1		A2		B1		B2		C1		C2	
RT	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
Cx32 (569bp)																		
β-actin(226bp)																		

Fig. 1 Expression of Cx32 in BM and hematopoietic spleen colonies. Total RNAs were extracted for RT-PCR from the liver (*Liv*) and BM of wild-type mice and CFU-S-9. Note that Cx32 expression was not detected in the BM but was detected in the liver, which is a positive control (see Materials and Methods). To obtain CFU-S, lethally irradiated wild-type mice were injected with BM cells from wild-type or Cx32-KO donor mice. After 9 days, total RNAs extracted from individual hematopoietic spleen colonies derived from wild-type BM cells or those from Cx32-KO BM cells were reverse-transcribed, followed by PCR and then loaded (lanes *A1*, *A2*, *C1* and *C2*). Also, total RNAs extracted from the colonies derived from wild-type BM cells obtained from lethally irradiated Cx32-KO recipient mice followed by repopulation with wild-type BM cells were similarly loaded (lanes *B1* and *B2*). RT(+) and RT(-): with or without avian reverse transcriptase, 2.5 U/20 μl, respectively (see Materials and Methods)

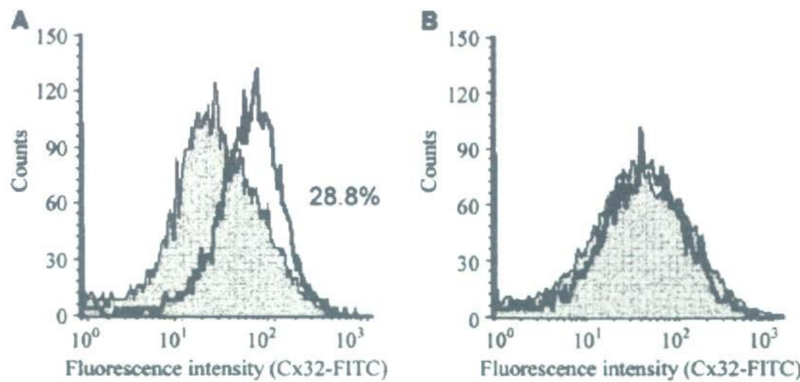

Fig. 2 Flow-cytometric analyses of Lin$^-$/c-kit$^+$ Cx32-positive cells from wild-type mice. Flow cytometry after BM cell separation was carried out by a combination of immunobead density gradient separation and immunomagnetic bead separation. Histogram of FITC-labeled anti-Cx32 antibody. Lin$^-$/c-kit$^+$ fraction (**a**) and Lin$^+$/c-kit$^-$ fraction (**b**) for wild-type BM cells (*open profile with bold line*) and same fractions for Cx32-KO BM cells (*shaded profile*), negative control. The Cx32-positive fraction shown in **a** calculated for the Lin$^-$/c-kit$^+$ fraction in wild-type BM cells is 28.8%

(*data not shown*). The findings described above suggest expression of Cx32 in the hematopoietic progenitor cells or stem cells alone; thus, further precise experiments were conducted.

Expression of Cx32 in Lin$^-$/c-kit$^+$ Hematopoietic Progenitor Cell Compartment

We determined whether Cx32-positive cells are consistently found in the HSC compartment. First, the Lin$^-$/c-kit$^+$ HSC-enriched fraction was obtained by the combination of immunobead density gradient separation for depleting lineage-positive cells and immunomagnetic bead separation for selecting c-kit$^+$ cells, followed by flow-cytometric analysis using the anti-Cx32 antibody. The separated Lin$^-$/c-kit$^+$ HSC fraction was 0.25% of the original unfractionated wild-type BM cells. The proportion of the Lin$^-$/c-kit$^+$ compartment (HSC compartment) is 90.2% of the Lin$^-$/c-kit$^+$ HSC-enriched preseparated fraction. Furthermore, the number of Lin$^-$/c-kit$^+$ compartments is 106.9-fold higher than the fraction of the original unfractionated BM cells. To determine which fraction Cx32-positive cells belong to, BM cells from wild-type mice and Cx32-KO mice with or without Lin$^-$/c-kit$^+$ HSC enrichment were stained with biotinylated antibody cocktail labeled with StAv-PerCP, c-kit-PE and Cx32-FITC. In wild-type BM cells, 28.8% of the Lin$^-$/c-kit$^+$ fraction was found to be Cx32-positive compared with the same fraction of BM cells obtained from Cx32-KO mice, which was used as the negative control (Fig. 2a). Together with the frequency data for the Lin$^-$/c-kit$^+$ HSC-enriched fraction, the fraction of Cx32-positive cells was calculated to be nearly 0.27% of the original unfractionated whole BM cells.

Whether the mature cell fraction, i.e., the Lin$^+$/c-kit$^-$ fraction, contains Cx32-positive cells, the fraction of the wild-type BM cells is compared with that of the control profile from the Cx32-KO mice. Because both fractions are nearly identical (Fig. 2b), few cells may be positive for Cx32 in the Lin$^+$/c-kit$^-$ fraction. The fraction of Cx32-positive cells is 0.0093% of the original unfractionated whole BM cells (*data not shown*).

