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p53 (TRP53) Deficiency-Mediated Antiapoptosis Escape after 5 Gy X Irradiation Still Induces Stem Cell Leukemia in C3H/He Mice: Comparison between Whole-Body Assay and Bone Marrow Transplantation (BMT) Assay

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Mice exposed to a lethal dose of radiation were repopulated with heterozygous $p53^{+/-}$ ($TRP53^{+/-}$) bone marrow cells and then exposed to doses of 1, 3 and 5 Gy 1 month later. This resulted in the transplanted bone marrow-specific diseases other than competitively induced nonhematopoietic neoplasms. Interestingly, the present study showed a high frequency of stem cell leukemia, i.e., leukemias characterized by a lack of differentiation due also to p53 deficiency, even after 5 Gy irradiation. The frequencies of stem cell leukemias (and those of total hematopoietic malignancies) were 16% (24%) at 1 Gy and 45% (75%) at 3 Gy. Furthermore, markedly high incidences of stem cell leukemias were observed at 5 Gy in $p53^{+/-}$ mice, i.e., 87% (100%) in the transplantation assay and 60% (83.3%) in the whole-body assay, whereas a conventional whole-body assay induced only 14% in wild-type mice. The high incidence of stem cell leukemias observed in this study using heterozygous p53-deficient mice agrees with results of a previous study of homozygous p53-deficient mice and is consistent with the high frequency of loss of heterozygosity in the p53 wild-type allele observed in leukemias. This suggests that the target cells for radiation-induced stem cell leukemias may be p53-deficient hematopoietic stem cells. © 2007 by Radiation Research Society

INTRODUCTION

In general, the incidence of radiation-induced leukemias after radiation exposure may be a function of the surviving fraction of hematopoietic stem cells[2] and the minimum number of presumptive hematopoietic stem cells required for the induction of one leukemia case (*1*). Because caloric restriction decreases the number of hematopoietic progenitor cells, presumed to be targets of leukemogenesis, our recent results were in good agreement with the decrease in the incidence of myeloid leukemia by 14% (8% compared to 22%) after 3 Gy X irradiation (*2, 3*). Classic studies of radiation-induced leukemia also showed a marked decrease in the incidence of leukemias after splenectomy (*4–6*), which was also interpreted to be a consequence of the decrease in the number of hematopoietic stem cells and/or progenitor cells. The incidence of radiation-induced leukemia increases with the dose of radiation up to 3 Gy but then starts to decrease at doses over 3 Gy and is only 2% or less at more than 6 Gy (*7, 8*) due to the decrease in the number of hematopoietic progenitor cells; thus this may also be the case at the stem cell level.

Previously, we examined the radiation-induced leukemias in homozygous p53 (TRP53)-deficient C3H/He mice. In that study, we found a high frequency of unusual stem cell leukemias with a trace of myeloblastic differentiation in the nonirradiated control group, the onset of which became significantly earlier when mice were irradiated with 3 Gy (*9*). Because the p53-homozygous deficiency generally caused an early onset of spontaneous and/or induced neoplasms even in the nonirradiated group, neither the relationship between radiation dose and the incidence of hematopoietic neoplasm nor the possible association with the induction of stem cell leukemias in terms of radiation effects was observed previously.

A heterozygous deficiency in the p53 tumor suppressor gene (*Trp53*) in mice, which provide a model of human Li-

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[2] An assay should be made by counting the number of hematopoietic progenitor cells, i.e., descendants of hematopoietic stem cells, which can be assayed only with a fraction of the long-term sustainability of lethally irradiated mice.

Fraumeni syndrome, greatly enhances predisposition to various malignancies characterized by reduced differentiation (*9–14*). Because of the slightly later onset of spontaneous and induced neoplasms in heterozygous p53-deficient mice than in homozygous p53-deficient mice, the former have been used for a more rapid carcinogenicity bioassay than the standard assay that uses wild-type mice. In this study, heterozygous p53-deficient C3H/He mice were used to compare the whole-body assay and the bone marrow transplantation (BMT) assay using mice repopulated with bone marrow cells from heterozygous p53-deficient mice. The BMT assay is an experimental tool for observing bone marrow-specific hematopoietic malignancies with the minimum competitive risk of developing neoplasms other than those arising from nonhematopoietic tissues. Three possibilities were hypothesized in this study: (1) a possible radiation dose-associated increase in the incidence of stem cell leukemias, (2) a possible relationship between stem cell leukemias and the loss of heterozygosity (LOH) for the remaining p53 allele, and (3) a possible induction of stem cell leukemias at a higher dose, 5 Gy, due to radioresistance, i.e., the p53 deficiency-mediated antiapoptosis escape of hematopoietic progenitor cells.

Wild-type mice were repopulated by transfusion of wild-type or heterozygous p53-deficient bone marrow cells after exposure to a lethal dose of radiation, followed by a whole-body exposure to increasing doses of radiation, to examine the incidence of stem cell leukemias throughout the lifetime of the mice.

MATERIALS AND METHODS

Mice

Mice lacking one allele of the p53 gene, which were derived by gene targeting in the embryonic stem cell line TT2 (*15*) and backcrossing with C57BL/6 mice, were kindly provided by Tsukada and Aizawa. These hybrid mice with heterozygous p53 deficiency were bred with normal C3H/HeNirsMs females in the Experimental Animal Care and Welfare Board-approved laboratory animal facility of the National Institute of Radiological Sciences (NIRS), Chiba, Japan.

