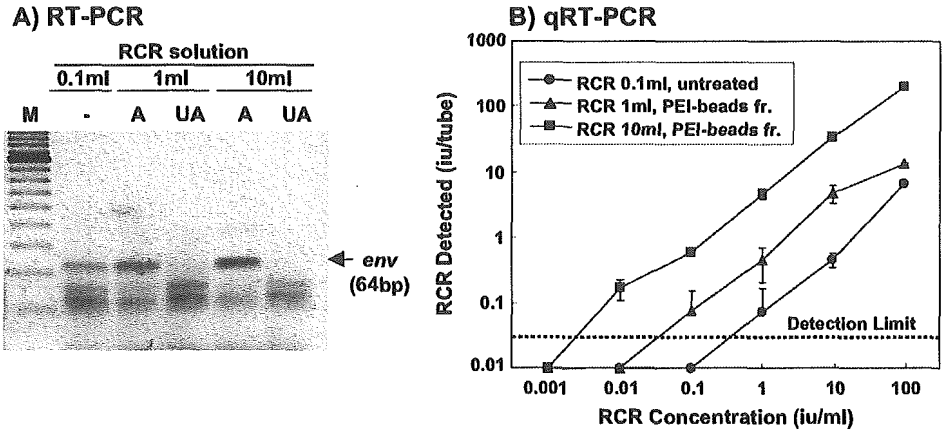

Fig. 3. Concentration of RCR by PEI-beads. (A) RCR solution (10^{-5} dilution) was fractionated with PEI-beads. Viral genome RNA extracted from the PEI-beads adsorbed fraction and unadsorbed supernatant were amplified with RT-PCR and analyzed by 5% agarose gel. M: 20 bp DNA ladder; −: untreated RCR solution; 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.

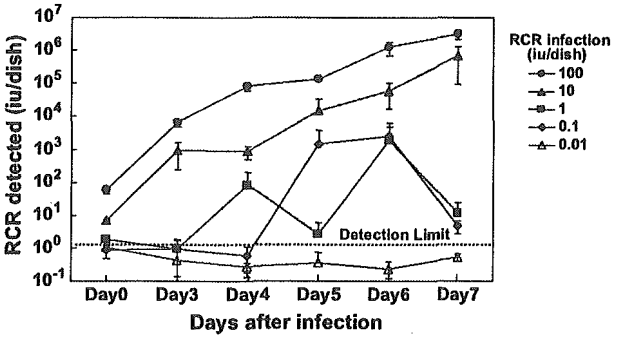

Fig. 4. RCR growth curve in *M. dunni* cells. *M. dunni* cells were infected with serial log dilutions of RCR solution. Culture supernatants were harvested at the indicated time, and RCR was concentrated by PEI-beads. Viral genome RNA was extracted from PEI-beads and the amount of RCR was determined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

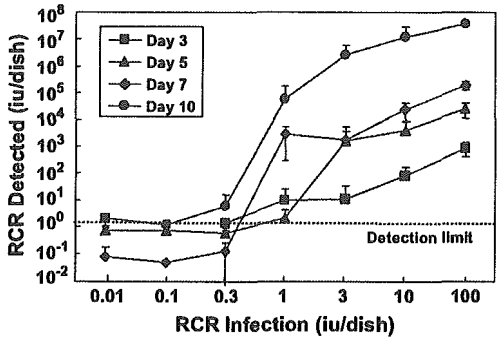

Fig. 5. Detection of RCR spiked in retrovirus vector supernatant by infectivity RT-PCR. *M. dunni* cells were infected with serial dilutions of RCR solution in 10^6 cfu/ml of ΨCRIP-LEGFP1 retrovirus vector supernatant. Cell culture supernatants of infected cells were harvested on day 3, 5, 7 and 10, 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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Detection of Replication-Competent Adenoviruses Spiked into Recombinant Adenovirus Vector Products by Infectivity PCR

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The presence of replication-competent adenovirus (RCA) in clinical lots of adenovirus vectors raises a variety of safety concerns. To detect RCA in adenovirus vector products, the cell culture/cytopathic effect (CPE) method has generally been preferred. However, it is difficult to evaluate the amount of RCA clearly and quantitatively by this method. In addition, the cell culture/CPE method requires large-scale cell culturing and a substantial amount of time. For the purpose of establishing a method to detect RCA more sensitively and rapidly, we developed the infectivity PCR, a hybrid method that combines the infectivity assay and quantitative PCR. This method allows RCA to be quantified by real-time quantitative PCR using primers and a probe designed for E1 DNA. By infectivity PCR, 1 pfu of RCA spiked into 10^9 particles of adenovirus vectors could be detected. In contrast, CPE was observed in the cells infected with 10^4 pfu of RCA spiked into 10^9 particles of adenovirus vectors. The glass-beads method was suitable for extracting DNA rapidly from the RCA-infected cells. These results showed that infectivity PCR combined with the glass-beads-based DNA extraction method was useful for the detection of RCA in adenovirus vector products.

Key Words: replication-competent adenovirus, adenovirus vector, infectivity PCR

Introduction

Recombinant adenovirus vector is one of the most promising vectors available for human gene therapy. In fact, adenovirus vector-based gene therapies now account for 26.9% of all clinical gene-therapy protocols [1]. Considerable efforts have been made to improve the potency of adenovirus vectors to make them more useful for gene therapy, e.g., regulation of target-cell specificity by modifying fiber protein [2–7], application of cassettes that enable the regulation of gene expression [8,9], and reduction of immunogenicity by deleting all viral genome sequences [10]. However, there have been few studies investigating the establishment of a system to ensure the safety of these vectors.

The vast majority of adenovirus vectors are constructed by inserting the therapeutic genes in place of the essential viral E1 sequence in the adenovirus. The generation of E1-deleted adenovirus vectors relies on the complementation functions present in HEK293 cells into whose genome E1 DNA has been inserted. However, HEK293 cells are prone to the generation of replication-competent adenovirus (RCA) as a result of recombination events between the vector DNA and the integrated adenovirus sequences present in the cells [11]. The presence of RCA in adenovirus vector products raises the possibilities of adenovirus infection, unintended vector replication due to the presence of wild-type helper function, and exacerbation of host inflammation response [12]. Because it is extremely difficult to avoid completely the emergence of RCA in adenovirus vector products by means of the current production technique, examining the level of RCA in each lot of adenovirus vector products is important [12]. In addition, examining for the presence of RCA in the patients who have been administrated adenovirus vectors

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is important to test for viral shedding during the clinical study.

As a method to detect RCA in adenovirus vector products, the cell culture/cytopathic effect (CPE) assay has generally been used [13,14]. In the cell culture/CPE assay, the vector products are infected into cells, the RCA are amplified, and the CPE induced by the RCA is observed. By this method, the presence of RCA is judged by microscopic observation, and thus the results may not always be accurate and quantitative. In addition, this method requires large-scale cell culturing and a substantial amount of time. As a sensitive method to detect viral DNA, PCR is thought to be useful [11,15]. However, because the infectivity of the viral DNA cannot be measured by PCR, the cell culture/CPE assay is still recommended by the FDA [12]. For all of the above reasons, there is need for a more sensitive, quantitative, and rapid method for the detection of RCA.