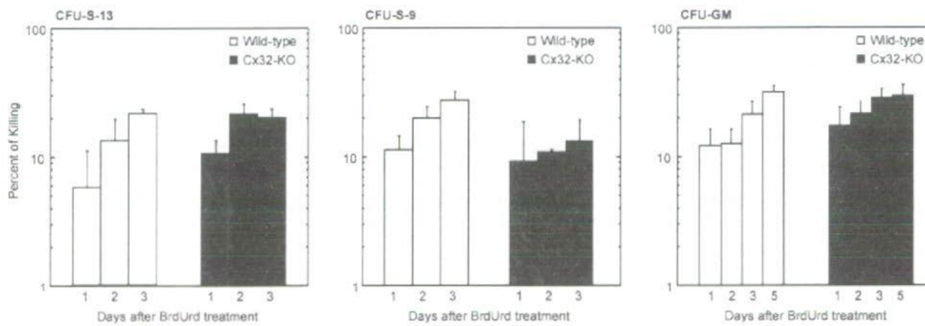

Fig. 3 The BrdUrd-labeled cells with an osmotic minipump purged by UVA light (BUUV) assay for evaluating the cycling fractions of the hematopoietic colonizing progenitor cells. Percent decreases in number of colonies compared with nonexposed control are shown along the ordinate axis (log.) vs. days for continuous labeling of BrdUrd with osmotic minipumps shown along the horizontal axis. CFU-S-13 (primitive hematopoietic progenitor cells), CFU-S-9 (differentiated progenitor cells) and CFU-GM (progenitor cells assayed by *in vitro* colonization) are shown. Each column represents 10 mice assayed for CFU-S-13 and six mice assayed for CFU-S-9

Function of Cx32 in Cell-Cycle Regulation in Hematopoietic Progenitor/Stem Cells

A significant decrease in the number of hematopoietic progenitor cells was observed in the Cx32-KO mice but without any significant difference in the decrease in BM cell number (Table 1), suggesting cell-cycle perturbation in the hematopoietic progenitor cells or stem cell compartment. Whether cell cycles are accelerated or decelerated in either the hematopoietic progenitor cell fractions or the hematopoietic stem cell compartment or both is not known. To characterize hematopoietic progenitor-specific cell cycle, the BUUV assay was conducted. To observe possible changes in the cell cycle in the hematopoietic stem cell compartment, the KSL fraction was assayed with Hoechst 33342 and possible changes in the ratio of G_0/G_1 were evaluated.

BUUV assay Hematopoietic stem cell-specific kinetics evaluation by continuous infusion of BrdUrd for cycling cells including hematopoietic progenitor cells followed by UVA exposure and hematopoietic progenitor colonization assay was conducted.

Results are shown in Figure 3. For CFU-S-13 (primitive hematopoietic progenitor cells), the incorporation of BrdUrd starts from a higher percentage with rapid increase in Cx32-KO mice, suggesting suppression of the cell cycle in these primitive hematopoietic progenitor cells with Cx32-mediated cell-cycle regulation in the wild-type steady state. This suppression may be attenuated in CFU-S-9, a differentiated progenitor cell compartment. For CFU-GM, the progenitor cells assayed by *in vitro* colonization also showed an accelerated cell cycle in Cx32-KO mice. The population doubling time calculated for each progenitor cell compartment is shown in Table 2.

Flow-cytometric analysis of KSL fraction Following the incorporation of the bioactive AT-rich DNA-binding dye Hoechst 33342, the lineage-depleted BM cells were analyzed by flow cytometry. The sizes of the $Lin^-/c\text{-}kit^+/Sca1^+$ (KSL) fraction obtained were 0.052% in the Cx32-KO BM cell compartment and 0.035% in wild-type BM cells (Table 3, Fig. 4a; $p = 0.0458 < 0.05$). The lineage-depleted BM cells were analyzed for their cell-cycle patterns by flow cytometry (Fig. 4b,c), and then G_0/G_1 was calculated for the $Lin^-/c\text{-}kit^+$ and KSL fractions for both the Cx32-KO and wild-type mice. The percentage of G_0/G_1 calculated for the $Lin^-/c\text{-}kit^+$ and KSL fractions were slightly lower in Cx32-KO mice (Table 4; 83.3% vs. 87.2% for Cx32-KO vs. wild-type for the $Lin^-/c\text{-}kit^+$ fraction, 89.2% vs. 91.5% for Cx32-KO vs. wild-type for

Table 2 Doubling times of hematopoietic progenitor cells

Progenitor cell	Genotype	Slope (%killing/day)[a]	*y* intercept (%)[a]	Population doubling[b] (h)	*r*
CFU-GM	Wild-type	0.255	9.09	28.3	0.973
	Cx32-KO	0.244	13.54	29.6	0.995
CFU-S9	Wild-type	0.440	7.62	16.4	0.986
	Cx32-KO	0.179	7.82	40.3	0.999
CFU-S13	Wild-type	0.659	3.16	11.0	0.988
	Cx32-KO	0.694	5.35	10.4	0.999

[a] Regression line: $y = b\ 10^{(ax)}$, where *x* is the duration after BrdUrd treatment (days), *y* is the percentage of killing, *a* is cell cycle velocity (coefficient) and *b* is the cycling ratio/unit time (coefficient)

[b] Doubling time (h) = (log2/a) × 24

Table 3 Incidence of hematopoietic stem cell fraction/femoral BM cells

Hematopoietic stem cell fraction	Wild-type	Cx32-KO	p*
Lin^{-}-c-kit^{+} fraction (%)	0.316 ± 0.007	0.412 ± 0.022	0.0010
KSL fraction (%)	0.035 ± 0.008	0.052 ± 0.011	0.0458

Each value is expressed as average (n = 3 for each genotype) ± standard deviation

* The difference between wild-type and Cx32-KO was calculated by t-test

the KSL fraction; $p = 0.0126$ and $p = 0.0556$, respectively). The results suggest that Cx32 may have a suppressive function on such a hematopoietic stem cell compartment, KSL, under the physiological condition of Cx32.

