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

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

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

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

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

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

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

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

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

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

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

One-Off Validation

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

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

Table 1. Recommendations: focus on biological systems.

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

Routine Validation

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

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

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

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

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

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

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

Validation as a Result of Procedural Changes

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

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

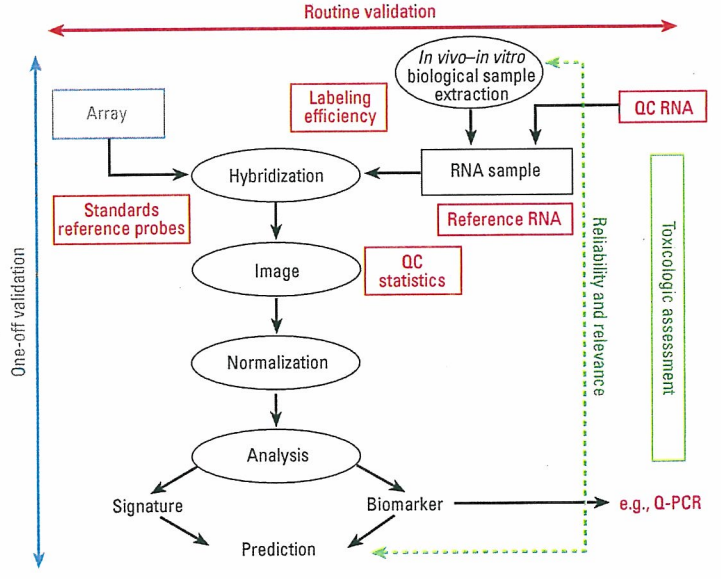

Figure 1. Scheme of the different steps in a toxicogenomics-based test. Three distinct levels were identified where validation is necessary: one-off validation (left), which should be performed once and is mainly related with the quality of the microarray and the instrumentation (blue); routine validation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

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

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

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

Data Management

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

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

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

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

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

Regulatory Acceptance of Validated Toxicogenomics-Based Methods

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

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

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

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

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

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

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

Table 2. Recommendations: focus on technology.

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

involves development of a standardized test method protocol and assessment of the protocol's within- and between-laboratory variability, predictive capacity/accuracy, usefulness and limitations, and adherence to performance standards.

Standards for comparison. As technologic advancements are made and new, modified, or revised toxicogenomics-type test methods are put forward for consideration, it will be necessary to have a means by which the performance of proposed methodologies can be compared with that of existing (traditional and nontraditional) methods, especially those that employ animals. The lack of an approach rooted firmly in high-quality science could jeopardize attempts to seek or gain regulatory acceptance of toxicogenomics-based test methods and strategies. Evaluations of test method performance might be based on comparisons made between particular parameters, as dictated by the specific intent for which the assay was developed. Examples include the following:

- *In vivo–in vivo* study comparisons to examine concordance of gene changes with such factors as onset, duration, severity, dose, age, possible temporal changes of effects, and species differences
- *In vitro–in vivo* study comparisons to explore gene changes associated with a critical event or end point in an *in vitro* cell-based assay and an established *in vivo* biomarker of toxicity
- *In vitro–in vitro* study comparisons to analyze the responses of human and animal cell systems to xenobiotics
- Technologic comparisons to evaluate the effects of proposed technical improvements (e.g., comparing gene changes using different techniques of array/platform preparation)

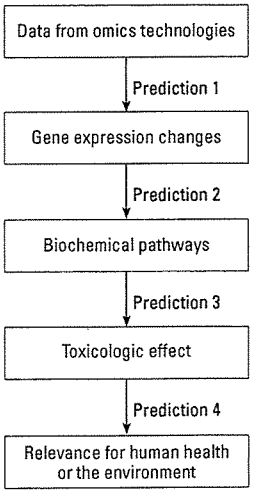

Figure 2. Process flow showing different independent prediction levels considered important in assessing validity of a toxicogenomics-based test method.

Accordingly, to determine the appropriate types of validation activity and comparison in a given situation, it is important that the specific purpose of the proposed methodology and a detailed description of all relevant procedures be clearly elaborated (Balls et al. 1995; Hartung et al. 2004; ICCVAM 1997, 2003).

Toxicogenomics data from in vitro *systems and data relevance.* At the present time, toxicogenomics data derived from *in vitro* systems have been considered to have limited utility in regulatory applications. However, a great deal of interest exists for the further development of *in vitro*–based toxicogenomics methods, for an examination of their potential applicability in the regulatory arena, and for an appraisal of their potential for contributing to improvements in animal welfare. It is anticipated that technologic advancements will ultimately facilitate the use of *in vitro*–based methods as adjuncts to or surrogates for *in vivo*–based methods. Possible areas where validated *in vitro*–based toxicogenomics test methods might play a future role include *a*) preliminary assessments (prescreens), *b*) complementary testing that might assist in obtaining additional (e.g., mechanistic) information, and *c*) surrogate tests that could help in the refinement, reduction, and replacement of animals used for omics-based or traditional testing methods. One exciting aspect of toxicogenomics technology is the prospect of being able to identify species differences and/or similarities in the response to a xenobiotic. Although this is not viewed as near-term prospect, it obviously has potential applications for hazard and risk assessment purposes and could also have an impact on previous regulatory decisions when the technology becomes sufficiently advanced to permit such uses for it.

Additional regulatory acceptance issues. In considering approaches to validation, achieving regulatory acceptance of toxicogenomics-based methods or acceptance of information/data derived from such methods is an important goal. Regulators will be asked to evaluate whether data submitted using omics technologies can be used in support of a particular or broader based toxicologic, pharmacologic, or physiologic premise. For example, experiments using microarrays demonstrated increased expression of a cluster of related genes that was associated with enhanced activity and production of a microsomal enzyme important in the metabolic activation of a chemical to a toxic entity, which in turn was associated with a histopathologic biomarker lesion in the liver with a known human cancer correlate. Each of the events in this example can be thought of as a sequence of separate critical steps or information levels (Figure 2) that progressively connect omics data (from microarrays) to gene expression changes (increased expression), to a biochemical pathway (liver enzyme induction leading to toxic metabolite formation), to a toxicologic effect *in vivo* (liver lesion) with human relevance (cancer). Moving between two levels involves a prediction of outcome linking both steps. At each of these prediction junctures, regulators would be looking for evidence to scientifically substantiate moving to the next step and whether the prediction linking the levels (e.g., in this example, prediction 1, 2, 3, or 4 in Figure 2) was adequately validated. Theoretically, with this type of system, validated links could be established between any two levels. Technologic advancements or new information could be independently incorporated into a given level and considered and evaluated for the specific relevant prediction juncture. In this way, each of the prediction levels can be assessed independently and the validity of the links determined.

In the future toxicogenomics-based test methods may be shown to have been adequately validated and technically suitable for certain specific purposes, but regulatory acceptability and implementation will depend partly on whether the methods validated can be used for a given regulatory agency or program, that is, they are applicable to the products that fall within their regulatory purview. Some regulatory bodies may have internal peer-review processes, specific regulatory mandates, and/or regulatory assessment procedures that also have a role in the determination of test method applicability in regulatory programs, even though a test method may have been appropriately validated.

