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An improved method for detection of replication-competent retrovirus in retrovirus vector products

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Abstract

Contamination by replication-competent retrovirus (RCR) is one of the most important safety issues of retrovirus vector products for gene therapy clinical research. To improve the sensitivity of RCR detection and to shorten the assay period, we have developed a novel RCR detection method (infectivity RT-PCR method) based on real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) in combination with virus infection and a novel virus concentration method using polyethyleneimine (PEI)-conjugated magnetic beads. In this method, permissive cells were infected with RCR samples, and amplified RCR in the culture supernatants was adsorbed by PEI-beads. Then RCR RNA extracted from PEI-beads was quantified by real-time RT-PCR. We demonstrated that 1 infectious unit (iu) of RCR spiked in 10^6 cfu/ml of vector products could be detected within 3 days, and the sensitivity for viral detection was increased 3- to 10-fold compared with the direct S + L− assay. By this method, the presence of retroviral vector interfered with RCR detection only slightly. In conclusion, infectivity RT-PCR conducted in conjunction with virus concentration using PEI-beads can detect RCR more sensitively and rapidly than the conventional infectivity assay.

1. Introduction

Retrovirus vectors are widely used in human gene therapy to treat genetic diseases, cancer, and other conditions. The retroviral vector products currently used in gene therapy clinical researches are replication-defective retroviruses, and the primary safety concern associated with the use of retroviral vector products is contamination by replication-competent retrovirus (RCR). RCR is the major risk factor for insertional mutagenesis, and exposure to retrovirus vector contaminated with a high titer of RCR has been shown to lead to lymphoma in rhesus monkeys [1].

The most likely source of RCR is the vector-packaging sequence. Since RCR can arise by homologous recombination during the production of retroviral vector supernatants, sensitive assays for the screening of RCR in vector products are required. The U.S. Food and Drug Administration (FDA) has developed guidelines for testing of RCR in clinical grade vectors and transduced cells, as well as for monitoring patients

Abbreviations: RCR, replication-competent retrovirus; RT-PCR, reverse transcription-polymerase chain reaction; PEI, polyethyleneimine; iu, infectious units; cfu, colony forming units; MLV, murine leukemia virus; AMLV, amphotropic MLV.

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treated with gene therapy protocols [2]. The FDA guidelines recommend that retrovirus vector products be tested for the presence of RCR by inoculation and passage of the test sample with a permissive cell line for a minimum of 5 passages in order to amplify any potential RCR present, followed by subsequent testing with an appropriate indicator cell assay. The PG-4 S + L− focus-forming assay and the marker rescue assays have been routinely used for the detection of RCR [3–7]. However, these conventional cell-based assays are known to have several disadvantages: the assays take a long time (weeks), visual evaluation of the results requires skill and is labor intensive, and the limited dynamic range requires many dilutions. Therefore, there is need for a more sensitive and rapid quantitative detection method for RCR.

Polymerase chain reaction (PCR) is a highly sensitive method for the detection of viral genomes [8]. It has been reported that PCR assays were capable of detecting one or more copies of RCR provirus in 500,000 cells [9]. PCR-based assay for RCR is used for biosafety monitoring of transduced cells with retroviral vectors [10] and of patients receiving retroviral gene therapy [9,11].

Moreover, the recently developed fluorescence-based real-time quantitative reverse transcription-PCR (RT-PCR) assay allows precise quantification of RNA genomes. Since quantitative RT-PCR can be performed in a short time with a wide dynamic range and high throughput, it is expected to be particularly suitable for quantifying RCR in viral stocks with high sensitivity. However, the PCR-based assay detects not only infectious virus genomes. In previous studies, PCR-based assays detected viral DNA fragments derived from packaging cell lines contaminated into retrovirus vector supernatants and caused false-positive findings [12,13]. Therefore, when quantitative RT-PCR is used for RCR detection, some process is required to distinguish infectious RCR RNA and viral DNA fragments prior to the quantitative RT-PCR assay. Infection of RCR into a permissive cell line is suitable for this purpose, because infectious RCR selectively replicates in cells without replication of viral DNA fragments and retrovirus vectors.

In addition, if RCR could be concentrated when preparing the sample for quantitative RT-PCR, it is expected that the sensitivity of RCR genome detection could be improved. In a previous study, our group demonstrated that polyethyleneimine (PEI)-conjugated magnetic beads efficiently adsorbed many types of viruses, with the exception of some non-enveloped viruses, and this novel virus concentration method using PEI-beads enhanced the sensitivity of virus detection by both PCR and RT-PCR [14].

In the present study, we have established a novel RCR detection method based on infectivity RT-PCR. Infectivity RT-PCR is a hybrid method that attempts to combine the best features of infectivity assays and quantitative RT-PCR. Samples are allowed to amplify in cell culture, as in conventional assays. Replication-competent retrovirus is quantified by real-time quantitative RT-PCR rather than by counting focuses. In addition, we applied a novel virus concentration method using PEI-beads to concentrate RCR in culture supernatants before quantitative RT-PCR. We demonstrated that this novel method could detect RCR more sensitively and rapidly than the conventional culture assays.

2. Materials and methods

2.1. Virus and cells

Hybrid Moloney/amphotropic *Murine leukemia virus* (MLV) obtained from ATCC (Manassas, VA; VR-1450; virus titer: $6.9 \pm 2.0 \times 10^7$ infectious unit (iu)/ml) was used as the RCR Reference Material. This hybrid virus, which was established by both the FDA and ATCC as an MLV RCR Reference Material, consists of Moloney MLV with a substitution of the *env* coding region from the 4070A strain of amphotropic MLV (AMLV), and represents a typical recombinant virus that could be generated in a retroviral packaging cell line containing coding sequences for an AMLV *env* [2].

Mus dunni cells (CRL-2017) and cat fibroblast PG-4 (S + L−) cells (CRL-2032) were obtained from ATCC. NIH/3T3 cells (JCRB0615) were obtained from the Japanese Cancer Research Resource Bank (Tokyo, Japan). ΨCRIP-P131 cells (RCB1088) were obtained from the RIKEN Cell Bank (Tsukuba, Japan). *M. dunni* cells and PG-4 (S + L−) cells were maintained in McCoy's 5A medium with 10% fetal calf serum (FCS). NIH/3T3 cells and ΨCRIP-P131 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum.

2.2. Preparation of recombinant retrovirus vector

The retrovirus vector plasmid pLEGFP-N1 (Clontech, Palo Alto, CA) contains the enhanced green fluorescent protein (EGFP) and neomycin resistance gene. ΨCRIP-P131 cells which contain the *gag/pol* gene of Moloney MLV and *env* gene of 4070A in different expression vectors were used as a high titer retrovirus vector-packaging cell line. ΨCRIP-P131 cells (1×10^6 cells) were transfected with pLEGFP-N1 (2 μg) by Effectene Transfection Reagent (Qiagen, Hilden, Germany). Two days after transfection, cells were trypsinized and replated. The next day, Geneticin (GIBCO-BRL, Grand Island, NY; final concentration 1 mg/ml) were added to each dish and cultured for an additional 2 weeks. Eighteen clones of neomycin-resistant cells were picked up, and a clone (ΨCRIP-LEGFP1) which showed the

highest EGFP expression when the NIH/3T3 cells were infected with the culture supernatants of cloned cells was used as a line of retrovirus vector-producing cells. For the preparation of retrovirus vector sample, ΨCRIP-LEGFP1 cells were cultured to subconfluence, the medium was replaced with fresh medium, and after 24 h of culture, the culture supernatants were collected as retrovirus vector samples (vector titer: 1×10^6 cfu/ml). Vector supernatants were stored at −80 °C until use.