All experimental protocols involving the laboratory mice in this study were reviewed by the externally established peer review panel, the Committee of the Ethics of the Research and Welfare of the Experimental Animals (ERWEA) of the NIRS, and were approved by the Animal Care and Use Committee at the NIRS. The experiments were performed according to the NIRS Guidelines for the Care and Use of Laboratory Animals. After 13 generations of backcrossing between heterozygous p53-deficient mice and C3H/HeNirsMs mice, heterozygous p53-deficient males and females were crossed to generate wild-type ($p53^{+/+}$) and p53-deficient ($p53^{+/-}$, $p53^{-/-}$) mice. The neonates were genotyped by targeted DNA screening by polymerase chain reaction (PCR) analysis using tissue obtained from the tail. The PCR primers used were the same as those used to detect the loss of heterozygosity (LOH) for leukemias induced in spleen tissues. Therefore, the band for the wild allele might be equivocal due to partial coverage of PCR primers for full-length p53; LOH might be detectable if the full-length p53 gene were examined. (See Table 1 in the Results.)

Eight-week-old C3H/HeNirsMs males bred in the NIRS were used as the recipient mice in the BMT assay and the assay of colony-forming units in the spleen.

Irradiation

Cells or mice were irradiated with an X irradiator (Shimadzu, Tokyo) operated at 200 kV and 20 A with 1.0-mm aluminum and 0.6-mm copper filters at a dose rate of 0.614 Gy/min and a 56 cm focal surface distance. During the irradiation, the mice were irradiated whole body, were not anesthetized, and were allowed to move freely in individual circular chambers.

Preparation of Bone Marrow Cells

The femoral bone marrow was harvested from $p53^{+/+}$ and $p53^{+/-}$ mice, and $p53^{-/-}$ mice if applicable, suspended in α-MEM, and then processed to obtain a single-cell suspension by repeated aspiration through a 27-gauge hypodermic needle.

Assay of Colony-Forming Units in Spleen (CFU-S)

The method of Till and McCulloch (*16*) was used to determine the number of CFU-S. Aliquots of bone marrow cell suspensions were used to evaluate the number of CFU-S. The number of bone marrow cells with or without irradiation was adjusted to that appropriate for producing nonconfluent spleen colonies, and the cells were then transplanted into lethally irradiated mice by injection through the tail vein. For the assay of spleen colonies, increasing doses of X radiation were given *in vitro* (irradiation conditions were similar to those for mice described above). Spleens were harvested 8 and 12 days after the injection and fixed in Bouin's solution. Macroscopic spleen colonies were counted under an inverted microscope at a magnification of 5.6×.

Irradiation for Induction of Leukemias

Twenty-four to 30 mice per group for the whole-body assay and 20 to 25 mice per group for the BMT assay were subjected to whole-body irradiation at doses of 0 (control), 1, 3 and 5 Gy and were observed throughout their lifetime. For the whole-body assay, mice were irradiated at 10 weeks of age. For the BMT assay, recipient mice grafted with heterozygous p53-deficient cells or those grafted with wild-type cells were subjected to a second irradiation 4 weeks after transplantation (see the next paragraph for the assay method).

Bone Marrow Transplantation (BMT) Assay

Eight-week-old male C3H/HeNirsMs mice were used as recipient mice. The mice were subjected to 9.45 Gy of whole-body X irradiation, as described above. Bone marrow cells were harvested from heterozygous p53-deficient C3H/HeNirsMs male donors, and 1×10^6 cells were injected into the tail vein of lethally irradiated wild-type C3H/HeNirsMs mice. The same number of wild-type bone marrow cells was also injected into lethally irradiated recipients.

Analysis by Fluorescence-Activated Cell Sorting (FACS) to Determine Stem Cell Leukemias

The leukemia cases negative for Thy-1.2, CD3, CD4, CD8, TL-2, IL-2, B220, sIgM, Mac-1 and Gr-1, and those positive for c-kit and CD44 were designated as stem cell leukemias and were confirmed immunohistopathologically. The immunophenotypic definition of stem cell leukemias has been fully described elsewhere (*9*); the analytical method is described briefly here. A single-cell suspension was prepared from leukemic spleens. One million leukemic spleen cells were suspended in phosphate-buffered saline (PBS) containing 0.3% bovine serum albumin and 0.1% NaN_3 and then incubated for 30 min with FITC-conjugated antibodies (anti-Thy1.2, anti-CD4, anti-TL-2, anti-IL-2 , B220, Gr-1 and anti-c-Kit antibodies), biotin-conjugated antibodies [anti-CD3, anti-CD8, anti-s-IgM (Zymed Lab., Inc., San Francisco, CA)], an anti-Mac-1 antibody (Caltag Lab, San Francisco CA), an anti-CD44 (*17*) (PgP-1: Becton Dickinson) antibody, and an anti-TER-119 monoclonal antibody (Becton

Dickinson). Data were analyzed using Lysis II software (Becton Dickinson). Cells from the thymus and mesenteric lymph nodes of mice with severe leukemia were also immunostained and analyzed as described above.

Histopathological Examination

Mice were observed three times daily with gentle care throughout their lifetime. Mice exhibiting anemia with pale appearance and/or hypothermia of extremities were isolated and euthanized humanely as soon as they showed signs of distress.

Blood samples collected from the circulating blood were examined immunohematologically and histopathologically for evidence of immunohematological and histopathological abnormalities. For histopathological examination, all the visceral organs including the thymus, spleen, sternum and femoral bone marrow were fixed in 4% neutral-buffered formalin for 24 h. The sternum and femoral bone marrow were decalcified in 7.5% formic acid for 72 h. After routine processing, paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and then examined under a light microscope. The histopathological classification of murine hematopoietic malignancies including stem cell leukemias was based on the authors' previous report (*9*) and the monograph (*18*) published by one of the present authors, along with the results of the FACS analysis.

Examination of Loss of Heterozygosity (LOH) for p53 Wild-Type Hemi-allele (PCR)

During the course of radiation-induced leukemogenesis, the wild-type allele of the p53 gene remaining in the heterozygous p53-deficient mice may be inactivated. The frequency of such LOH was evaluated in mice with radiation-induced leukemias. The band corresponding to the wild-type allele indicated that there was no complete loss of the allele in most of the leukemia cases with LOH, but the band showed a weak signal due to stromal tissue contamination by leukemic cells whose wild-type allele signals were noticeable weaker than those of the positive control band or paired mutated p53. Whenever possible, tissues were obtained immediately after death and the p53 hemi-allele derived from the heterozygous p53-deficient mice with leukemia was determined using the same PCR primers used in the genotyping of the mice for breeding. The PCR primers used to detect the p53 gene in this study cover exon 1 and its vicinity.

