and the US FDA Investigational New Drug Annual Report. These reports overlap slightly in content but differ substantially in purpose, scope and timing of data-lock points, creating costly inefficiency and redundant work for sponsors. Because of the gap in reporting periods and the difference in purposes between these annual reports, it has been pointed out that, for example, EU regulators might receive different safety messages regarding a particular investigational drug at different timepoints from FDA regulators.[1,2]

These issues prompted a new initiative by CIOMS for developing a unique, standardized content and format for periodic safety reports on investigational drugs. In August 2006, the CIOMS VII Working Group published *The Development Safety Update Report (DSUR): Harmonizing the Format and Content for Periodic Safety Reporting During Clinical Trials*.[1] The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) step 2 consensus guideline (E2F) on the DSUR, based on the CIOMS proposals, was issued for public comment in June 2008.[2]

In this present current opinion article, while we argue for the significance of harmonized periodic safety reports during development phases, we present extensive discussion on the draft ICH E2F guideline and the CIOMS VII Working Group report to help improve the current, malfunctioning risk communication and to promote safety risk management during clinical development programmes. In particular, we discuss the following subjects:

- effective use of DSURs to improve the current risk communication system;
- efficiency brought about by introduction of integrated periodic safety reporting throughout the life-cycle of a drug.

Furthermore, we hope that our discussion will attract greater public attention to the regulatory system on drug development safety, and trigger wider discussion among the international regulatory community as well as the representatives of trial investigators and ethics committees, on the basis that although these parties are primarily responsible for managing the safety of individual patients, they have thus far rarely been involved with the bigger picture.

1.2 Comparison between the Report of the CIOMS VII Working Group and the Draft International Conference on Harmonisation (ICH) E2F Guideline

DSURs are intended to be a common standard report to "notify regulators and other interested parties (e.g. ethics committees) at regular intervals of the evolving safety profile of an investigational drug and actions proposed or being taken to address safety concerns" during clinical development.[2] The CIOMS VII Working Group further stated that "by design, [DSURs] will enable a seamless transition for communicating safety information to relevant stakeholders, starting at the early clinical development stage and [...] continuing throughout the post-approval period". The DSUR table of contents was developed in alignment with that of established PSURs for marketed drugs (table I).[2,3] Where possible, commonalities in the table of contents between the proposed DSUR and the PSUR were retained. Furthermore, the concept of safety risk management during development is fully reflected in the detailed instructions in the proposed DSUR guideline, in accordance with the proposal by the CIOMS VI Working Group, 'Management of Safety Information from Clinical Trials'.[4]

Several recommendations made by the CIOMS VII Working Group were not reflected in the draft ICH E2F guideline (table II). For example, both the CIOMS VII Working Group and the draft ICH E2F guideline recognize the value of providing an executive summary of a DSUR to ethics committees and trial investigators where the local legislation requires, although only the CIOMS VII Working Group suggests disclosure of the full report upon request. Additionally, one chapter of the CIOMS VII Working Group report is devoted to the goal of a single periodic safety report covering the lifecycle of a drug from development to post-launch, and incorporating the current PSURs within its scope. However, a compromise on this

Table I. Comparison of table of contents between the Development Safety Update Report (DSUR) proposed by the International Conference on Harmonisation (ICH) E2F draft guideline and the current Periodic Safety Update Report (PSUR) for marketed drugs[a] (reproduced by kind permission of the Council for International Organizations of Medical Sciences.[1] © CIOMS)

Proposed contents of the DSUR[2]	Corresponding contents of the PSUR[3]
Title page	Title page
Executive summary	Executive summary
Table of contents	Table of contents
1. Introduction	1. Introduction
2. Worldwide marketing authorization status	2. Worldwide market authorization status
3. Update on actions taken in the reporting period for safety reasons	3. Update of regulatory authority or MAH actions taken for safety reasons
4. Changes to reference safety information	4. Changes to reference safety information
5. Status of clinical trials ongoing and completed during the reporting period	7. Studies 7.1 Newly analysed company-sponsored studies 7.2 Targeted new safety studies planned, initiated or continuing during the reporting period 7.3 Published safety studies
6. Estimated exposure 6.1 Cumulative subject exposure in clinical trials (phase I–IV) 6.2 Patient exposure from marketed setting	5. Patient exposure
7. Presentation of safety data from clinical trials 7.1 General considerations 7.2 Interval line listings of serious adverse reactions (SARs) 7.3 Cumulative summary tabulations 7.4 Deaths in the reporting period 7.5 Subjects who dropped out in association with any adverse event in the reporting period	6. Presentation of individual case histories 6.1 General considerations 6.2 Cases presented as line listings 6.3 Presentation of the line listing 6.4 Summary tabulations 6.5 MAH's analysis of individual case histories
8. Significant findings from clinical trials during the reporting period 8.1 Completed trials and any interim analyses 8.2 Ongoing clinical trials 8.3 Other therapeutic use of investigational drug 8.4 New safety data related to combination therapies	7. Studies 7.1 Newly analysed company-sponsored studies 7.2 Targeted new safety studies planned, initiated or continuing during the reporting period 7.3 Published safety studies
9. Relevant findings from non-interventional studies	
10. Relevant findings from other studies	
11. Safety findings from marketing experience	Included in point 6. 'Presentation of individual case histories'
12. Other information 12.1 Non-clinical data 12.2 Long-term follow-up 12.3 Literature 12.4 Other DSURs 12.5 Significant manufacturing changes 12.6 Lack of efficacy 12.7 Phase I protocol modifications	8. Other information 8.1 Efficacy-related information 8.2 Late-breaking information
13. Late-breaking information	
14. Overall safety assessment 14.1 Evaluation of the risks 14.2 Benefit-risk considerations 14.3 Conclusions	8. Other information 8.3 Risk management programmes 8.4 Benefit-risk analysis report 9. Overall safety evaluation 10. Conclusion
15. Summary of important risks	Not explicitly covered by PSUR according to ICH E2C (R1)

a Contents are numbered according to the applicable ICH guidelines.

MAH = Marketing Authorization Holder.

Table II. Comparison regarding expected audience of Development Safety Update Reports (DSURs) and separation from Periodic Safety Update Reports (PSURs) for the postmarketing phase

Reports and guidelines	Ethics committee and trial investigators as recipients of DSUR	DSUR as a separate document from PSUR
CIOMS VII report[1]	Executive summary provided where national legislation requires periodic submission of safety information on an investigational drug to ethics committees, institutional review boards or investigators, and a full document delivered upon request	Ultimate goal is to implement an integrated life-cycle periodic safety report covering the scope of the DSUR and PSUR, but current recommendation is to create two stand-alone documents – one for investigational drugs during development (DSUR) and one for postmarketing (PSUR)
ICH E2F draft guideline[2]	Executive summary only for submission to ethics committees and other stakeholders, if required by local regulations No description on full report	No description for integration with the PSUR Create two stand-alone documents – one for investigational drugs during development phase (DSUR) and one for postmarketing (PSUR)
Our proposal	Executive summary submitted to all ethics committees and participating investigators across the board A full report readily available upon request to trial investigators and ethics committees	Introduce a single, life-cycle periodic safety report pertaining to both development and postmarketing as soon as possible Require update of outdated PSUR guidelines to comply with most recent concept of risk management

ICH = International Conference on Harmonisation.

point appears to have been reached, considering "the significant and complex challenges a unified safety update report would present, such as requiring changes to existing practices and requirements", presumably the current, long-standing PSUR practice. Thus, the temporal focus of the CIOMS VII Working Group is a stand-alone DSUR in a step-by-step approach towards their ultimate goal.[1] In contrast, the draft ICH E2F guideline included no suggestion regarding an integrated DSUR-PSUR report. We consider the above points as being of high relevance to effective risk communication and rational use of resources. In this current opinion article, we discuss two proposals regarding DSUR recipients and the DSUR as a separate document from the PSUR.

2. Proposals

2.1 Ethics Committees and Investigators as Development Safety Update Report (DSUR) Recipients

The CIOMS VII Working Group considers that "the DSUR is intended for submission exclusively to regulatory authorities. However, where national legislation requires periodic submission of safety information on an investigational drug to Ethics Committees, Institutional Review Boards, or investigators, the CIOMS VII Working Group recommends that only the DSUR Executive Summary be provided, with a full DSUR available upon request".[1] Furthermore, the ICH E2F draft guideline recommends that "where local authorities ask for periodic submission of safety information on an investigational drug to ethics committees, institutional review boards, or investigators, the DSUR executive summary should suffice, supplemented with line listings of serious adverse reactions (SAEs) as warranted".[2] Thus, even though they are primarily responsible for managing the safety of trial participants, not all the ethics committees and trial investigators around the world receive the executive summary, and a full report might be available only if requested.

Currently, safety information regarding an investigational drug can be relayed to investigators and ethics committees in several ways, namely as an IB and an expedited individual case summary report (ICSR).[5] Recently, the CIOMS VI Working Group proposed periodic reporting with interval line listings of SUSARs, as a substitute for a barrage of expedited ICSRs.[4] Safety risk communication in clinical trials should be established by maintaining transparency of safety data, rapidity and consistency of assessment, and periodicity and clarity of messages throughout all

development programmes. However, whether the current communication tools satisfy all of these criteria is unclear.