The widespread use of omics technologies will also bring about increasing demands on the regulatory community in terms of training of regulatory personnel in areas such as potential applications; data QC, analysis, and interpretation; statistical analysis; limitations of the technology; and how the information might be incorporated into safety, hazard, and risk assessment processes. To satisfy these needs, regulatory agencies have been engaging in developing and implementing training procedures, hiring scientists with the necessary technical knowledge and experience, establishing centers of excellence and dedicated laboratories focused specifically on the various omics and related informatics areas [e.g., National Center for Toxicological Research (U.S. FDA), NCT (NIEHS), Minister of Health Labour and Welfare–National Institute of Health Sciences Project in Japan, Netherlands Genomics Initiative, and EMBL–EBI, where informatic scientists are working with experimental practictioners and the MGED Society to ensure that transcriptomic experiments can be mapped on to regulatory toxicology studies]. In addition the regulatory arena has found that maintenance of open lines of communication with appropriate external scientists facilitates cooperation and the sharing of technical aspects, skills,

and practical experiences that help to broaden the collective knowledge base. Regardless, as the technology evolves further and finds wider application and acceptance, it will be necessary to address such fundamental matters as *a*) the generation, management, and interpretation of massive amounts of data; *b*) the consequent complex questions that will undoubtedly arise (e.g., what constitutes an adverse effect as identified using the technology; how does a given gene pattern correlate with a particular toxic end point or relate to onset, duration, and severity of effects, and to age, dose, and species?); and *c*) the limitations to the technology. Addressing such issues efficiently will warrant an ongoing dialogue between regulators and practitioners and a willingness to share relevant experiential and theoretical knowledge. Standard submission and presentation formats compatible with electronic data submission likely would need to be developed. Programs and staff would need to learn how information from the new technologies might be incorporated in regulatory practices and decision-making processes and would also have to face possible incongruities between toxicogenomics-derived data and existing or future submissions of conventional toxicity data. A number of regulatory authorities have already begun to contemplate and make provisions for this enormous and challenging task, but others may not yet have committed the resources to do so.

The recommendations related to regulatory acceptance and use of toxicogenomics-based test methods are listed in Table 3.

Conclusions

This workshop was organized as a result of the rapid growth and technologic advancements in the field of toxicogenomics; the promise it offers for numerous scientific arenas, especially human health and the environment; and the interest demonstrated by regulatory agencies as well as by the industrial sector. Consequently, it has become apparent that a considerable effort needs to be invested in the appropriate validation of both the technology alone and those test methods that incorporate the technology. The workshop provided a platform for technical experts in the field to become cognizant of the validation principles and regulatory issues to be encountered and for regulators and principal validation bodies to gain a better sense of those technologic aspects that would lend themselves to standardization, harmonization, and validation. Thus, this workshop was an important initiative that fostered an exchange of information fundamental to the ultimate adoption of toxicogenomics-based test methods for regulatory decision-making purposes. It is envisioned that the conclusions and recommendations that resulted will be a basis for future validation considerations for test method applications of toxicogenomics technologies in the regulatory arena and evaluating their potential utility for hazard/safety/risk assessments.

Several aspects of the validation of toxicogenomics that were identified as needing further exploration to help facilitate regulatory acceptance of future toxicogenomics-based test methods are as follows:

- Conduct toxicogenomics-based tests and the associated conventional toxicologic tests in parallel to *a*) generate comparative data supportive of the use of the former in place of the latter or *b*) provide relevant mechanistic data to help define the biological relevance of such responses within a toxicologic context
- Determine and understand the range of biologic and technical variability between experiments and between laboratories and ways to bring about greater reproducibility
- In the short term, favor defined biomarkers that are independent from technology platforms, and therefore are easier to validate; in the longer term, focus on pathway analysis (i.e., system biology approach) rather than just on individual genes
- Harmonize reference materials, QC measures, and data standards and develop compatible databases and informatics platforms that are key components of any validation strategy for a toxicologic method; this can only be achieved by promoting partnerships and collaborations among ongoing initiatives in toxicogenomics, standardization, and validation
- Determine performance standards for toxicogenomics-based test methods that will serve as the yardsticks for comparable test methods that are based on similar operational properties
- Define further the modular validation scheme that would allow keeping up with methodologic improvements and innovations without having to repeat the entire validation process but would, however, integrate ECVAM and ICCVAM principles of validation and acceptance.

Table 3. Recommendations: focus on regulatory acceptance of toxicogenomics-based methods.

- Build on and/or learn from previous and ongoing efforts in toxicogenomics, standardization, validation, and harmonization efforts where possible (e.g., MIAME, ICCVAM, ECVAM, NCT, EMBL–EBI, ILSI–HESI, U.S. FDA, U.S. EPA, OECD)
- Fund pilot programs to test possible validation strategies and processes
- Identify training needs and assist in developing training vehicles and ways of presenting the state-of-the-science to regulators and the regulated community (including electronic means)
- Maintain transparency of validation processes
- Explore additions, amendments, and revisions to ICCVAM and ECVAM validation guidance that would accommodate new and rapidly changing technologies
- Implement the modular approach to validation to accommodate existing knowledge and future technical developments
- Establish performance standards for toxicogenomics-based test methods and have them accommodate rapid technologic advancements and procedural modifications
- Explore, develop, and support sector-spanning worldwide harmonization entities
- Create confidence among regulators by involving them early on in discussions and various scientific forums that would facilitate application of the technology for regulatory purposes
- Encourage industry and other parties to share data, in part, to support validation comparisons
- Promote high-quality science in supporting the use and development of the technology for regulatory purposes to further protection of human health and the environment
- Consider opportunities for synergy between QSAR, pharmacokinetic, and pharmacodynamic modeling, and other *in silico* efforts and the toxicogenomics communities

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はじめに
Introduction

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解読された全ゲノムの塩基配列を利用して明らかにしうることは，ゲノム構造に拘束された決定論的な生命の枠組みと，遺伝子発現の可塑性によって展開する確率論的な生命の多様性である．前者ではミレニウム計画などで傾注された薬物代謝における SNPs の発見やテーラーメイド創薬戦略の研究が進んだ一方，後者でいまゲノムと環境の相互作用によって展開される生物の多様なエピジェネティクスとしての環境生体応答学がおもしろくなっている[1]．トキシコゲノミクス[2]はどちらかといえば後者に属し，それはトランスクリプトミクスやプロテオミクス，インフォマティクスなどの総体によって描きだされるトキシコパノミクス(toxicopanomics)とでもよぶべき新領域である．

トキシコパノミクスは物質と生体の応答学であるが，生体側からみる手法をトランスクリプトミクスと考えれば，生体内生成物の側からみる解析手法は主としてプロテオミクスによる．前者では DNA チップなどの高密度集積アレイ解析を用い，後者では GC マスなどの蛋白定量解析手技を適用する．ちなみに代謝に注目すると，前者は，メタボノミクス的手法，後者では代謝物に焦点をあてたメタボロミクスを中心に探査することになる．

この世界ではゲノム科学が取り組んできた帰納的解析にとどまらず，遺伝子発現や蛋白発現をフェノタイプとして用いることによって演繹的に後生的な予測が可能となる．オミクス領域を積極的にそうした予測のツールに発展させようとする点に，ここで取り上げるトキシコパノミクスの，決定論的な応用としての“マイクロアレイ診断”と異なった特徴がある．遺伝子発現情報は塩基配列情報にとどまり転写の経路や蛋白がフェノタイプとしてもつ情報の多様性(complexity)をカバーしないから予測は限定的になる．エピジェネティクスに対する予測能は未知である．そうした限界にもかかわらずこのものの生命の安全性や環境科学へ果たす役割には大きな期待が寄せられている．

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Expression of IAP-Family Proteins in Adult Acute Mixed Lineage Leukemia (AMLL)

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Inhibitor of apoptosis protein (IAP)-family proteins suppress apoptotic signaling in normal/neoplastic cells in various settings. To determine the apoptosis-resistant mechanism in adult acute mixed lineage leukemia (AMLL) with biphenotypic blasts responsible for resistance against chemotherapy, the expression levels of IAP-family proteins in AMLL bone marrow cells were analyzed by quantitative RT-PCR. The overall expression levels of IAPs were higher than those in control, AML, and ALL cells. A significant difference for the expression of survivin was observed between AMLL and AML ($P < 0.05$), and differences between AMLL and ALL were significant for the expression of survivin ($P < 0.05$), NAIP ($P < 0.05$), and XIAP ($P < 0.05$). These findings suggest that higher expression of various IAPs is associated with the chemotherapy-resistant nature of this specific type of leukemia. Am. J. Hematol. 78:173–180, 2005. © 2005 Wiley-Liss, Inc.