2.3. RCR concentration by PEI-beads

PEI-beads were made by coupling of PEI (MW 70,000; Wako Pure Chemical Inc., Tokyo, Japan) with magnetic beads (IMMUTEX-MAG™; mean diameter: 0.8 μm; JSR Inc., Tokyo, Japan) by the 1-ethylene-3-(3-dimethylaminopropyl)carbodiimide coupling method as described previously [14]. RCR concentration using PEI-beads was done as follows: various dilutions of RCR solution were prepared from RCR Reference Material diluted with DMEM. Then 1 or 10 ml of each RCR dilution was incubated with 100 μl of PEI-beads for 10 min at room temperature. Then the complexes of virus and PEI-beads were trapped by a magnetic field (for 1 ml: Magnetic Trapper™, Toyobo Co., Tokyo, Japan; for 10 ml: Dynal MOC-1™, Dynal AS, Oslo, Norway). The virus genome was extracted from the PEI-beads adsorbed fraction (the whole volume) or unadsorbed supernatant (100 μl) with an SMI-TEST EX R&D Kit (Genome Science Laboratories, Fukushima, Japan). Extracted nucleic acids were dissolved in 50 μl of DNase/RNase-free distilled water, and 10 μl of this solution was used for the quantitative RT-PCR reaction.

2.4. Real-time quantitative RT-PCR

The real-time quantitative RT-PCR for RCR was monitored on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The reaction was carried out in a 50 μl reaction mixture containing 10 μl of extracted sample, 1 μM each of the forward and reverse primer, 0.2 μM of TaqMan probe, and 25 μl of TaqMan One-Step RT-PCR Master Mix Reagents with 1.25 μl of 40× MultiScribe and RNase Inhibitor (Applied Biosystems). The reaction conditions were as follows: the viral RNA was reverse-transcribed into cDNA for 30 min at 48 °C, then heat-inactivated for 10 min at 95 °C; PCR was then performed for 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Standard curves were generated from RCR RNA extracted from RCR Reference Material in each RT-PCR assay and validated using linear regression analysis. The RCR genomes were quantified in infectious units (iu). One infectious unit of RCR measured by quantitative RT-PCR means that the sample contains virus genome RNA equivalent to 1 iu of RCR Reference Material. The sequences of the primer pair and the probe used were as follows: forward primer (AMLVenv-1018F): 5′-GCG GTC GTG GGC ACT TAT A-3′; reverse primer (AMLVenv-1082R): 5′-TGT TGG GAA GTG GCC GTA C-3′; TaqMan probe (AMLVenv-1040TM): 5′-(FAM)-ATC ATT CCA CCG CTC CGG CCA-(TAMRA)-3′. These sequences were designed to detect the *env* gene of 4070A AMLV using Primer Express Ver 1.0 Software (Applied Biosystems). The amplified product is predicted to be 64 base pairs (bp) in length.

2.5. Amplification of RCR by culture cells

M. dunni cells were plated in 60-mm dishes at 2×10^5 cells/dish and cultured overnight. Culture medium was replaced with 1 ml of polybrene solution (16 μg/ml) as well as 1 ml of virus solution and incubated for 4 h at 37 °C. Cells were washed with 1 ml of medium 3 times and incubated with 5 ml of fresh culture medium. Culture supernatants were collected at the indicated days for RCR concentration using PEI-beads and detected by quantitative RT-PCR.

2.6. S + L− focus-forming assay

The PG-4 cells were plated in 6-well plates at a concentration of 2×10^5 cells/well and incubated at 37 °C in 5% CO_2 overnight. On the day of infection, the medium was discarded, 1 ml of DEAE-Dextran (20 μg/ml in medium) was added to each well, and the cells were incubated for 30 min at 37 °C. Then 1 ml of test sample was added to each well and the cells were incubated for 2 h at 37 °C. Finally, the samples were replaced with 2 ml of fresh culture medium and cultured at 37 °C in 5% CO_2. Foci of transformed cells were examined microscopically on day 3 and day 7.

3. Results

3.1. Detection of RCR RNA by real-time quantitative RT-PCR

We first established the detection method of RCR RNA by real-time quantitative RT-PCR. Serial log dilutions of RCR solution were prepared, and viral genome RNA extracted from 100 μl of each RCR solution was analyzed by TaqMan quantitative RT-PCR. Forward and reverse primers as well as the TaqMan probe used for the detection of RCR were designed to detect the AMLV *env* sequence that exists in the RCR genome but not in the retroviral vector sequence (Fig. 1). Fig. 2 shows the standard curve generated from an amplification plot of the quantitative RT-PCR assay for RCR. A linear relationship was observed between the threshold cycle (C_T, the PCR cycle at which the

fluorescence of amplification first exceeds baseline) and the log-transformed input retroviral RNA genomes. The linearity of the standard curve was obtained at a range of 10^{-1}–10^{6} iu of RCR in 100 μl of the sample with a correlation coefficient of 0.998. The standard curve was reproducible for repeated assay (data not shown). Since C_T could not be calculated from virus solutions having concentrations below 0.1 iu, the detection limit of the quantitative RT-PCR for RCR was 0.1 iu.

3.2. Concentration of RCR by PEI-beads

In order to detect very low titers of RCR in the culture supernatants of infected cells, we tried to concentrate retrovirus particles using PEI-beads. One and 10 ml of RCR solution (10^{-5} dilution of RCR in DMEM) were incubated with 100 μl of PEI-beads, and fractionated into the PEI-beads adsorbed fraction and the unadsorbed supernatant fraction. Viral genome RNA extracted from each fraction was applied to RT-PCR and analyzed by agarose gel electrophoresis. As shown in Fig. 3A, RCR *env* RNA was detected by RT-PCR from the PEI-beads adsorbed fraction but not from the unadsorbed supernatant, indicating that RCR was efficiently adsorbed in the PEI-beads. When the starting volume of virus solution used for concentration was increased from 1 to 10 ml, the amounts of RCR RNA obtained in the PEI-beads adsorbed fraction were increased without any change in the unadsorbed fraction. To analyze the concentration of virus with PEI-beads quantitatively, serial log dilutions of RCR solution were fractionated with PEI-beads, and the amounts of RCR RNA in the adsorbed fraction and the unadsorbed fraction were quantified by real-time RT-PCR (Table 1, Fig. 3B). When solutions containing low concentrations of RCR were applied to PEI-beads, all of the retrovirus particles in the viral solutions were efficiently collected in the PEI-beads fraction. On the other hand, when a solution containing high concentration of RCR was applied to the PEI-beads, unadsorbed

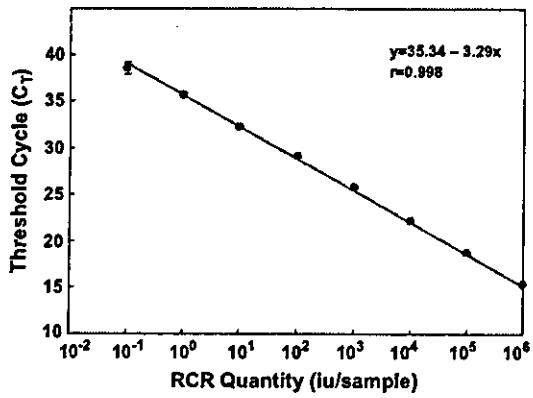

Fig. 2. Standard curve for the determination of RCR quantity generated from an amplification plot of real-time quantitative RT-PCR. Serial dilutions of RCR solution were analyzed by quantitative RT-PCR. A standard curve was generated from the amplification plot of RCR using real-time quantitative RT-PCR. The correlation coefficient is 0.998. Data are the mean ± S.D. (n = 3).

viruses were detected in the supernatant (Table 1). As a result, RCR treated with PEI-beads were maximally concentrated about 10-fold from 1 ml of virus solution and 100-fold from 10 ml of virus solution compared to direct extraction from 100 μl of original virus solutions, and at the same time, the assay sensitivity was increased about 10- and 100-fold, respectively (Table 1, Fig. 3B). These results clearly demonstrated that PEI-beads efficiently adsorbed RCR, and that this novel virus concentration method is useful for improving the sensitivity and lowering the limits of RCR detection.