An IB is a comprehensive compilation of findings on an investigational drug, and is submitted to an ethics committee when seeking approval to begin a new trial. It is developed and reviewed for update annually according to Good Clinical Practice standards, and updated as significant new information becomes available by sponsors, although the requirements for annual updating is subject to local regulations.[4] However, a revised IB does not necessarily contain the latest safety information from ongoing clinical trials, because such results are not declared as 'locked'. A complete set of results from a clinical trial is included in the revised IB only after the data has been validated and analysed. Occasionally, annual revision may thus be delayed until analysis completion or 'when significant results become available' (figure 1), reducing the periodicity of IBs. Furthermore, this 'dictionary' contains information accumulated from early developmental stages; too much to be efficiently processed. Readers struggle to identify important updates in safety information and the relevant risk assessments. Thus, an IB lacks periodicity in communicating newly emerging risks associated with an investigational drug, and clarity in effectively conveying the sponsor's perspective on those risks. Nonetheless, an IB is submitted annually to ethics committees for continuing review of clinical trial activities, despite the above disadvantages. In fact, submission of revised IBs to regulatory authorities during an ongoing study is not necessarily required.

When a SUSAR case associated with an investigational drug is reported to the sponsor, an expedited ICSR is issued according to existing ICH standards.[5] The report is delivered to

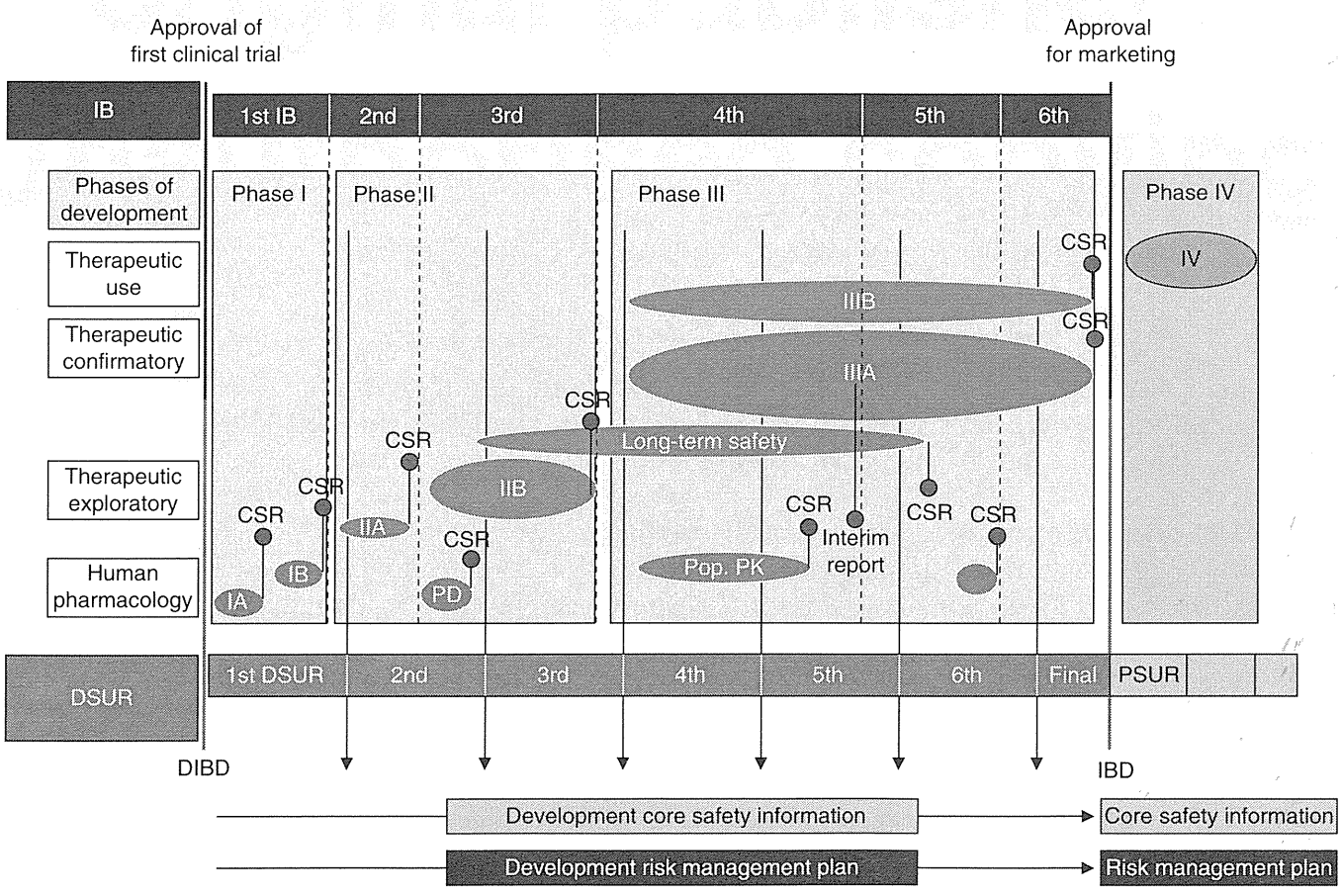

Fig. 1. Periodicity of Development Safety Update Reports (DSURs) and Investigators' Brochures (IBs) in a model development programme. Vertical dotted lines indicate data-lock points for IB revisions, and vertical solid lines indicate data-lock points for each DSUR. **CSR** = Clinical Study Report; **DIBD** = Development International Birth Date; **IBD** = International Birth Date; **PD** = pharmacodynamics; **PSUR** = Periodic Safety Update Report; **Pop. PK** = population pharmacokinetics.

regulators, trial investigators, and ethics committees within a locally determined timeframe. As reported by the CIOMS VI Working Group,[4] in this era of global multi-tiered and parallel development, ethics committees and trial investigators are overwhelmed with paperwork for processing ICSRs for various investigational drugs dispatched one after another from pharmaceutical companies. The original intent to expeditiously convey important risk information is lost, and investigators may overlook relevant safety findings. The CIOMS VI Working Group proposed replacing sporadic ICSRs with periodic interval line listings of unblinded SUSAR cases, along with a brief summary from the sponsor on the up-to-date safety profile.[4] This appears to improve upon current practices with regard to reducing the burden on investigators and ethics committees to process a large quantity of ICSRs while keeping trial investigators and ethics committees abreast of recent unblinded SUSAR cases, but remains to be implemented with a new global consensus on harmonization.

An ICSR also fails to serve as a common tool with global integrity that provides a sponsor's safety assessment on one particular case. Expectedness of adverse events varies depending on the reference documents adopted by local regulations in each country, including an IB, a Summary of Product Characteristics and a package insert. In a multinational study, this difference may result in regulators in different countries receiving ICSRs with different expectedness for a particular case, or even no report at all. To address this issue, the CIOMS VI Working Group proposed using a single reference safety information document for expectedness assessment, namely a Development Core Safety Information (DCSI), which is comparable to company core safety information for postmarketing. The CIOMS VII Working Group report and ICH draft guideline also recommend appending a DCSI to DSURs, and describing any significant changes occurring during the reporting period in the DSUR. Furthermore, by attaching the DCSI to IBs, the recency of IBs may be enhanced because of the more frequent, prompt and independent updates required of DCSIs.[4,6]

The current safety communication tools described above lack either reporting periodicity, data transparency, clarity or risk message integrity. In contrast, a DSUR has accurate periodicity because of the predetermined annual data lock and efficiency in documenting a sponsor's latest comprehensive and integrated perspective on the safety of an investigational drug while focusing on relevant recent data showing any changes from the previous knowledge. The recency of a DSUR would be ensured by its completeness in listing recent SUSAR cases from ongoing clinical trials reported during the covered period in the cumulative tabulation of SAE incidences once the case files are closed, without having to wait for the data lock of the concerned trial. In addition, the focus on interval safety in a DSUR, in contrast to an IB, would be preferable to continuing review of ongoing development activities, and therefore we argue for its submission to all ethics committees and participating investigators across the board (table II).

Transparency in safety communication may be undermined by providing only an executive summary, because of the summary's inherent lack of supporting evidence on which the safety assessments are based. This limitation may be overcome by making relevant information (namely the remaining periodic reports) available to not only clarify events occurring during the interval, but also to bestow a sense of easy access to relevant materials. In the EU, both the ethics committees and regulatory authorities are the target audience of annual safety reports under the applicable regulation. DSURs are developed as replacements for annual safety reports and there would therefore be no definitive reason to stop distributing the equivalent to ethics committees in those countries. It is true that an executive summary may be sufficient during initial review to allow ethics committees to judge whether or not further discussion is necessary. When the committees regard further discussion on the sponsor's recommended actions as necessary, a full DSUR can provide immediate access to the supporting data. Moreover, such reference information on important risks and similar event cases may greatly aid investigators in the early

and appropriate management of adverse event cases and in enhancing their sensitivity to usually overlooked but significant non-SAEs. Objections to distributing full reports stem mainly from the additional burden that reviewing a new voluminous report would pose on investigators. We therefore suggest that sponsors make full DSURs readily available upon request during the conduct of clinical trials via methods such as web distribution under appropriate security control and electronic documentation with hyperlinking, which ensures quick retrieval of documents as the need arises, and easy access from the executive summary to supporting data in the main body of the DSUR without a significant increase in paperwork. Additionally, to further improve development risk communications, it may be worthwhile to conduct a survey on the receptivity and usefulness of DSURs for safety management at study sites after the start of DSUR distribution to reflect feedback from trial investigators and study sites.