Statistical Analysis

The data were analyzed using the generalized Wilcoxon test for determining the statistical significance of differences.

RESULTS

Survival Curves after Exposure to Graded Increasing Doses of Radiation: Whole-Body Assay and BMT Assay

Figure 1A shows the survival curves for groups of 10-week-old mice subjected to whole-body irradiation. The mean survival time for the control group (0 Gy) was 380 ± 19 days. With increasing doses of radiation (1, 3 and 5 Gy), the mean survival time decreased to 323 ± 24, 212 ± 13 and 152 ± 9 days, respectively ($P = 0.1$, $P < 0.001$ and $P < 0.0001$, respectively, compared to the 0-Gy control group by the generalized Wilcoxon test). Because p53-deficient mice have higher incidences of various nonepithelial tumors other than hematopoietic neoplasms, a BMT assay focusing on the induction of hematopoietic neoplasms was designed to examine the incidence of C3H/He-specific myeloid leukemias after exposure to increasing doses of radiation. The experimental protocol for the BMT assay in this study using heterozygous p53 deficiency is similar to that used in a previous study of homozygous p53-deficient mice (*9*). One million bone marrow cells were transfused into 6-week-old lethally irradiated C3H/He mice followed by another whole-body irradiation with increasing doses to induce leukemia when they were 10 weeks old.

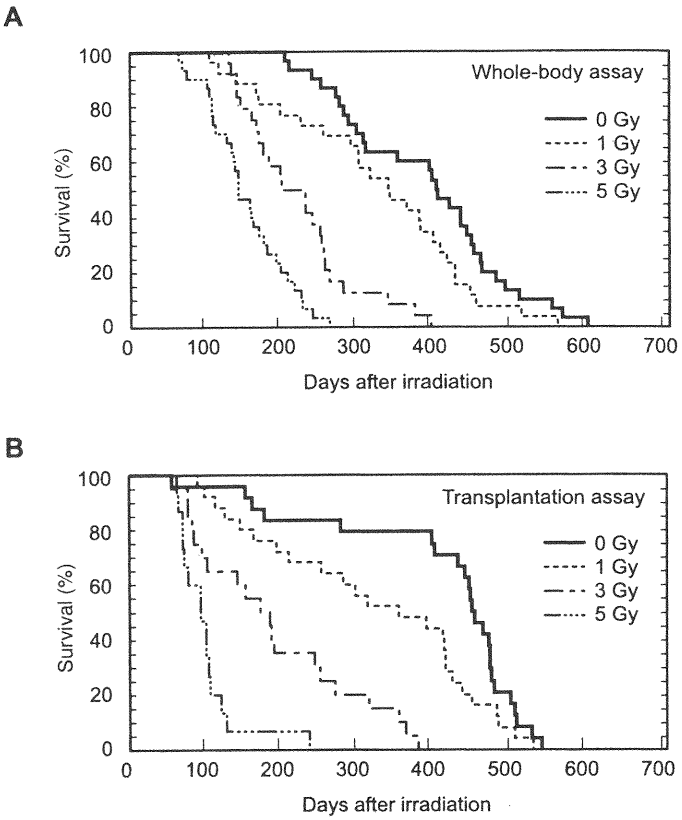

FIG. 1. Survival curves for mice irradiated with 1, 3 and 5 Gy and for nonirradiated mice. Panel A: Whole-body assay; 10-week-old mice were irradiated and observed throughout their lifetime. Panel B: Transplantation (BMT) assay (*9*): Recipient mice that had been lethally irradiated with 9.45 Gy were repopulated with 1×10^6 bone marrow cells from heterozygous p53-deficient mice. The mice were allowed to recuperate for 4 weeks before X irradiation. After irradiation, they were observed throughout their lifetime.

Figure 1B shows the survival curves for the groups in the BMT assays that were exposed to increasing doses of radiation and for the 0-Gy control group. The mean survival time for the 0-Gy control group was 402 ± 27 days. With increasing doses of radiation (1, 3 and 5 Gy), the mean survival time decreased to 318 ± 28, 188 ± 23 and 99 ± 11 days, respectively ($P = 0.0168$, $P < 0.001$, and $P < 0.0001$, respectively, compared to the 0-Gy control group). The survival curve for the 5-Gy-irradiated group in the BMT assay (Fig. 1B) shows earlier deaths than in the corresponding group in the whole-body assay shown in Fig. 1A ($P < 0.0001$). However, the survival curves for other groups exposed to less than 3 Gy had slightly lower death rates than the corresponding groups in the whole-body as-

