A

B

C


FIGURE 2. Cytokines and liver enzyme levels in serum after the systemic administration of Ad-L2 or AdK7-L2 into mice. Blood samples were collected by inferior vena cave at 3 h (A) or 24 and 48 h (B) after i.v. administration of Ad-L2 or AdK7-L2 (1.0×10^{11} VP for A or 3.0×10^{10} VP for B). The livers were collected after 48 h following the injection (3.0×10^{10} VP) (C). A, IL-6 and IL-12 levels in the serum were measured by ELISA. B, AST levels in the serum were measured using a Transaminase-CII kit. C, Paraffin sections of the livers were prepared. Each section was stained with H&E. Data represent the means ± SD of four mice.
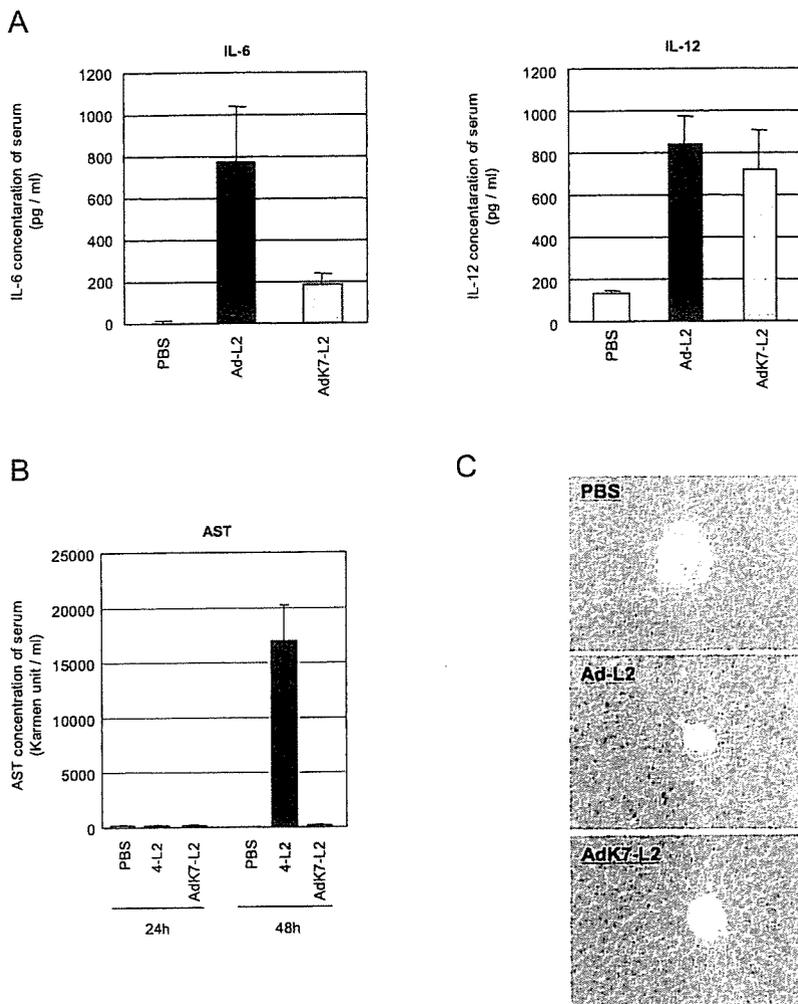
Cell sorting of splenic cells

Splenic conventional DC, plasmacytoid DC, and B cells, which were $CD11c^+B220^-$, $CD11c^+B220^+$, and $CD11c^-B220^+$ cells, respectively, were sorted by FACS Aria (BD Biosciences). Total RNA samples were isolated from each cell by the reagent ISOGEN, and RT-PCR analysis was then performed as described above.

Results

This study was undertaken to elucidate the biological mechanism in the innate immune host responses toward i.v. administered Ad vector. The relationship between the innate immune response and liver toxicity by systemic administration of the Ad vectors was also examined.