It should be noted that the objective of the above-mentioned periodic line listings proposed by the CIOMS VI Working Group, which consist of only expeditiously-reported, unblinded SUSAR cases along with brief updates on the emerging safety profile, differs from that of DSURs. Although DSURs present a transparent overview of interval safety findings for an investigational drug, the CIOMS VI Working Group's proposed line listings lack content essential to ensuring the transparency of a periodic safety report, such as information regarding all SUSAR cases occurring during the period, and tabulated cumulative incidences of SAEs, available safety analyses by study from ongoing or completed clinical trials and information on risk management during the relevant period; these important elements are all available in DSURs.

In summary, we recommend that executive summaries of DSURs should be distributed to all participating study sites around the world on the grounds that they are the most effective tool for strengthening risk communication, and that sponsors should make full DSURs readily available upon request. Further discussion on the role and structure of IBs is required if DSURs are to be submitted to ethics committee review sessions.

2.2 A Single, Life-Cycle Periodic Safety Report Format Pertaining to Development and Post-Authorization Phases

The ICH E2F draft guideline states that "some overlap is expected between the DSUR and PSUR" and contains no description of a single model of periodic safety reporting throughout the life-cycle of a drug (table II).[2] In contrast, the CIOMS VII Working Group presents their goal of "the eventual development and implementation of a single, integrated life-cycle safety report that incorporates the scope of the current DSUR and PSUR, and avoids duplication of information, unnecessary burden and confusion for both sponsors and regulators".[1] We strongly agree with the CIOMS VII Working Group's perspective and further suggest that integrated reporting throughout development and postmarketing be implemented as soon as possible when the periodic safety reports during the development phase start.

Collection of safety data starts before human exposure to an investigational drug and continues into the postmarketing phase. The nature and quantity of available data evolve depending on the developmental stages. All relevant safety-related data should be evaluated within a continual and comprehensive context throughout the life-cycle of the drug, considering the differences in data sources and clinical stages to relate a simple, specific and clear message to all concerned parties. From the perspective of continuing and consistent safety assessment, separating periodic safety reporting by authorization status would not be of great significance as the status varies by country and marketing authorization would be nothing but a landmark during the life-cycle of the drug. Safety data can be collected concurrently from clinical trials and postmarketing experiences for a fair amount of time after the initial marketing authorization. In this peri-approval period, the content of PSURs in any country will be similar to that expected for DSURs in other countries where clinical trials are still ongoing. Both reports deal with a large amount of information from pre-approval clinical trials, along with a comparable portion from emerging postmarketing experiences. The proposed

DSURs and the current PSURs differ with regard to items, order and headings due to differences in applicable guidelines (table I).[1] If a sponsor must create a DSUR in parallel with a PSUR for the same reporting interval, cross-validate them and submit these reports on different schedules according to local regulatory requirements in the recipient countries, inefficiency is inescapable. In addition, it may be undesirable and confusing for a regulatory body to receive two periodic safety reports regarding the same medicinal agent in countries where clinical trials are ongoing after the initial market launch. Therefore, a single, common, integrated periodic update report for each active substance should be pursued to relate a simple, specific and clear safety message with global consistency.

With the effort of the CIOMS VII Working Group, the proposed DSUR format is modelled after the current PSUR format to promote smooth transition to PSURs during the peri-approval period, contributing to the discussion on a common format for integrated report. The CIOMS VII Working Group also expressed their strong belief in the efficiency and utility provided by integration of DSURs and PSURs, and provided the framework for a model integrated periodic safety report, which could accommodate the presentation of both elements for early development compounds, such as non-clinical or clinical pharmacological data, and elements for postmarketing, such as interval line-listings of SUSAR cases for approved indications (table III).[1] As specifying the data sources and authorization status would ensure that readers appropriately understand the significance and quality of various safety findings, refinement in this regard may be necessary for implementation. Thus, we recommend that the international regulatory community should make every effort to realize harmonized life-cycle reporting as soon as possible, rather than maintain parallel production of a separate DSUR and PSUR. In case a sponsor restarts a new development programme for an approved product for an additional indication, for which the development programme has terminated, it would be much more efficient and prudent to use a single integrated periodic

Table III. Proposed table of contents for a model integrated periodic safety report (reproduced by kind permission of the Council for International Organizations of Medical Sciences.[1] © CIOMS)

- Title page
- Table of contents
- Executive summary
- Introduction
- Worldwide marketing authorization status
- Update of regulatory authority, trial sponsor or MAH actions taken for safety reasons (actions taken during the reporting period)
- Changes to reference safety information (DCSI and CCSI when relevant)
- Patient exposure
  - market use
  - clinical trials
- Individual case histories from marketing experience (excluding clinical trials)
  - clinically significant individual case histories
  - line listings (only for special types of reports, such as SUSARs, and by exception)
  - summary tabulations (including spontaneous and solicited reports)
- Clinical studies
  - inventory and status of worldwide interventional clinical trials (all phases; approved and non-approved indications, dosage forms, populations)
  - results from interventional clinical trials
    - completed (synopsis of results)
      - approved uses
      - unapproved uses
    - ongoing (synopsis of results if interim analysis conducted during reporting period)
      - approved uses
      - unapproved uses
  - line listing (only for SUSARs)
  - summary tabulations (all serious adverse events from interventional clinical trials; cumulative)
  - observational and epidemiological studies (including use of registries)
    - completed
    - ongoing
  - targeted new safety studies
    - completed
    - ongoing
    - planned
- Other information
  - efficacy-related information
  - chemistry, manufacturing and formulation issues

Continued next page

Table III. Contd

non-clinical findings
literature sources
Late-breaking information
Overall safety evaluation
discussion on (i) marketed use experience; and (ii) investigational use
Summary of important issues (problem list; update of those previously identified and any new ones)
identified risks
potential risks
important missing data (needed to resolve outstanding issues/risks)
New actions recommended
Conclusions
Appendices

CCSI = Company Core Safety Information; **DCSI** = Development Core Safety Information; **MAH** = Marketing Authorization Holder; **SUSAR** = Suspected Unexpected Serious Adverse Reaction.

report than compiling two different but similar reports in parallel, to communicate a single, consistent safety message. The cost of inefficiency and redundancy from having both reports would ultimately fall on consumers and society, and therefore we believe that such duplication should be avoided in line with the ICH's objective of "a more economical use of human, animal and material resources, and the elimination of unnecessary delay in the global development and availability of new medicines whilst maintaining safeguards on quality, safety and efficacy, and regulatory obligations to protect public health".[7] Therefore, we believe that the international regulatory community would be better off undertaking the new initiative for integrated periodic safety reporting immediately.

3. Main Points to Consider

3.1 Preparing for Distribution of DSURs

Several important points regarding the contents of DSURs should be discussed before distribution to ethics committees and investigators. First, concern exists regarding presenting unblinded SUSAR cases in DSURs in terms of statistical validity. We agree with the CIOMS VI recommendation that all SUSAR cases should be unblinded[4,5] and presented in DSURs. In fact, with the aid of adequate allocation methods, disclosing treatment information regarding isolated SUSAR cases would rarely affect the statistical validity of the results from comparative studies. To ensure unbiased comparison of safety results, cumulative incidences of SAEs should be tabulated by treatment arms, including unblinded placebo cases in DSURs.[1,2]

The second point concerns aggregate safety review from clinical trial data. When designing a development plan for an investigational drug, a prospectively planned aggregate safety review within the sponsoring company should be discussed from the perspective of safety risk management.[4] The ICH E2F draft guideline showed the summary tabulation of SAEs across the programme using a simple summation of cases across studies in its appendix.[2] To obtain more useful statistics for early identification of emerging safety risks, we suggest incorporating into the development plan a carefully planned, prospective meta-analysis, which should be performed at a timing to yield sufficient power to effectively detect important safety signals. Drug exposure, doses and characteristics of populations studied should be considered whenever appropriate.[4,8] The results of these meta-analyses should be reported and discussed within the context of overall safety evaluation in a DSUR when available, ultimately aiding in assessing individual case reports and in interpreting results from each clinical trial.[9] Thus, DSURs include the results of periodic assessment of aggregate safety data, and any subsequent changes to the reference safety information should be reflected in the DCSI. Consequent actions taken for risks are determined in the Development Risk Management Plan, ensuring appropriate risk management throughout the development programme (figure 1).