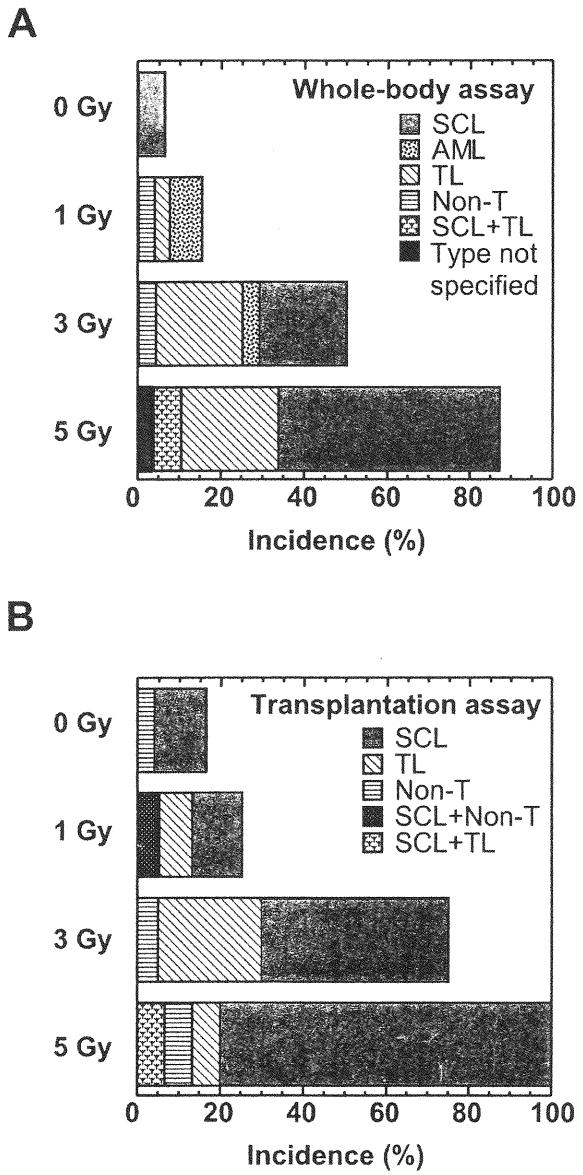

FIG. 2. Incidences of hematopoietic neoplasms in heterozygous p53-deficient mice irradiated with 1, 3 and 5 Gy X rays and in nonirradiated mice. The diagnosis for each hematopoietic malignancy was made on the bases of immunocytochemical analysis, fluorescence-activated cell sorting, and histopathological analysis. Panel A: Whole-body assay; panel B: transplantation (BMT) assay. SCL, stem cell leukemia; AML, acute myeloid leukemia; TL, thymic lymphoma; Non-T, nonthymic lymphoma.

say (statistically insignificant). The differences in the fatal disease spectra in the BMT and whole-body assay groups may have been the result of different p53 environments of the host tissue as well as factors attributable to a lethal whole-body irradiation prior to the BMT.

p53 Deficiency Induces Stem Cell Leukemias: Immunocytological and Histopathological Examinations

The incidences of hematopoietic neoplasms in each experimental group are shown in Fig. 2A for the whole-body assay and in Fig. 2B for the BMT assay. As observed previously, stem cell leukemias that developed with a trace of myeloid differentiation were examined immunocytologically to determine their cytological origin (*9*). The leukemia cases negative for Thy-1.2, CD3, CD4, CD8, TL-2, IL-2, B220, sIgM, Mac-1 and Gr-1 and positive for c-kit and CD44 were designated as stem cell leukemias. The incidences of stem cell leukemias increase with the dose of radiation as shown in Fig. 2A and B; however, the dose–incidence relationship was much clearer in the BMT assay (Fig. 2B).

LOH in Leukemia Tissue of Heterozygous p53-Deficient Mice

The normal allele of the p53 gene remaining in heterozygous p53-deficient mice was examined in tissues from those mice that developed leukemias. Figure 3 shows an example of a PCR analysis of a leukemic mouse with the wild-type p53 allele. Except for lanes 7, 9 and 10, the wild-type p53 allele, which corresponds to the lower band with a weaker signal than the band corresponding to the p53 mutated allele for knockout strategy (p53 mut-signal), was regarded as essentially deleted in all leukemic tissues (M, molecular marker; A-C, positive control for PCR; D, negative control). Results for LOH showed good agreement with the characteristics of stem cell leukemias determined by immunocytochemical and histopathological examinations. Lanes 7 and 10 correspond to samples from the same mouse with angiosarcoma originating from the spleen, and lane 9 corresponds to the sample from a mouse with myelogenous leukemias other than stem cell leukemias. Table 1 shows the data from all the stem cell leukemia cases in which LOH for the p53 hemi-allele could be examined. Despite the fact that nearly half of the cases could not be examined randomly, 86% of the leukemia cases in the whole-body assay groups and 92% of the mice in BMT assay groups showed LOH for the remaining p53 allele. (Refer to an unpaired staining intensity comparison between the mutated and wild-type p53 alleles at the same mobilities for the positive control, lane C in Fig. 3.)

Cumulative Incidence of Leukemias and Dose Response

When one compares the incidences of stem cell leukemias in the whole-body assay and BMT assays (Fig. 4A and B, respectively), the curves for the BMT assay show a higher incidence of stem cell leukemias than those for the whole-body assay, owing in part to possible exclusion of the competitive induction of neoplasms other than hematopoietic neoplasms. The incidences of ordinary myeloid leukemia in wild-type mice at each dose are shown in Fig. 5 as closed circles with a solid line. In contrast, a high incidence (87%) of stem cell leukemias was seen in the heterozygous p53-deficcient 5-Gy-irradiated group in the BMT assay, whereas a low incidence of leukemias was observed in the wild-type 5-Gy-irradiated group in the whole-body assay (14%). No leukemias developed as shown by