Gene transduction and Ad vector accumulation in vivo

In this study we used the conventional Ad vector (Ad-L2) and a fiber-modified Ad vector containing a polylysine (K7) peptide (AdK7-L2), both of which express luciferase under the control of the CMV promoter. First, we examined luciferase production in the organ and the biodistribution of viral DNA after i.v. administration of AdK7-L2 (1.0×10^{10} VP) into mice compared with Ad-L2 (see Fig. 3). The vector dose of 1.0×10^{10} VP was selected because this dose did not induce any apparent toxicity (IL-6 and AST production) with either Ad-L2 or AdK7-L2. When a higher dose (3.0×10^{10} or 1.0×10^{11} VP) was used, only Ad-L2 and not AdK7-L2 showed toxicity (described later), which does not reflect an exact comparison of the transduction efficiency. The Ad type 5-based vector delivers the foreign gene predominantly in the liver after i.v. injection into mice (29, 30). Interestingly, AdK7-L2 mediated ~6-fold higher liver transduction

than Ad-L2 (Fig. 1A). In contrast, the luciferase production in the heart, lung, kidney, and spleen in response to AdK7-L2 was similar to that in response to Ad-L2. To examine the biodistribution of Ad-L2 and AdK7-L2 in mice, the amounts of Ad DNA in each organ 48 h after the injection of Ad vectors were measured with the TaqMan fluorogenic detection system. More AdK7-L2 DNA accumulated in the liver than Ad-L2 DNA (Fig. 1B), although the amounts of AdK7-L2 DNA in the heart, lung, kidney, and spleen were less than those of Ad-L2 DNA. In particular, the amounts of AdK7-L2 DNA in the spleen were ~56-fold less than those of Ad-L2 DNA. The data regarding luciferase production (Fig. 1A) and the amounts of Ad DNA in most organs (Fig. 1B) showed discrepancies. Luciferase production in the liver was >2 log order higher than that in other organs, while the amounts of Ad DNA in liver were not as striking among the organs compared with luciferase production. This difference is likely due to the difference in the amount of nonspecific viral uptake among the organs. Reduced spleen accumulation of AdK7-L2 DNA, compared with Ad-L2 DNA, was also observed at a dose of 1.0×10^{11} VP (data not shown).

Serum cytokines and AST levels

The systemic administration of Ad vectors results in the initiation of strong innate immune responses and inflammation in animals and humans (1), and this toxicity limits the utility of Ad vectors for gene therapy. To evaluate the innate immune response and liver toxicity of each Ad vector, we measured the levels of IL-6, IL-12, and AST in serum. Because IL-6 in the serum and hepatic toxicity
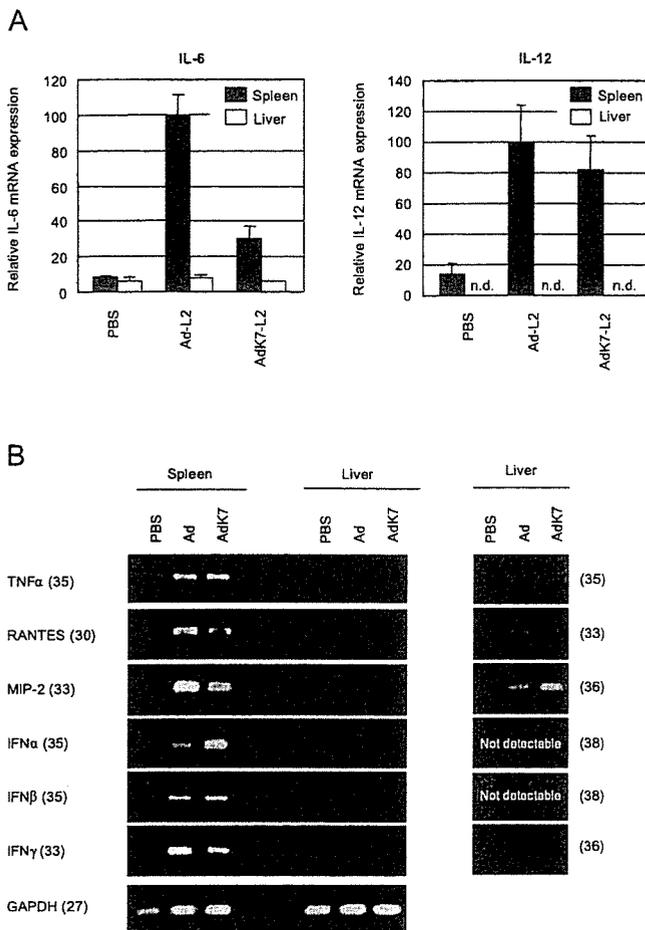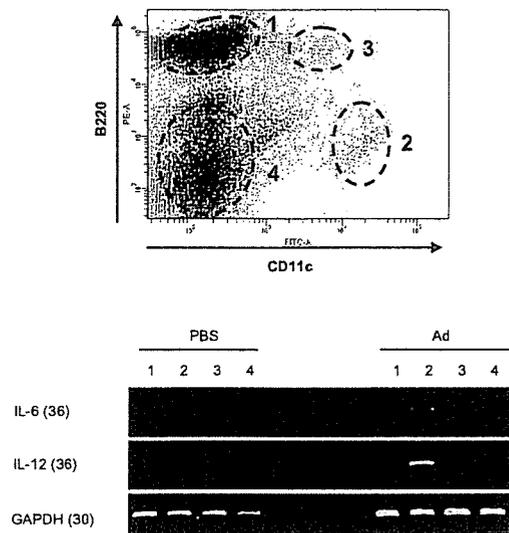
FIGURE 3. Cytokine, chemokine, and IFN mRNA levels in liver and spleen after the systemic administration of Ad-L2 or AdK7-L2 into mice. Total mRNA samples were isolated from liver and spleen at 3 h after i.v. administration of Ad-L2 or AdK7-L2 (1.0×10^{11} VP). After the reverse transcriptase reaction, IL-6 and IL-12 cDNA were measured with the quantitative TaqMan PCR assay (*A*). The expression of TNF-α, RANTES, MIP-2, IFN-α, IFN-β, and IFN-γ was measured by semiquantitative RT-PCR assay (*B*). All data represent the means ± SD of four mice. Cycle number is given in parentheses.