Discussion

The role of Cx32 in steady-state hematopoiesis was analyzed in this study. This is the first observation of a Cx gene, namely *Cx32*, that is expressed in hematopoietic stem/progenitor cells. The functions of Cx32 in hematopoiesis were also investigated. In Cx32-KO mice, the numbers of various hematopoietic progenitor cells in the BM were lower than those in wild-type mice, suggesting a beneficial role of Cx32 for maintaining hematopoiesis during the steady state. Because the cell-cycle analyses of the hematopoietic stem cells, namely, the Lin^{-}/c-kit^{+}/$Sca1^{+}$ KSL, or the progenitor cells, Lin^{-}/c-kit^{+} fractions, suggested a slightly but significantly higher incidence of a dormant stem cell fraction in wild-type mice, the physiological role of Cx32 is probably to maintain

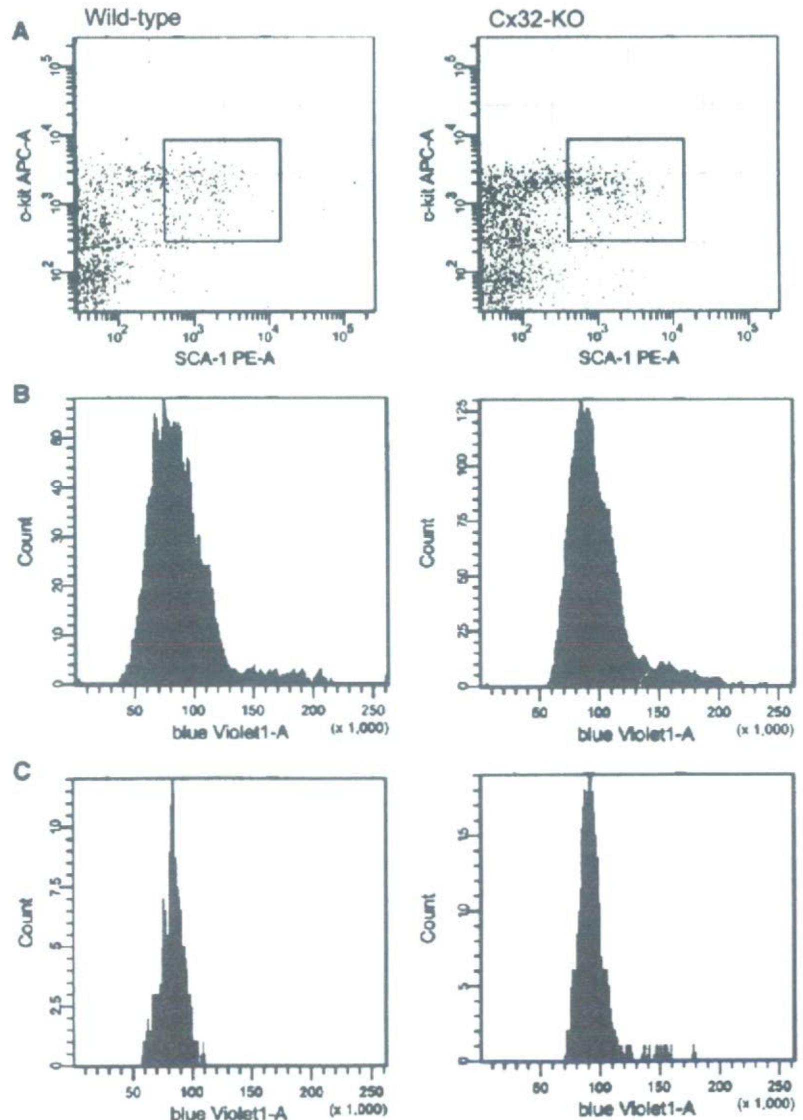

Fig. 4 **a** Two-dimensional expression shown by flow-cytometric analysis between c-kit and Sca1 expression on cells gated by lineage-negative fractions: wild-type and Cx32-KO mice. *Box* represents the c-kit^{+}/$Sca1^{+}$ fraction; thus, it is equivalent to the KSL fraction. **b**, **c** Flow-cytometric histograms showing reaction to Hoechst 33342 for Lin^{-}/c-kit^{+} fraction (**b**) and Lin^{-}/c-kit^{+}/$Sca1^{+}$ (=KSL) fraction (**c**)

Table 4 G_0/G_1 ratio of hematopoietic stem cell fraction

Hematopoietic stem cell fraction	Wild-type	Cx32-KO	p*
Lin$^-$/c-kit$^+$ fraction (%)	87.2 ± 0.76	83.3 ± 1.75	0.0126
KSL fraction (%)	91.5± 2.53	89.2 ± 1.82	0.0556

Each value is expressed as average (n = 3 for each genotype) ± standard deviation

* The difference between wild-type and Cx32-KO was calculated by t-test

the quiescence of the primitive hematopoietic stem cell compartment, thereby maintaining the stemness of the cells in the fraction.