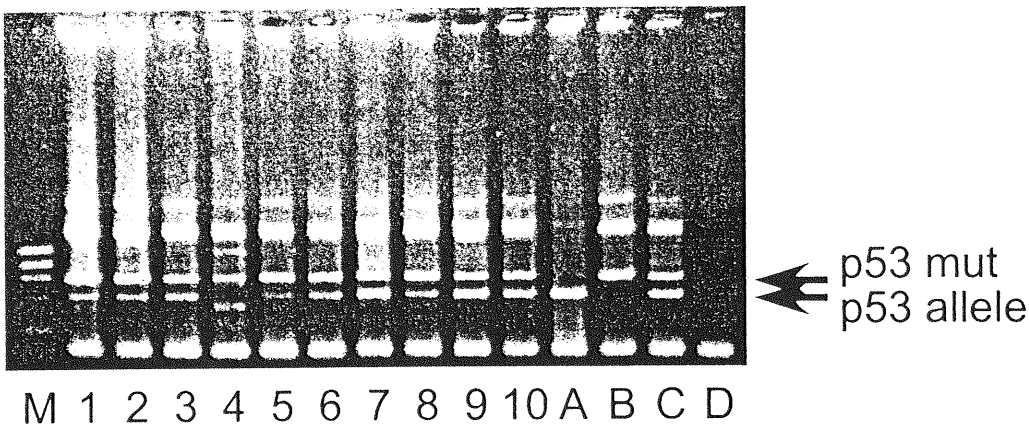

FIG. 3. An example of PCR data for wild-type p53 allele in each leukemia case. M indicates molecular marker; A–C, positive controls for PCR; D, negative control. Lanes 7, 9 and 10 show two bands corresponding to a p53 mutated allele for knockout strategy (p53 mut) and a p53 wild-type allele (p53 allele). Lanes 1–6 and 8, corresponding to samples from stem cell leukemia cases, show weak signals of the lower bands. Lanes 7 and 10 correspond to two samples from the same mouse with an angiosarcoma originating from the spleen, and lane 9 corresponds to a sample from a mouse with myelocytic leukemia other than stem cell leukemia.

the BMT assay of wild-type mice and as indicated by the open circles on the horizontal baseline. In this BMT assay, mice repopulated with wild-type bone marrow cells did not show any leukemia and died mostly of nephrosclerosis induced by a lethal dose of radiation.

Surviving Fractions of Hematopoietic Progenitor Cells after Exposure to Increasing Doses of Radiation

The survival of hematopoietic progenitor cells after exposure to radiation was found to be higher for p53-deficient cells than for wild-type cells on the basis of spleen colony-forming units [assay of CFU-S, both day 12 immature colonies (Fig. 6A: CFU-S late) and day 8 mature colonies (Fig. 6B: CFU-S early)]. As shown in Fig. 6, the survival curves for both day 12 immature colonies and day 8 mature colonies derived from the bone marrow of p53-deficient mice ($p53^{+/-}$ and $p53^{-/-}$) are flatter than those colonies derived from the bone marrow of wild-type mice ($p53^{+/+}$). This increased survival of the p53-deficient progenitor cells is assumed to be due in part to prevention of apoptosis as well as cell cycle arrest. This increased survival due to p53 deficiency might be responsible for the induction of stem cell leukemias at a higher incidence in a dose-dependent manner up to 5 Gy.

TABLE 1
Frequency of Loss of Heterozygosity in p53 Hemi-allele[a]

Assay	Number of cases[b]	Number examined (A)[c]	Number showing loss of wild allele[c] (B)	Ratio[c] (%)
Whole body	26	14	12	86
BMT	30	12	11	92

[a] Whenever tissues were obtained immediately after death, LOH was examined in mice with radiation-induced leukemia (See Materials and Methods).

[b] All leukemia cases observed in present study (from 0 to 5 Gy radiation exposure).

[c] Loss of wild allele = LOH. Frequency of LOH, (B/A) × 100 (%).

DISCUSSION

p53-deficient mice develop undifferentiated immature hematopoietic neoplasms (*9*), and in C3H/He mice, we previously observed a high frequency of stem cell leukemias with a trace of myelogenous differentiation in homozygous p53-deficient mice with or without radiation exposure (*9*). In the present study, as shown in Figs. 2A and B, regardless of the assay used, whole-body irradiation at increasing doses resulted in a high, dose-dependent frequency of stem cell leukemias. The incidence of stem cell leukemias was higher in the BMT assay, possibly owing to the deletion of competitive risks other than hematopoietic malignancies. Thymic lymphomas were also observed in all the irradiated groups. A few cases of double hematopoietic neoplasms (stem cell leukemias and thymic lymphomas) were observed in the 5-Gy-irradiated groups, implying that the loss of dose dependence at high doses may be because of competition between stem cell leukemias and thymic lymphomas. The mechanism underlying the loss of differentiation in neoplasms deficient in the p53 gene has been discussed elsewhere (*9, 19–22*).

Because most of the leukemias that developed in a previous study using homozygous p53-deficient mice were stem cell leukemias, the high incidence of stem cell leukemias observed in heterozygous p53-deficient mice in the present study suggests a high frequency of loss of the remaining heterozygous p53 allele in mice that developed leukemias. This possibility was supported by the observation of 86% LOH in leukemias in the whole-body assay and 92% LOH in leukemias in the BMT assay. In other words, among the cases of hematopoietic neoplasms that were examined for LOH, the cases showing LOH were all diagnosed as stem cell leukemias. In the present study, al-