In the present study, we established an infectivity PCR method for detecting RCA. Infectivity PCR is a hybrid method that combines the best features of the infectivity assay and PCR. By this method, the virus is allowed to replicate in the cell culture, as in the traditional cell culture/CPE assay, and the amount of virus replicated in the cells is determined by quantitative PCR rather than by observing CPE. For efficient extraction of the DNA from RCA-infected cells, a novel glass-bead method was developed. Our results demonstrated that the infectivity PCR method combined with glass-beads-based DNA extraction was superior to the classical cell culture/CPE method for detecting RCA.

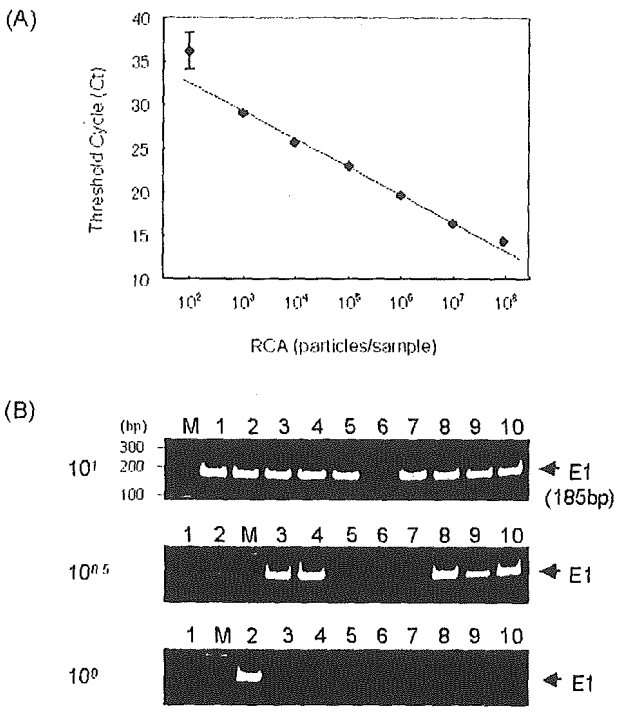

FIG. 1. Detection of RCA by PCR. Viral genome DNA was extracted from serial log dilutions of RCA and amplification of each sample was performed by (A) real-time quantitative PCR or (B) nested PCR. (A) Standard curve for the determination of RCA quantity generated from the amplification plot of real-time quantitative PCR. Data are the means ± SD of triplicate amplifications. (B) Detection of a low copy number of RCA by nested PCR ($n = 10$). The particle numbers in the samples were 10^1, $10^{0.5}$, and 10^0. M, molecular weight marker.

RESULTS

Quantification of RCA by Real-Time Quantitative PCR

As a first step in establishing an infectivity PCR method, we developed a real-time quantitative PCR that can quantify the copy number of the RCA genome. We designed four pairs of primers and probes for the detection of E1 DNA, which is included in the RCA genome but not in the adenovirus vectors. Among them, the Ad5dE1-1035F and Ad5dE1-1105R primers and the Ad5dE1-1058TM probe worked well for quantifying the RCA genome. Fig. 1A shows the standard curve with the starting quantity of RCA on the x axis and the threshold cycle (Ct denotes the PCR cycle at which the threshold line intercepts the amplification curve) on the y axis. The threshold cycle and the log-transformed concentration showed a high, inverse correlation in a linear fusion from 10^8 to 10^3 particles.

Detection of RCA by Nested PCR

Since the nested PCR method is known to be suitable for detecting low concentrations of DNA, we applied it here to detect the DNA extracted from 10^1, $10^{0.5}$, or 10^0 particle(s)/tube of RCA. When nested PCR was performed in 10^4 tubes, the amplification succeeded in the ratios of 9/10 for 10^1 particles, 5/10 for $10^{0.5}$ particles, and 1/10 for 10^0 particles (Fig. 1B). Although DNA concentration cannot be quantitatively determined by nested PCR, it can be estimated from the hit rate of the amplification. This estimation assumes that, when low concentrations of samples are transferred from a stock tube to PCR tubes, viral particles will not necessarily be present in all the reaction tubes. If the PCR is optimized, then as little as one copy of DNA can be amplified, and the relationship between the hit rate in the PCR and the average copy number in the PCR tubes can be calculated as follows: 1/10 for 0.105, 2/10 for 0.233, 3/10 for 0.357, 4/10 for 0.511, 5/10 for 0.693, 6/10 for 0.916, 7/10 for 1.20, 8/10 for 1.61, and 9/10 for 2.30 [16,17]. In our experiments, half of the extracted DNA was subjected to the nested PCR; therefore, the hit rate of the PCR and the RCA copy number were 1/10 for 0.5, 5/10 for 1.58, and 9/10 for 5. Although the hit rate in this experiment seemed to be slightly lower than the theoretical values, this does not

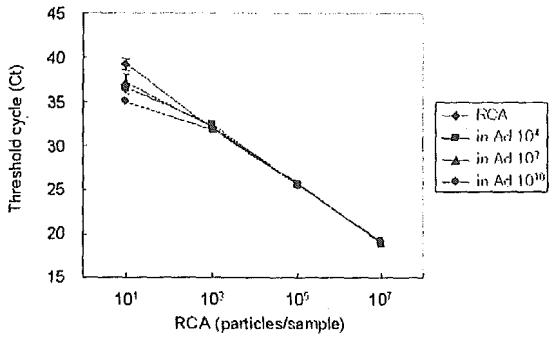

FIG. 2. Detection of RCA spiked into adenovirus vectors by real-time quantitative PCR. 10^1, 10^3, 10^5, or 10^7 particles of RCA were spiked into 0, 10^4, 10^7, or 10^{10} particles of adenovirus vectors (AdHM4LacZ). Viral DNA was extracted from each sample, and E1 DNA was detected by real-time quantitative PCR. Data are the means ± SD of triplicate amplifications. The background Ct (Ad without any RCA) was 39.1 for 10^4 particles of adenovirus vectors, 39.2 ± 1.6 for 10^7 particles, and 35.2 ± 0.04 for 10^{10} particles.

necessarily invalidate the results, since DNA may have been lost during the DNA extraction, and the detection limit may have been higher than one copy. These results demonstrated that the nested PCR could be used for the detection of lower concentrations of RCA and that the detection limit might be fewer than 10^1 particles.