3.3. Amplification of RCR in cell culture for infectivity RT-PCR

For the screening of RCR in retrovirus vector products, it is necessary to detect very less amounts of RCR among large amounts of retrovirus vectors. In our preliminary study, however, viral *env* DNA sequences derived from a packaging cell line were used to

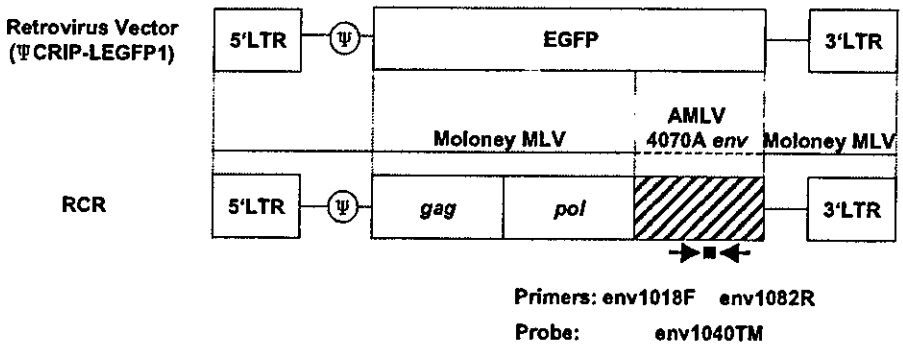

Fig. 1. Structure of RCR and retrovirus vector used in this study. The open bars represent Moloney MLV genome, the gray bar represents the expression cassette for the EGFP gene, and the striped bar represents the AMLV 4070A *env* gene. Black arrows and a small black square underneath the RCR genome indicate the location of the primers and a probe for RCR detection, respectively.

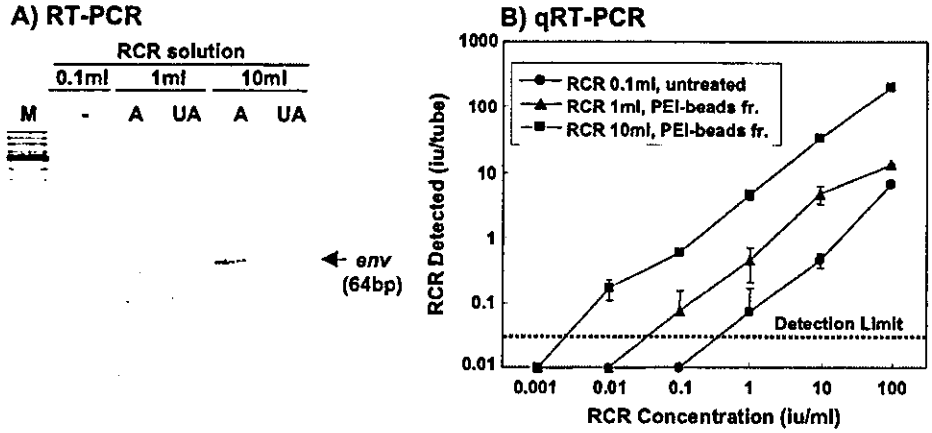

Fig. 3. Concentration of RCR by PEI-beads. (A) RCR solution (10^{-5} dilution) was fractionated with PEI-beads. Viral genome RNA extracted from the PEI-beads adsorbed fraction and unadsorbed supernatant were amplified with RT-PCR and analyzed by 5% agarose gel. M: 20 bp DNA ladder; –: untreated RCR solution; A: PEI-beads adsorbed fraction; UA: PEI-beads unadsorbed supernatant fraction. (B) One or 10 ml of serial dilutions of RCR solution was incubated with PEI-beads. Viral genome RNA extracted from the PEI-beads adsorbed fraction and untreated RCR solution was analyzed by real-time quantitative RT-PCR.

contaminate ΨCRIP-LEGFP1 retrovirus vector supernatants and detected using the same conditions used for the detection of RCR RNA (data not shown). Then, in order to detect only infectious RCR in retrovirus vector products by quantitative RT-PCR, we developed an infectivity RT-PCR. We first infected *M. dunni* cells with solutions containing various titers of RCR and cultured for several days. The replicated RCR in culture supernatants was then concentrated by PEI-beads and quantified by real-time RT-PCR.

Fig. 4 demonstrates the time course of the detection of RCR by infectivity RT-PCR. When *M. dunni* cells were infected with 10 or 100 iu of RCR, the viruses were linearly amplified from day 3 to day 7 (Fig. 4), and all 3 dishes had detectable amounts of virus even on day 2 (Table 2). When the cells were infected with 1 or 0.1 iu of RCR, amplification of RCR could be detected in more than one of the dishes after day 2 and day 5, respectively, though the level of amplification varied widely between the dishes (Table 2). RCR could not be amplified when the cells were infected with 0.01 iu of RCR. The same RCR solutions were also examined by direct S + L– assay using PG-4 cells (Table 2). We could not detect any focuses after 3 days of infection. On day 7, only when cells were infected with 100 iu of RCR, focuses were observed in 100% of wells. However, infection with 10 or 1 iu of RCR induced focus formation in only 1/2 or 1/6 of infected wells, respectively. These results demonstrated that infectivity RT-PCR was able to detect RCR more rapidly and 10- to 100-fold more sensitively than conventional S + L– assay.

3.4. Detection of RCR in retrovirus vector supernatant by infectivity RT-PCR

Finally, various amounts of RCR spiked in 10^6 cfu of retrovirus vector supernatant were examined by infectivity RT-PCR with RCR concentration by PEI-beads

Table 1
Quantitative analysis of RCR concentration using PEI-beads

RCR dilution	RCR quantity (iu/sample)				
	RCR 0.1 ml	RCR 1 ml		RCR 10 ml	
	Untreated	Adsorbed fr.	Unadsorbed fr. (0.1 ml)	Adsorbed fr.	Unadsorbed fr. (0.1 ml)
10^{-1}	2.0×10^6	4.0×10^6	4.9×10^1	6.3×10^6	6.3×10^7
10^{-2}	9.6×10^4	1.4×10^6	–	3.8×10^6	4.4×10^3
10^{-3}	3.7×10^3	3.4×10^4	–	7.2×10^5	1.5×10^0
10^{-4}	4.8×10^2	3.3×10^3	–	6.6×10^4	–
10^{-5}	2.4×10^1	1.2×10^2	–	2.6×10^3	–
10^{-6}	2.1×10^0	6.9×10^0	–	1.2×10^2	–
10^{-7}	–	3.8×10^{-1}	–	1.0×10^1	–
10^{-8}	–	–	–	5.0×10^{-1}	–
10^{-9}	–	–	–	–	–

Serial log dilutions of RCR solution (RCR Reference Material; original concentration: 6.9×10^7 iu/ml) were fractionated with PEI-beads. The amounts of RCR RNA extracted from the PEI-beads adsorbed fraction and unadsorbed fraction were quantified by real-time RT-PCR. –: Under detection limit.

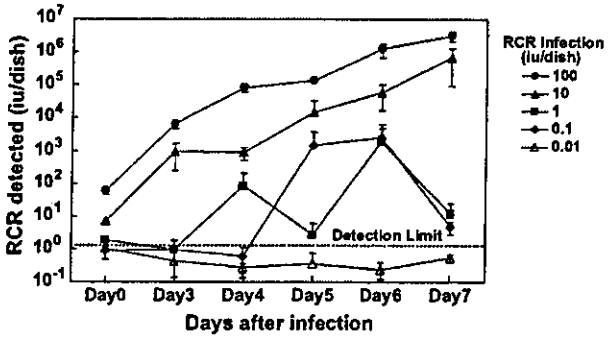

Fig. 4. RCR growth curve in *M. dunni* cells. *M. dunni* cells were infected with serial log dilutions of RCR solution. Culture supernatants were harvested at the indicated time, and RCR was concentrated by PEI-beads. Viral genome RNA was extracted from PEI-beads and the amount of RCR was determined by real-time quantitative RT-PCR. Data are the mean ± S.D. ($n = 3$).