Various Cxs are expressed in the stromal cells of the fetal liver (i.e., Cxs 43, 45, 30.3, 31 and 31.1) and the BM (i.e., Cxs 43, 45 and 31) (Cancelas et al., 2000). However, the contribution of Cxs to hematopoiesis was determined only on the basis of the effect of Cxs via stromal cell dependence; consequently, no Cxs were previously found in hematopoietic stem cells or progenitor cells (Krenacs & Rosendaal, 1998). However, in our recent study, interestingly, Cx32-KO mice exposed to benzene showed hematopoietic impairment more than wild-type mice; furthermore, the site of this impairment was not identified in either hematopoietic progenitor cells or stromal cells (Yoon et al., 2004).

Thus, we first determined whether hematopoietic progenitor cells express Cx32 molecules. As reported elsewhere (Yoon et al., 2004; Nelles et al., 1996), no Cx32 was detected in unfractionated BM cells by either RT-PCR or cell sorter analysis with an immunofluorescence antibody against Cx32 in this study (Figs. 1, 2). However, interestingly, hematopoietic spleen colonies, derived from hematopoietic progenitor cells and consisting of relatively immature hematopoietic cells, were found to express Cx32. This observation was also consistent with the immunohistochemical reaction of cells in the colonies with the anti-Cx32 antibody, in which Cx32-positive cells were only found along the border of each colony (*data not shown*). Subsequent flow-cytometric analysis using the anti-Cx32 antibody after performing the combination of immunobead density gradient separation and immunomagnetic bead separation showed that the most Cx32-positive fraction belonged to the HSC-enriched fraction, i.e., the Lin$^-$/c-kit$^+$ fraction (28.8% of the fraction) (Fig. 2a). It was calculated as only 0.27% with respect to the unseparated BM cells. Because RT-PCR or Northern blotting possibly detects >1% of expressing cells, these findings are in good agreement with a previous report on the absence of Cx32 expression in unseparated BM tissue (Cancelas et al., 2000). A hematopoietic disadvantage in progenitor cells associated with Cx32 deficiency was further evident because all progenitor cells from the BM of Cx32-KO mice showed ~20% decrease in the numbers of CFU-S-13, CFU-S-9 and CFU-GM. Thus, it can be concluded that Cx32 is required for maintaining normal hematopoiesis, specifically during the maturation of hematopoietic stem cells to progenitor cells.

BM transplantation in different combinations of the donor and recipient, which were repopulated with BM cells from either wild-type or Cx32-KO mice, showed a small number of spleen colonies in the groups repopulated with Cx32-KO BM cells (*data not shown*). Interestingly, the colonies derived from the same Cx32-KO BM cells were significantly smaller, regardless of the genotype of the recipients, i.e., wild-type or Cx32-KO mice, presumably owing to the lack of Cx32 expression in the hematopoietic progenitor cells.

Whether Cx32 is also functional in differentiated mature blood cells is, however, questionable despite the observation that the numbers of white blood cells and platelets in the peripheral blood were significantly lower in Cx32-KO than in wild-type mice (Table 1). It is interesting to calculate the probability of Cx32-positive cells on the basis of the ratio of the number of Cx32-positive BM cells to the Lin$^+$/c-kit$^-$ fraction, i.e., only 0.0093% of the unfractionated original BM cells (*data not shown*). Because our repeated analysis failed to detect Cx32 expression in mature blood cells, the decreased numbers of white blood cells and platelets in the Cx32-KO mice may reflect the shortage of immature progenitor cell compartments, possibly due to the lack of Cx32 at the level of the stem and progenitor cells.

Flow-cytometric cell cycle analyses of the Lin$^-$/c-kit$^+$/Sca1$^+$, KSL fraction with Hoechst 33342 and the BUUV assay for colony-forming progenitor cells showed that the cell cycle of the hemopoietic stem cell fractions, i.e., the Lin$^-$/c-kit$^+$/Sca1$^+$, KSL or Lin$^-$/c-kit$^+$ fraction, seems to be maintained in the quiescence state, thereby maintaining the stemness of the cells, although consequent molecular regulations of these fractions are not yet known.

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RESEARCH ARTICLE

An Assessment of Integrated Risk Assessment

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ABSTRACT

In order to promote international understanding and acceptance of the integrated risk assessment process, the World Health Organization/International Programme on Chemical Safety (WHO/IPCS), in collaboration with the U.S. Environmental Protection Agency and the Organization for Economic Cooperation and Development, initiated a number of activities related to integrated risk assessment. In this project, the WHO/IPCS defines integrated risk assessment as a science-based approach that combines the processes of risk estimation for humans, biota, and natural resources in one assessment. This article explores the strengths and weaknesses of integration as identified up to this date and the degree of acceptance of this concept by the global risk assessment/risk management community. It discusses both opportunities and impediments for further development and implementation.