Measurement of RCA in Adenovirus Vector Products

Because, in practice, it is often necessary to detect very slight amounts of RCA in high concentrations of adenovirus vectors, we next tried to quantitate the amount of RCA spiked into adenovirus vectors. We extracted viral genome DNA from 10^1, 10^3, 10^5, or 10^7 particles of RCA spiked into 10^4, 10^7, or 10^{10} particles of adenovirus vectors and then measured the amount of RCA by real-time quantitative PCR. As shown in Fig. 2, when the RCA concentration was higher than 10^3 particles/sample, the amount of RCA could be measured without major interference by coexisting adenovirus vectors. However, when the spiked RCA concentration was 10^1 particles, the Ct value seemed to be lowered by the presence of adenovirus vectors. From these results, although RCA was thought to be detectable even in the presence of adenovirus vectors, the adenovirus vectors used in this experiment might have contained a certain amount of E1 DNA. We therefore examined several lots of adenovirus vectors for the presence of E1 DNA by nested PCR. When 10^{10} particles of adenovirus vectors were subjected to the nested PCR, E1 DNA was detected in all seven lots tested. When 10^9 particles of adenovirus vectors were tested, E1 DNA was detected in four of the seven lots tested (data not shown).

Because we could not distinguish whether the E1 DNA detected in adenovirus vectors was derived from RCA or HEK293 cells, we tested for the presence of HEK293 cell-derived DNA in the vector products by PCR. For this purpose, we used primers designed for the sequence of the pregnancy-specific glycoprotein (PSG) gene, in which the adenovirus E1 gene is inserted into the HEK293 cell genome [18]. In all lots examined, PSG DNA was detected in 10^{10} particles of adenovirus vectors, meaning that the adenovirus vector products contained HEK293 cell-derived DNA as impurities (Fig. 3A). E1 DNA was barely detected in 10^{10} particles of adenovirus vectors (Fig. 3B). These results showed that adenovirus vectors can be subjected to the direct measurement of RCA by PCR, although the results are complicated by the presence of residual HEK293 cell DNA. To separate RCA from HEK293 cell-derived DNA, we attempted to establish an infectivity PCR method for the measurement of RCA in adenovirus vectors. In the infectivity PCR assay, RCAs are expected to be selectively amplified in the cells that support their growth.

Comparison of the Amount of RCA in Cells and Cultured Medium from RCA-Infected Cell Culture

To determine a suitable source for the PCR template in infectivity PCR, we compared the amounts of E1 DNA in the cells and cultured medium from the RCA-infected cell culture. We infected HeLa cells with serially diluted RCA and cultured them. We extracted DNA from the cells and cultured media and then subjected it to real-time quantitative PCR. In this experiment, we used one-third of the cells or one-hundredth of the supernatant from each dish for DNA extraction. A 100-fold higher amount of RCA was

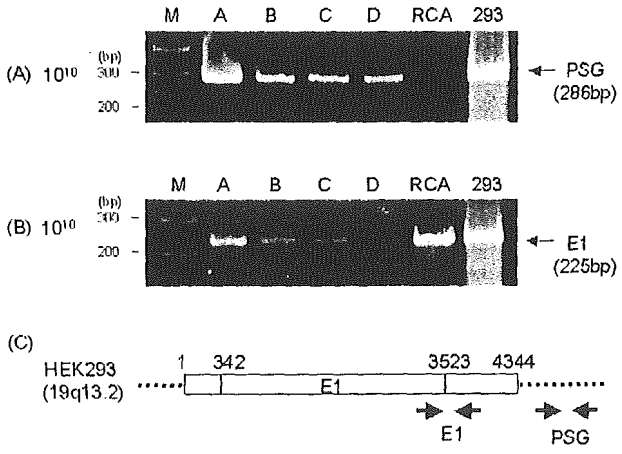

FIG. 3. Detection of pregnancy-specific glycoprotein (PSG) or E1 DNA in adenovirus vectors by PCR. Viral DNA was extracted from 10^{10} particles of adenovirus vectors and then (A) 293 cell-derived pregnancy-specific glycoprotein DNA or (B) E1 DNA was detected by PCR. DNA from HEK293 cells was used as a positive control. Lane A, AdHM4LacZ (Lot 0516); lane B, AdHM10LacZ-3 (Lot 0529); lane C, AdHM10LacZ-4 (Lot 0529); lane D, AdHM10LacZ-5 (Lot 0529), lane RCA, replication-competent adenoviruses; lane 293, genomic DNA extracted from HEK293 cells; lane M, molecular weight marker. (C) E1 DNA inserted into the chromosome of HEK293 cells and the positions of the PCR primers used are shown.

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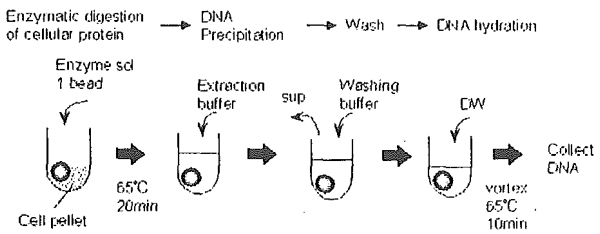
(A)



(B)

Method	EX R&D	Glass Beads
Raw data	8.9×10^8	4.5×10^8
	3.4×10^8	4.9×10^8
Average	6.15×10^8	4.7×10^8

FIG. 4. DNA extraction using glass beads. (A) Procedure of DNA extraction from cell pellets using glass beads. (B) Comparison of the efficiency of DNA extraction. DNA was extracted from RCA-infected HeLa cell lysate by the EX-R&D reagent or glass-beads method. The copy number of E1 DNA in each sample was determined by real-time quantitative PCR.

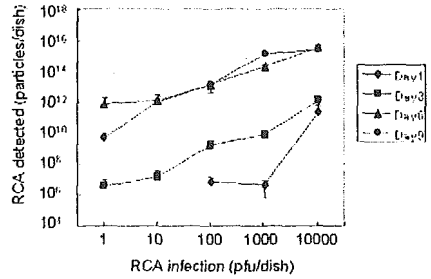
detected in the DNA extracted from the cells compared with that from the cultured medium (data not shown). Therefore, we considered the cells to be a suitable source for further examination of the propagation of RCA.

In the preparation of DNA from RCA-infected cells, cellular genomic DNA caused high viscosity and disturbed the subsequent PCR. However, cellular genomic DNA could be digested by nuclease after freezing and thawing without damaging the viral DNA, because the viral DNA is protected by capsid proteins. By this procedure, we could extract viral DNA from more than 1×10^6 cells and use it as a template for the PCR, although this procedure was laborious. We considered that glass beads, which were initially developed for extracting cellular DNA, might be used to simplify this step, and therefore we examined a glass-beads-based DNA extraction method (Fig. 4A). The protocol for the glass-beads method is simple and less time-consuming than extracting DNA after freezing and thawing followed by nuclease treatment. For comparing the efficiency of DNA extraction using glass beads to that using the SMI TEST EX-R&D, we extracted DNA from cells that contain the same amount of RCA. We used the SMI TEST EX-R&D as an example of a method that can extract DNA with high efficiency [19]. As shown in Fig. 4B, we detected equal amounts of RCA in DNA extracted by these two methods, meaning that glass beads are useful for extracting DNA rapidly, with an efficiency similar to that of the previous method.