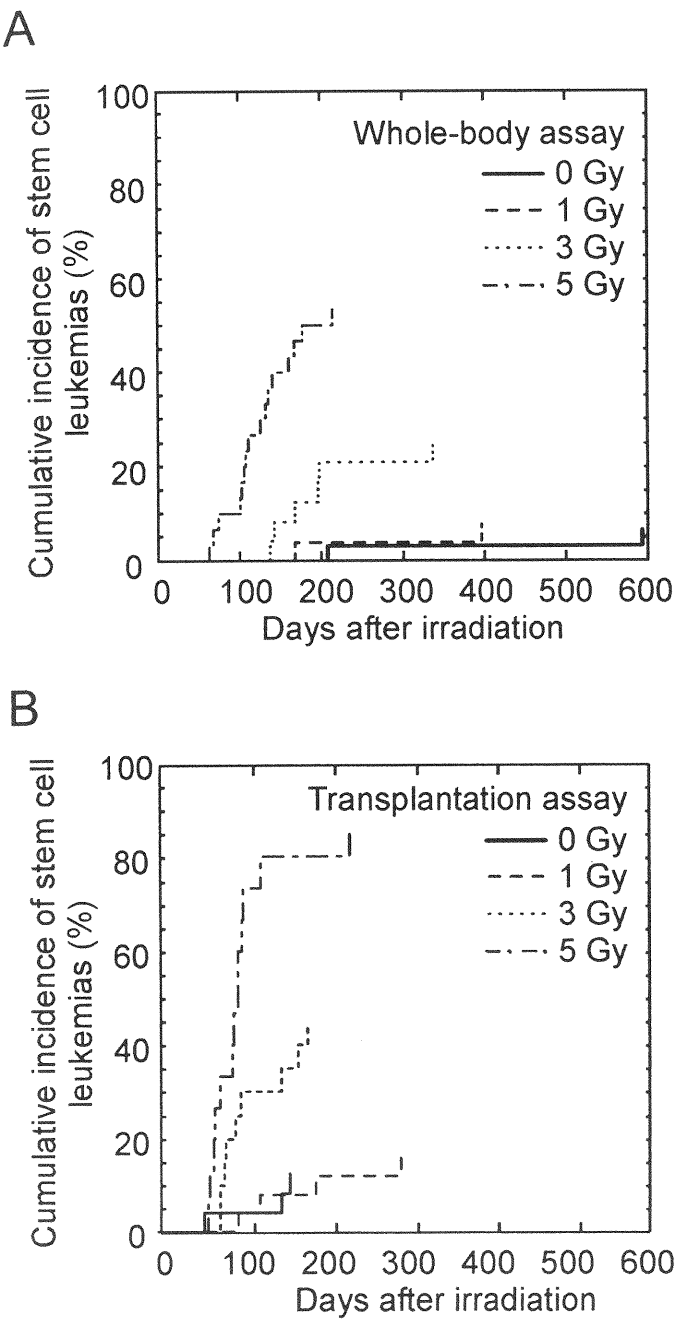

FIG. 4. Cumulative incidences of stem cell leukemias in heterozygous p53-deficient mice exposed to 1, 3 and 5 Gy X rays and in nonirradiated mice. Panel A: Whole-body assay; panel B: transplantation (BMT) assay.

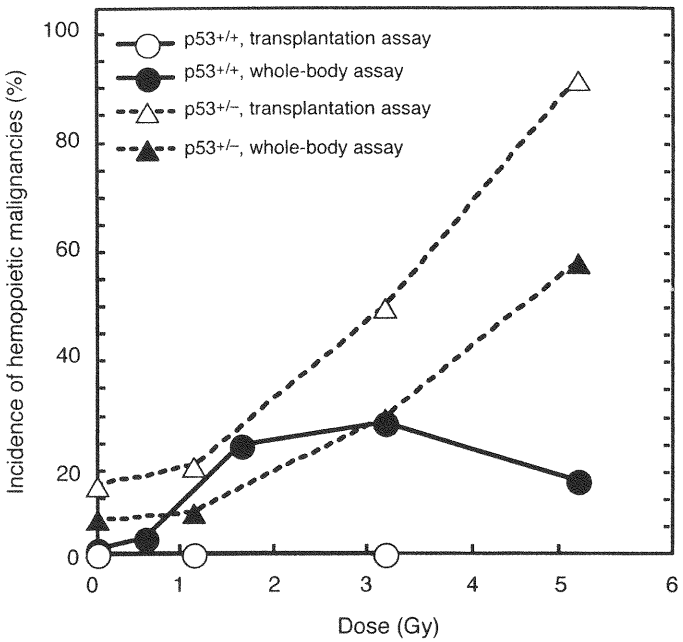

FIG. 5. Dose–response relationship of hematopoietic malignancies for different assays and different p53 genotypes. Data for wild-type mice are from Seki *et al.* (*8*). No hematopoietic malignancies were observed in the wild-type group subjected to the BMT assay.

though not all the stem cell leukemia cases showed LOH, the frequency might be higher, because the PCR primers used in the present study cover a limited part of exon 1 (*23*). Together with the other evidence observed, these results suggest that the stem cell leukemia may require complete inactivation of p53 and that LOH is readily achieved by radiation exposure in a dose-dependent manner in our C3H/He mice.

A few cases of nonhematopoietic soft tissue tumors and leukemias other than stem cell leukemias were observed, neither of which showed any deletion or mutation of the wild-type p53 allele in heterozygous p53-deficient bone marrow. The mechanisms underlying these cases were not examined in this study.

Myeloid leukemia cases in C3H/He mice lack a part of chromosome 2 in 49 out of the 52 cases examined (94%) regardless of radiation exposure (*24*). However, interestingly, none of the leukemia tissues examined showed evidence of the deletion in chromosome 2 (*25*). In the stem cell leukemia cases that developed in the p53-deficient mice in this study, the deletion of a part of chromosome 2 might be skipped because of the rapid development of leukemias, but this possibility has not been fully clarified in this study.

Because an increase in the dose of radiation exponentially decreases the number of hematopoietic progenitor cells, exposure to a dose of more than 5 Gy will not yield a high frequency of leukemias, but instead it will induce a significant decrease in the incidence of leukemias; this is in agreement with the loss of hematopoietic progenitor cells (Fig. 6). Conversely, damaged p53-deficient progenitor cells escaped from the pathway leading to apoptosis after exposure to increasing doses of radiation, and their survival curves indicate resistance to radiation (Fig. 6). Therefore, such surviving damaged hematopoietic progenitor cells are to be targets of leukemogenesis at an even higher radiation dose, for example, 5 Gy. The high incidences of stem cell leukemias observed in both the whole-body assay and BMT assay in heterozygous p53-deficient mice at 5 Gy are possibly attributable to p53-deficient bone marrow cells escaping radiation-induced DNA damage (*26, 27*) and apoptosis (*28–31*). This was observed for the first time in the present