The major emerging opportunities for an integrated approach stem from the increasing societal and political pressure to move away from vertebrate testing leading to a demand for scientific integrated approaches to *in vitro* and *in vivo* testing, as well as to computer simulations, in so-called Intelligent Testing Strategies. In addition, by weighing the evidence from conventional mammalian toxicology, ecotoxicology, human epidemiology, and eco-epidemiology, risk assessors could better characterize mechanisms of action and the forms of the relationships of exposures to responses. It is concluded that further demonstrations of scientific, economic and regulatory benefits of an integrated approach are needed. As risk assessment is becoming more

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mechanistic and molecular this may create an integrated approach based on common mechanisms and a common systems-biology approach.

Key Words: integrated risk assessment, environment, health.

INTRODUCTION

In a complex world, the call for integrated analysis, broadly to be taken as a holistic approach towards problem solving, is an understandable objective. This is by no means different in the world of risk assessment. Risk-based decision-making requires that decision-makers and stakeholders are informed of all risks that are potentially significant and relevant to their decision. Different permutations of this view can be observed in various organizations and institutions.

In the European Union (EU), some regulatory frameworks, notably that on industrial chemicals and biocides, already requires a partly integrated risk assessment (EC 2003a). This requirement also extends to a legislative proposal for implementing a new EU chemicals policy dealing with the registration, evaluation, authorization, and restriction of chemicals (REACH, EC 2006).[1] Political pressure has further led to a European Environment and Health Strategy (EC 2003b) proposing an integrated approach involving closer cooperation between health and environment research. This strategy aims at the development of an EU system integrating information on the state of the environment, the ecosystem, and human health, taking into account mixture effects, combined exposure, and cumulative effects. The strategy is connected to the European Environment and Health Action Plan 2004–2010 (EC 2004) and builds on the aims of the Commission's Sixth Environment Action Programme, a specific target of which is that levels of pollution in Europe should not give rise to deleterious effects on human or environmental health.

In order to promote international understanding and acceptance of the integrated risk assessment process, the World Health Organization/International Programme on Chemical Safety (WHO/IPCS), in collaboration with the U.S. Environmental Protection Agency (USEPA) and the Organization for Economic Cooperation and Development (OECD), initiated a number of activities related to integrated risk assessment. In this project, WHO/IPCS defines integrated risk assessment as a science-based approach that combines the processes of risk estimation for humans, biota, and natural resources in one assessment. A generic framework for integrated risk assessment and four case studies were evaluated at an April 2001 IPCS/EC scientific workshop, held in Ispra, Italy (Suter 2003). More than 60 participants representing diverse international and national organizations, academic institutions, and industry attended this workshop. The workshop identified: (1) the benefits and obstacles to integrated risk assessment; (2) research needed to facilitate the implementation of integrated risk assessment, and (3) mechanisms and actions that can be taken to facilitate the practical application of integrated risk assessment by regulatory bodies. As a follow-up, a case study on nonylphenol was developed and presented during a symposium on integrated risk assessment at the

[1]Editor's note: REACH is a new EU regulation that would require producers and importers of chemicals to register them along with the information needed to use them safely.

10th International Congress of Toxicology (ICTX) in Tampere, Finland, in July 2004 (Bontje *et al.* 2005).

This paper explores the strengths and weaknesses of integration identified to date and the degree of acceptance of this concept by the global risk assessment/risk management community. It discusses both opportunities and impediments for further development and implementation. The conclusions are based on the expertise and experience of the authors, who were members of the WHO/IPCS working group and authors of the integrated risk assessment case studies. However, the true nature and magnitude of benefits will become apparent only when integrated assessment is implemented in routine regulatory risk assessments and when testing, data generation, and model development are conducted to support integrated assessments.

BENEFITS OF INTEGRATION

There are several fundamental reasons for adopting integrated risk assessment as a primary environmental decision-support tool (WHO/IPCS 2001). Most important are the inherent interdependence of risks to humans and nonhuman species that results from commonalities in the sources and routes of exposure, the conservative nature across species of many toxic mechanisms, and the fact that the quality of human life is inextricably linked to that of the environment and *vice versa* (Suter *et al.* 2003a, 2005). Integrated analyses can take advantage of these commonalities by sharing assumptions, quantification schemes, information and data sets, model-based evaluations, and characterization methods to develop coherent expressions of risk to human and nonhuman receptors. Further, the broader understanding of environmental processes gained by combining knowledge of human health and ecology in an integrated risk assessment can lead to identification of risks previously unexpected. The holistic nature of the integrated process itself promotes more efficient and efficacious assessments of risk and the decision processes they support.

Despite the soundness of such reasoning, the chemical management community will require demonstration of the tangible benefits of integrated risk assessment to accept this as a way of doing business. To facilitate this, the IPCS project conducted two activities to identify and communicate the benefits of integrated risk assessment. The first involved development of case studies that illustrated how assessments might be conducted following the IPCS framework, and evaluated whether there were specific components of each assessment that would benefit from integration. The case studies were reviewed in a workshop setting to elicit the opinions of members of the international risk assessment/risk management community about the benefits of integrated risk assessment (Munns *et al.* 2003). The second was to commission an actual risk assessment of a chemical of international interest, nonylphenol, which could be used to demonstrate benefits directly (available at www.who.int/ipcs/).