Infectivity PCR for the Detection of RCA

For examining the infectivity PCR, we used RCA in the absence of adenovirus vectors as a preliminary experiment. HeLa cells were infected with serial log-diluted RCA (0, 0.1, 1, 10, 100, 1000, 10,000 pfu) in triplicate and cultured. After culturing them for 1, 3, 6, or 9 days, we harvested the cells and extracted the DNA using glass beads. We then measured the amount of RCA in each sample by real-time quantitative PCR. As shown in Figs. 5A and 5B, we detected RCA in all of the triplicate samples that had been infected with more than 1 pfu of RCA. RCA was not detected in the samples that had been infected with 0.1 pfu of RCA. Therefore, we concluded that at least 1 pfu of RCA was detectable by this assay. In parallel with this infectivity PCR assay, we observed the cells by microscopy and tested for CPE (Fig. 5C). On day 6, we observed weak signs of CPE in the cells that had been infected with 1000 pfu of RCA, and we clearly observed CPE in the cells that had been infected with 10,000 pfu of RCA. On day 9, we observed slight CPE in the cells that had been infected with 100 pfu of RCA, and we clearly observed CPE in the cells that had been infected with 1000 or 10,000 pfu of RCA. These results showed that the sensitivity of infectivity PCR was 100 or 1000 times higher than that of the CPE assay. In addition, by the infectivity PCR method, RCA could be detected at an earlier time point than by the CPE assay. Since A549 cells are often used for CPE assay, we also examined infectivity PCR using A549 cells. The viability of the cells decreased later than day 6, and it was difficult to test the CPE. The amount of RCA amplified in A549 cells tended to be lower than that in HeLa cells(data not shown). Therefore, we used HeLa cells for further examination.

(A)



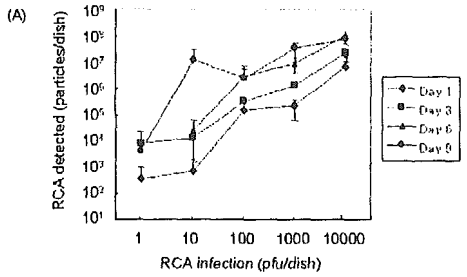
(B) Infectivity PCR

pfu/dish	Day1	Day3	Day6	Day9
10000	3/3	3/3	3/3	3/3
1000	3/3	3/3	3/3	3/3
100	3/3	3/3	3/3	3/3
10	0/3	2/3	3/3	3/3
1	0/3	1/3	2/3	2/3
0.1	0/3	0/3	0/3	0/3
0	0/3	0/3	0/3	0/3

(C) CPE method

pfu/dish	Day1	Day3	Day6	Day9
10000	0/3	0/3	2/3	3/3
1000	0/3	0/3	3/3	3/3
100	0/3	0/3	0/3	3/3
10	0/3	0/3	0/3	0/3
1	0/3	0/3	0/3	0/3
0.1	0/3	0/3	0/3	0/3
0	0/3	0/3	0/3	0/3

FIG. 5. Comparison of RCA detection by CPE assay and infectivity PCR. HeLa cells were infected with serial dilutions of RCA in medium. CPE was observed and cells were harvested on days 1, 3, 6, and 9. The viral DNA was extracted by glass beads and then the amount of RCA was determined by real-time quantitative PCR. (A) RCA growth curve in HeLa cells. Data are the means ± SD ($n = 3$). (B) The ratio of E1 DNA-positive samples is indicated ($n = 3$). (C) The number of CPE-positive samples is indicated ($n = 3$).



(B) Infectivity PCR (TaqMan PCR)

pfu/dish	Day1	Day3	Day6	Day9
10000	3/3	3/3	3/3	3/3
1000	3/3	3/3	3/3	3/3
100	3/3	3/3	3/3	3/3
10	1/3	2/3	3/3	3/3
1	1/3	1/3	0/3	2/3
0.1	0/3	0/3	0/3	0/3
0	0/3	0/3	0/3	0/3

(D) CPE method

pfu/dish	Day1	Day3	Day6	Day9
10000	0/3	0/3	0/3	1/3
1000	0/3	0/3	0/3	0/3
100	0/3	0/3	0/3	0/3
10	0/3	0/3	0/3	0/3
1	0/3	0/3	0/3	0/3
0.1	0/3	0/3	0/3	0/3
0	0/3	0/3	0/3	0/3

(C) Infectivity PCR (Nested PCR)

pfu/dish	Day1	Day3	Day6	Day9
1	2/3	2/3	1/3	2/3

FIG. 6. Comparison of the detection of RCA spiked into adenovirus vectors by CPE assay and infectivity PCR. HeLa cells were infected with serial dilutions of RCA spiked into 10^9 particles of adenovirus vectors. CPE was observed and cells were harvested on days 1, 3, 6, and 9. The viral DNA was extracted by glass beads, and then the amount of RCA was determined by real-time quantitative PCR or nested PCR. (A) RCA growth curve in HeLa cells. Data are the means ± SD (n = 3). (B and C) The ratio of E1 DNA-positive samples is indicated (n = 3). (D) The number of CPE-positive samples is indicated (n = 3).

Infectivity PCR for the Detection of RCA Spiked into Adenovirus Vector Products

Finally, we applied the infectivity PCR method for detecting RCA spiked into adenovirus vectors (Fig. 6). We infected 1.5×10^6 HeLa cells with 0, 0.1, 1, 10, 100, 1000, or 10,000 pfu of RCA spiked into 10^9 particles of adenovirus vectors (AdHM10LacZ-3). Because 10^9 particles of the adenovirus vector were equivalent to 5×10^7 infectious units, the multiplicity of infection (m.o.i.) was about 33 in this assay. We harvested the cells on days 1, 3, 6, and 9, and then we extracted the DNA using glass beads. By real-time quantitative PCR, we detected RCA in the samples that had been infected with more than 1 pfu of RCA (Figs. 6A and 6B). We detected no RCA in the samples infected with 0.1 pfu of RCA. Therefore we concluded the detection limit of this assay to be 1 pfu. This result was the same as that in the assay performed in the absence of adenovirus vectors. The absolute value of the amplified RCA was lower than in the assay without adenovirus vectors. When we also examined the presence of RCA by nested PCR (Fig. 6C), we detected E1 DNA in the samples infected with 1 pfu of RCA, but we did not detect it in those infected with 0.1 pfu of RCA, meaning that the detection limit was still 1 pfu. We observed a slight sign of CPE on day 9 in only one of the three dishes that had been infected with 10,000 pfu of RCA (Fig. 6D). CPE appeared to be suppressed by the presence of adenovirus vectors. From these results, when we measured RCA spiked into adenovirus vectors, the sensitivity of the assay was almost 10,000 times higher by infectivity PCR than by CPE assay. Thus the RCA contamination in adenovirus vectors could be detected with high sensitivity and short-term cell culture by the infectivity PCR method reported here.