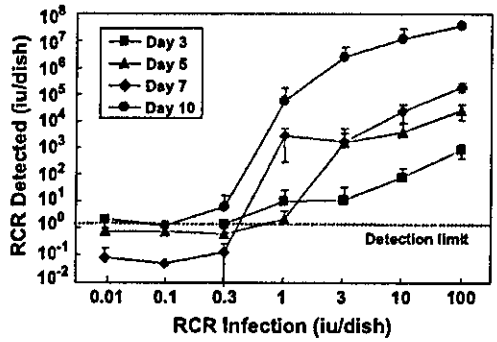

Fig. 5. Detection of RCR spiked in retrovirus vector supernatant by infectivity RT-PCR. *M. dunni* cells were infected with serial dilutions of RCR solution in 10^6 cfu/ml of ΨCRIP-LEGFP1 retrovirus vector supernatant. Cell culture supernatants of infected cells were harvested on day 3, 5, 7 and 10, and then RCR was concentrated by PEI-beads. The amount of RCR genome RNA extracted from the PEI-beads adsorbed fraction was determined by real-time quantitative RT-PCR. Data are the mean ± S.D. ($n = 5$).

(Fig. 5, Table 3). The amount of RCR was evaluated on 3, 5, 7, and 10 days after infection. Infectivity RT-PCR was able to detect 1 iu of RCR on day 3, and 0.3 iu of RCR on day 10 (Fig. 5). The detection ratio of 100% could be achieved for 10 iu on day 3 and 3 iu on day 5. We could not detect any *env* DNA from the culture supernatant of *M. dunni* cells after infection and cultivation of retrovirus vector supernatant (data not shown). When the same RCR samples were evaluated by direct S + L− assay, we could not detect any focuses on day 3, and focuses could be detected at 1 iu on day 7, although 100 iu was required for 100% detection (Table 3). Therefore, it is demonstrated that infectivity RT-PCR improved the level of sensitivity for the detection of RCR in retrovirus vector products 3- to 10-fold and shortened the assay period compared with the conventional S + L− assay.

4. Discussion

In the present study, we have developed a novel RCR detection method based on an infectivity RT-PCR and a virus concentration method using PEI-beads. Real-time quantitative RT-PCR is a suitable alternative to conventional RCR detection by infectivity assays because it is not only a quantitative but also a more sensitive method. However, viral *env* DNA derived from packaging cells was also detected in retrovirus vector supernatants used in this study under the same conditions used to detect RCR RNA (data not shown). Although RCR spiked in retrovirus vector supernatants was concentrated with PEI-beads, *env* DNA was also detected in the PEI-beads adsorbed fraction (data not shown). The mechanism of virus-adsorption by PEI-beads remains unclear, but it is hypothesized that the positive charge field of the PEI molecule might tightly interact with the negative charge of surface lipids or negatively charged proteins on viruses [14]. It is possible that PEI-beads adsorbed RCR particles as well as negatively charged DNA fragments. Therefore, to detect only infectious RCR by quantitative RT-PCR, infection and replication of virus in permissive cells is inevitable. The method of amplifying a virus in a permissive cell line, as used in infectivity RT-PCR, is also a common method to increase the assay sensitivity for virus detection, and is often used before conventional indicator cell

Table 2
Comparison of sensitivity of RCR detection by direct S + L− assay and infectivity RT-PCR

RCR infection (iu/dish)	Direct S + L− assay		Infectivity RT-PCR					
	Day 3	Day 7	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
100	− (0/6)	+ (6/6)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)
10	− (0/6)	± (3/6)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)	+ (3/3)
1	− (0/6)	± (1/6)	± (1/3)	± (1/3)	± (1/3)	± (1/3)	+ (3/3)	± (2/3)
0.1	− (0/6)	− (0/6)	− (0/3)	− (0/3)	− (0/3)	+ (3/3)	± (2/3)	+ (3/3)
0.01	− (0/6)	− (0/6)	− (0/3)	− (0/3)	− (0/3)	− (0/3)	− (0/3)	− (0/3)

Serial log dilutions of RCR solution were evaluated by direct PG-4 (S + L−) assay or infectivity RT-PCR conducted in conjunction with virus concentration using PEI-beads. Data are presented as positive assays (dishes or wells) over the total number of assays performed. +: All the dishes or wells were positive for RCR; ±: at least one dish or well was positive; −: none of the replicates were positive.

Table 3
Comparison of direct S + L– assay and infectivity RT-PCR on RCR detection spiked in retrovirus vector supernatant

RCR infection (iu/dish)	Direct S + L– assay		Infectivity RT-PCR			
	Day 3	Day 7	Day 3	Day 5	Day 7	Day 10
100	– (0/5)	+ (5/5)	+ (5/5)	+ (5/5)	+ (5/5)	+ (5/5)
10	– (0/5)	± (4/5)	+ (5/5)	+ (5/5)	+ (5/5)	+ (5/5)
3	– (0/5)	± (2/5)	± (1/5)	+ (5/5)	± (3/5)	+ (5/5)
1	– (0/5)	± (1/5)	± (2/5)	± (2/5)	± (4/5)	± (3/5)
0.3	– (0/5)	– (0/5)	– (0/5)	– (0/5)	– (0/5)	± (1/5)
0.1	– (0/5)	– (0/5)	– (0/5)	– (0/5)	– (0/5)	– (0/5)
0.01	– (0/5)	– (0/5)	– (0/5)	– (0/5)	– (0/5)	– (0/5)

Serial dilutions of RCR in 10^6 cfu/ml of ΨCRIP-LEGFP1 retrovirus vector supernatant were evaluated by direct PG-4 (S + L–) assay or infectivity RT-PCR conducted in conjunction with viral concentration using PEI-beads. Data are presented as positive assays (dishes or wells) over the total number of assays performed. +: All the dishes or wells were positive for RCR; ±: at least one dish or well was positive; –: none of the replicates were positive.

assays. In infectivity RT-PCR, the indicator cell assay was replaced by quantitative RT-PCR subsequent to the amplification of viruses.

Concentration of retrovirus particles is a simple method to increase the sensitivity of RCR detection. Several approaches to concentrate viruses have been tried in an attempt to enhance the sensitivity of virus genome detection [15–17]. Ultra-centrifugation is widely used for virus concentration, although it is associated with loss of infectivity of MLV [18]. Centrifugation at high-speeds for long duration has been used for concentration of retrovirus vectors [19,20], but this method is very time-consuming and not suitable for virus screening. Polyethylene-glycol (PEG) precipitation is a simple and easy method to concentrate several viruses, but the excess amount of PEG hampers the PCR reaction. In the present study, we have demonstrated that PEI-beads efficiently concentrated RCR in proportion to the volume of virus solution used for the assays. Virus concentration with PEI-beads is a simple and rapid method and is suitable for multiple sample preparation for quantitative RT-PCR.

By the combination of infectivity RT-PCR and virus concentration with PEI-beads, we have developed a novel RCR detection method. We demonstrated that 1 iu of RCR spiked in 10^6 cfu/ml of vector products could be detected within 3 days, and the sensitivity for viral detection was increased 3- to 10-fold compared with the direct S + L– assay. By this method, the presence of retroviral vector interfered with RCR detection [5] only slightly. As a result, this method can detect infectious RCR more rapidly and more sensitively and less labor intensive than conventional cell assays. However, the detection sensitivity was not additively improved as expected from the data of quantitative RT-PCR and virus concentration by PEI-beads. We consider that the limiting step of the detection of RCR by infectivity RT-PCR is the initial infection of the permissive cells with the virus, and thus it is difficult to improve the sensitivity after the replication step. In this case, improvement of the infection process may increase the detection sensitivity. We used polybrene for enhancing viral infectivity, as is done in conventional infectivity assays, but the effect was limited. It has been reported that spinoculation, in which RCR samples are inoculated under centrifugation, increased the sensitivity of RCR detection by the S + L– assay and marker rescue assays [6]. Alternatively, co-precipitation of retrovirus vector with calcium phosphate [21] or complexation with polybrene and chondroitin sulfate C [22] has been shown to increase the transduction efficiency. Utilizing these methods may be useful for increasing the infectivity sensitivity of RCR detection by infectivity RT-PCR.

The RCR detection method described here was designed to specifically detect infectious AMLV RCR in retrovirus vector products. The same strategy should be applied to RCRs other than AMLV by using primers and a probe designed to detect the specific RCR RNA. Furthermore, the infectivity (RT-) PCR strategy may be applicable to the detection of other replication-competent viruses. We have demonstrated that the infectivity PCR method was superior to the conventional cell culture/CPE method for detecting replication-competent adenovirus and useful for the detection of RCA in adenovirus vector products [23].