3.2 Preparing for Integrated Periodic Safety Reports

Some established practices will be challenged by introducing an integrated DSUR/PSUR model.[1] It may take more years to realize integrated

periodic safety reports, considering the possible difficulties discussed below and the time needed for the DSUR guidelines to reconcile EU Annual Safety Reports and US Investigational New Drug annual reports. The likely barriers to establishing the new processes for integrated safety reports include the need for further harmonization in the relevant local regulations and international guidelines regarding postmarketing safety, and the need to fix the existing chasm in evaluation processes between the departments responsible for development and those for postmarketing safety within the regulatory agencies.[10] Should the regulatory community stop advancing further simplification and improvement of periodic safety reporting processes once the new regulation on separate DSURs is set in place; however, the consequence would predictably be costly, as discussed in section 2.2. Introduction of globally harmonized DSURs will also challenge Japanese regulators and commercial sponsors, in particular, as no annual reports during clinical development phases are currently in place in Japan, and post-approval local periodic safety reports are required to have a harmonized PSUR attached in English. To avoid further burdening sponsors with its unique requirements, Japan's peculiar regulatory system must be harmonized before DSUR implementation.[1]

Postmarketing safety risk management was publicly introduced with the implementation of the 2005 ICH E2E guideline, *Pharmacovigilance Planning*.[11] However, the most recent working PSUR guideline [ICH E2C(R1), 2003] is obsolete in current practice because of its insufficiency in specific instructions regarding contents for risk management.[3] In contrast, the draft DSUR guideline provides detailed instructions for several risk-related sections, particularly as provided under the headings "Overall Safety Evaluation" and "Summary of Important Risks" (table I).[2] When the outdated PSUR guideline is updated, its contents will be expanded to convey important risk information in a globally harmonized manner

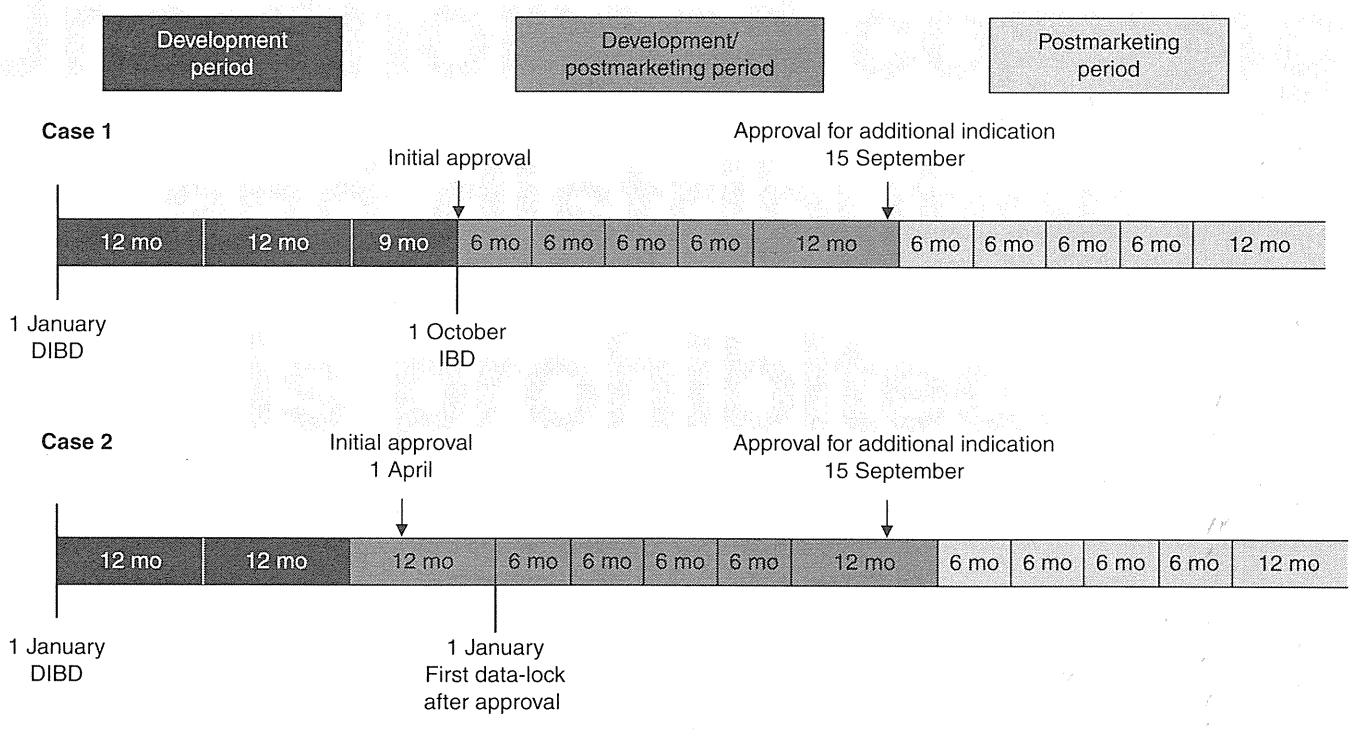

Fig. 2. Data-lock points and intervals in a model transition from development phase to postmarketing for a single, life-cycle periodic safety report. Case 1: When the first marketing approval is granted within 6–12 months after the last Drug Safety Update Report (DSUR) data-lock point, the data-lock point for subsequent integrated periodic safety reports after the first approval is changed to the date of first marketing approval, namely the International Birth Date (IBD). Case 2: When the first marketing approval is granted within 6 months after the last DSUR data-lock point, the data-lock point for the subsequent integrated periodic safety reports after the first approval remains as the date of approval of the first clinical trial, namely the DIBD (Development International Birth Date).

at the latest level of regulatory science, as indicated in the model integrated periodic safety reports proposed by the CIOMS VII Working Group (table III).[1] This expectation also supports the concept of a single integrated periodic report.

Synchronization of reporting intervals represents another outstanding issue. We recommend simple alignment of reporting intervals for integrated reports with the current PSUR intervals of 6 months for the 2 years immediately following approval of a new indication, and with those stipulated by the applicable local regulations thereafter (figure 2). Considering the current PSUR intervals and the suggestion for DSURs by the ICH E2F guideline, keeping intervals to less than 12 months would be necessary for the effective periodic evaluation of the flood of postmarketing information immediately after first launch.[2] The model transition from DSUR to PSUR in the CIOMS VII Working Group report permits a maximum interval of 18 months for a DSUR in the peri-approval period,[1] but this duration appears too long for integrated reports to efficiently convey emerging safety information immediately after launch. We also suggest that use of the Development International Birth Date and International Birth Date as a data-lock point for integrated reports should be determined as a matter of convenience, rather than being bounded by the convention of the existing PSUR regulations, to permit a 6-month to 1-year interval in transitioning from a pure DSUR-type report to a DSUR/PSUR-type report (figure 2). Allocation of a sponsor's resources to an approved product during this peri-approval period would typically cover the anticipated workload for frequent reporting.

4. Conclusions

The DSUR is a comprehensive and concise document fit for communicating risk information of investigational drugs, including safety-related data and actions for patient protection. Distributing the executive summary of DSURs and ensuring immediate accessibility of full reports to all ethics committees and trial investigators worldwide is strongly encouraged as it may strengthen the clarity of current risk communication and enhance risk mitigation actions by providing a rationale. On taking into consideration both the challenges of introducing an integrated periodic safety report and the anticipated unnecessary burden and confusion likely to arise from simultaneously having two similar kinds of periodic reports for one medicinal agent, we believe that pursuing an approach to a single, life-cycle integrated periodic safety report is worthwhile. Additionally, updating the outdated PSUR guideline to the latest regulatory scientific level following the concept of risk management is introduced is urged.

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SHORT REPORT

Familiarity and Prudence of the Japanese Public with Research into Induced Pluripotent Stem Cells, and Their Desire for its Proper Regulation

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Abstract The lack of knowledge of current public attitudes towards basic research into induced pluripotent stem cells (iPSCs) is a serious problem when considering appropriate ways of governance regarding research and its clinical applications. We therefore conducted an internet-based survey to determine public opinion regarding the research and development of iPSCs and regenerative medicine (RM). A total of 14,908 valid responses were collected, which revealed that the Japanese public were familiar with the terms iPSCs and RM, and many of them had received information about iPSCs and RM through the television and newspapers. They also generally accepted the need for extra funding for research into iPSCs, but also decided to adopt a "wait and see" approach and thought that research and development of iPSCs and RM should be conducted under proper governance in accordance with an international regulatory framework. It will be necessary to discuss an internationally consistent regulatory system and effective mechanisms for information flow.

Keywords iPS cell · Regenerative medicine · Public attitude · Internet-based survey · Governance

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Introduction

Science news from Japan and the United States in 2007 regarding the successful generation of induced pluripotent stem cells (iPSCs) through the direct reprogramming of somatic cells in vitro [1, 2] traveled all over the world and impacted upon not only scientific fields, but also the public sphere. In Japan, iPSCs have been reported and discussed in all forms of media. Following a global trend to encourage the research and development of iPSCs and derived products in light of their expected impact, importance and benefits, the Japanese Ministry of Education, Sports, Culture, Science and Technology (MEXT) decided to invest 10 billion yen into promoting the research and development of iPSCs over a 5-year period beginning March 2008 [3].