Discussion

The infectivity PCR assay was developed for detecting RCA in adenovirus vectors (Fig. 7). The cell culture/CPE method in common use involves an infection of HeLa or A549 cells, harvesting, blind passage on fresh cells, and the search for CPE (Fig. 7A). It usually takes more than 4 weeks. In the method we reported here, HeLa cells are infected with adenovirus vector products and cultured for 3 or more days. The cells are harvested and viral DNA is extracted using glass beads, and then the amount of E1 DNA is measured by real-time quantitative PCR. At least 1 pfu of RCA spiked into adenovirus vectors can be detected by this method. The infectivity PCR method thus provides a more sensitive, rapid, and simple means of testing the quality of adenovirus vectors than the traditional CPE assay.

As a sensitive method for the detection of viral genome, PCR has been widely used. Real-time quantitative PCR is sensitive and highly reproducible over a wide dynamic range in addition to having high-throughput capacity. In our study, 10^3 to 10^8 particles of RCA could be detected quantitatively by real-time quantitative PCR. Nested PCR is known to be suitable for the detection of smaller amounts of DNA. By nested PCR, fewer than 10^1 particles of RCA could be detected. While the PCR method has advantages in terms of sensitivity, it has a disadvantage in that not only infectious virus but also uninfectious viral particles or DNA fragments are detected. In our experiments, we used primers and a probe for the E1 sequence that is present in RCA but not in adenovirus vectors. However, in addition to RCA, HEK293 cells in which the adenovirus vectors had been propagated had the E1 sequence in their genome. Although the adenovirus vectors had been purified by CsCl ultracentrifugation after treating the HEK293 cell lysate with nuclease, HEK293 cell-derived E1 DNA was detected in adenovirus vector products, and thus if the E1 DNA was detected in adenovirus vector products by PCR, it is not clear whether the origin is RCA or HEK293 cells. The presence of cellular E1 DNA in adenovirus vector products might not be limited to our sample, because purification by CsCl ultracentrifugation is the method used for the adenovirus vectors for clinical studies [20], and cellular DNA has been detected in adenovirus reference materials [21]. On the other hand, the fact that the ratio of infectious titer to particle concentration was 1:8.6 (see Materials and Methods) means that not all of the RCA particles

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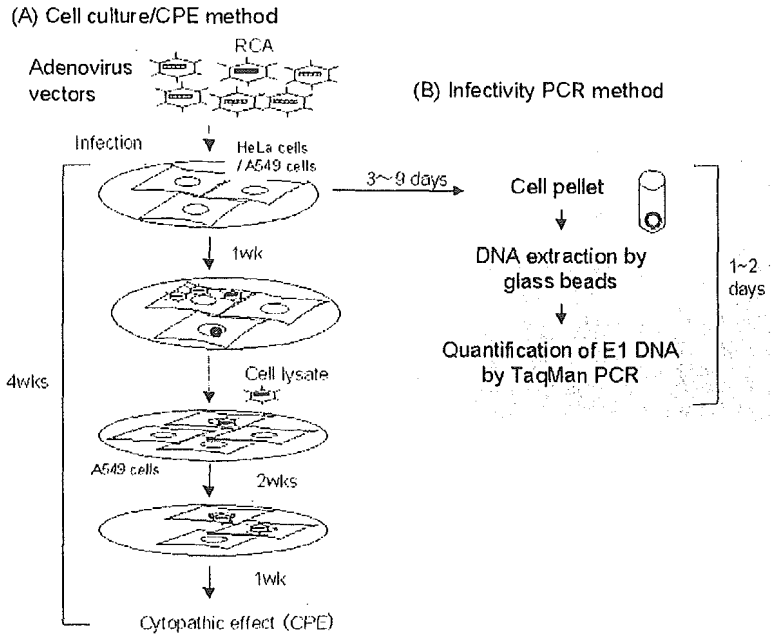

FIG. 7. (A) The cell culture/CPE method in common use involves an infection of HeLa or A549 cells, harvesting, blind passage on fresh cells, and the search for CPE. It usually takes more than four weeks. (B) In infectivity PCR method, HeLa cell are infected with adenovirus vector products, and cultured for three or more days. The cells are harvested and viral DNA is extracted using glass beads, and then the amount of E1 DNA is measured by real-time quantitative PCR.

were infectious. Therefore, infection of the adenovirus vectors into cells was thought to be indispensable for the selective amplification of the infectious RCA particles.

In the infectivity PCR assay, RCA could be detected with a detection limit of 1 pfu irrespective of the presence of adenovirus vectors. On the other hand, CPE was observed in the cells that had been infected with 10^2 to 10^4 pfu of RCA without adenovirus vectors or 10^4 pfu of RCA spiked into adenovirus vectors. Therefore, for the detection of RCA spiked into adenovirus vectors, the infectivity PCR was shown to be almost 10,000 times more sensitive than the CPE assay.

Since nested PCR was able to detect smaller amounts of DNA than real-time quantitative PCR, nested PCR was expected to contribute to an increase in the sensitivity of the infectivity PCR assay. However, the detection limit of the assay was still 1 pfu even when nested PCR was used. This might have been because, at lower concentrations of RCA, there was a threshold in the steps of infection or amplification in the cells, and thus the RCA was not linearly amplified.

The infectivity PCR was also shown to have the advantage of requiring fewer cells than the CPE assay. It is known that too high an input m.o.i. may lead to suppression of RCA outgrowth by the vector [12,22]. Because adenovirus vectors are prepared as high-titer stocks, a large-scale cell culturing, e.g., roller-bottle culture, is required to test the presence of RCA at a low m.o.i. Although the presence of adenovirus vectors seemed to interfere with the CPE, RCA could be detected by real-time quantitative PCR with the same detection limit of 1 pfu even in the presence of adenovirus vectors. This means that infectivity PCR can be performed at a higher m.o.i. than the CPE assay and therefore may contribute to a decrease in the number of the cells required for the assay.

The FDA currently recommends that adenovirus vector preparations contain <1 RCA in 3×10^{10} particles. Since, in our method, 10^9 particles of adenovirus vectors can be tested using one 10-cm-diameter plate, 3×10^{10} particles of adenovirus vectors can be tested using 30 plates. If 3×10^{10} particles are tested in the standard culture method with blind passage and come out positive, the only possible conclusion would be that there was >1 pfu in 3×10^{10} particles. Our method has the potential to be much more effective and quantitative than the standard culture method, because each plate can be tested separately, and then the actual level of RCA contamination can be estimated using the Poisson distribution.

With a goal of ensuring the safety of gene therapy, official guidelines have been published for the testing of replication-competent retroviruses (RCR) [23]. These guidelines, entitled *Supplemental Guidance on Testing for Replication-Competent Retrovirus in Retroviral Vector-Based Gene Therapy Products and During Follow-Up of Patients in Clinical Trials Using Retroviral Vectors*, provide information on RCR testing during manufacture (including timing), amount of material to be tested, and general testing methods. However, with respect to RCA in adenovirus vectors, the only available guidelines are the descriptions included within the *Guidance for Human Somatic Cell Therapy and Gene Therapy* [12]. In these guidelines, the cell culture/

CPE method is recommended for testing the presence of RCA. Although it is recommended that the assay sensitivity should be validated by spiking the test inocula with increasingly smaller numbers of wild-type adenovirus particles, there are currently no detailed guidelines for the RCA test. With regard to the safety concerns of adenovirus vector-based gene therapy, most research has focused on the immune response against capsid proteins [24]. In addition, preclinical safety studies are inherently limited in their assessment of RCA-related risks, since there are no animal models that support extensive replication of human wild-type adenovirus. This fact may have limited the attention paid to establishing an RCA detection method. Our method reported here could be used to test the RCA contamination in clinical lots of adenovirus vectors or to detect RCA in patients undergoing adenovirus vector-based gene therapy. Together with the recent efforts to develop cell lines that can propagate adenovirus vectors without emergence of RCA [25–27], this method might improve the safety of adenovirus vector-based gene therapy.