FIGURE 4. IL-6 and IL-12 mRNA levels in splenic CD11c-positive cells after the systemic administration of Ad-L2 into mice. Total mRNA samples were isolated from sorted splenic cells 3 h after i.v. administration of Ad-L2 (1.0×10^{11} VP). The expression levels of IL-6 and IL-12 mRNA were measured by RT-PCR assay. *Lane 1*, B cell (B220$^+$CD11c$^-$); *lane 2*, conventional DC (B220$^-$CD11c$^+$); *lane 3*, plasmacytoid DC (B220$^+$CD11c$^+$); *lane 4*, other cells (B220$^-$CD11c$^-$). Cycle number is given in parentheses.

analysis was detected at a dose of $>1.0 \times 10^{11}$ or 3.0×10^{10} VP, respectively, these doses were used.

IL-6 levels in response to AdK7-L2 were one-fourth of those with Ad-L2 (Fig. 2*A*). In contrast, there was no difference in serum IL-12 levels between Ad-L2 and AdK7-L2. Thus, IL-6 and IL-12 appear to be produced by a different mechanism. TNF-α in the serum after the injection of Ad-L2 or AdK7-L2 could not be detected (data not shown). Ad-L2 led to high levels of serum AST at 48 h after injection, while AdK7-L2 did not induce AST (Fig. 2*B*). At 24 h, neither Ad-L2 nor AdK7-L2 induced AST. In histological analysis, degranulation or denucleation occurred in hepatocytes from Ad-L2, while AdK7-L2 did not induce hepatocyte toxicity (Fig. 2*C*). The results using AdK7-L2 were similar to those in the untreated mice (Fig. 2, *B* and *C*), suggesting that AdK7-L2 does not show any liver toxicity. These results suggest that AdK7-L2 shows less IL-6 production and almost no liver toxicity.

Cytokines mRNA levels in liver and spleen cells

Ad vectors induce the expression of various cytokines and chemokines in the innate immune responses by effector cells such as macrophages and DC (15, 17, 31–33). Liver and spleen are two

major organs responsible for the location of immune cells. We attempted to determine which organ (liver or spleen) produces cytokines, chemokines, and IFNs (IL-6, IL-12, TNF-α, RANTES, MIP-2, IFNα, IFNβ, and IFN-γ) by quantitative real-time RT-PCR or semiquantitative RT-PCR analysis. IL-6 and IL-12 mRNA levels were not induced in the liver after i.v. administration of Ad vectors (Fig. 3*A*). This result was also checked by the result that specific IL-6 and IL-12 mRNA bands were not detected in the liver by RT-PCR analysis (data not shown). Expression of TNF-α, RANTES, MIP-2, IFN-α, IFN-β, and IFN-γ mRNA was also detected mainly in the spleen, not the liver (Fig. 3*B*). IL-6, MIP-2, and IFN-γ mRNA levels in the spleen in response to AdK7-L2 were lower than those in response to Ad-L2. In the liver, TNF-α, RANTES, MIP-2, and IFN-γ mRNA were detected by a high cycle number of PCR after Ad (Ad-L2 or AdK7-L2) injection, whereas IFN-α and IFN-β could be not detected (Fig. 3*B*).