In conclusion, infectivity RT-PCR conducted in conjunction with virus concentration using PEI-beads can detect infectious RCR more sensitively and rapidly than the conventional infectivity assay. This novel method would be useful for detecting RCR in retrovirus vector products.

Acknowledgements

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CHANGES IN JAPANESE PHARMACEUTICAL AFFAIR LAW AND QUALITY REGULATIONS

by Yukio Hiyama

The revised Pharmaceutical Affair Law (rPAL) was passed by the Diet in July 2002 and its implementation is due in April 2005. The major changes include revision of the approval system and enhancement of post-marketing safety measures, especially for medical devices and biologics.

Under the changes, revisions of Quality Regulations will have impact on:

1) Market Approval Holder's responsibility for the quality management;
2) Drug Master File system to support Common Technical Document (CTD) based applications (*CTD based application became effective in July 2003);
3) Consolidation of the legal positioning of GMP;
4) Revision of GMP standards.

Under the old pharmaceutical product approval system, approval (Manufacturing or Importation) was granted to manufacturers if the product was made domestically or to importers if made overseas. That meant that Japanese pharmaceutical companies had to manufacture their own products while foreign pharmaceutical companies did not have to do so. This was seen as a huge discrimination against the domestic industry. For importers, there was a quality assurance system called Good Import Practices (GMPI), which required foreign pharmaceutical companies to comply with GMP. However, under GMPI, Japanese inspectors did not normally inspect foreign manufacturing facilities.

Under the new law, the two-tier system will disappear and become one system, Market Authorization. The new law allows all parties to subcontract manufacturing activities and requires market approval holders to ensure product quality no matter where products are made.

In order for market authorization holders to ensure product quality, the new law requires applicants to provide full details of product design and manufacturing processes which could be developed and manufactured by others. The Common Technical Document (CTD) based application, which became mandatory in July 2003, gives guidance to applicants on what should be described in pharmaceutical development and in manufacturing processes. Those areas were poorly described in old non-CTD based applications. The reason why they were not described well is that most manufacturing processes were not subject to assessment/review in the old system and so this is not part of the new approval system. In order to promote CTD based applications, a Master File system will be created. Manufacturers will probably register master files for active pharmaceutical ingredients, new kinds of additives and specialized processes.

Approval matters are provided in the approval document, which is generally 10 to 20 pages long. Specifications and test methods occupy the majority of the document whilst manufacturing processes are described in less than one page. To change the approval matters, partial changes of applications must be submitted and reviewed. Partial change review currently takes one year.

However, much more detailed description about the manufacturing process is desperately needed under the new law, with the help of a CTD based application. In order to make the new review/regulatory system effective, a notification system for non-critical approval matters has been established. To change those non-critical approval matters, the Market Authorization holder notifies the government with the required information within a month or so. Although no assessment will be done at the time of notification, rationale and data for the changes will be subject to GMP inspection at a later date. Active discussion is taking place to determine what portion of the manufacturing processes should be approval matters and what should be notification items. Probably principles and end points of the critical manufacturing steps with key operational parameters will become approval matters. Only principal and quality end points for each manufacturing step would be subject to pre-approval review. (see **Figure**).

GMP compliance becomes a core requirement for the Market Authorization system. GMP compliance checks

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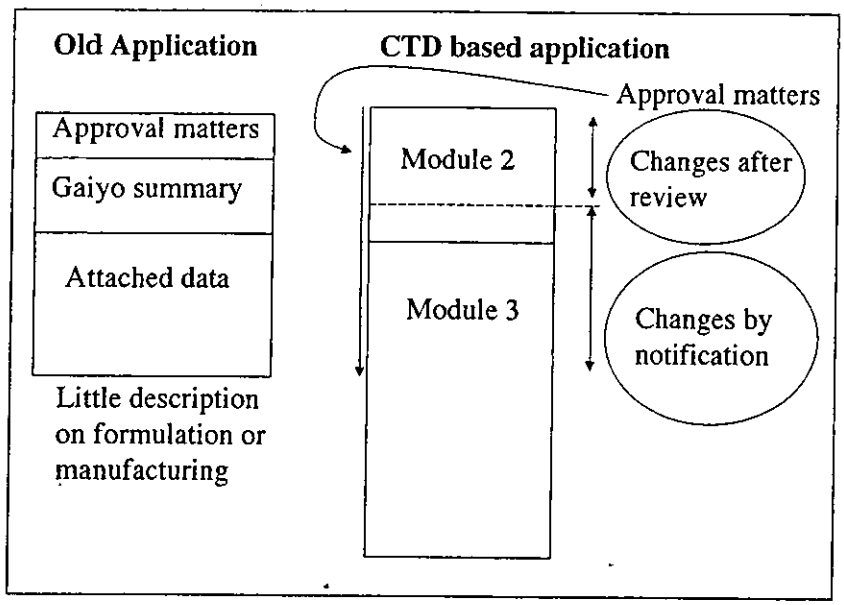

Figure. Structure of CMC portion of drug application. In the old system, specifications and test methods occupy the majority of the portion. Changing approval matters currently takes one year. In the new CTD based application, the portion of approval matters is expanded to include core information on the manufacturing process. To change non-critical approval matters, companies need only submit a notification.

for each new application will be conducted prior to approval. This requires Industry to finalize technology transfer before approval and gives the regulatory authority an opportunity to check on the qualification of the final manufacturing process against the description in the application document.

The rPAL and its regulations are major undertakings both to Authorities and Industry. In order to streamline the review and inspection systems, the Pharmaceuticals and Medical Devices Agency has been established in April 2004 by combining the review function under NIHS and the inspection function of the Kiko. Several Health Science Grant-sponsored study groups have been formed to establish comprehensive GMP guidance documents, GMP inspection policies and a scope document for approval matters. In 2002-2003, the GMP guidance study group focused on quality systems, regulatory system issues, technology transfer and laboratory control. The quality system subgroup for example, recommended that Market Authorization holders should have a declaration statement of responsibilities/authorities, organization, and resources in their quality system.

The technology transfer subgroup made the following recommendations: a development report should be written and transferred to the manufacturer; product specifications should be set by reflecting critical functional attributes, which should be clearly defined at the Product Quality Design stage; the specifications with rationale should be in the development report, which should be available for review.

Draft GMP-related guidance documents with an inspection policy will be published and a framework of approval matters set by Summer 2004.

The regulation changes give opportunities for a complete description of the Quality System in the applications and for better knowledge transfer/ management both in the Regulatory Authority and in Industry. Training of reviewers and inspectors would be a challenge for the Authority while most companies will need to change undesirable mindsets, which were presumably created by the old system.

Because expectations created by rPAL have a strong link with the new ICH quality topics, i.e. Pharmaceutical Development and Quality Risk Management, we hope that international collaboration and learning such as discussion at the ICH will be helpful in implementing the new Pharmaceutical Affair Law.

再生医療分野における指針・ガイドライン：再生医療の適正かつ効果的な推進を目指して

Guidelines on Regenerative Medicine in Japan : Approaches for Appropriate and Effective Promotion of Regenerative Medicine

Keywords
再生医療
細胞組織医薬品等→用語解説106頁
品質・安全性
指針・ガイドライン

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Summary

There are many approaches for producing and evaluating novel biologicals, including cell/tissue-based products used in regenerative medicine. To have such products contribute more significantly to human health care, it is essential that suitable measures based on sound scientific principles and approaches should be taken by physicians, manufacturers and control authorities to assure the quality, safety, and efficacy of these products. In addition to this, relevant aspects with respect to emerging technologies, public concerns, as well as the protection of individual rights are essential elements that must be taken into account.

In this article, Japanese guidelines on the quality and safety of cell/tissue-based products, as well as on ethics in regenerative medicine are described.