Key words: IAP; apoptosis; AMLL; AML; ALL; bone marrow

INTRODUCTION

The regulation of apoptotic cell death has a profound effect on the pathogenesis and progression of hematological malignancies. Acute mixed lineage leukemia (AMLL) is a relatively rare group of hematological malignancies that exhibits the expansion of biclonal or biphenotypic blasts in peripheral blood [1,2]. According to FAB criteria, AMLL may present as ALL or as one of the AML subtypes, often as M1 [2]. AMLL has a high incidence of clonal chromosomal abnormalities, the most common being the t(9;22)(q34;q11) (Ph chromosome) and structural abnormalities involving 11q23 [2]. Recently, molecular analysis revealed that the *mixed lineage leukemia* (*MLL*) gene rearrangement occurs in AMLL cases and also in a fraction of AML/ALL patients [3]. One characteristic feature of AMLL as well as *MLL* gene-rearranged leukemia is a poor patient prognosis associated with lower sensitivity to chemotherapeutic procedures [2,4]. Resistance against chemotherapy might result from the resistance to apoptosis-inducing drugs such as steroids and Ara-C [5,6]. Regarding the complicated mechanisms that regulate apoptosis in the bone marrow of acute leukemias and myelodysplastic syndromes (MDS), we previously showed that a variety of apoptosis-related molecules are active in hematopoietic cells [7–13]. However, the associated parameters and molecules involved in apoptosis in AMLL are unclear.

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IAP-family proteins, including survivin, block apoptosis induced by a variety of triggers [14,15]. Although the biochemical mechanism by which IAP-family members suppress apoptosis is under debate, survivin is known to bind directly to and inhibit caspase-3 and -7, which act as terminal effectors in apoptotic protease cascades [15,16]. The expression of survivin is ubiquitous in fetal tissues but is restricted during development and is negligible in the majority of terminally differentiated adult tissues [17,18]. However, an analysis of the differences in gene expression between normal and tumor cells reveals that survivin is a protein whose gene is most consistently overexpressed in tumor cells relative to normal tissue [19]. Survivin is prominently expressed in transformed cell lines and in many human cancers, including hematopoietic cell tumors [20]. It is also usually detected in the cytoplasm of tumor cells and is therefore widely regarded as a cytoplasmic protein [17,21,22]. However, several studies have shown the nuclear accumulation of survivin in gastric cancer cells [23] and lung cancer cells [24]. We recently reported that ALL cells principally exhibited the nuclear localization of survivin, while CLL cells exhibited cytoplasmic distribution [13]. Although the significance of this nuclear-cytoplasmic expression in tumor cells is still controversial, the subcellular localization of survivin should also be clarified for AMLL subjects.

We also reported that survivin exhibited higher levels of expression in acute lymphocytic leukemia (ALL) and that chronic lymphocytic leukemia (CLL) cases exhibited significant over-expression of survivin and cIAP2 [13]. In acute myelogenous leukemia (AML) cases, some of these IAP-family proteins, such as NAIP and XIAP, are expressed at significantly higher levels [25]. To focus on the contribution of IAPs to the expansion of blasts in AMLL, we examined cases of AMLL that exhibited biphenotypic proliferation of blasts. The expression levels of survivin tended to be high in AMLL samples compared with control bone marrow, AML, and ALL subjects. The expression of other IAPs, including cIAP1, cIAP2, NAIP and XIAP, which suppress apoptosis by inhibiting caspase and procaspase [26–29], was also observed in these samples. The significance of IAP-family proteins in resistance against chemotherapy in AMLL is discussed.

MATERIALS AND METHODS

Patients

Fresh-frozen and formalin-fixed paraffin-embedded bone marrow-aspirated samples from 13 individuals with no hematological disorders were used as normal controls (male/female 5:8; age, median 52 years, range: 25–84 years), 9 patients with AML (8 with M2 and 1 with M1 according to the FAB classification, male/female 5:3; age, median 41 years, range: 19–78 years), 7 patients with ALL (male/female 2:5; age, median 58 years, range: 46–87 years), and 8 patients with AMLL with biphenotypic blasts (male/female 4:4; age, median 50 years, range 17–73 years) were examined. To rule out the influence of aging on bone marrow cells, age-matched control cases were analyzed. Flow-cytometic analysis was routinely performed for CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD13, CD14, CD16, CD19, CD20, CD33, CD34, CD41a, CD56, and HLA-DR. Among them, the data for CD19, CD13, and CD33 were tabulated to demonstrate the biphenotypic nature of blastic cells in AMLL samples (Table I). Diagnoses were based on Catovsky's standard clinical and laboratory criteria [2] including cell morphology [30,31]. All samples were collected at the time of the initial aspiration biopsy and stored at −80°C. We selected the adult M1 or M2 AML samples and adult

TABLE I. Summary of Cases With Adult AMLL

Case no.	Age (years)	Sex	Blast (%)	Cell markers (%)			Chromosome abnormality
				CD19	CD13	CD33	
1	40	F	90.2	98.7	58.3	99.4	45,XX,der(12)t(12;22)(p13;q11) −22
2[a]	57	M	4.2	31.2	50.2	55.0	46,XY
3	67	M	94.4	95.2	67.8	0.9	36,XY,−3,−3,−5,−7,−9,−13,−15,−16,−17,−20
4	61	F	95.7	95.9	6.4	56.6	46,XX
5	17	M	96.4	99.8	53.8	50.6	46,XY
6	21	F	76.0	97.3	75.2	64.0	47,XX,+8
7	43	F	92.0	97.9	67.6	83.1	46,XX,i(8)(q10)del(9)(?q), der(9)del(9)(p22)t(9;22)(q34;q11),der(22)t(9;22)
8	73	M	69.2	89.2	45.4	13.5	46,XY,del(20)(q11.2)

[a]For case 2, material for flow-cytometric analysis was not sufficient at the time of initial diagnosis, although the diagnosis was confirmed as AMLL at the time of second biopsy. For the second biopsy sample, the blast count accounted for more than 90% of the bone marrow cells and consisted of more than 90% CD19-positive cells and more than 50% CD13/CD33-positive cells.

ALL samples for the comparison with AMLL. The AML, ALL, and AMLL samples exhibited the proliferation of blastic cells accounting for more than 80% of all bone marrow cells. The patients were not infected with viruses including HTLV-1 and had not been treated with therapeutic drugs prior to the study.

The procedures followed were in accord with the ethical standards established by the ethics committee of Tokyo Medical and Dental University.

Double Staining for Myeloid and Lymphoid Cell Markers

The phenotype of leukemic cells in AMLL was confirmed by double immunostaining using the formalin-fixed paraffin-embedded bone marrow samples. Sections were deparaffinized and incubated with monoclonal antibody against CD20 or CD79a (DAKO, Glostrup, Denmark) and polyclonal antibody against myeloperoxidase (DAKO). Next, the sections were treated with peroxidase-conjugated anti-mouse IgG followed by a DAB development system and then with alkaline phosphatase-conjugated anti-rabbit IgG (DAKO) followed by development with an alkaline phosphatase–nitroblue tetrazolium chloride–5-bromo-4-chloro-3-indolyl phosphatase development system (DAKO).