We next identified the cell types responsible for the IL-6 and IL-12 expression in the spleen after i.v. administration of the Ad vector (Ad-L2). Spleen cells were sorted by FACS Aria based on the expression of CD11c and B220 in conventional DC (CD11c$^+$B220$^-$), plasmacytoid DC (CD11c$^+$B220$^+$), and B cells (CD11c$^-$B220$^+$ cells). IL-6 and IL-12 mRNA were mainly detected in the splenic conventional DC. Only a faint band of IL-12 mRNA was also detected in the splenic plasmacytoid DC (CD11c$^+$B220$^+$) (Fig. 4). These results suggest that splenic conventional DC are major effector cells of innate immune response (at least IL-6 and IL-12 production) against systemically administered Ad vectors.

Elimination of IL-6 signaling reduces liver toxicity

It has previously been shown that TNF-α is likely to be involved in host responses to Ad vectors in vitro and in vivo (34). Recently, Shayakhmetov et al. (35) have reported that IL-1 signaling, not TNF-α signaling, is involved in Ad vector-associated liver toxicity after i.v. administration. However, the mechanism of liver toxicity

FIGURE 5. Effects of serum IL-6 on serum AST levels and liver luciferase production after the systemic administration of Ad-L2 into mice. C57BL/6 mice were i.p. administered 100 μg per mouse of anti-IL-6R Ab (clone D7715A7), which was specific for blocking IL-6 signaling, or rabbit IgG as a control (clone; R3-34). Ad-L2 or AdK7-L2 (3.0 $\times$ 10^{10} VP) was i.v. injected into the mice 1.5 h later. Blood samples and liver tissue were collected 48 h after the injection of Ad-L2. The AST levels in the serum were measured using a Transaminase-CII kit. Luciferase production in the liver was measured by a luciferase assay system. All data represent the means $\pm$ SD of three to four mice. *, $p <$ 0.01.
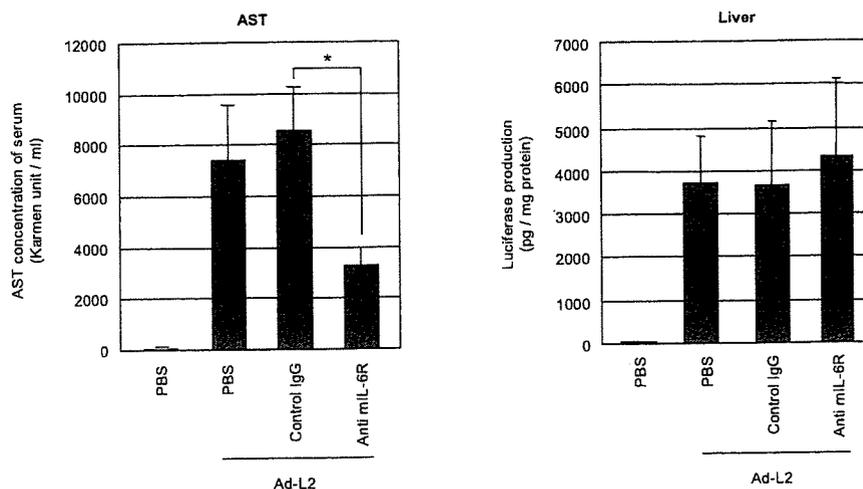
after i.v. Ad administration is poorly understood. In the present study, although AdK7-L2 mediated higher luciferase expression and a higher accumulation of viral DNA in the liver than Ad-L2, it remains unclear why AdK7-L2 showed almost background levels of liver toxicity while Ad-L2 showed high toxicity. As reported previously, inflammatory cytokines, chemokines, and IFNs could be the mediators responsible for liver toxicity (2). IL-6 levels in the serum were the most strikingly different between AdK7-L2 and Ad-L2. Furthermore, IL-6 stimulated acute phase protein (serum amyloid A, fibrinogen, α_1-anti-trypsin, and α_1-acid glycoprotein) in rat and human hepatocytes (36, 37). Therefore, we next examined the effects of serum IL-6 on liver toxicity (Fig. 5). To do this, we used an anti-IL-6R Ab that inhibits the signal through the IL-6 receptor. The IL-6 receptor system consists of two functional molecules, an 80-kDa ligand-binding chain (IL-6R) and a 130-kDa nonligand-binding but signal-transducing chain (gp130). The anti-IL-6R Ab blocks the binding of IL-6 to the IL-6R (38, 39). The anti-IL-6R Ab or the control Ab was i.p. injected 1.5 h before the injection of Ad-L2. The AST levels in the serum and luciferase production in the liver were determined 48 h later. Administration of anti-IL-6R Ab significantly ($\sim$2-fold) reduced Ad vector-mediated AST levels in the serum compared with PBS or the control Ab (Fig. 5A). Importantly, anti-IL-6R Ab injection did not interfere with luciferase production in the liver (Fig. 5B). These results suggest that IL-6 signaling is involved in liver toxicity after i.v. administration of an Ad vector.