Four case studies involving chemical and nonchemical stressors were developed initially: persistent organic pollutants (POPs) (Ross and Birnbaum 2003), organotin (tributyltin and triphenyltin) compounds (Sekizawa *et al.* 2003), organophosphorous pesticides (Vermeire *et al.* 2003), and ultraviolet (UV) radiation (Hansen *et al.* 2003). The case studies were illustrative only; rather than actual assessments of risk, they described how integration might be accomplished over the entire assessment process,

highlighting integration approaches and the information that would be needed to conduct integrated risk assessments. Their development and subsequent evaluation during the workshop served to identify benefits of integration that were uniquely associated with each risk problem, as well as those shared commonly across problems. The POPs case study, for example, illustrated that similarities in the biological effects experienced by upper food chain receptors (cetaceans, humans) could be used to enhance understanding of the risks experienced by all exposed species. The value of nonhuman receptors as sentinels of human risk was communicated clearly in the organotin case study. Both the organotin and UV case studies illustrated how integrated risk assessment can be used to identify the potential for cascading, indirect effects of the stressors on ecosystem and human welfare. Coherent use of multiple lines of evidence and decision criteria, and particularly in interpretation of assessment results against those criteria, was suggested to lead to more balanced risk management decisions by the organophosphorous pesticide case study. Among the benefits evident in all case studies were the advantages accrued by acknowledging common sources and pathways of chemical transport, fate, and exposure. Such advantages would lead to substantial gains in assessment efficiency and completeness.

In the WHO integrated risk assessment (IRA) approach described here, the emphasis is on communalities and shared resources with regard to human and ecological risk assessment. Other important connotations are, for instance, the integration across endpoints into one measure of risk or integration of exposure via different routes and pathways. It should therefore be emphasized that, in view of the multiple definitions used, the word "integration" should be used with caution (Suter *et al.* 2003b).

The IPCS nonylphenol assessment provided an additional demonstration of benefits (Bontje *et al.* 2005). This assessment used existing information about sources of nonylphenol, its exposure to humans and nonhumans, and the health and ecological effects of this chemical, to attempt an integrated risk assessment following the IPCS framework. Although resource limitations prevented completion of a comprehensive analysis of benefits, specific advantages of integration were identified through this effort. Included were enhanced coherence of assessment results through the harmonization of exposure pathways and the exposure concentrations experienced by human and nonhuman receptors, a reduction in assessment uncertainties as a result of confirmation of mechanisms of action through evaluation of information across species, and improved characterization of exposure-response relationships through use of broader data sets. The evaluation also suggested that overall risk assessment costs would be reduced through use of common sets of information, exposure models, and data analysis approaches. Examples were common stressor characteristics, sources and releases, distribution pathways, transport and fate and mode of action data as well as common approaches towards exposure-response analysis, uncertainty analysis and risk characterization and communication. If the integrated risk assessment for nonylphenol was used in a regulatory context, these benefits likely would lead to improved quality in the decisions regarding the management of risks of nonylphenol.

In addition to these examples, the DDT case as described by Sekizawa and Tanabe (2005) also illustrates the benefits of integrating human health and wildlife risk assessments. In particular, the discovery of the loss of some populations of birds

indicated that the effects of DDT could be more serious than was suggested by conventional toxicity tests. The IPCS has evaluated DDT and its derivatives in two occasions independently, once for health aspects, and the other for environmental aspects, in its Environmental Health Criteria series (IPCS 1979, 1989). The Joint FAO/WHO Meeting on Pesticide Residues (JMPR), an international expert committee associated with IPCS, has reviewed toxicological aspects of DDT and its derivatives several times from 1963 to 2000 (WHO 2001). In the IPCS document for ecological risk assessment on DDT and its derivatives, it was mentioned that there is a fundamental difference in approaches between toxicologists and ecologists concerning the appraisal of the potential threat posed by chemicals (IPCS 1989). For example, toxicologists are said to be preoccupied with any adverse effects on individuals, whereas ecotoxicologists are concerned primarily with the maintenance of population levels of organisms in the environment.

Formerly, an acceptable daily intake (ADI) of 0–0.02 mg/kg bw was allocated in 1984 for combination of DDT, DDD, and DDE, principally based on human studies where no overall change was observed in liver functions in workers exposed to 0.05–0.25 mg/kg bw. Old findings of eggshell thinning suggested that this effect might be induced by hormonal disturbance. However, by the accumulation of knowledge in *in vitro* and *in vivo* studies in experimental animals, together with supporting evidence from wildlife observations, a new evaluation was developed. Recent concern about endocrine disrupting chemicals and progress of research in related areas has given us impetus to integrate and assess data on exposure and potential effects in humans and wildlife which may share similar exposure pathway and show potential effects to both humans and wildlife through similar mode of actions. The ecological finding that DDT biomagnified in food chains and affected species that fed at higher trophic levels, particularly in aquatic food chains, led investigators to study differential exposure of human populations to DDT and other organochlorine compounds based on dietary differences with possible differential health effects (Sekizawa and Tanabe 2005).

The evaluations conducted to date by the IPCS project as well as the DDT case suggest substantial benefits to be gained by the integration of ecological and human health risk assessments. The most important of these include increased assessment efficiency, cost effectiveness of assessment activities, coherence of assessment results, and predictive and diagnostic capability. These benefits should translate directly into a higher quality of assessment relative to ecological and human health assessments conducted independently, thereby increasing the usefulness of risk assessment to environmental policy and decision-making. Because of institutional inertia, most risk assessors and managers are likely to continue their established practices. The transition to integrated assessment must be driven by innovative assessors and managers who appreciate the potential benefits and are willing to demonstrate that there are benefits in routine practice.