Identification of Apoptotic Cells

To identify apoptotic cells, the terminal deoxy-transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method was used as previously described [10]. Briefly, tissue sections were deparaffinized and incubated with proteinase K (prediluted, DAKO) for 15 min at room temperature. After the tissues were washed, TdT, fluorescein isothiocyanate (FITC)-dUTP and -dATP (Boehringer Mannheim, Mannheim, Germany) were applied to the sections, which were then incubated in a moist chamber for 60 min at 37°C. Anti-FITC-conjugated antibody-peroxidase (POD converter, Boehringer Mannheim) was employed to detect FITC-dUTP labeling, and color development was achieved with DAB containing 0.3% hydrogen peroxide solution. The sections were then observed under a microscope and the proportion of TUNEL-positive cells was determined by dividing the number of positively stained cells by the total cell number (count of more than 1,000 cells).

Preparation of RNA and Quantitative Assay for IAP-Family Proteins Using TaqMan RT-PCR

RNA was extracted from frozen bone marrow samples of control subjects with no hematological disorders, AML, ALL and AMLL patients using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's directions. For quantitative RT-PCR, fluorescent hybridization probes and a TaqMan PCR Core Reagents Kit with AmpliTaq Gold (PerkinElmer Cetus, Norwalk, CT) were used with an ABI Prism 7900HT Sequence Detection System (PerkinElmer, Foster City, CA). Oligonucleotides as specific primers and TaqMan probes for the IAP-family and glutaraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were synthesized at a commercial laboratory (PerkinElmer Cetus). The primers and TaqMan probes used were as follows. The sequence of the forward primer for survivin mRNA was 5′-TGCCTGGCAGCCCTTTC-3′ and that of the reverse primer was 5′-CCTCCAAGAAGGGCCAGTTC-3′; the TaqMan probe was 5′-CAAGGACCACCG CATCTCTACATTC-3′. For cIAP1 mRNA, the forward primer was 5′-CAGCCTGAGCAGCTTGCAA-3′ and the reverse primer was 5′-CAAGCCACCAT CACAACAAAA-3′; the TaqMan probe was 5′-TTT ATTATGTGGGTCGCAATGATGATGTCAAA-3′. For cIAP2 mRNA, the forward primer was 5′-TCCGTCAAGTTCAAGCCAGTT-3′ and the reverse primer was 5′-TCTCCTGGGCTGTCTGATGTG-3′; the TaqMan probe was 5′-CCCTCATCTACTTGAA CAGCTGCTAT-3′. The forward primer for NAIP mRNA was 5′-GCTTCACAGCGCATCGAA-3′ and the reverse primer was 5′-GCTGGGCGGATGCT TTC-3′; the TaqMan probe was 5′-CCATTTAAAC CACAGCAGAGGCTTTAT-3′. The forward primer for XIAP mRNA was 5′-AGTGGTAGTCCTGTTT CAGCATCA-3′ and the reverse primer was 5′-CCGCACGGTATCTCCTTCA-3′; the TaqMan probe was 5′-CACTGGCACGAGCAGGGTTTCTT TATACTG-3′. Finally, the forward primer for GAPDH mRNA was 5′-GAAGGTGAAGGTCG GAGT-3′ and the reverse primer was 5′-GAA GATGGTGATGGGATTTC-3′; the TaqMan probe was 5′-CAAGCTTCCCGTTCTCAGCC-3′. The conditions for one-step RT-PCR were as follows: 30 min at 48°C (stage 1, reverse transcription), 10 min at 95°C (stage 2, RT inactivation and AmpliTaq Gold activation), and then 40 cycles of amplification for 15 sec at 95°C and 1 min at 60°C (stage 3, PCR). The expression of survivin and other IAP-family proteins was quantitated according to a method described elsewhere [13]. Briefly, the intensity of the reaction was evaluated from the quantity of total RNA in Raji cells (ng) corresponding to the initial number of PCR cycles to reveal the linear increase in reaction intensity (threshold cycle) for each sample on a logarithmic standard curve. Data on the quantity of RNA (ng) for the IAP family was normalized using the data for GAPDH in each sample, and then the ratio to the mean value of control subjects was calculated and compared.

Immunohistochemistry for Survivin and Proliferating Cells

Tissue sections (4 μm thick) of bone marrow from the control, AML, ALL, and AMLL cases were cut on slides covered with adhesive. The sections were deparaffinized, and endogenous peroxidase was quenched with 1.5% hydrogen peroxide in methanol for 10 min. Antibodies were applied to identify survivin and to characterize proliferating cells. The primary antibodies included polyclonal rabbit antibody against human survivin (SURV 11-A, Alpha Diagnostic International, Inc., San Antonio, TX) and monoclonal antibody Ki-67 (DAKO). All sections were developed using biotin-conjugated secondary antibodies against rabbit IgG or mouse IgG followed by a sensitive peroxidase-conjugated streptavidin system (DAKO) with DAB as the chromogen. Negative control staining was performed using rabbit or mouse immunoglobulin of irrelevant specificity substituted for the primary antibody. The proportion of Ki-67-positive cells was determined in the same way as the proportion of TUNEL-positive cells.

Statistical Analysis

Statistically significant differences in the quantitative analysis were determined using the Mann–Whitney *U*-test for comparisons between the control, AML, ALL, and AMLL samples.

RESULTS

Clinicopathological Characteristics of Cases With Acute Mixed Lineage Leukemia

To determine the clinicopathological characteristics of cases with AMLL, the clinical data for cases including laboratory findings are summarized in Table I. As indicated by the flow-cytometric data, bone marrow blasts in these cases exhibited a high frequency of B-cell lineage antigen (CD19) and myeloid cell marker (CD13 and/or CD 33) expression. Thus, blasts of these cases were "biphenotypic." Chromosomal abnormalities were identified in 5 cases (cases 1, 3, 6, 7, and 8), and the Philadelphia chromosome was identified in two cases (cases 1 and 7). Although abnormalities involving chromosome 11q were identified in two cases (cases 1 and 8), the molecular rearrangement of the *mixed lineage leukemia* (*MLL*) gene located on chromosome 11q23 [32,33] was not observed at the chromosome level.

In spite of AML- and ALL-directed therapy (cytarabine, vincristine, etoposide, adriamycin, predonin, etc.), five patients failed to exhibit complete hematological remission, having blast persistence in bone marrow above 10%. Although complete remission could be induced by chemotherapy in four cases (cases 3, 6, 7, and 8), relapse with leukemic blast proliferation occurred within 6 months in two cases (cases 3 and 6, Table II). Overall, most cases exhibited a poor prognosis and the survival times after diagnosis were shorter than 14 months for 5 cases. However, one patient who received a bone marrow transplant (case 6) and the other patients who received chemotherapy (cases 7 and 8) lived.