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はじめに

生命科学や関連技術の進歩の延長線上に人々が期待する大きな成果に，画期的な医薬品・医療機器や医療技術の開発がある。その成果が優れていればいるほど，保健衛生面で人類に恩恵をもたらす共通の資産としての価値が高くなる。これらの医薬品・医療機器や医療技術は，科学的には，生命科学や関連技術の進歩を集学的に統合化して得られる結晶であるが，その過程において，いかに個々の科学的要素を充実させ，最も効率的かつ最大限に活用できるか，また，最終目標である品質・有効性・安全性においていかに望ましいものとするかが，必須の課題である。一方，社会的な存在としての医薬品・医療機器や医療技術という視点でみると，特に先端的製品や技術であればあるほど，その開発や適用に当たって，倫理的妥当性，社会的理解や認知，経

済的妥当性などの課題にいかに適正に対処し，クリアするか，その結果，人類の資産である優良な医薬品・医療機器や医療技術を，いかに速やかに誕生させ，医療の場に提供し，いかに適正，使用するかが，重要課題である。トータルとして，基礎研究・基盤技術研究から臨床応用・実用化に至るまでの過程をいかにスムーズに効率よく行うかというポイントは，図に示したような各要素をそれぞれの連携・調整を取りつつ満たすことにかかっており，規制環境の整備も含め，最終目標を目指した統合的アプローチが必要となる[1]。このためには，研究開発，評価，使用の各局面において，再生医療の推進を目指す企業，学界，公的研究機関，規制当局のいずれもが密接な連携，情報共有を図り，それぞれの立場においてそれぞれの機能を最大限に発揮しながら，最も望ましい形の共通の目的実現を目指した科学的思考やアプローチを実践すること，すなわち情報や認識の共有化がキーポイントになる。

これらのさまざまな局面で，各種指針・ガイドラインや品質・有効性・安全性にかかわる評価科学の適切な適用が重要な役割を演ずることになる。再生医療に限らず，指針・ガイドラインは本来，科学技術の所産を最も望ましい形で，かつ迅速，効率的に臨床の場にもたらすために，望ましい考え方やアプローチ法，適切な試験項目や試験，作成すべきデータを示すものである。再生医療分野は医療技術的にも新しく，経済的妥当性，社会的理解・認知，倫理的妥当性の面でも現時点では確たる答えがすべて用意されているとは限らない分野である。このような分野では，健康被害や倫理問題などが発生することのないよう特に慎重に配慮する一方で，先端科学技術の「より望ましい形での国民生活への還元」ということの意義を踏まえ，これを推進することは極めて重要であるとの認識をもつ必要がある。指針・ガイドライン類は，新たな医療技術を1日でも早く国民のもとに提供するための流れをより適正，円滑に推進するためのものであって，結果的にブレーキをかけるためのものとして利用してはならない。このため，公的な指針・ガイドラインの作成・運用に際しては，その時点での科学的かつ合理的な根拠に基づいて，社会的な合意を得つつ行われる必要がある。不確実な要素を多く含む技術的および行政的諸課題に対しては，学際的に可能なアプローチを含む統合的アプローチや国際的動向も加味した上で，社会的に最適な選択肢を決定し，歩を進めることが肝要である。

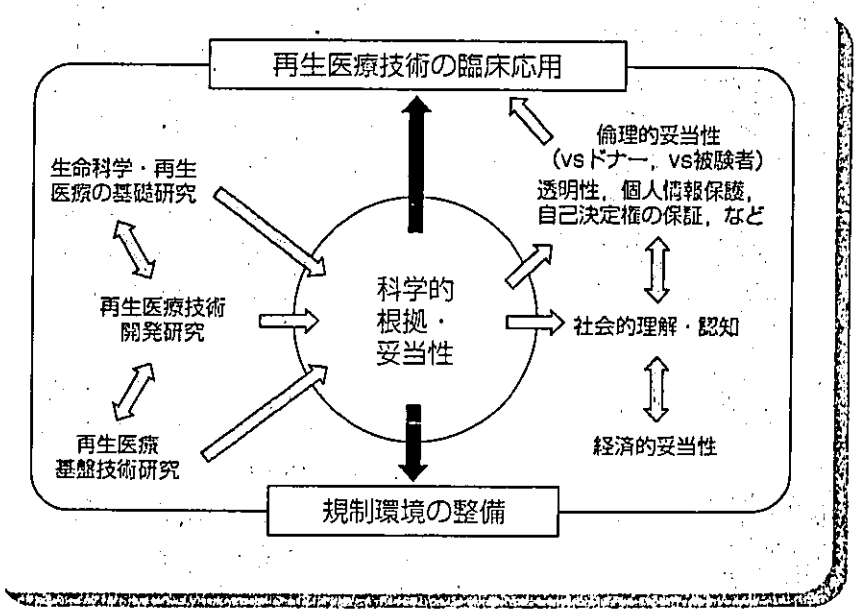

図　再生医療の実現化に向けて統合的アプローチが有用となるさまざまな局面[1]

以下に再生医療に関係すると思われる既存の指針・ガイドラインについて概説するが，これらは必要なすべてを包含したものではなく，また，科学技術などの進歩や社会情勢の変化に対応して，適宜，見直しの対象ともなるべきものである。

品質・安全性面における指針・ガイドライン

再生医療に用いられる細胞組織製品に特徴的な最重要課題は，①ウイルスなどの感染性物質の伝播を可能なかぎ

り防止すべきであること，および，②製品ごとにその特質を個別に考慮した品質・安全性確保のための適切な方策をケースバイケースで採用すべきであること，の二点である[2),5)]。

2003年5月に告示された「生物由来原料基準」[6)]は，医薬品，医薬部外品，化粧品および医療機器（以下，医薬品等）に使用されるヒト，その他の生物（植物を除く）に由来する原料または材料（添加剤，培地などとして製造工程において使用されるものを含む）について，製造に使用される際に講ずべき必要な措置に関する基準を定めることにより，医薬品等の品質，有効性および安全性を確保することを目的としたものである。ヒトまたは動物の細胞や組織に由来する再生医療用の医薬品等は，「生物由来原料基準」[6)]においては「人細胞組織製品」もしくは「動物細胞組織製品」として分類されており，それぞれ「人細胞組織製品原料基準」（表1）および「動物細胞組織製品原料基準」の中で，ドナーの適格性，原材料の採取の方法，記録の保管などについて規定されている。「生物由来原料基準」[6)]は，厳密には薬事法上の医薬品等のみを直接の規制対象としていることから，医師/医療機関の責任により実施される臨床研究（薬事法上の承認申請の意志をもたずに実施される研究。その研究結果報告書を承認申請資料として用いることは原則不可）に用いられる細胞組織製品は規制対象に該当しないと考えられるもの[7)]，薬事法上の医薬品等では「生物由来原料基準」[6)]の遵守が義務付けられていること，および「ヘルシンキ宣言」（「11. ヒトを対象とする医学研究は，一般的に受け入れられた科学的原則に従い，科学的文献の十分な知識，他の関連した情報源及び十分な実験並びに適切な場合には動物実験に基づかなければならない」）[8)]を踏まえると，再生医療の臨床研究を実施するに当たっても，この「生物由来原料基準」[6)]に可能なかぎり準拠することは当然であると期待される。

同様に，直接的には薬事法上の医薬品等を適用対象としているものの，その開発段階も含めた細胞組織製品の品質および安全性の確保を目的として，2000年に厚生省から「ヒト又は動物由来成分を原料として製造される医薬品等の品質及び安全性確保について」という通知[9),10)]が出されており，その別添1として「細胞・組織利用医薬品等の取扱い及び使用に関する基本的考え方」（以下，「基本的考え方」）[9)]，別添2として「ヒト由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針」（以下，「指針」）[10)]が示されている。このうち，「基本的考え方」[9)]は，製品の由来がヒトか動物かを問わず，細胞組織製品全般に共通して品質・安全性を確保するために必要な基本的考え方を示したものである。「基本的考え方」[9)]には，「生物由来原料基準」[6)]で規定されている内容に加えて，製造工程に関するGMP（Good Manufacturing Practice）の概念など，重要な考え方が明記されており（表2下線部），「生物由来原料基準」[6)]と併せて日本版cGTP（current Good Tissue Practice）と捉えることができるであろう。