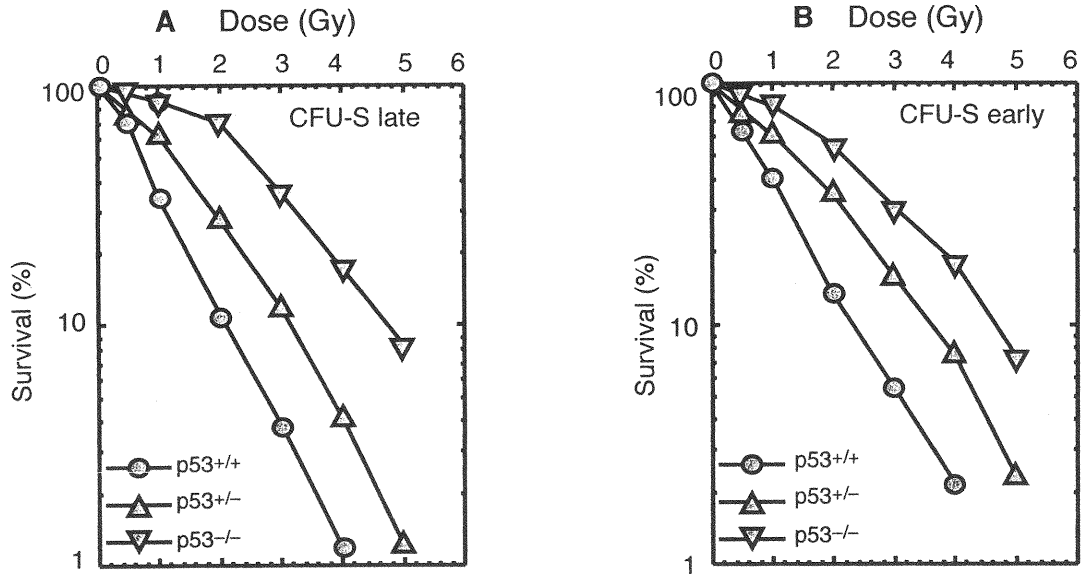

FIG. 6. Radiation survival curves for hematopoietic progenitor cells, colony-forming units in spleen (CFU-S) with p53 deficiency. Bone marrow suspensions were exposed to increasing doses of radiation *in vitro*. Panel A: CFU-S late, counted on day 12; panel B: CFU-S early, counted on day 8.

study. It is of interest that the incidences of stem cell leukemias at 5 Gy were extremely high in both the whole-body and BMT assays. As shown in Fig. 2B, because of the nullification of competitive risk, the incidence of hematopoietic neoplasms at 5 Gy is 100%. This presumably is due to an increase in the incidence of targeted hematopoietic progenitor cells that lack differentiation due to p53 deficiency (*9, 19–22*), resulting in an increase in the incidence of stem cell leukemias. In contrast, with increasing radiation dose in the p53-deficient mice, hematopoietic target stem cells and/or progenitor cells may undergo p53-independent apoptosis. Although have no data for irradiation with 6 Gy or higher, the incidence of leukemias in heterozygous p53-deficient mice may peak at 5 Gy.

The present leukemogenicity studies using p53-deficient mice have elucidated much more clearly the nonthreshold incidence of leukemogenicity than those using incidence curves for wild-type mice (*32*). Conversely, leukemogenesis in wild-type mice is presumably and potentially prevented by p53 gene expression not only at high doses of radiation but also at low doses, as supported by a conventional radiation-induced leukemogenicity curve showing a linear-quadratic relationship.

Assaying leukemogenicity by BMT eliminates the competitive induction of neoplasms other than hematopoietic neoplasms, which occur at an increased frequency in heterozygous p53-deficient mice. Thus the increase in the risk of radiation-induced leukemogenesis with increasing dose was clearly visible in our model in which the "first hit" was genetically engineered. therefore, our model may be a good one for studying for the promotion stage of leukemogenesis. Sporadic nullification of the heterologous p53 allele in somatic cells is also observed in senescent animals and humans. In this regard, the radiation-induced risk of hematopoietic leukemogenesis in p53-deficient mice may be not only a theoretical model but also a practical tool for studying the possible mechanism(s) of senescent tumorigenesis in animals and humans, although p53 alteration generally is not involved in experimental models. Moreover, the present results suggest that using ex.p53ER^{TAM} for tamoxifen-derived p53 gene alteration (*33*) may be useful for developing other experimental models to examine an earlier part of leukemogenesis and/or leukemogenesis of differentiated leukemias. Furthermore, because p53 deficiency tends to induce spontaneous neoplasms other than leukemias, the BMT assay is a useful experimental tool for high-risk neoplastic modeling analogous to the p53-deficient system modeling used in the leukemogenicity bioassay.

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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);

- EURISKED studies the interaction between endocrine disruptors with estrogenic actions; and
- FIRE: aims at an improved, integrated risk assessment of brominated flame retardants for human health and wildlife.

Together, these projects represent more than 60 research laboratories in Europe, with a total budget of more than 20 million Euros. Research on endocrine disruptors is believed to cross traditional borders between human health and environmental research and include themes important to integrated risk assessment such as combined effects, human and non-human receptors, and multi-pathway exposure.

In 2003, a further research call on integrated risk assessment resulted in two projects, NoMiracle and ERApharm, with 54 partners and a total budget of 12.8 million Euros. NOMIRACLE (Novel Methods for Integrated Risk Assessment of Cumulative Stressors in Europe, www.dmu.dk/International/Environment + and + society/ NOMIRACLE/), will develop methodological systems to analyse interactions between environment and health, and integrated risk assessments methods for the evaluation of cumulative effects, interactions between stressors and their influence on human health. ERApharm (Environmental Risk Assessment of pharmaceuticals, http://www.erapharm.org/summary.html) aims to advance existing knowledge and methods for evaluating potential risks, which human and veterinary pharmaceuticals pose to the environment.