Double Immunostaining for Myeloid and Lymphoid Cell Markers on AMLL Cells

To confirm the biphenotypic nature of blasts in the AMLL samples, double immunostaining for myeloid and lymphoid cell markers was performed. The majority of AMLL cells exhibited positive signals for B-cell markers such as CD20 or CD79a, while the myeloid cell marker (myeloperoxidase) was partially observed for many of the cases examined

TABLE II. Treatment and Outcome of Cases With Adult AMLL*

Case no.	First treatment	Response and status	Second treatment	Response status	Survival (months)
1	A-VVV	Failure	H-CPM/VP-16	Failure	3
2	H-CPM/VP-16, H-AraC + MIT	Failure	TBI + CPM	Failure	5
3	DCM, H-AraC + MIT	CR, relapse	A-VVV, H-AraC	Failure	6
4	L-AdVP, MVP	Failure	B-VVV, H-CPM/VP-16, H-AraC, L-AdVP	Failure	11
5	AdVP	Failure	A-VVV, VP-16, CAG	Failure	14
6	DC, A-VVV, H-CPM/VP-16	CR, relapse	H-AraC + MIT, BMT	CR and alive	>6
7	A-VVV	CR	H-AraC + MTX	CR and alive	>6
8	CAG	CR	DC	CR and alive	>9

*Abbreviations: A-VVV, AraC (cytarabine) + VCR (vincristine) + VLB (vinblastine) + VP-16 (etoposide); H-CPM, high-dose CPM (cyclophosphamide); H-AraC, high-dose AraC; MIT, mitoxantrone; TBI, total body irradiation; DCM, DNR (daunorubicin) + AraC + 6-MP (mercaptopurine); CR, complete remission; L-AdVP, L-Asp (L-asparaginase) + ADR (doxorubicin) + VCR + PDN (predonin) + CPM; MVP, MIT + VP-16 + PDN; B-VVV, BHAC (enocitabine) + VCR + VLB + VP-16; CAG, AraC + ACR (acurabicin) + G-CSF (lenograstim); DC, DNR + AraC; BMT, bone marrow transplantation; MTX, methotrexate.

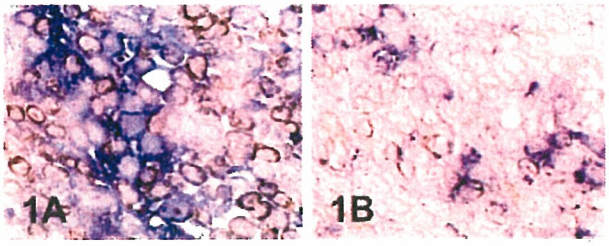

Fig. 1. Double immunostaining for a B-cell marker (CD79a) and myeloid cell marker (myeloperoxidase) in cases with AMLL (A, case 8; and B, case 6; original magnification 400×). Note that the majority of blasts stained positively for CD79a (brown) and a portion of them also stained positive for myeloperoxidase (blue) in both cases. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(Fig. 1A for case 8 and Fig. 1B for case 6). These findings were consistent with the flow-cytometric analytical data shown in Table I.

Apoptotic Frequency and Proliferation Activity of Acute Mixed Lineage Leukemia

To identify the apoptotic and proliferative cells present in the bone marrow samples, the TUNEL method and immunohistochemistry for Ki-67 were performed on paraffin-embedded sections. As expected from our previous studies [7,10], the frequency of apoptosis was significantly lower in AML (median, 0.769; range, 1.06–0.219) ($P < 0.001$) and ALL bone marrow cells (median, 0.543; range, 1.18–0.072) ($P < 0.01$) than control cells (median, 2.03; range, 2.81–0.848), and the proliferative cell ratio in AML/ALL bone marrow (median, 39.7; range, 47.8–32.4/median, 45.9; range, 71.9–34.2) was significantly higher than that in control cases (median, 19.2; range, 24.3–10.0) ($P < 0.0001$ and $P < 0.001$, respectively). As shown in Table III, AMLL cells exhibited a tendency similar to AML and ALL cells in that the apoptotic ratio (median, 0.176; range, 1.69–0.021) was significantly lower than the control ($P < 0.01$) and the proliferative cell ratio (median, 26.7; range, 49.1–18.3) was significantly higher ($P < 0.01$). However, AMLL cells exhibited a relatively lower apoptotic index and also significantly lower proliferative index compared with the AML ($P < 0.05$) or ALL samples ($P < 0.05$).

Expression of IAP-Family Proteins Determined by Real-Time Quantitative PCR

To quantitate the mRNA expression levels of the IAP-family members in AMLL cells, real-time quantitative RT-PCR was performed using bone marrow samples from control, AML, ALL, and AMLL cases.

TABLE III. Apoptotic Frequency and Proliferation Activity of Bone Marrow Cells From Control and Acute Leukemia Cases*

	$TUNEL^+$ cell ratio (%)	$Ki\text{-}67^+$ cell ratio (%)
Cases	Median (max-min)	Median (max-min)
Control	2.03 (2.81–0.848)[a,b,c]	19.2 (24.3–10.0)[d,e,f]
AML	0.769 (1.06–0.219)[a]	39.7 (47.8–32.4)[d,g]
ALL	0.543 (1.18–0.072)[b]	45.9 (71.9–34.2)[e,h]
AMLL	0.176 (1.69–0.021)[c]	26.7 (49.1–18.3)[f,g,h]

*Values indicate the median, maximum, and minimum. Differences were significant between the TUNEL-positive cell ratio for control and AML ([a]$P < 0.001$), control and ALL ([b]$P < 0.01$), and control and AMLL ([c]$P < 0.01$) as seen by the Mann–Whitney U-test. The Ki-67-positive cell ratio exhibited significant differences between control and AML ([d]$P < 0.0001$), control and ALL ([e]$P < 0.001$), control and AMLL ([f]$P < 0.01$), AML and AMLL ([g]$P < 0.05$), and ALL and AMLL ([h]$P < 0.05$) as seen by the Mann–Whitney U-test.

As shown in Fig. 2, the expression of survivin ($P < 0.05$), cIAP1 ($P < 0.05$), NAIP ($P < 0.01$), and XIAP ($P < 0.01$) exhibited significant up-regulation in AMLL compared with the controls. The mRNA for survivin ($P < 0.05$) showed significantly higher levels of expression in AMLL than AML, while the expression levels of survivin ($P < 0.05$), NAIP ($P < 0.05$), and XIAP ($P < 0.05$) in AMLL were significantly higher than those in ALL.

In summary, survivin expression in AMLL was significantly higher than the expression in control, AML, and ALL. The expression level of cIAP1 in AMLL was significantly higher than that in control, but similar with the expression in AML and ALL. Regarding cIAP2, the AMLL cases exhibited stronger expression than the control, AML, and ALL samples although the differences were not significant. NAIP expression in AMLL was significantly higher than control and ALL. The expression level of XIAP in AMLL was significantly higher than control and ALL but similar with AML. No remarkable differences were found between IAP protein expression and patients' age, sex, phenotype, or genotype for AMLL, although further analysis would be necessary because the number of cases was rather small.

These results indicate that the overall expression of IAP-family proteins in AMLL subjects tended to be higher than that for the control, AML, or ALL samples. Specifically, survivin expression in AMLL was significantly higher than that for the control, AML, and ALL samples.