一方，「指針」[10)]は，ヒト由来細胞・組織加工医薬品等の品質および安全性確保のための基本的な要件を定めたものである（表3）。この「指針」[10)]においては，当該医薬品等の臨床試験（薬事法上の承認申請の意志をもって実施される治験）をわが国で実施するに当たり，臨床試験依頼者は事前に厚生労働大臣に対して本指針に適合することの確認を求めることとされているが，その際の確認申請資料に記載すべき内容を具体的に明らかにしたものである。本文書の適用対象は，ヒト由来の細胞・組織加工医薬品等に限定されているものの（ここでいう「加工」の定義は表4を参照），当該医薬品等の臨床研究を実施する場合および他の細胞組織製品を用いて臨床研究/臨床試験を実施する場合でも，その内容は十分活用できるであろう。臨床研究/臨床試験に用いられる細胞組織製品の品質および安全性の確保のためには，上記「生物由来原料基準」[6)]および「基本的考え方」[9)]を踏まえながら，「指針」[10)]で具体的にあげられている事項について十分理解・考慮しなければならないのである。

なお，細胞組織製品の製造過程で人為的に遺伝子操作を行うケースでは，その導入遺伝子から発現する蛋白質に何らかの作用を期待する場合，厚生労働省や文部科学省の指針[11)12)]の適用対

1．人細胞組織製品［人に由来する原料又は材料（血液及び血液から製造される成分を除く。）から構成される医薬品又は医療用具をいう。以下同じ。］の原料又は材料として用いる細胞及び組織については，採取するために必要な衛生管理を行うのに十分な人員及び設備を有する施設で採取されたものでなければならない。

2．人細胞組織製品の原材料として用いる細胞又は組織を採取するに当たっては，次に掲げる措置が講じられていなければならない。

ア）当該細胞又は組織を採取する過程における病原微生物その他疾病の原因となるものによる汚染を防止するために必要な措置が講じられていること。

イ）採取された細胞又は組織について，必要に応じて感染症に関する最新の知見に照らして適切な検査が行われ，病原微生物その他疾病の原因となるものに汚染されていない旨が確認されていること。

3．ドナーは，次のいずれにも該当し，人細胞組織製品の原材料として用いる細胞又は組織を提供するにつき十分な適格性を有するものでなければならない。なお，人細胞組織製品の使用の対象者とドナーが同一の者である場合は必ずしもドナースクリーニングを必要としない。

ア）当該細胞又は組織を採取するに当たって，それらの利用の目的に応じ，問診，検診，検査等により，細菌，真菌，ウイルス等の感染が否定されていること。

イ）ア）の検査項目及び検査方法が感染症等に関する最新の知見に照らして適切なものであること。

ウ）ア）の検査項目，検査方法等に応じた再検査がウインドウピリオドを勘案して適切な時期に行われていること。

4．上記のほか次に掲げる疾病等について，問診，検診，検査等を行うとともに，輸血又は移植医療を受けた経験の有無等を勘案して，ドナーとしての適格性があると判断されていなければならない。

ア）梅毒トレポネーマ，クラミジア，淋菌，結核菌等の細菌による感染症

イ）敗血症及びその疑い

ウ）悪性腫瘍

エ）重篤な代謝及び内分泌疾患

オ）膠原病及び血液疾患

カ）肝疾患

キ）伝達性海綿状脳症及びその疑い並びにその他の痴呆症

5．細胞又は組織の採取を行う者が，ドナーとなる者に対して，ドナースクリーニングの実施前に細胞及び組織の利用目的，個人情報の保護，その他採取に関する事項について当該者の理解を得るよう，文書を用いて十分に説明し，自由な意思による同意を文書により得たものでなければならない。なお，説明に当たっては，同意の拒否及び撤回の権利があり，拒否又は撤回することにより当該者が不利益を受けないことが明らかにされていなければならない。

6．ドナー本人が説明を受け同意を与えることが困難な場合又は単独で完全な同意を与える能力を欠いている場合において，下記の要件を満たす場合に限り，代諾者（本人に対して親権を行う者，配偶者及び後見人その他これらに準じる者であって，本人に代わって説明を受け，本人に代わって同意をする権限を有するものをいう。以下同じ。）の同意により細胞又は組織の採取を行うことができる。

ア）当該ドナーからの細胞又は組織の採取が人細胞組織製品の品質，有効性及び安全性の確保の観点等から必要とされる合理的理由があること。

イ）代諾者がドナーの意思及び利益を最もよく代弁できると判断される者であり，かつ，代諾者の同意に際しては，ドナーと代諾者の関係についての記録が作成され，同意書とともに保存されていること。

ウ）細胞又は組織を採取する者は可能な限りドナーにその理解力に応じた説明を行うとともに，ドナー本人からも同意を得るように努めること。

エ）採取を行う施設の倫理委員会等において，当該ドナーからの細胞又は組織の採取の科学的及び倫理的妥当性が審査され，了承されていること。

表 1-1 「人細胞組織製品原料基準」[6]

7. 死体から細胞又は組織の提供を受ける場合には，遺族に対して5.に従って説明し同意を得たものでなければならない。細胞又は組織の採取は，当該ドナーが細胞又は組織の提供を生前に拒否していない場合に限る。また，ドナーに対する礼意の保持に留意したものでなければならない。
8. 手術等で摘出された細胞又は組織を利用する場合においても，5.及び6.に従って同意を得たものでなければならない。なお，この場合にあっては，当該手術等が細胞又は組織の採取の目的を優先して行われたものであってはならない。
9. ドナーからの細胞又は組織の採取が無対価で行われたものでなければならない。ただし，細胞又は組織の提供により生じるドナーの負担につき，交通費等実際にかかった費用を勘案しつつ，倫理委員会等の了承を得た上で，適切な補填がなされることは，この限りではない。
10. 細胞組織製品の原材料となる人の細胞又は組織についての，品質及び安全性の確保上必要な情報が確認できるよう，次に掲げる事項が記録され，保存されていなければならない。
ア）当該細胞又は組織を採取した施設
イ）当該細胞又は組織を採取した年月日
ウ）ドナースクリーニングのための問診，検診，検査等による診断の結果及び状況
エ）当該細胞又は組織を採取する作業の経過
オ）倫理委員会等の審議結果
カ）同意説明文書及び同意文書
キ）ドナーに関する識別番号
ク）ア)からキ)に掲げるもののほか，人細胞組織製品の品質及び安全性の確保に関し必要な事項

表1-2 「人細胞組織製品原料基準」[6]

象にもなり，臨床研究/臨床試験の実施前には確認申請が必要となる。動物培養細胞をフィーダー細胞として利用する場合も含めて，製造過程で動物由来の細胞・組織を用いる際には，厚生労働科学研究費補助金 厚生労働科学研究事業によりまとめられた感染性物質に関する指針[14][15]も参考になるであろう。また，2002年の薬事法改正に伴い，企業の依頼により実施される従来型の臨床試験（治験）に加えて，2003年7月からは新たに医師/医療機関主導型の臨床試験（治験）が認められているが[16]，このような臨床試験においても，「医薬品の臨床試験の実施の基準」(Good Clinical Practice：GCP)[16][17]を遵守することはもちろんのこと，「治験薬の製造管理及び品質管理基準並びに治験薬の製造施設の構造設備基準」(治験薬GMP)[18]への準拠も求められる。

倫理面における指針・ガイドライン

一般に，臨床研究/臨床試験を実施する際の国際的な倫理的規範として「ヘルシンキ宣言」[8]が存在し，さらに臨床研究に関しては2003年7月に告示された「臨床研究に関する倫理指針」[19]，臨床試験（治験）に関しては「医薬品の臨床試験の実施の基準」[16][17]を遵守することとされている。これらは被験者に対する倫理面での配慮を定めたものであるが，再生医療に特徴的なこととして，上記「生物由来原料基準」[6]および「基本的考え方」[9]にも明記されているとおり，被験者への倫理的配慮に加えてドナーに対する倫理的配慮も忘れてはならない。