The importance of adequate governance of advanced science has been discussed previously [4, 5], and this applies to stem cell research. Many factors need to be

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Table 1 List of questions

Question sentence
1.Do you know about iPS cells?
2.Do you know about RM?
3.How would you like to find out about iPS cells or RM?
4.How long do you think it will take to realize RM?
5.Which idea regarding research and development into iPS cells and RM is closest to your opinion?
6.What do you think about the cost of research into iPS cells and RM?
7.What do you think about the regulatory framework regarding the medical applications of research and development into iPS cells and RM?
8.Which of the following ideas about the use of iPS cells to make germ cells is closest to your opinion?
9.Do you think that research into iPS cells and RM is necessary?
10.Do you mean 'Is there anyone you know who would like to know more about RM?', or 'Is there anyone you know who would like to receive RM?'
11.Do you have any opportunity to talk about iPS cells or RM in your daily life?
12.Would you be prepared to be actively involved in iPS cell research?
13.What is your attitude towards research into iPS cells or advanced life sciences?
14.How old are you
15.What is your occupation?
16. What is your sex?

considered in order to ensure proper governance, including social climate and public opinion. Several studies concerning public opinion about stem cell research have been conducted [6–8], but few large-scale investigations have focused on public attitudes towards iPSCs. One exception was a telephone-based survey conducted by a research group at Virginia Commonwealth University (VCU). They asked 1,000 Americans their opinions regarding stem cells, including iPSCs [8]. To the best of our knowledge, no subsequent large-scale investigations into public attitudes towards iPSCs have been conducted, despite the rapid movement forward on governance, funding decisions and iPSC research and development, and the rapid broadcasting of iPSC issues by the mass media. Considering that iPSCs have opened a new dialogue with respect to the basic research and clinical applications of pluripotent stem cells (PSCs), the lack of knowledge on current public attitudes towards iPSCs is a serious problem. We therefore conducted an internet-based survey in conjunction with the Asahi newspaper, one of the most prestigious newspapers in Japan with a circulation of approximately eight million [9]. This survey was open to the public and sought to determine their opinions regarding the research and development of iPSCs and regenerative medicine (RM).

Table 2 Demographics of respondents

%(n)	Male	Female	Total
Under 19	0.5% (72)	0.3% (52)	0.8% (124)
20's	1.5% (219)	2.3% (344)	3.8% (563)
30's	5.9% (882)	6.8% (1021)	12.8% (1903)
40's	16.3% (2435)	11.1% (1694)	27.7% (4129)
50's	17.6% (2625)	10.0% (1488)	27.6% (4113)
60's	15.1% (2249)	4.9% (731)	20.0% (2980)
Over 70	6.3% (945)	1.0% (151)	7.4% (1096)
Total	63.2% (9427)	36.8% (5481)	100% (14908)

Methods

Questionnaire

This was a web-based survey. A questionnaire was sent to members of the Asahi newspaper portal site and readers of the Asahi newspaper. Because public attitudes towards iPSCs and RM in Japan are unknown, we designed the questions to determine the public attitude towards and their recognition of iPSCs and RM. This research focused on the public recognition of iPSCs and RM and their opinions concerning the future prospects for and necessary regulation of these techniques, and their willingness to be actively involved. The questionnaire contained 16 questions, including three questions concerning the respondents' backgrounds and a question allowing free

Table 3 Public familiarity with iPSCs and RM

% (n)		Do you know RM?		Total
		Yes	No	
Do you know iPS cell?	Yes	72.1% (10743)	1.7% (246)	73.7% (10989)
	No	15.3% (2275)	11.0% (1644)	26.3% (3919)
	Total	87.3% (13018)	12.7% (1890)	100% (14908)

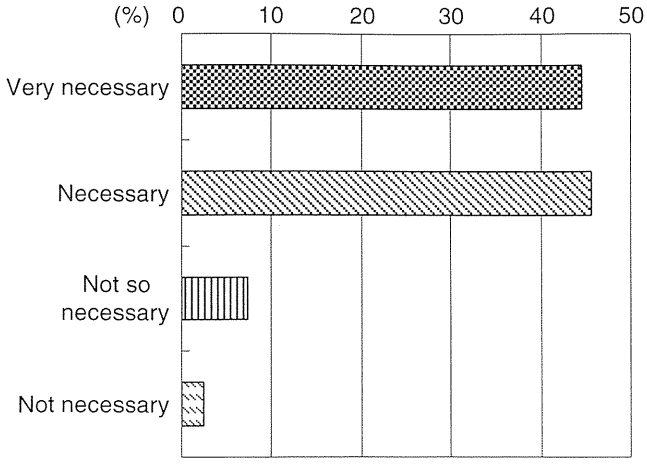


Fig. 1 Do you think that research into iPS cells and RM is necessary?

description of the respondents' images of iPSCs and advanced life science research (Table 1). The results of this aspect of the questionnaire are not discussed here. We provided a brief introduction to the current situation of iPSCs and RM research, where we aimed to offer information on both the potential benefits and risks of iPSCs and RM, based on the opinions of scientists and the information broadcast in the media. All the questions required the respondents to choose one answer that best described their attitude. A total of 14,908 valid responses were collected in the 5-day period from the 5th to 9 September 2008. We are unable to present all the data here, but report on some of the significant results regarding the current public attitudes to iPSCs and RM in Japan.

To reduce confusion and prevent the questionnaire being too diffuse, we did not ask about people's recognition of research into stem cells or embryonic stem (ES) cells. Although many Japanese people seem to be aware of the terms ES cells and stem cells, it seemed likely that they would not know the difference between different types of stem cells. It was therefore decided that this research would focus on iPSCs and RM, which are the most significant and popular topics with the public in the field of advanced life sciences. A detailed investigation of public attitudes towards other types of stem cell research will follow.

Potential Biases

The nature of internet-based research is associated with a potential for bias. While it has the advantages of low cost, rapid and easy collection of answers, easy limitation of target respondents, etc., possible disadvantages include a bias towards wealthy and more highly-educated respondents (particularly towards older people who can easily access the internet) [10]. In addition, the respondents were all readers of the Asahi newspaper and internet users, and were therefore possibly more conscious of social problems and had easier internet access than would have been the case had respondents been sampled at random. There were also possible biases in terms of age and sex ratios (Table 2). Respondents under the age of 20 were in a minority, and our results may therefore not adequately reflect the opinions of younger members of the public towards iPSC and RM. However, considering the large number of respondents (14,908), it seems likely that the results of this survey reflect the general Japanese public opinion towards iPSCs and RM.

Results

Demographics of Respondents

The demographics of the respondents with regard to age and sex are shown in Table 2. Of the total 14,908 respondents, 63.2% were male and 36.8% were female. Concerning their ages, 0.8% were <19 years, 3.8% were 20–29 years, 12.8% were 30–39 years, 27.7% were 40–49 years, 27.6% were 50–59 years, 20.0% were 60–69 years and 7.4% were over 70 years.

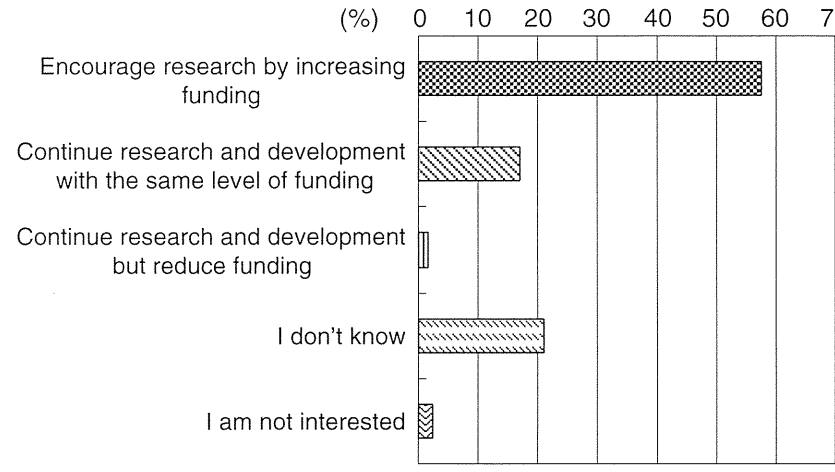


Fig. 2 What do you think about the cost of research into iPS cells and RM?

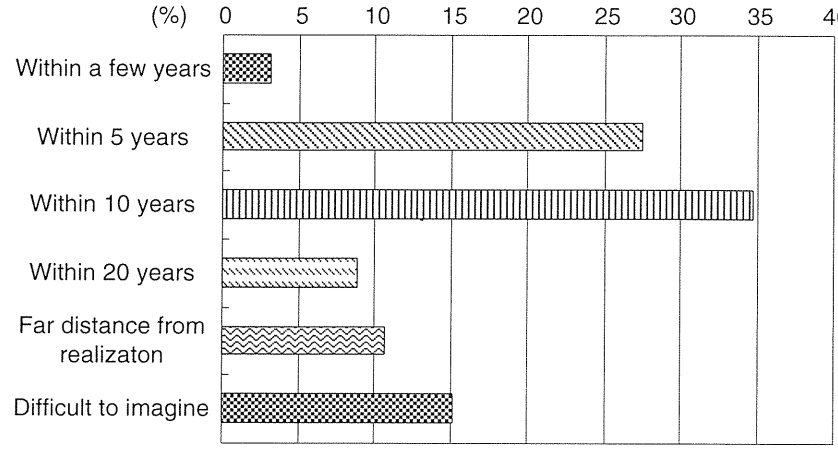


Fig. 3 How long do you think it will take to realize RM?