Materials and Methods

Cells. HeLa cells were obtained from the Japanese Cancer Research Resource Bank (Tokyo, Japan) and maintained in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS). HEK293 cells were obtained from Clontech (Palo Alto, CA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS.

Replication-competent adenovirus. The seed RCA (adenovirus type 5) was purchased from ATCC (Manassas, VA). For the amplification of the RCA, HeLa cells were infected with the RCA and harvested when CPE was observed. The cell lysate was prepared by four cycles of freezing and thawing and then was added to another dish of HeLa cells. After the amplification was repeated, the cell lysate from five 150-mm-diameter dishes was subjected to RCA purification by CsCl ultracentrifugation. The particle concentration and infectious titer of RCA were measured according to the standard operating procedure for adenovirus reference material (ATCC VR1516). For determining the particle concentration, RCA was diluted with the excipient solution (20 mM Tris, 25 mM NaCl, 2.5% glycerol (w/v), pH 8.0). After incubation for 15 min at room temperature, the absorbance value at 260 nm was measured. The particle concentration was determined as 5.826×10^{11} particles/ml. For determining the infectious titer, HEK293 cells were seeded in 96-well plates at 4×10^4 cells/well. After culturing for 1 day, medium was replaced with 200 μl of medium containing 5×10^7 to 1.28×10^{10} times diluted RCA stock. The plates were incubated for 60 min, and then the medium was replaced with 200 μl of fresh medium. On day 10, each well was examined for signs of CPE using a light microscope. The infectious titer was calculated as the normalized adjusted standard titer [28] and determined to be 6.767×10^{10} NIU/ml. The ratio of infectious titer to particle concentration was 1:8.6.

After we had prepared our in-house RCA standard, the Adenovirus Reference Material (ATCC VR-1516) developed under the guidance of the Adenovirus Reference Material Working Group and the U.S. Food and Drug Administration was distributed [21]. The ratio of infectious titer to particle concentration of the reference material is 1:8.3. The ratio of our in-house RCA was 1:8.9 (5.8×10^{11} particles/ml vs 6.5×10^{10} IU/ml) when these values were measured using the Adenovirus Reference Material as a reference. Since the ratios for our in-house standard and the reference material were similar, our in-house RCA standard was considered to have qualities similar to those of the reference material.

Adenovirus vector. Adenovirus vectors were prepared as described previously [29]. In brief, the plasmid harboring β-galactosidase in the E1-deleted region of the adenovirus, pAdHM10LacZ, was digested with *PacI*. The linearized plasmid was transfected into subconfluent HEK293 cells plated in a 60-mm dish using SuperFect (Qiagen, Valencia, CA) according to the manufacturer's protocol. Ten days later, cells were harvested and adenovirus vectors were released by four cycles of freezing and thawing. The vectors were amplified by further infecting into HEK293 cells and then purified by CsCl step gradient ultracentrifugation followed by CsCl linear gradient ultracentrifugation.

Real-time quantitative PCR. The ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) was used for detecting real-time quantitative PCR products. The DNA extracted from RCA standard or RCA-infected cells was dissolved in 20 μl of distilled H_2O. Ten microliters of the DNA sample was used as template in a subsequent real-time quantitative PCR with 0.5 μM each primer, 0.16 μM TaqMan probe, and 25 μl of TaqMan universal PCR master mix (Applied Biosystems). The PCR was initially denatured at 95°C for 10 min and then subjected to cycles of 95°C for 15 s and 60°C for 1 min. The reaction was carried out for 50 cycles. A calibration curve was generated for the DNA extracted from purified RCA and validated using linear regression analysis. The sequences of the primers and probe used were as follows: Ad5dE1-1035F, TCCGGTCCTTCTAACACACCTC; Ad5dE1-1105R, ACGGCAACTGGTTTAATGGG; and Ad5dE1-1058TM probe, FAM-TGAGATACACCCGGTGGTCCCGC-TAMRA. These sequences were designed using Primer Express software version 1.0 (Applied Biosystems), and it was confirmed that they amplified the products of desired molecular weight.

Nested PCR for E1 DNA. The primers used for the first PCR were Jzp5-Ad3473F, CGCTGAGTTTGGCTCTAGCGAT, and Jzp6-Ad3698R, CATCACATTCTGACGCACCC. The primers for the second PCR were Jzp5-2-Ad3483F, GGCTCTAGCGATGAAGATACAG, and Jzp6-2-Ad3668R, GGGCATGCGCGTTGTCAAAT. The amplification conditions for the PCR consisted of 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, followed by a final incubation at 72°C for 7 min. Two microliters of the 50-μl reaction mixture from the first PCR was subjected to a second PCR. For the second PCR, the number of amplifications was set to 25.

PCR for pregnancy-specific glycoprotein gene. To detect the DNA derived from HEK293 cells, a pregnancy-specific glycoprotein gene adjacent to the E1 gene in HEK293 cells was selected as a target. The primers used for the PCR were PSG3-293-5281F, CTCATGCCTGCCTCTTTCACT, and PSG4-293-5567R, AGAGCCATCCA CACAATGTGC.

Nucleic acid extraction using SMI TEST EX-R&D. Cells were harvested and centrifuged at 2000 rpm for 5 min and then suspended in PBS(−). After four cycles of freezing and thawing, the nucleic acids derived from the cells were digested by incubating with DNase I (0.2 mg/ml), RNase A (0.2 mg/ml), and $MgCl_2$ (10 mM) at 37°C for 30 min. Then DNA was extracted using SMI TEST EX-R&D (Genome Science Laboratories, Fukushima, Japan) according to the manufacturer's instructions. When DNA was extracted from purified RCA or adenovirus vectors, the samples were subjected directly to DNA extraction using SMI TEST EX-R&D reagents.

Nucleic acid extraction using glass beads. Glass beads (GSB 07) approximately 7 mm in diameter were obtained from Nippon Rikagaku Kikai Co. (Tokyo, Japan). The glass beads were treated with 30% hydrogen fluoride (HF) solution for 1 h and then extensively washed with distilled water. The HF-treated glass beads were used for the extraction of viral genome.