特定の技術および原材料に関して，わが国が策定した医学・生命科学研究全体に係る倫理指針類として，クローン技術，特定胚およびヒトES細胞に関する指針などがすでに公表されており，さらに現在，専門の委員会を設けて審議中のものもある（表5）。また，この他にも関係学会などで独自に作成された指針類もあるので，再生医療研究を実施する際には十分留意されたい[23][24]。

1．本文書の目的，基本原則，定義
・細胞組織製品は，細胞・組織に由来する感染症の伝播などの危険性を完全には排除し得ないおそれがあることから，他の治療薬や治療法と比較して有用性が同程度以上と判断されるときにのみ使用

2．細胞・組織採取について
・適切な衛生管理。知識・技術をもった人員の確保
・倫理委員会での事前調査・審議
・ドナーからのインフォームドコンセントの取得。無対価での提供
・ドナーおよびドナー動物の選択基準および適格性
・感染性物質による汚染を防ぐために必要な措置・検査の実施
・記録

3．製造段階における安全性確保対策
・独立した作業区域の設置。複数のドナーからの細胞・組織を同一室内で同時期に取り扱うことや，交叉汚染を引き起こす可能性のある保管方法の禁止
・標準操作手順書の作成および遵守。製造工程に関する記録
・採取した細胞・組織および試薬などの受け入れ試験・検査。製品の試験・検査。感染性物質による汚染の危険性の排除
・最新技術の反映

4．職員および組織ならびに管理体制（職員の教育訓練，健康管理）など

5．使用段階における安全性確保対策
・ドナーや最終製品の試験・検査結果の医療機関に対する提供
・患者からのインフォームドコンセントの取得
・患者などの試料の保存。患者などに関する情報の把握

6．個人情報の保護

7．見直し

※生物由来原料基準では明記されていない内容に下線を付した。

表2 「細胞・組織利用医薬品等の取扱い及び使用に関する基本的考え方」[9]の概略

おわりに

再生医療の臨床研究/臨床試験を実施するに当たって，たとえば細胞組織製品の品質・安全性に関する「基本的考え方」[9]や「指針」[10]をはじめ，本稿で紹介した指針・ガイドライン類はそれぞれの作成時点での知識や情報に基づくものであることから，これらを未来永劫固定化された規制と捉えることは不適切である。基礎研究・基盤技術研究や非臨床試験も含めて，個々の細胞組織製品について実施される試験の内容やその成績の評価に際しては，品質および安全性の確保，そして国民に対する先端科学技術の迅速な還元という最終目的を常に意識しながら，倫理面への配慮も含めた統合的アプローチにより柔軟かつ合理的に対応していくことが重要である。このようなアプローチおよび種々の事例の蓄積から，再生医療のさらなる進展が図られるものと期待される。

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1．本文書の目的，定義
2．利用目的，製造方法および安定性
①原材料となる細胞・組織および製造方法について
・原材料となる細胞・組織の特性と適格性の確認。採取した細胞・組織の一部保管。感染性物質の不活化/除去。加工した細胞の特性解析
・培地や細胞・組織の処理に用いる試薬などの全成分について，感染性物質の否定も含めて適格性を明らかにし，必要な品質規格を設定。培養・加工時の血清の使用は可能なかぎり避ける。これが避けられない場合，血清由来感染性物質の混入・伝播の防止および使用血清の一部を保管
・細胞・組織に人為的に遺伝子を外部から導入する場合における詳細は文献11も参照
②細胞・組織以外の原材料について
・細胞・組織以外に最終製品の一部を構成する原材料がある場合，当該原材料の品質および安全性ならびに細胞に及ぼす影響を検討。当該原材料の特性に応じて文献12を参考に必要な規格を設定
・細胞・組織と患者の適用部位を隔離する目的で非細胞・組織成分を用いる場合，次の項目を参考に効果・安全性を確認：免疫隔離の程度，栄養成分および排泄・分泌物の拡散，細胞・組織由来の生理活性物質の膜透過キネティクスと薬理効果，患者由来の生理活性物質の細胞・組織への有害作用
③細胞・組織の同一性および均一性の確認，品質管理，製品の安定性の確認
・次に示す一般的な品質管理試験項目を参考に必要な規格を設定：細胞の回収率・生存率，同一性の確認，細胞・組織由来の目的生理活性物質の量/力価，無菌性およびマイコプラズマ，エンドトキシン，製造工程由来不純物，細胞の純度，細胞・組織由来の目的外生理活性物質の種類および量/力価，力学的適合性，感染性物質
3．非臨床安全性試験，効力/性能を裏付ける試験，体内動態
・特に次の項目について必要に応じて動物および *in vitro* での試験を実施し，安全性を確認：加工細胞の性質の変化，細胞・組織が産生する各種生理活性物質の定量および患者への影響，患者の正常細胞・組織に対する製品の影響，望ましくない免疫反応が生じる可能性，（最終製品が大量に生産される場合には）一般毒性試験
4．臨床試験（外国における開発状況も含める）
①治験計画の概要
・製品適用後の有効性/安全性評価期間・項目は十分検討して決定。免疫学的事項も含める
5．確認および報告

表3 「ヒト由来細胞・組織加工医薬品等の品質及び安全性の確保に関する指針」[10]の概略

1．細胞・組織の人為的な増殖
例）ドナーから採取した細胞・組織を体外で培養・増殖させた後，それを患者に適用する場合。
2．細胞・組織の活性化などを目的とした処理
①薬剤処理
例）ドナーから採取した未分化の細胞の体外での培養に際し，分化誘導物質を添加して目的とするステージの細胞に分化させた後，それを患者に適用する場合。
②生物学的特性の改変
例）採取した細胞を体外で培養する際に，サイトカインや抗原で人為的に刺激することによって細胞の生物学的特性を目的のものに改変した後，それを患者に適用する場合。
③細胞・組織の遺伝子工学的改変
例）採取した細胞・組織に体外で遺伝子導入を行った後，それを患者に適用する場合（このような場合は「遺伝子治療」の範疇にも属することに注意）。
3．非細胞・組織とのハイブリッド化
例）採取した細胞・組織の体外での培養に際し，特定の効果を期待して人為的に添加された非細胞・組織成分が，最終製品においても含有されている場合。一例としては，採取した細胞を培養用マトリクス上で培養し，それにより得られた増殖細胞の貼りついたマトリクス全体を患者に適用する場合。
4．カプセル化
例）細胞・組織加工医薬品・医療機器の本質である細胞・組織が適用患者などに直接的に接触しないよう，非細胞・組織成分を用いて当該細胞・組織が隔離されるような剤型として最終製品が製造されている場合。一例としては，目的のペプチド・蛋白を産生・分泌する動物細胞を患者に適用する際に，そのまま適用したのでは免疫反応の惹起や人獣共通感染症病原体の混入が懸念されるため，目的のペプチド・蛋白が透過するような材質のカプセルで当該細胞をくるみ，それを患者に適用する場合。
5．その他

注：上記の区分は各々独立したものではなく，品目ごとに複数の区分に該当する場合もある。

表4 「ヒト由来細胞・組織加工医薬品等」[10]における「加工」の具体例

<臨床研究>
・臨床研究に関する倫理指針（厚生労働省，2003）[18]
・ヒト幹細胞を用いた臨床研究の在り方（厚生労働省 厚生科学審議会 科学技術部会 ヒト幹細胞を用いた臨床研究の在り方に関する専門委員会，審議中）
<臨床試験>
・医薬品の臨床試験の実施の基準（厚生労働省，1997，2003）[16][17]
・生物由来原料基準（厚生労働省，2003）[9]
・細胞・組織利用医薬品等の取扱い及び使用に関する基本的考え方（厚生省，2000）[9]
<研究全般>
・ヒトに関するクローン技術等の規制に関する法律（法律第146号，2000）[20]
・ヒトES細胞の樹立及び使用に関する指針（文部科学省告示第155号，2001）[21]
・特定胚の取扱いに関する指針（文部科学省告示第173号，2001）[22]
・ヒト胚の取扱いに関する基本的考え方（中間報告書）（総合科学技術会議 生命倫理専門調査会，2003）[23]

表5 再生医療に関するわが国の倫理指針類

mhlw.go.jp/general/seido/kousei/i-kenkyu/index.html）
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