STATUS OF ACCEPTANCE, OPPORTUNITIES FOR FURTHER DEVELOPMENT

Searching worldwide for applications in regulatory risk assessment of the concept of integrated risk assessment as defined by WHO/IPCS, it soon becomes clear that

Table 1. Regulation of chemicals in the EU.

New chemicals	Directive 67/548/EEC*
Existing chemicals	Regulation 793/93*
Pesticides	Directive 91/414/EEC
Biocides	Directive 98/8/EC
Veterinary drugs	Directive 2004/28/EC
Human drugs	Directive 2004/27/EC
Feed additives	Directive 70/524/EEC
Food additives	Directive 89/107/EEC
Cosmetics	Directive 76/768/EEC
Packaging material	DirectiveCS/PM/1025
Novel foods	Directive 258/97

*To be replaced in June, 2007 by the Regulation on Registration, Evaluation, Authorisation and restriction of Chemicals, REACH (EC 2006).

this is not a common phenomenon. This section will describe the experience in the EU, Japan, and the United States. In addition, the role of integrated risk assessment in emergency response functions (ERF) will be discussed. This will lead to a consideration of emerging opportunities to promote further development and acceptance.

State-of-Practice

European Union

An overview of the legislation on chemicals in the EU is given in Table 1. Risk assessments carried out for food additives, cosmetics, packaging material, and novel foods only cover human endpoints, but in all other cases risk assessments for both human health and environment are required. However, for human pharmaceuticals, the environmental impact should not constitute a criterion for refusal of a marketing authorization. With regard to pesticides, biocides, new and existing chemicals, one element of integration in the conceptual model is secondary poisoning of predatory mammals and birds. The characterization of this risk is based on comparison of the measured or modelled intakes of predators to predicted no-effect levels that are, in the absence of specific data, often extrapolated from mammalian toxicity data (such as a No-Observed-Adverse Effect Level for rats). In addition, for new and existing substances, measured or modelled concentrations in environmental compartments are used to derive an estimated daily intake that is compared to predicted no-effect levels, again derived from mammalian toxicity data. Both the risk assessment for predators and for humans exposed via the environment may be refined using toxicokinetic data.

An impetus towards increased integration of human health and environmental risk assessment could come within the scope of REACH. Central in this new policy is the requirement for the chemicals industry to demonstrate that the manufacture, use and disposal of chemicals are safe to humans and the environment. In the assessment, a central position is taken by the so-called exposure scenarios, defined as the set of conditions that describe how the substance is manufactured or used during its life cycle and how the manufacturer controls, or recommends downstream users

to control, exposure to humans and the environment. Industry will be required to develop exposure scenarios for workers and consumers, exposed directly and via the environment, as well as exposure scenarios for ecosystems. These scenarios should consider manufacture, use, service life, and waste disposal. In these exposure scenarios, risk management measures to be taken should be based on an integrated assessment of the risks, *e.g.*, abatement techniques recommended for the protection of workers should not lead to unacceptable risks for the environment and vice versa.

The European Environment and Health Strategy, introduced earlier, calls for an integrated approach with regard to information, research, environmental, and health concerns, and understanding of the cycle of pollutants, intervention and stakeholders. The strategy, among others, aims for "linking . . . environmental, health and research information to enable an integrated approach showing the cycle of a pollutant, assessing global exposure and associated health effects and identifying the most productive action routes." The European Research Area will have "to deepen this integration by fostering collaboration and the development of a common vision and research programs." This seems to give ample opportunity toward integrated approaches as defined in this article.

Japan

In Japan, the Ministry of the Environment (MOE) has been performing Initial Risk Assessment of Chemicals for both health and ecological aspects since 1997. The assessment is performed to set priorities for further investigations on chemicals. The ministry has been conducting extensive environmental monitoring since 1974, a part of which was published in English as Chemicals in the Environment (*e.g.*, MOE 2003). The Ministry of Health, Labour and Welfare (MHLW) addresses health aspects of exposure of the general public to chemicals in foods, consumer products, drinking water, indoor air, and for exposure in occupational situations. Assessment in each category is performed considering specific and independent exposure scenarios. The Research Center for Chemical Risk Management was established in 2004 in the National Institute of Advanced Industrial Science and Technology, which is affiliated to the Ministry of Economy, Trade and Industry (METI). It has been developing risk assessment documents on chemicals, while developing tools for risk assessment. The ministries and the research center have not yet incorporated the idea of integrated risk assessment in their activities and are operating independently without systematic coordination. However, there are new movements towards integration in Japan. One is triggered by implementation of the Pollutant Release and Transfer Register stipulated in the "Law Concerning Reporting of Releases to the Environment of Specific Chemical Substances and Promoting Improvements in Their Management" of 1999, in which amounts of chemicals released in the environment are estimated by manufacturing plants or local governments depending on their use pattern. That information is shared by society to reduce release of pollutants to the environment effectively. In this law, delivery of material safety data sheets is requested together with the information on the amount of release of chemicals in the environment. The major concerns are for protection of human health, but attempts are also made to make use of the information in estimating effects on the organisms in the environment.