Immunohistochemical Detection of Survivin in the Bone Marrow of AMLL Subjects

To investigate the distribution of survivin, immunohistochemical staining was performed on bone

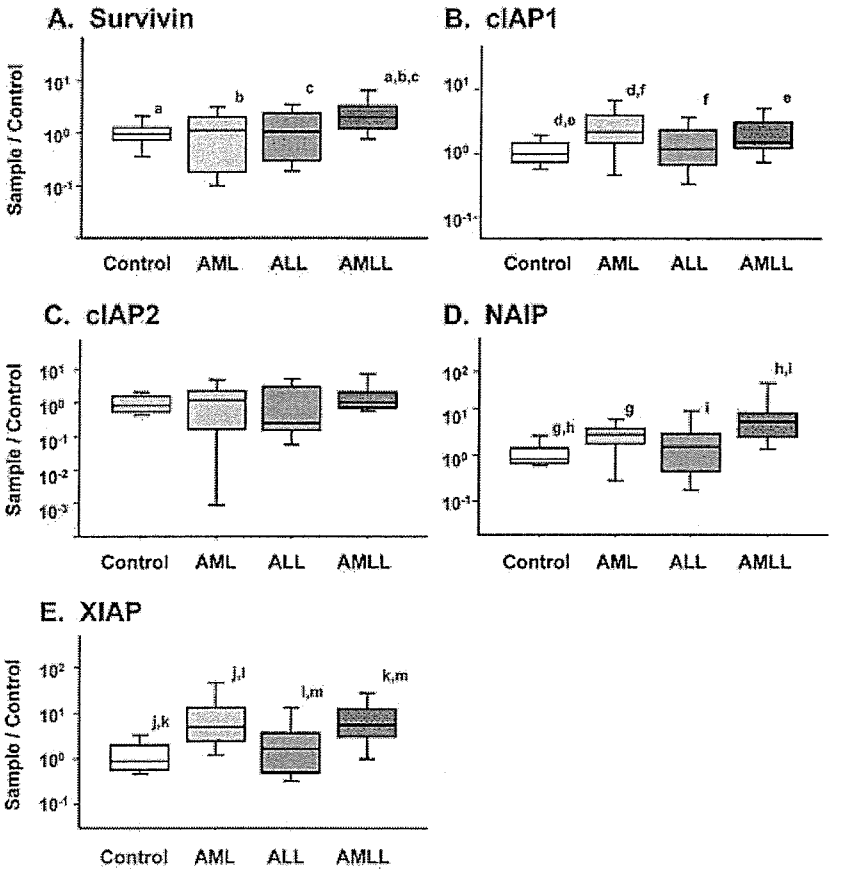

Fig. 2. Expression of IAP-family proteins in control bone marrow and acute leukemias determined by real-time quantitative RT-PCR. The relative intensity was calculated as (intensity of the reaction of IAP-family members [total Raji RNA, ng])/(intensity of the reaction of GAPDH [total Raji RNA, ng]). The intensities of the expressions from the AML, ALL, and AMLL samples are indicated as the ratios to the intensity of the control subjects. The box–bar graphs indicate the value of the control, AML, ALL, and AMLL cases: the bars indicate the 90th and 10th percentiles, and the box indicates the 75th to 25th percentiles. Differences were significant between samples as seen by the Mann–Whitney *U*-test as follows: (A) survivin—control and AMLL ($^{a}P < 0.01$), AML and AMLL ($^{b}P < 0.05$), and ALL and AMLL ($^{c}P < 0.05$). (B) cIAP1—control and AML ($^{d}P < 0.01$), control and AMLL ($^{e}P < 0.05$), and AML and ALL ($^{f}P < 0.05$). (C) cIAP2—differences were not significant. (D) NAIP—control and AML ($^{g}P < 0.05$), control and AMLL ($^{h}P < 0.01$), and ALL and AMLL ($^{i}P < 0.05$). (E) XIAP—control and AML ($^{j}P < 0.01$), control and AMLL ($^{k}P < 0.01$), AML and ALL ($^{l}P < 0.05$), and ALL and AMLL ($^{m}P < 0.05$).

marrow samples from AMLL subjects. As we previously showed [13], survivin was detected in only a few scattered myeloid cells in the control bone marrow samples and subcellular localization was mainly cytoplasmic but partly nuclear. The staining pattern and intensity in the control bone marrow was constant between different samples. All of the AMLL samples showed positive staining for survivin, although the staining intensity and frequency varied for each case. At the cellular level, survivin signals in AMLL cells were predominantly localized in the nucleus and also weakly in the cytoplasm (Fig. 3A). However, one case exhibited prominent cytoplasmic staining with mildly positive staining in the nucleus (Fig. 3B). The tissue sections that reacted with preimmune rabbit antibody of nonrelevant specificity showed no significant staining for any of the samples (not shown).

DISCUSSION

AMLL blasts are expected to possess more immature or intermediate characters of AML and ALL blasts because they express both myeloid and lymphoid phenotypes. Regarding the expression of survivin in myeloid neoplasms, previous studies have revealed the significant expression of survivin in AML [34,35]. Adida et al. [35] reported that survivin expression frequently occurs in AML, detecting it in 60% of a series of 125 patients analyzed, and survivin expression was found to be an unfavorable prognostic factor. In contrast, in lymphoid neoplasms, several

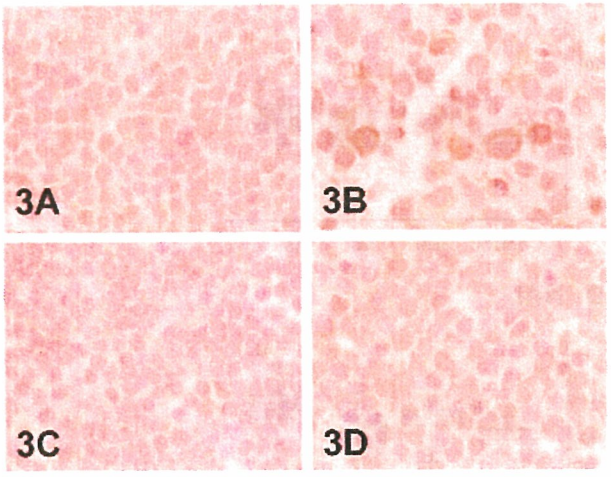

Fig. 3. Immunostaining for survivin in the bone marrow of AMLL (A, case 5; and B, case 8) in comparison with AML (C) and ALL (D) (original magnification 400×). Development was performed using the peroxidase–DAB system (brown) with hematoxylin counterstaining. Note the positive signals in the nucleus as well as the cytoplasm of AMLL cells (A) in contrast to the cytoplasmic staining (B). AML (C) and ALL (D) cases exhibited nuclear and partial cytoplasmic staining. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

studies investigated the dynamics of survivin expression in association with cell proliferation. The in vitro data on mononuclear cells from peripheral blood or bone marrow indicated that B-CLL cells expressed survivin in concert with CD40 and that survivin was the only IAP whose expression was induced by the CD40 ligand (CD40L) [36]. CD40 belongs to the TNF receptor superfamily [37], and its stimulation rescues B-CLL cells from apoptosis and induces proliferation [38]. We recently found that ALL as well as CLL cells exhibited significant expression of survivin and cIAP2 [13]. Thus, both in myeloid and lymphoid neoplasms, IAPs are expressed and seemed to influence the prognosis of patients. Therefore, we can imagine that IAPs would have functions also in AMLL blasts; however, little is known about the potential roles of survivin and other IAPs in the pathogenesis of AMLL.

A major problem with leukemia treatment is drug resistance to chemotherapeutic agents, which may already be present upon diagnosis or after chemotherapy for minimal residual blasts. Resistance originates from genetic or epigenetic mutations during growth of the leukemic clone. Anti-apoptosis mechanisms, alterations of tumor suppressor genes, altered immunogenicity, and drug-resistance mechanisms act in combination [39]. AMLL exhibits strong resistance against chemotherapy, resulting in poor patient prognosis [40,41]. In the present study, expression levels of IAPs in AMLL blasts were higher than those in control samples. Furthermore, several IAPs, such as survivin, NAIP, and XIAP, exhibited stronger expression in AMLL compared with conventional acute leukemias. Thus, the IAP expression level is one criterion that can be used to explain the strong drug resistance in this category of leukemia. The IAP might function probably via the inhibition of caspase-dependent apoptotic signaling. Although we have yet to clarify the caspase-independent pathway of apoptosis in AMLL, the findings of the present study suggest that the regulation of IAPs may become a possible target of AMLL therapy in the future.