Recognition of iPSCs and RM by the Japanese Public

There was surprisingly high recognition by the public of the terms iPSCs and RM. The term iPSCs was recognized by 73.7% of the respondents, while 87.3% recognized the term RM. The results of a cross analysis of recognition between iPSCs and RM are shown in Table 3. This result indicates that the majority of Japanese readers of the Asahi newspaper were aware of these terms.

Public Attitudes to iPSCs and RM in Japan

Concerning the necessity for research and development of iPSCs and RM, 44.6% of respondents thought it "very necessary" and 45.7% thought it "necessary" (Fig. 1). In addition, 57.6% believed it should be encouraged with more research funding, while 17.0% believed it should be continued with the same level of funding (Fig. 2). This suggests that many respondents accepted the necessity for continued research and into iPSCs and RM, and the possible need for extra funding. With respect to sex-and age-related responses, there was a tendency for older and male respondents to be more positive supporters of iPSC and RM research (data not shown). A similar tendency was identified when respondents were asked about the prospect of RM becoming a reality; a total of 65.4% said they believed it would be possible "within 10 years" (Fig. 3). Regarding progress and competition in research into iPSCs and RM, 34.9% of the respondents thought that Japan should establish a pioneering research system, and 51.4% of the respondents believed that research and development should progress with international cooperation (Fig. 4).

Concerning respondents' willingness to cooperate with research and development of iPSCs and RM, 21.6% said they would like to cooperate by offering cells and/or blood, but 69.4% said they would like to wait and see the results of further research. Only 9.1% of respondents said they did not wish to cooperate (Fig. 5).

With respect to the collection of information regarding iPSCs and RM, most people said they gained their information from the TV or internet, or from newspapers (73.5%). Contrary to this, 12.4% answered that they would like to search for information using the internet, and 6.0% said they would like to be informed through books or by attending

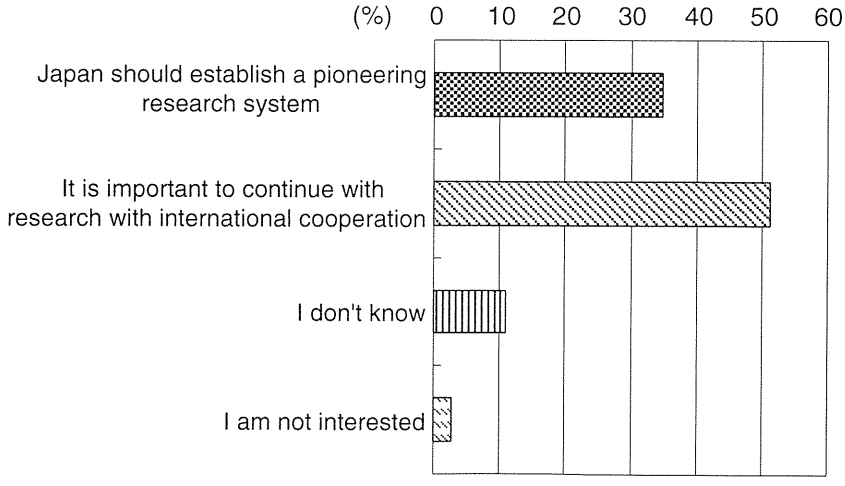


Fig. 4 Which idea regarding research and development into iPS cells and RM is closest to your opinion?

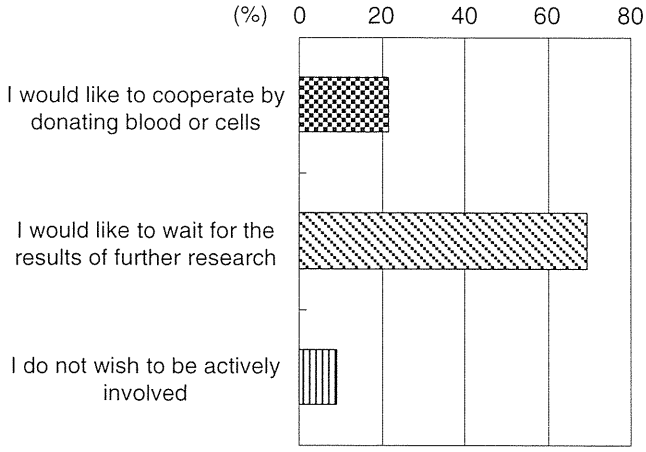


Fig. 5 Would you be prepared to be actively involved in iPS cell research?

seminars (Fig. 6). 60.0% of respondents had no experiences of conversations about iPSCs or RM.

We also determined opinions about the regulatory framework governing research and development into iPSCs and RM for medical applications. 63.0% of respondents thought that Japan should have a regulatory framework based on international guidelines, while 23.0% thought that Japan should establish a proper regulatory system specifically for the Japanese situation (Fig. 7). The ethical issues surrounding the use of iPSCs to produce germ cells caused 30.3% of participants to respond that they believed the production of germ cells from iPSCs should be banned, while 58.4% thought it should be allowed to progress as long as it was carefully managed and monitored within a regulatory framework (Fig. 8).

Discussion

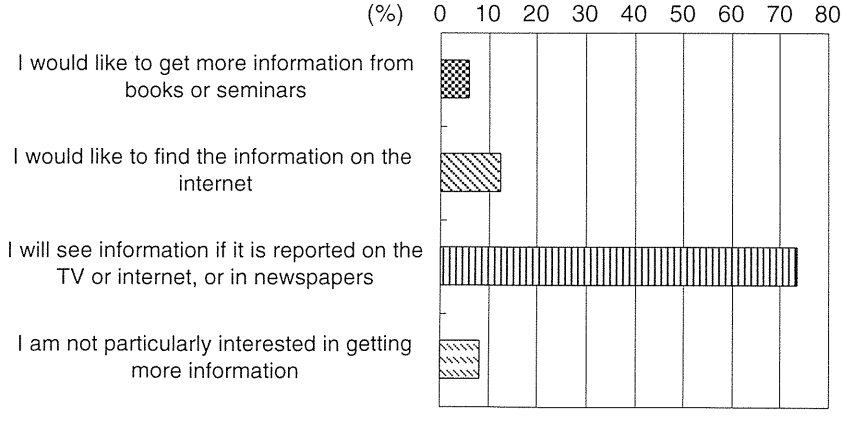
Public Familiarity with and Prudence Regarding iPSCs

The Japanese public generally accepted the necessity for extra funding and research into iPSCs and RM, and believed that RM would be realized in the near future (Figs. 1, 2 and 3). This high level of recognition of a new type of stem cell was not found in a previous study in the United States [8]. Although the results of these two studies cannot be directly compared, the differences suggest a rapid change in social recognition. Although further studies are necessary, it seems likely that the rapid increase in mass-media broadcasting of iPSCs topics over the past 2 years may be responsible for this increased recognition [11].

It is worth noting that the Japanese public was not motivated to actively cooperate with the research and development of iPSCs and RM by offering their blood or cells at this stage (Fig. 5), but preferred to adopt a "wait and see" approach. Although it is difficult to judge the true meaning of the answers to this survey, the "wait and see" option favored by the public seems to suggest a prudent approach. Interestingly, this implies that a high level of recognition and acceptance of the necessity for research into iPSCs and RM does not directly impact the public's motivation to actively cooperate. In order to investigate this point further, more detailed research into sources of information is needed, given that many people received their information on iPSCs and RM through the media (Fig. 6), and the amount and nature of this information is likely to affect public opinions. A previous study pointed out the possible correlation between public opinion and mass-media broadcasts regarding the issue of genetically modified food in Japan [12]. This aspect will be the subject of future research.

With regard to the sources of information, it should be emphasized that over 70% of the public received most of their information concerning iPSCs and RM solely from the television and newspapers (Fig. 6). Thus the cooperation between researchers, regulatory agencies and journalists is a critical factor when considering the flow of information. Active and effective disclosure of the latest developments in iPSC and RM research, including the risks involved, should be considered, because a balanced information supply is the basis for appropriate governance by the natural and social scientists, policymakers, journalists, and the public. The

Fig. 6 How would you like to find out about iPS cells or RM?



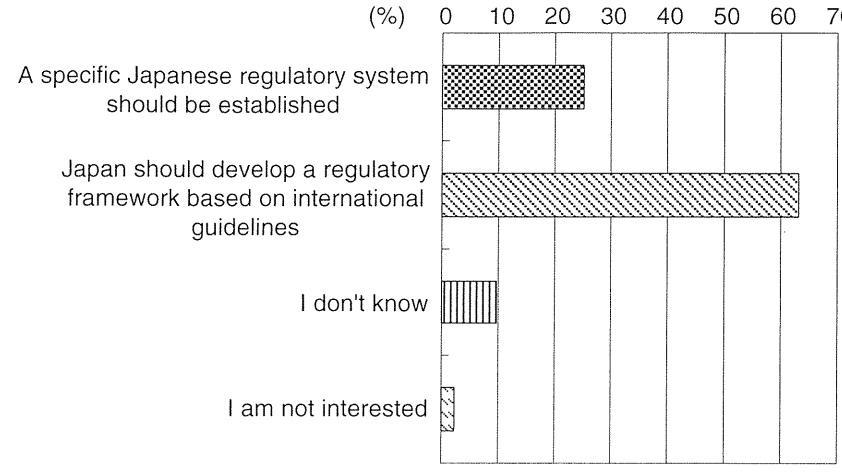


Fig. 7 What do you think about the regulatory framework regarding the medical applications of research and development into iPS cells and RM?

current situation, with increased interactions between scientists and journalists, works in a positive direction [13].