In addition to opportunities focused specifically on chemicals, increasing international attention is focusing on problems associated with cyanobacterial blooms as they affect drinking water supplies, agricultural production, recreational opportunities and other ecosystem services, and the condition of aquatic and terrestrial communities. An integrated approach to risk assessment of these blooms was considered at the recent International Symposium of Cyanobacterial Harmful Algal Blooms (Orme-Zavaleta and Munns, submitted). The approach outlined during the symposium considered the direct and indirect effects of cyanotoxins as well as effects that can result from increased algal biomass and other bloom-associated stressors. It also considered socioeconomic risks resulting from loss of ecosystem services and other insults to human well-being. Although not yet finalized at the time of this writing, the symposium report will recommend a number of research directions that will not only support integrated assessments of risks of cyanobacterial blooms, but also development and acceptance of integrated risk assessment as a decision-support methodology.

ISSUES OF DEVELOPMENT AND IMPLEMENTATION

The impediments to the development and implementation of integrated risk assessment are, in general, not regulatory in nature. In most nations, the laws that control pollution to assure the quality of air, land, and water call for protecting both human health and the environment which may imply that assessments take on a holistic approach. Rather, the impediments are institutional and technical.

Institutional Impediments

Some new governmental entities such as the EU and the Republic of South Africa are availing themselves of the opportunity to base their new environmental management on integrated approaches. However, most governments, such as those of the United States, Canada, and Japan, are implementing decades-old laws, using established regulations, in well-entrenched institutional structures. Although the laws might have been enforced in an integrated manner and there is some impetus toward that direction in various assessment activities, they were not. Instead, risks to human health and to nonhuman populations and ecosystems have been assessed separately by distinct groups within regulatory organizations. Over time, they have developed separate approaches and methods that have been incorporated into guidance documents.

Failure to integrate assessments is most obvious in the development of different approaches to the analysis of exposure-response relationships. For example, in the U.S. human health benchmarks such as reference doses are estimated by applying factors of 10 to No Observed Adverse Effects Levels to account for extrapolations between species and between typical humans and sensitive subpopulations and for uncertainties such as from minimal data sets. Quantitative uncertainty analysis of human exposure-response benchmarks is currently precluded by policy. In contrast, quantitative uncertainty analysis may be applied to ecological exposure-response analyses along with probabilistic extrapolation models such as species sensitivity distributions (USEPA 2004). Similarly, toxicity testing of effluents and ambient media is commonly used in ecological risk assessments but not in human health assessments. These discrepancies in assessment methods illustrate the degree of separation of human health and ecological risk assessment in practice and suggest that integrated assessment will require overcoming the policies that create technical barriers.

The opportunities for integration are more obvious in the modeling of chemical transport, fate, and exposure, and the use of common transport and fate models for human health and ecological assessments is common. However, even here institutional barriers operate. For example, the accumulation of chemicals by fish and shellfish has recently been addressed by USEPA guidance for water quality criteria to protect human health (USEPA 1998). Clearly, this is also an important issue for ecological risk assessment. It would have required little additional effort to address issues related to wildlife (*e.g.*, consumption of whole fish versus filets) and publish integrated guidance.

The opportunities for integration could be increased by one important change in risk assessment practices, the weighing of multiple lines of evidence to characterize exposure, response and risk. For example, in the United States toxicological benchmarks for human health are based on a single critical study. That critical study is usually a rodent chronic toxicity test. If, rather than a single critical study, all relevant toxicological data may be used, it would be possible to use data from nonstandard mammalian tests as well as tests of birds and fish. The test data could be interpreted in the light of observations of ecological effects in the field such as deformed piscivorous birds and diseased marine mammals. 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. However, the policy of using a single critical study inhibits that weighing of the entire body of human health and ecological data.

Finally, the institutional tradition of considering only direct effects on human health reduces the opportunities for integration. Although ecological risk assessments consider indirect effects of chemicals including loss of food resources and habitat structure, human health risk assessors do not consider how human health and well-being are affected by damage to the environment. Indirect effects on health have been little studied but could include reduced consumption of wholesome foods such as fish due to contamination, loss of the health benefits of outdoor recreation, and the health effects of stress due to loss of livelihood when resources are destroyed or stress due to perceptions of inhabiting a degraded environment. Even when indirect health effects cannot be demonstrated, reduced environmental quality can result in reduced quality of life. Finally, as ecosystems are degraded, services of nature such as water purification and pollination are lost, resulting in economic costs. None of these effects of chemicals on humans are routinely assessed, because they are not institutionally recognized as risks to humans. If they were, assessments of those risks to human health, well-being and quality of life would necessarily be integrated with ecological risk assessments.

Technical Impediments

Much of the interest in integrated risk assessment of chemicals is associated with the possibility of using the increasing knowledge of toxicokinetics and toxicodynamics to assess effects on various species using a common mechanistic framework. For example, estrogenicity, cholinesterase inhibition, and the "dioxin-like" toxicity of Ah receptor agonists are toxicodynamic mechanisms common to all vertebrates. Similarly, except for the lung/gill dichotomy, the basic routes of toxicokinetics are common to all vertebrates. However, case studies performed by the WHO/IPCS integrated risk assessment program have shown that the benefits of this approach are constrained by the types of data and models that are currently available. That is, knowledge of shared mechanisms will benefit mechanistic assessments more than the current risk assessment approaches that treat effects phenomenologically. Greater benefits will come as the growth of mechanistic knowledge and testing methods leads to new cellular and molecular tests, molecular modeling, and organism simulation modeling. Increasing knowledge of the genomics, proteomics and metabonomics of a wider range of species holds out the promise of being able to address effects by modeling when testing is not practical. As a result, it should become practical to assess risks to taxa such as cetaceans, amphibians, and reptiles that are not now routinely considered. The primary impediment to realizing this vision is the tremendous complexity of the necessary assessment models and their data requirements. However, because of the importance of these techniques to biomedical science, the necessary molecular methods and computational tools are developing rapidly (Waters *et al.* 2003).

There are dangers in attempting to implement this vision of integrated risk assessment. The greatest danger is that ecological risk assessments will become too focused on the effects that other species have in common with humans and will