In addition to its anti-apoptotic function, survivin also helps regulate cell-cycle progression during mitosis [20]. The highly proliferative activity of AMLL bone marrow cells as well as AML/ALL cells might be associated with survivin expression. As for the expression of IAPs in AML/ALL, the present study found strong expression in some cases and control levels in others, suggesting that AML/ALL cases are heterogeneous in terms of IAP expression.

The human *MLL* gene is involved in about 50 different chromosomal translocations associated with the acute leukemia phenotype [42]. Although chromosomal rearrangement involving chromosome 11q23 was not identified, the cases in the present study were not examined for the presence of *MLL* gene rearrangement by PCR analysis at the DNA level. Further studies are necessary to clarify the interaction of the *MLL* gene and IAP-family genes in association with apoptotic signaling in AMLL blasts.

In conclusion, we showed that strong expression of IAPs, especially survivin and NAIP, occurs in AMLL. Further studies are warranted to clarify the regulatory mechanisms of IAP expression in AMLL in association with drug resistance in this leukemia.

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DNA-dependent protein kinase enhances DNA damage-induced apoptosis in association with Friend gp70

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Abstract

Friend leukemia virus (FLV) infection strongly enhances γ-irradiation-induced apoptosis of hematopoietic cells of C3H hosts leading to a lethal anemia. Experiments using *p53* knockout mice with the C3H background have clarified that the apoptosis is *p53*-dependent and would not be associated with changes of cell populations caused by the infection with FLV. In bone marrow cells of FLV + total body irradiation (TBI)-treated C3H mice, the p53 protein was prominently activated to overexpress *p21* and *bax* suggesting that apoptosis-enhancing mechanisms lay upstream of p53 protein in the signaling pathway. Neither of DNA-dependent protein kinase (DNA-PK)-deficient SCID mice nor ataxia telangiectasia mutated (*ATM*) gene knockout mice with the C3H background exhibited a remarkable enhancement of apoptosis or p53 activation on FLV + TBI-treatment indicating that DNA-PK and ATM were both essential. ATM appeared necessary for introducing DNA damage-induced apoptosis, while DNA-PK enhanced *p53*-dependent apoptosis under FLV-infection. Surprisingly, viral envelope protein, gp70, was co-precipitated with DNA-PK but not with ATM in FLV + TBI-treated C3H mice. These results indicated that FLV-infection enhances DNA damage-induced apoptosis via p53 activation and that DNA-PK, in association with gp70, might play critical roles in modulating the signaling pathway.

Keywords: DNA damage; Apoptosis; Friend virus; DNA-PK; ATM

1. Introduction

p53 has important roles in the cellular response to DNA damage-inducing agents such as ionizing irradiation [1–3]. Ionizing irradiation induces DNA-double strand breaks in cells, and then the stabilization and accumulation of p53 protein by phosphorylation of the N-terminal serine residues, leading to a disruption of MDM2 interaction which negatively regulates p53 [4,5]. In response to DNA damage, p53 protein is also activated by phosphorylation or acetylation allowing conformational changes convenient for the DNA binding domain to play a role. Activated p53 binds to specific DNA sequences and acts as the transcription factor whose target genes are mainly involved in cell cycle arrest and apoptosis. Although the mechanisms for the activation of p53 after DNA-double strand breaks are still unclear, the catalytic subunit of DNA-dependent protein kinase (DNA-PK) and ataxia telangiectasia mutated (ATM) kinase are candidates for the upstream activator or the regulator of p53 [6,7]. These proteins have homology and are members of the phosphatidylinositol 3 (PI3) kinase family that can phosphorylate p53 in vitro. Wang et al. [8] have proposed that DNA-PK and ATM are similar in the selective activation of p53, but dissimilar in that DNA-PK selects for apoptosis but not cell cycle arrest, and ATM for cell cycle arrest but not apoptosis. However, others have demonstrated that not DNA-PK but

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ATM functions as the major activator of p53 in response to DNA damage in vivo [9–15]. On the other hand, the main role of DNA-PK in vivo is thought to promote the rejoining of DNA breaks by non-homologous end-joining [16,17]. Therefore, the control mechanisms of these kinases, linking DNA damage to *p53*-dependent apoptosis, are still controversial.

Viral infection has been known to have various effects on the apoptotic signaling pathways of cells, negatively and positively. For example, viral FLICE-inhibitory proteins prevent apoptosis induced by death receptors [18] and the *ets-2* transcription factor inhibits apoptosis through a *bcl-xL*-dependent mechanism [19]. In contrast, HIV-infection causes apoptosis in $CD4^+$ T cells via various pathways [20], and Moloney murine leukemia virus is shown to enhance thymocyte apoptosis [21]. HTLV-I Tax expression promotes anti-apoptotic or apoptotic processes in T cells according to the experimental conditions [22].

Recently we found that Friend leukemia virus (FLV) infection strongly enhanced DNA damage-induced apoptosis in mice of an FLV-susceptible C3H strain [23]. Mice were infected with FLV and then treated with split low dose γ-irradiation (total body irradiation: TBI). Surprisingly, FLV + TBI-treated C3H hosts died within 10 days after TBI treatment, while mice just infected with FLV survived for more than 40 days. The hematopoietic cells, especially erythroid cells of FLV + TBI-treated C3H mice, revealed frequent apoptosis causing lethal anemia in these mice. Experiments using *p53* knockout mice with the C3H background clarified that the apoptosis was *p53*-dependent and would not be associated with changes of cell populations caused by the infection with FLV [23]. Regarding apoptosis and FLV infection, an enhancement of anti-apoptotic signaling has been observed in transformed cell lines [24,25] as well as primary erythroblasts [26]. However, effects of FLV-infection on pro-apoptotic signaling have been unknown. In the present study, we used C3H·SCID mice in which the activity of DNA-PK would be deficient [27–29] and *ATM* knockout mice with the C3H background to elucidate the mechanisms behind the enhanced apoptotic signaling in C3H cells after FLV + TBI-treatment. The results indicated that enhanced apoptosis in vivo required both DNA-PK and ATM. To further demonstrate the regulatory mechanisms of *p53*-dependent apoptotic pathways, these PI3 kinases as well as viral protein were analyzed and the relation between FLV-infection and the enhanced DNA damage-induced apoptosis was discussed. The mechanism of enhanced *p53*-dependent apoptosis in the present system might aid in generating a novel gene therapy model using *p53* by controlling the *p53*-dependent cell death.

2. Materials and methods

2.1. Mice

Eight to ten-week-old male C3H/He mice (C3H, $H\text{-}2^k$, $Fv\text{-}2^s$), C3H·SCID mice and *ATM* knockout mice with the C3H background (C3H·$ATM^{-/-}$) were bred from our colony at the Animal Production Facility of the National Institute of Radiological Sciences in Chiba. Methods for the generation of the knockout construct and $ATM^{-/-}$ mice were described elsewhere [30]. The SCID and $ATM^{-/-}$ mice with the C3H background were generated by crossing CB.17·SCID and 129/Sv $ATM^{-/-}$ mice to the C3H strain of mice, respectively, followed by backcrossing through more than 20 generations. All of the mice were reared and treated in accordance with the guidelines governing the care and use of laboratory animals at the National Institute of Radiological Sciences (approval numbers 1997-4 and 1997-17) and also the guidelines established by the Animal Experiment Committee of the Tokyo Medical and Dental University.