For nucleic acid extraction, cells were harvested in 10-ml tubes and centrifuged at 2000 rpm for 5 min. After the medium was removed, one glass bead and 250 μl of extraction solution (200 mM Tris–HCl (pH 8.0), 200 mM NaCl, 1% *N*-lauroylsarcosinate, 1% SDS, 2% 2-mercaptoethanol, and 1 mg/ml proteinase K) were added to each tube, and then the samples were stirred for 10 s. After incubation at 65°C for 20 min, 2 ml of 70% isopropyl alcohol was added. The samples were incubated at room temperature for 1 min with gentle stirring. The solution was removed, and the beads were washed with 2 ml of 75% ethanol twice. Then the beads were

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dried at room temperature. DNA was eluted from each bead by adding 50 μl of distilled H_2O and incubating at 65°C for 5 min. Twenty microliters of the sample was assayed for E1 DNA.

Infectivity PCR using HeLa cells. HeLa cells (1.5×10^6) were seeded in 100-mm-diameter dishes. One day later (day 0), the cells were infected with 1 to 10^4 pfu of RCA spiked into 10^9 particles of adenovirus vectors by incubating at 37°C for 2 h in 1 ml of MEM containing 1% FCS. After infection, the medium was replaced with MEM containing 10% FCS. On days 1, 3, 6, and 9, the cells and supernatant were harvested. DNA was extracted, and the amount of E1 DNA was measured by real-time quantitative PCR. The experiments were done in triplicate.

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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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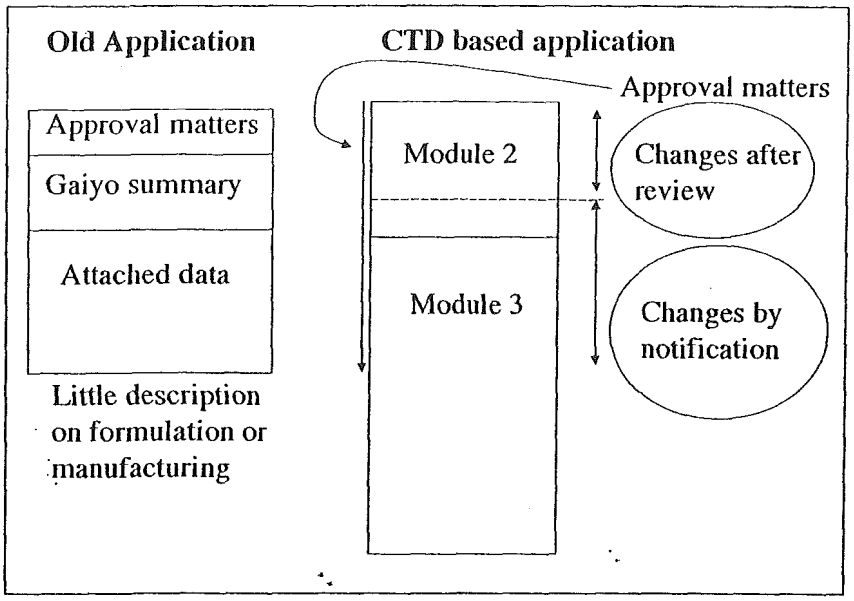

Figure. Structure of CMC portion of drug application. In the old system, specifications and test methods occupy the majority of the portion. Changing approval matters currently takes one year. In the new CTD based application, the portion of approval matters is expanded to include core information on 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.

OPINION

DDS 19巻2号，平成16年

バイオ創薬の新たな展開と効果的な推進に向けて

早川堯夫

現在は創薬史上に類のない時機である．ゲノム解読後のゲノム科学をベースにした創薬，幹細胞学の発展などをベースにした細胞治療や再生医療用の製品開発，その他の生命科学や先端技術の進展を背景にした創薬など，いずれも熾烈な国際的競争が展開されている．医薬品などが疾病の予防，診断，治療を通して保健衛生の向上に寄与するものであり，それゆえに人類に恩恵をもたらす共通の資産であるという本質を考えれば，創薬が国際競争により推進されることは，当然望ましいことである．わが国としては，科学技術立国を目指すということも含めて，米・欧に伍して，産・官・学あげてこの課題に取り組む必要がある．

創薬は，シーズ探索・発見と，およびそれをもとにした医薬品候補の探索・選択・最適化，製法の検討，品質・有効性・安全性評価という二つのステージに大別される．

ゲノム科学をベースにした創薬の場合，第1のステージは，疾患や薬物の作用，生体の恒常性維持に関係する新規遺伝子や蛋白質の探索とその機能解明である．このためには，各種ゲノミクス，プロテオミクス，バイオインフォマティクスなどの包括的・網羅的なアプローチや，これらにより絞り込み，推定された遺伝子や蛋白質機能の実証的な解析・確認が必要とされる．しかし，キーとなる“機能の実証的解析・確認”は，適切な技術基盤が必ずしも十分に開発，整備されておらず律速段階となっている．したがって，この点をブレークスルーすれば，米・欧に匹敵する“新規日の丸遺伝子や蛋白質”を見いだすことも可能であり，わが国独自の技術開発や研究の進展に期待したい．

第2のステージは，明らかにした遺伝子や蛋白質の機能に基づく創薬である．その際，機能が明らかにされた新たな遺伝子，蛋白質，関連機能分子自体が医薬品候補(有効成分)となるケースや，新たに機能解明された遺伝子や蛋白質を分子標的としてこれらを制御できるもの，たとえば，アンチセンスやsiRNAなどの核酸，抗体類，分子標的化学合成品，テーラーメード型製品などが医薬品候補となるケースが考えられる．第2ステージで最も重要なことは，有効性・安全性確保の観点から最終的にあるべき薬剤の姿を想定しながら開発を進めることであり，そこでDDS研究の果たす役割は大きい．

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医薬品は有効成分によって第1の特性を与えられるが，DDS技術によって第2の特性を与えられる．それは，臨床目的に応じた薬物治療の最適化，究極的には，必要な場で，必要な時間，必要な濃度で有効成分が作用するという特性の賦与である．蛋白質性医薬品，核酸医薬品，遺伝子治療薬，分子標的薬などは有効成分において画期的なものであるとともに，DDS研究による適切な特性の賦与により最も有効に活用される先端的医薬品となるものが多い．また，細胞をベースにした製品のあるものは，適用された生体側とのコミュニケーションにより効能効果を示すという理想的な薬剤を目指すものである．最適なDDSは集学的に統合化して達成されるが，わが国には充実した研究基盤があり，世界をリードできる潜在力がある．

新規遺伝子・蛋白質機能解明や再生医学・細胞治療に有用な細胞の開発と，DDS研究の推進・統合により，わが国のバイオ創薬が効果的に推進され，国益に適うとともに，平和的で素晴らしい国際貢献にもなることを心から期待したい．

TOPICS

バイオ医薬品の現状と将来

早川 堯夫・石井 明子

バイオ医薬品とは，生命現象の分子的解明を基にバイオテクノロジーなどの先端技術を応用して製造される医薬品を指す．具体的には，①組換え細胞や培養細胞などの細胞基材より生産されるタンパク質性医薬品(ホルモン，酵素，サイトカイン，血液凝固因子，ワクチン，抗体など)，②遺伝子治療薬，③細胞治療薬・医療機器，④トランスジェニック(Tg)動物／植物由来タンパク質性医薬品，⑤Tg動物由来細胞治療薬など，⑥核酸医薬品(アンチセンス，リボザイム，siRNA，デコイ，DNAワクチン)などがあげられる．