Requirement for Governance

It is important to recognize that the Japanese public thought that research and development of iPSCs and RM should be conducted under proper governance, in accordance with an international regulatory framework (Fig. 7). In addition, approximately half of the respondents thought that it was important to progress with research and development with international cooperation (Fig. 4). Thus the establishment of proper governance at the international level needs to be discussed, and discussions relating to the regulatory framework for research and development into PSCs, including iPSCs, are currently being conducted at the highest levels worldwide. For example, several international guidelines concerning general research and the clinical application of PSCs were presented by the International Society for Stem Cell Research (ISSCR) [14, 15]. However, the ISSCR is an academic society and their proposed guidelines cannot be legally enforced in all countries. Moreover, the pace of regulatory developments differs between countries. In January of this year, the United States FDA approved safety evaluations regarding the clinical applications of ES cells [16]. International consensus guidelines are required to cover the safety aspects of such research and its medical applications.

In order to formulate such international guidelines, it is imperative to actively involve regulatory agencies to oversee safety testing, risk evaluation of tumorigenicity and clinical research procedures for cellular therapeutics. Current reports that have discussed the tumorigenicity risk of PSCs have emphasized the importance of appropriate preclinical studies and have pointed out the necessity for rapid discussions on evaluating safety standards and the effectiveness of PSCs in clinical applications [17, 18]. Yamanaka, one of the key researchers into iPSCs and RM, pointed out the risk of cancer due to iPSC implantation in long-term mouse experiments. He emphasized the importance of long-term safety and tumorigenicity risk evaluations [19]. Concerning this problem, a detailed description of regulatory systems will be

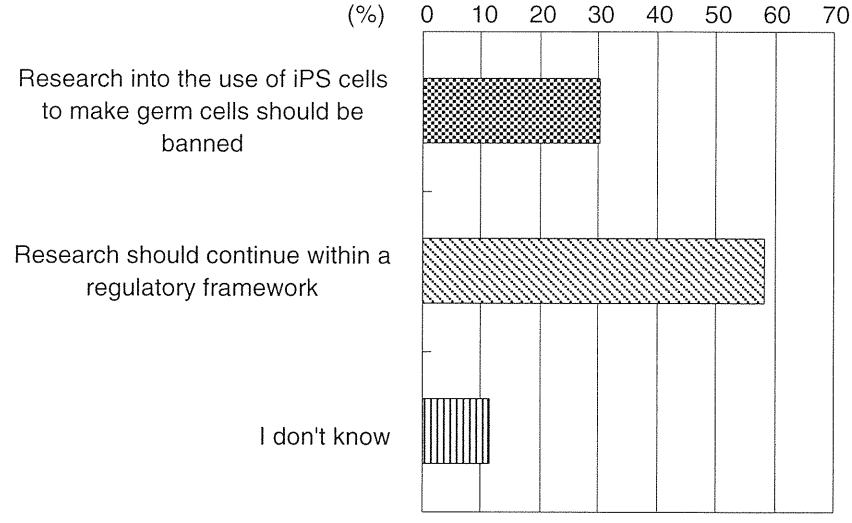


Fig. 8 Which of the following ideas about the use f iPS cells to make germ cells is closest to your opinion?

needed, considering the potential problems that can arise from imperfect articulation of regulatory guidelines concerning the clinical applications of RM and biologics derived from stem cells [20].

In addition to the safety aspects, ethical aspects are also important. In this study, subjects were asked about the issue of making germ cell from iPSCs. More than half of the respondents thought that research should progress within a carefully established regulatory framework (Fig. 8). In Japan, a MEXT sub-committee approved the production of germ cells from PSCs for basic research in December 2008, though the fertilization of germ cells derived from PSCs is banned [21]. In the present study, we were unable to investigate in detail the public opinion on research concerned with making germ cells from iPSCs. In addition, this investigation was conducted over a different time period from that when the political decision on moderating research into making germ cells from iPSCs was made. Further research into the changes in public opinion and social, political, and scientific changes is needed.

Thus, in order to address the public requirement for the development of proper regulatory frameworks, it will be necessary to establish an international consensus on regulatory frameworks that has domestic legal authority to govern stem cell research and its application in each country. An internationally consistent regulatory system will promote future worldwide research and development into iPSCs and RM

Conclusion

The results of this study show that the Japanese public was familiar with the terms iPSCs and RM, and that they generally accepted the necessity of research into iPSCs. At the same time, they decided to adopt a "wait and see" approach, and thought that research and development into iPSCs and RM should be conducted under the proper regulations, in accordance with an international regulatory framework. This seems to demonstrate the prudence of the Japanese public. In order to address the public requirements, it will be necessary to discuss an international consensus on regulatory frameworks that have an impact on domestic stem cell research and its application in each country. It is also important to consider the methods of information flow concerning iPSCs and RM, and further research into this issue will be conducted.

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Enzyme-Linked Immunosorbent Assay for Detection of Filovirus Species-Specific Antibodies▽

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Several enzyme-linked immunosorbent assays (ELISAs) for the detection of filovirus-specific antibodies have been developed. However, diagnostic methods to distinguish antibodies specific to the respective species of filoviruses, which provide the basis for serological classification, are not readily available. We established an ELISA using His-tagged secreted forms of the transmembrane glycoproteins (GPs) of five different Ebola virus (EBOV) species and one Marburg virus (MARV) strain as antigens for the detection of filovirus species-specific antibodies. The GP-based ELISA was evaluated by testing antisera collected from mice immunized with virus-like particles as well as from humans and nonhuman primates infected with EBOV or MARV. In our ELISA, little cross-reactivity of IgG antibodies was observed in most of the mouse antisera. Although sera and plasma from some patients and monkeys showed notable cross-reactivity with the GPs from multiple filovirus species, the highest reactions of IgG were uniformly detected against the GP antigen homologous to the virus species that infected individuals. We further confirmed that MARV-specific IgM antibodies were specifically detected in specimens collected from patients during the acute phase of infection. These results demonstrate the usefulness of our ELISA for diagnostics as well as ecological and serosurvey studies.

Ebola virus (EBOV) and Marburg virus (MARV) belong to the family *Filoviridae* and cause severe hemorrhagic fever in primates (20). While MARV consists of a single species, *Lake Victoria marburgvirus*, four distinct EBOV species are known: *Zaire ebolavirus* (ZEBOV), *Sudan ebolavirus* (SEBOV), *Côte d'Ivoire ebolavirus* (CIEBOV), and *Reston ebolavirus* (REBOV). The phylogenetically distinct *Bundibugyo ebolavirus* (BEBOV) was recently identified in Uganda and was proposed to be a new species of EBOV (Fig. 1) (31).

EBOV and MARV are filamentous, enveloped, single-stranded, negative-sense RNA viruses. The virus genome encodes seven structural proteins, nucleoprotein (NP), polymerase cofactor (VP35), matrix protein (VP40), glycoprotein (GP), replication-transcription protein (VP30), minor matrix protein (VP24), and RNA-dependent RNA polymerase (L). EBOV also expresses at least one secreted nonstructural glycoprotein (sGP) (20). GP is responsible for receptor binding and fusion of the viral envelope with host cell membranes (11, 22, 35) and has an important role in the pathogenesis of filovirus infection (3, 23, 36). GP is the main target of neutralizing antibodies, and most of the known ZEBOV-specific monoclonal antibodies (MAbs) show little cross-reactivity to other filovirus species (24, 27, 34).

Serological diagnostic methods based on enzyme-linked immunosorbent assays (ELISAs) using the recombinant EBOV and MARV NP antigens have been developed to detect filovirus-specific antibodies (5, 17). Using a ZEBOV NP antigen, NP-specific antibodies were broadly detected in animals infected with ZEBOV, SEBOV, CIEBOV, or REBOV (17), indicating strong cross-reactivity among EBOV species. It is predicted, however, that the antibody response to GP is more species specific due to the larger genetic variability with this protein, which is supposed to be the main target of the host humoral immune response. Therefore, in this study we developed a filovirus species-specific ELISA using recombinant GP antigens to serologically distinguish filovirus species.

MATERIALS AND METHODS

Plasmids. Viral RNA extracted from the supernatant of Vero E6 cells infected with ZEBOV, SEBOV, CIEBOV, BEBOV, REBOV, or MARV strain Angola was used for the cloning of the respective GP cDNAs lacking the transmembrane domain and cytoplasmic tail. The cDNAs of truncated EBOV and MARV GPs with a C-terminal histidine (His) tag (His-EBOV-GP and His-MARV-GP, respectively) were cloned into a pATX vector. Finally, the cDNA fragments of His-EBOV-GP and His-MARV-GP were inserted into the mammalian expression vector pCAGGS/MCS, which contains the chicken β-actin promoter (13). All clones were confirmed by sequencing prior to expression.