2.2. Viral infection and total body irradiation

An NB-tropic Friend leukemia virus (FLV) complex, originally from Dr. C. Friend, was prepared as described earlier [31,32] and injected i.p. into mice at a highly leukemogenic dose of 10^4 PFU/mouse [33]. On day 7 after inoculation with FLV, 8–10-week-old mice were treated with 3 Gy of total body irradiation (TBI). A dose of 3 Gy TBI was delivered from a GAMMA-CELL-40 at a dose rate of 1.12 Gy/min. Sham-treated mice that were not irradiated were also prepared in each experiment.

2.3. Detection of apoptotic cell

Fresh bone marrow tissue was mounted in an OCT compound (Sakura, Tokyo, Japan), frozen with liquid nitrogen and cut to make 8–10 μm-thick frozen sections. To identify apoptotic cells on frozen tissue sections by terminal deoxytransferase (TdT)-mediated dUTP nick end labeling (TUNEL), an in situ cell death detection kit, fluorescein (Boeringer Mannheim, Mannheim, Germany) was used as described previously [34]. Briefly, frozen sections were fixed with a 4% paraformaldehyde solution for 20 min, washed with phosphate-buffered saline (PBS), incubated in 0.1% sodium citrate–0.1% Triton X-100 for 2 min, washed with PBS and then incubated with fluorescein isothiocyanate (FITC)-labeled dUTP and TdT at 37 °C for 60 min. Sections were then observed by fluorescence microscopy and the TUNEL-positive cell ratio was determined by dividing the cell number of positively stained cells by the total cell number (counting more than 1000 cells).

2.4. Antibodies

The mouse monoclonal anti-p53 antibody Pab421 (Oncogene Research Product, Cambridge, MA) was used for immunoprecipitation. Cocktails of the mouse monoclonal anti-DNA-PK antibodies 18-2, 25-4, and 42-psc (NeoMarkers, San Jose, CA) and the rabbit polyclonal

anti-ATM antibody Ab-3 (Oncogene Research Product) were used for immunoprecipitation or as primary antibodies for immunoblotting. The mouse monoclonal anti-p53 antibody Pab240 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal phospho-p53 (Ser15) antibody (Cell Signaling Technology Inc., Beverly, MA), goat polyclonal antibody for Friend MuLV (ATCC, Manassas, VA), goat polyclonal anti-Moloney MuLV gp70 antibody (Quality Biotech, Camden, NJ) which is known to cross-react with F-MuLV gp70 [34], goat polyclonal anti-Raucher MuLV gp70 antibody (ATCC) which is expected to cross-react with F-MuLV gp70 and rabbit polyclonal anti-actin antisera (Sigma Chemicals) were used as primary antibodies for immunoblotting. Horseradish peroxidase-conjugated anti-mouse IgG antibody (Dakopatts, Glostrup, Denmark), horseradish peroxidase-conjugated anti-rabbit antisera (Dakopatts), and horseradish peroxidase-conjugated anti-goat IgG antibody (Dakopatts) were used as secondary antibodies for immunoblotting.

2.5. Immunoprecipitation and immunoblotting analysis

The bone marrow cells from each experimental group of mice were suspended in Iscove's modified Dulbecco's medium (IMDM; Sigma Chemicals, St. Louis, MO) containing 10% fetal bovine serum and pelleted. Cell lysates were prepared by incubating cell pellets on ice for 15 min in ice-cold lysis buffer containing 10 mM Tris–HCl, pH 7.5, 5 mM EDTA, 1% Nonidet P-40, 0.02% NaN_3, 1 mM PMSF, 0.1% aprotinin, 100 μM leupeptin, and 100 μM TPCK (Sigma Chemicals). Supernatants were separated from debris by centrifugation at 12,000 rpm for 5 min at 4 °C. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Cell lysates which contained 100 μg of protein were incubated with antibodies and protein A-sepharose beads (Amersham Life Science, Buckinghamshire, England). The resulting immunoprecipitates or whole cell lysates of 50–100 μg were subjected to 6–12.5% SDS–PAGE. Gels were transferred electrophoretically to nitrocellulose membranes (Schleicher and Schull, Dassel, Germany). The membranes were blocked in 10% skim milk in PBS, incubated with primary antibodies, and after washing, were incubated with peroxidase-conjugated secondary antibodies. Bands in the washed membrane were detected with an enhanced chemiluminescence (ECL) system (Amersham Life Science) as described previously [35]. In a part of gp70 experiments, cell lysate was divided into two fractions, cytoplasmic and nuclear fraction, according to the protocol by Dignam et al. [36], and then, these fractions were used for immunoprecipitation and immunoblotting.

2.6. Reverse transcription (RT)-polymerase chain reaction (PCR)

To determine the activation of *p21* and *bax*, known as downstream molecules of p53, and to examine the expression of *DNA-PK* and *ATM* genes at the mRNA level, an RT-PCR was performed for each experimental group. The RNA was extracted from the bone marrow using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's directions. Tissue RNA (100 ng) was used as a template for the amplification. Complementary (c)DNA was synthesized using Rous-associated virus reverse transcriptase (Takara Biomedicals, Kyoto, Japan). The PCR was performed as described elsewhere [34]. Oligonucleotides as specific primers for *p21* and *bax* were synthesized by a commercial laboratory (Life Technologies Oriental, Tokyo, Japan). As a control reaction, *β-actin* was also detected in each run. The sequences of primers were as follows: *p21*: 5′ PCR primer AATCCTGGTGATGTCCGACC, 3′ PCR primer TTGCAGAAGACCAATCTGCG; *bax*: 5′ PCR primer CCAGCTCTGAACAGATCATG, 3′ PCR primer AGCTCCATATTGCTATCCAG; *DNA-PK*: 5′ PCR primer GAATTCACCACAACCCTGCT, 3′ PCR primer GCTTTCAGCAGGTTCACACA; *ATM*: 5′ PCR primer TTACGATGGCAACAGCAGAG, 3′ PCR primer TCCAGTTCTCGCTGAACCTT; *β-actin*: 5′ PCR primer TGGAATCCTGTGGCATCCATGA, 3′ PCR primer ATCTTCATGGTGCTAGGAGCCAG. The expected sizes of the PCR products were 461 bp for *p21*, 187 bp for *bax*, 188 bp for *DNA-PK*, 225 bp for *ATM*, and 175 bp for *β-actin*. *ϕX174/Hae*III-cut DNA was run in parallel as a molecular size marker.

2.7. Kinase assays

Kinase assays were performed according to the protocol of Shangary et al. [37] with our modification. Cell lysates were incubated with anti-DNA-PK or anti-ATM antibody for 2 h on ice and then, mixed with 25 μl of protein A-sepharose beads rocking at 4 °C for 1 h. The immunoprecipitates obtained with anti-DNA-PK or anti-ATM antibody was centrifuged, washed three times, and used for kinase assays. The immunoprecipitate was mixed with the substrate, 1 μg of p53 protein (p53 (1–393), Santa Cruz Biotechnology, Santa Cruz, CA), along with 5 μM cold ATP in kinase buffer (50 mM HEPES, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 0.2 mM EGTA, 0.1 mM EDTA, and 1 mM dithiothreitol). The kinase reaction was carried out at room temperature for 30 min and terminated by adding an equal volume of SDS sample buffer followed by heat inactivation. The reaction products were subjected to 10% SDS–PAGE and transferred onto a nitrocellulose membrane. The membrane was processed for immunoblotting using the phospho-p53 (Ser15) antibody as described above and then, analyzed.

2.8. Densitometric analysis

The densities of the bands were measured by densitometric analysis with an ImageQuant scanning imager (Molecular Dynamics, Sunnyvale, CA). The relative intensities of the bands were calculated by comparing the density of the sample with that of the control.