Another new movement is triggered by incorporation of the idea of risk analysis in food safety as formulated by the FAO/WHO for the Codex Alimentarius, in which integration of risk assessment, risk management, and risk communication is deemed imperative. Some progress is being made in cooperation among risk assessment authorities, *i.e.*, Food Safety Commission of the Cabinet Office, and risk management offices in the MHLW and the Ministry of the Agriculture, Forestry, and Fisheries, and further in involvement of consumers, manufacturers, and other concerned partners in risk communication.

A new framework was incorporated in the Law Concerning the Examination and Regulation of Manufacture of Chemical Substances, a forerunner in 1972 to regulate manufacture of chemicals before marketing. Originally, a major concern of the law was protection of human health, but beginning in 2003, the law also examines and regulates manufacture of chemicals considering effects on organisms in the environment. This year, MHLW, METI, and MOE started the "Japan Challenge Program," a joint initiative of the government with manufacturers and importers of chemicals to facilitate safety information collection of High Production Volume (HPV) chemicals in accordance with OECD SIDS initial assessment. Collected safety information will be available to the public on the Internet. Major environmental concern has focused on the effects of pesticides to the neighbouring fishery activities in the Agricultural Chemicals Control Law, although an expert committee was convened to consider development of a method to assess a wider range of effects to organisms in the environment. These changes indicate a broader concern for not only human health protection, but also for environmental effects.

United States

A recent analysis of the USEPA's risk assessment practices, based on typical historic and current practice (USEPA 2004), states, "EPA uses risk assessment as a tool to integrate exposure and health effects or ecological effects information into a characterization of the potential for health hazards in humans or other hazards to our environment." The document addresses the issues of conservatism, the nature of uncertainty and variability, defaults and extrapolations and the use of site- and chemical-specific data. Although human and ecological risk assessments are discussed separately, it is acknowledged that "many of the principles and practices in human health assessment also apply to ecological health assessment." This separate discussion reflects the fact that human health and ecological risks are analyzed separately in practice. However, the WHO framework has inspired an exploratory integrated assessment of the mode of action of Bisphenol A (Euling and Sonawane 2005).

ERF

In Emergency Response Functions (ERF) all over the world one or more executive bodies work together to prevent, evaluate and reduce the risks of incidents. One element in the evaluation is the characterization of risks during and after the incident. In many countries, the risk assessment will pertain to both humans and the environment. Monitoring and modeling activities will provide exposure values that can be used to estimate the risks and the consequences of risk reduction measures

to both humans and the environment. Sampling strategies need to take account of this integrated approach toward exposure assessment. For example, in case of fires, exposure modeling and sampling and analysis of air, water, soil, and crops should not only be planned to answer questions regarding risks for fire workers, residents, bystanders, *etc.*, but also risks to nonhuman species and livestock.

Emerging Opportunities

What incentives can be identified for promotion of integrated risk assessment of chemicals? In the European Union (EU), one of the objectives of the 6th Environmental Action Program is "To achieve an environment where the levels of anthropogenic chemicals do not give rise to significant risks to, and impacts on, human health and the environment." The target set, *i.e.*, the assessment of all chemicals in a step by step approach with clear target dates and deadlines, leads up to the new chemicals policy REACH. The European Environment and Health Strategy subsequently calls for an integrated approach, meaning integration of information, research and Community policies, intervention and communication, as well as an integrated understanding of the cycle of pollutants. Although the scope for this integration is wider and the strategy focuses on the influence of environmental stressors on human health, it also clearly promotes Integrated Risk Assessment goals. For instance, the strategy aims at setting up an integrated environment and health monitoring system for the systematic and comprehensive collection of data over time and calls for research to achieve a better fundamental understanding of environment and health issues. Searching for commonalities in human and environmental risk assessment is at least a useful element in this type of research.

The call for further research in the area of risk assessment, even more pressing under REACH in view of the demand to assess more chemicals, raises concerns for increased vertebrate testing. At the same time, the political ambition is to reduce the number of animals used for testing, pressing the development and validation of alternative methods. Integrated approaches to *in vivo*, *in vitro* and *in silico* testing can contribute significantly in diminishing *in vivo* testing. Therefore, the societal and political pressure to move away from vertebrate testing is an important driver for integrated risk assessment (Briggs 2003; Bradbury *et al.* 2004; Höfer *et al.* 2004). A report of the EU Joint Research Centre has calculated that for REACH the cost and animal saving potential of Integrated Testing Strategies over a period of 11 years can be around 1 billion Euros and 1.5 million animals (van der Jagt *et al.* 2004).

Several ongoing or planned research activities with specific integrated risk assessment topics can be identified. In addition to the European Strategy described above, several research projects have become part of the EU research programs, first of all in the CREDO cluster (http://www.credocluster.info/). CREDO stands for "Coordinating European Environmental and Human Health Research into Endocrine Disruption." Four projects funded by the European Union form the core of the CREDO cluster:

- EDEN adopts an integrative approach to assess human and wildlife exposures to endocrine disruptors, mechanisms, and low-dose/mixture-evaluations;
- COMPRENDO addresses endocrine disruption in human and wildlife species, focusing on androgenic/anti-androgenic compounds (AACs);