MAbs. Hybridoma cells producing EBOV GP-specific MAb ZGP42/3.7 (IgG1) (24, 26), which recognizes a linear epitope on GP comprising the sequence GEWAFWENKKN, and MARV GP-specific MAb AGP127-8 (IgG1) were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma) and RPMI medium (Sigma), respectively, supplemented with fetal calf serum (FCS) and antibiotics. Mouse ascites were obtained by a standard procedure, and MAbs were purified from ascites fluid using protein A-agarose columns (Bio-Rad). The S139/1 monoclonal antibody (IgG2a), which binds to the hemagglutinin of influenza A viruses (37), was used as a negative control.

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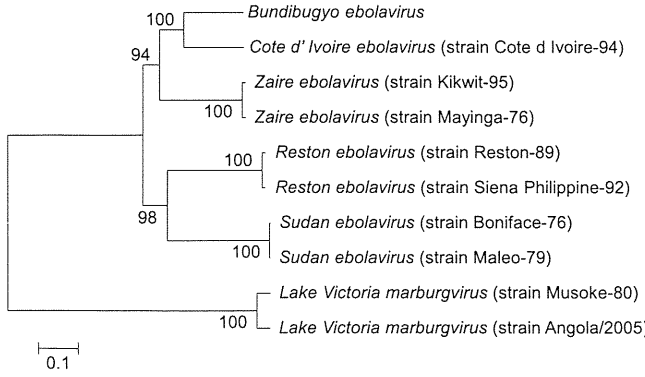

FIG. 1. Phylogenetic analysis of filovirus GP amino acid sequences. The phylogenetic tree was constructed by using the neighbor-joining method. For the construction of this tree, we used 10 GP amino acid sequences, each comprising a whole GP amino acid sequence. Numbers at branch points indicate bootstrap values (1,000 replicates).

Sera and plasma. Five-week-old female BALB/c mice were immunized twice intraperitoneally with 100 μg virus-like particles (VLPs) (14, 21) in 3-week intervals, and the serum samples were collected 7 to 10 days after the second immunization. Convalescent-phase plasma samples were collected from cynomolgus macaques vaccinated and/or infected with EBOV as described previously (27). ZEBOV convalescent-phase human plasma (patients 2 to 7) and serum (patients 1 and 8) samples were obtained 51 to 135 days after the onset of ZEBOV infection during the 1995 outbreak in Kikwit, Democratic Republic of the Congo (25). SEBOV convalescent-phase patient serum samples (patients 9 and 10) were collected about 2 months after onset during the Ebola hemorrhagic fever outbreaks in Uganda in 2000 associated with SEBOV (2). These EBOV-infected human samples were kindly provided by T. G. Ksiazek (Centers for Disease Control and Prevention). MARV-infected human blood samples (patients 11 to 21) were collected within a few days after the onset of symptoms from admitted patients from the 2004-2005 outbreak in Angola (29). Blood collections during outbreak investigations were approved under the special response protocol established between the World Health Organization and national authorities.

Expression and purification of His-EBOV-GP and His-MARV-GP. Human epithelial kidney 293T cells cultured in high-glucose DMEM containing 10% FCS and antibiotics were transfected with pCAGGS vectors expressing His-EBOV-GP (pCHis-ZEBOV-GP, pCHis-SEBOV-GP, pCHis-CIEBOV-GP, pCHis-BEBOV-GP, or pCHis-REBOV-GP) or His-MARV-GP (pCHis-MARV-GP) using TransIT LT1 (Mirus). Forty-eight hours after transfection, the supernatants were collected, and the recombinant GPs were purified by using the Ni-nitrilotriacetic acid (NTA) purification system (Invitrogen) according to the manufacturer's instructions. The majority of contaminant protein was removed with wash buffer containing 15 mM imidazole. Finally, bound proteins were collected with elution buffer containing 250 mM imidazole. To monitor inevitable nonspecific reactions (i.e., nonspecific antibodies) to FCS-derived impurities in each GP preparation, control antigens (FCS-derived proteins nonspecifically bound to the Ni beads) were prepared by using the Ni-NTA column under the same conditions. The eluted protein was concentrated by using Amicon Ultra 4 spin columns (Millipore) and dialyzed against phosphate-buffered saline (PBS) at 4°C overnight. Purified His-EBOV-GP and His-MARV-GP were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue. Western blotting was performed by using ZGP42/3.7, AGP127-8, and anti-His MAbs (Covance).

Antigens prepared from cell lysates and VLPs. Membrane lysates of 293T cells transfected with pCAGGS expressing full-length GP were prepared by using the Mem-PER eukaryotic membrane protein extraction reagent kit (Pierce) according to the manufacturer's instructions. To generate VLPs, 293T cells were transfected with plasmids expressing major viral structural proteins, GP, NP, and VP40 (10, 33). After 48 h, supernatants were overlaid on 25% sucrose and ultracentrifuged at 28,000 × *g* at 4°C for 1.5 h. The VLPs were recovered from the pellet and disrupted with 0.05% Triton X-100 in the presence of 30 mM potassium chloride for the use of ELISA antigens. The GP amounts in the membrane lysates and VLPs were quantified by Western blotting using MAb ZGP42/3.7 or AGP127-8, and the GP concentrations of each preparation were calculated based on the standard band intensities provided by known concentrations of His-GP. Membrane lysates or supernatants of 293T cells transfected with empty pCAGGS vectors were used to prepare control antigens for ELISA using cell lysates or VLPs, respectively.

ELISA. ELISA plates (Nunc Maxisorp) were coated with the GP antigens (100 ng of GP/50 μl/well) or control antigens in PBS at 4°C overnight and then washed with PBS containing 0.05% Tween 20 (PBST). Unspecific binding of the antibodies was avoided by blocking with 3% skim milk (150 μl/well) for 2 h at room temperature. Monkey plasma samples were preincubated with 2% FCS to absorb antibodies to FCS components, since they were exposed to FCS by the injection of the vaccines or viruses diluted in DMEM containing FCS. After washing three times with PBST, 50 μl of appropriately diluted serum or plasma samples or the GP-specific MAb in PBST containing 1% skim milk was added and incubated for 1 h at room temperature. After washing three times with PBST, the bound antibodies were detected by using the following secondary antibodies conjugated with horseradish peroxidase diluted in 1% skim milk in PBST: goat anti-mouse IgG (Jackson ImmunoResearch), goat anti-monkey IgG (Rockland), goat anti-human IgG (Jackson ImmunoResearch), or donkey anti-human IgM (Jackson ImmunoResearch). After incubation for 1 h at room temperature and three PBST washes, 50 μl of 3,3′,5,5′-tetramethylbenzidine (Sigma) was added to each well, and the mixture was incubated for 15 min at room temperature. The reaction was stopped by adding 1 N sulfuric acid to the mixture, and the optical density (OD) at 450 nm was measured.

Phylogenetic analysis. Phylogenetic analysis was based on whole amino acid sequences of filovirus GPs. The sequences were analyzed by using GENETYX (Genetyx Corp., Japan) for Windows software, version 7. A phylogenetic tree was constructed by using the neighbor-joining bootstrap method (1,000 replicates) in MEGA 4.0 software (28). Amino acid sequences of ZEBOV strain Mayinga-76, ZEBOV strain Kikwit-95, SEBOV strain Boniface-76, SEBOV strain Maleo-79, CIEBOV strain Côte d'Ivoire-94, BEBOV, REBOV strain Reston-89, REBOV strain Siena Philippine-92, MARV strain Musoke-80, and MARV strain Angola/2005 used in phylogenetic analyses were obtained from GenBank under accession numbers Q05320, P87666, Q66814, Q66798, Q66810, ACI28624, Q66799, Q89853, P35253, and Q1PD50, respectively.

Statistical analyses. OD values higher than 3 standard deviations above the averages of negative-control samples at a 1:100 dilution were considered positive. To test the specificity of each reaction, ELISA data (i.e., OD values) were analyzed by using one-way analysis of variance (ANOVA). The differences between OD values were compared by using the two-sided *t* test with the Bonferroni-Holm correction for multiple comparisons (4). All statistical analyses were performed with the computer program R (version 2.2.8).

RESULTS

Expression and purification of recombinant EBOV and MARV GPs. The expression and secretion of His-EBOV-GP and His-MARV-GP in the supernatants of 293T cells transfected with a plasmid encoding His-GP were confirmed by immunoblotting using anti-GP and anti-His MAbs (data not shown). These recombinant GPs were purified as described in Materials and Methods. All purified His-GPs were detected by SDS-PAGE and immunoblotting using anti-GP and anti-His MAbs as prominent protein bands of the predicted size of the transmembrane anchor-minus EBOV and MARV GPs (Fig. 2). These purified GPs were used as antigens for the ELISA described in the following experiments.

Sensitivity of the GP-based ELISA. The sensitivity of the purified GP-based ELISA was tested by using anti-EBOV-GP MAb ZGP42/3.7 and anti-MARV-GP MAb AGP127-8. Serial 10-fold dilutions of the antibodies (10^{-5} to 10^{2} μg/ml) were prepared, and the reactivity to each GP antigen was examined (Fig. 3a to c). The negative-control MAb, S139/1, did not bind to any His-GPs in the ELISA. At concentrations ranging from 0.1 μg/ml to 100 μg/ml, ZGP42/3.7 reacted with all His-EBOV-GPs but not His-MARV-GP, whereas AGP127-8 reacted specifically with His-MARV-GP but not any of the His-EBOV-GPs. The detection limit for specific antibodies using this assay