1980年代以降，まず，遺伝子組換え技術などを応用して，従来の手法では入手困難であったヒト型のタンパク質や微量活性タンパク質が組換え大腸菌や動物細胞から生産され，医薬品として臨床に供された．その後さらに，ヒト型のモノクローナル抗体，遺伝子治療薬や細胞治療薬などがバイオ医薬品として開発されてきた(表1)[1]．

ポストゲノム時代を迎えて，生命現象の維持に関与し，あるいは疾患に関連する新たな遺伝子やタンパク質の探索および機能解明が熾烈な国際競争となっている．機能が明らかにされた新たな遺伝子やタンパク質に医療上の有用性が期待される場合には，それ自体あるいは誘導体を有効成分とする遺伝子治療薬やタンパク質性医薬品が開発される．また，新機能遺伝子で改変された細胞が細胞治療や再生医療に活用されることも考えられる．さらに，疾患関連タンパク質などを分子標的として制御する抗体医薬品や，特定の遺伝子発現を制御する塩基配列を有する各種核酸医薬品など

表1　わが国で臨床応用されているバイオ医薬品など(分類と代表的な効能・効果)

●細胞基材由来タンパク質性医薬品
酵素：t-PA/ウロキナーゼ(急性心筋梗塞)，グルコセレブロシダーゼ(ゴーシェ病)
血液凝固因子：血液凝固第Ⅶ/Ⅷ因子(血友病)
ホルモン：インスリン(糖尿病)，成長ホルモン(下垂体性小人症)，ソマトメジンC(高インスリン血症，成長障害)，ナトリウム利尿ペプチド(急性心不全)，グルカゴン(低血糖時の救急処置)
ワクチン：A／B型肝炎ワクチン(A／B型肝炎の予防)
サイトカイン：インターフェロンα(B／C型慢性肝炎，腎癌)，インターフェロンβ(多発性硬化症)，インターフェロンγ(腎癌，菌状息肉症)，エリスロポエチン(腎性貧血)，G-CSF(癌化学療法による好中球減少)，インターロイキン-2(血管肉腫，腎癌)，bFGF(褥瘡，皮膚潰瘍)
モノクローナル抗体：抗HER2抗体(転移性乳癌)，抗CD20抗体(リンパ腫)，抗RSウイルス抗体(RSウイルス感染)，抗TNFα抗体(関節リウマチ，クローン病)，抗CD25抗体(腎移植後の急性拒絶反応)
●遺伝子治療薬(臨床研究および計画段階のもの：20プロトコール)
ベクター(件数)：アデノウイルス(9)，レトロウイルス(6)，センダイウイルス(1)，プラスミド(2)，リポソーム(2)
対象疾患(件数)：癌(14)，遺伝性疾患(3)，血管関連疾患(3)
●細胞治療薬など(治験および臨床研究段階のもの)
培養皮膚(皮膚潰瘍，熱傷など)，樹状細胞(多発性骨髄腫，前立腺癌)，軟骨細胞(軟骨損傷など)，リンパ球から細胞傷害性T細胞(CTL)を誘導し癌治療など，骨髄細胞より軟骨/骨芽細胞/血管/皮膚などに分化させ適用，角膜の再生

0917-138x/04/¥500/論文/JCLS

の開発も期待される．

一方，幹細胞などを基にした細胞治療や再生医療のための各種細胞・組織製品，バイオ製品(たとえば細胞)と医療機器あるいは異なるバイオ製品を組み合わせた複合型の製品，癌ワクチン，異なるタンパク質の機能ドメインを組み合わせた製品，糖鎖改変タンパク質，新規担体の利用などによる製剤学的工夫を施された製品なども開発されると想定される．

新規バイオ医薬品の開発・臨床応用においては，急速な学問と技術の進歩に応じた品質・安全性確保策という科学面での課題はもとより，適正な規制・基準の設定，ヒトの遺伝子や細胞・組織を操作し，個人の遺伝情報を扱ううえでの社会的理解や認知，および倫理的妥当性の確保という課題があり，関係者の英知を結集して解決していく必要がある[2,3]．

文献

1) 早川堯夫，他：先端的バイオロジクス開発の現状と新たなバイオ創薬に向けての課題．医薬品研究 33(11)：693-729, 2002.＜遺伝子組換えを含めた最新技術を応用した医薬品開発の現状と今後の展望が書かれている＞
2) 早川堯夫，他：生物薬品の開発の現状とトランスレーショナルリサーチへの条件．医学のあゆみ 200(7)：539-543, 2002.＜先端技術を応用した医薬品開発における探索的臨床研究の意義や課題について述べられている＞
3) 早川堯夫：バイオテクノロジー応用医薬品．内藤周幸(編)：臨床試験 2003，pp157-179，薬事日報社，2003.＜バイオテクノロジーを応用して生産した医薬品について，医薬品の特徴と臨床試験実施上の留意点が述べられている＞

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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。[2]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。

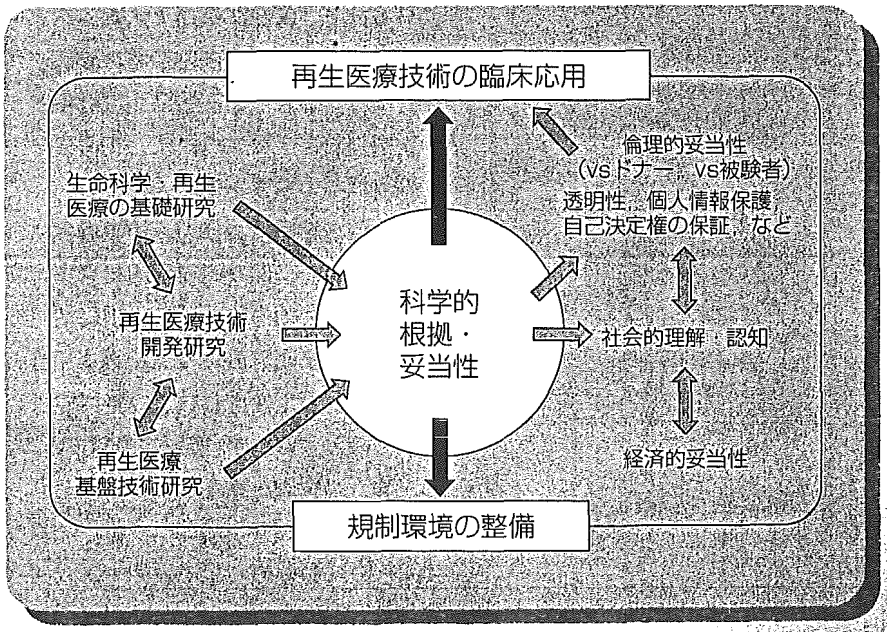

図　再生医療の実現化に向けて統合的アプローチが有用となるさまざまな局面[1]

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