Discussion

In this study we found that the fiber-modified Ad vector containing the K7 peptide, which has high affinity with heparin sulfate, shows much lower serum IL-6 and liver toxicity than the conventional Ad vector. This improved characteristic is likely involved with the reduced biodistribution of the vector to the spleen compared with that of the conventional Ad vector. RT-PCR analysis showed that the spleen, not the liver, is the major site of cytokine, chemokine, and IFN (IL-6, IL-12, TNF-α, RANTES, MIP-2, IFN-α, IFN-β, and IFN-γ) production and that splenic conventional DC are the major effector cells of the innate immune response (at least IL-6 and IL-12 production) after i.v. administration of Ad vectors. We also showed that IL-6 signaling is involved in part with liver toxicity in response to Ad vectors. Importantly, this fiber-modified Ad vector containing the K7 peptide maintained higher transduction efficiency in all the organs examined, and the liver transduction was higher than that of the conventional Ad vector. Although there have been some reports that modified Ad vectors such as the pe-

gylated Ad vector (18–21), the Ad vector containing the Ad type 35 fiber shaft and knob (40), and the triple mutant Ad vector with ablation of CAR, α_v integrin, and HSG binding (22) show decreased innate immune response and liver toxicity, these types of vector lose their transduction activity in vivo. To our knowledge, this is the first report of an Ad vector that maintains high transduction efficiency in vivo with reduced toxicity.

The fiber-modified Ad vector containing the K7 peptide has been developed to overcome the limitations imposed by the CAR dependence of Ad infection. Expanded and efficient gene transfer has been reported based on the use of mutant fiber proteins containing a stretch of lysine residues (23–25). However, there has been no report on the difference in gene transfer activity and toxicity in vivo between the conventional Ad vector and the fiber-modified Ad vector containing the K7 peptide. We have demonstrated that the fiber-modified Ad vector containing the K7 peptide mediates $\sim$6-fold higher mouse liver transduction in response to i.v. administration than the conventional Ad vector (Fig. 1A). The amounts of fiber-modified Ad vector DNA in the liver after i.v. administration were also 5-fold higher than those with the conventional Ad vector (Fig. 1B). It has been reported that the interaction between the Ad type 5 fiber and the HSG of a hepatocyte is involved in the accumulation in the mouse liver and the cynomolgus monkey liver of systemically administered Ad vectors (41, 42). This fiber-modified Ad vector might mediate more efficient gene transduction through a much higher affinity for HSG. In contrast, the amounts of fiber-modified Ad vector DNA in the spleen after i.v. administration were 56-fold lower than those of the conventional Ad vector (Fig. 1B). Biodistribution of viral DNA reflects the total of receptor-mediated uptake and nonspecific uptake. Luciferase production in the cells mainly reflects receptor-mediated uptake. We previously reported that most Ad DNAs are taken up in the liver nonparenchymal cells, not parenchymal cells, after i.v. administration (22). In this study, the conventional Ad vector would also be taken up in the macrophages and DC by nonspecific uptake, resulting in significantly higher Ad DNA and lower luciferase production in the spleen. In contrast, the fiber-modified Ad vector would be taken up more in the liver via receptor-mediated uptake and nonspecific uptake, resulting in significantly lower Ad DNA in the other organs, especially the spleen. Even though the amount of AdK7-L2 uptake in the spleen, heart, lung, and kidney was less than that of Ad-L2 uptake, the amount of receptor-mediated uptake in these organs would be similar between Ad-L2 and AdK7-L2, suggesting that these vectors showed similar levels of luciferase production in the organs other than the liver.

The initiation of inflammatory innate immune responses occurs after the systemic administration of Ad vectors to animals and humans, and this toxicity limits the utility of Ad vectors for gene therapy. Increased cytokine/chemokine production after the injection of Ad vectors has been reported to be due to the introduction of input Ad vectors to Kupffer cells in the liver and DC (15, 17, 43–46). Detailed analysis of the organs responsible for the expression of cytokines, chemokines, and IFNs by RT-PCR suggests that their production can mainly be attributed to spleen cells (especially splenic conventional DC), not liver cells (Figs. 3 and 4), which is consistent with the recent report of Bart et al. (47). Therefore, interference with spleen distribution of the Ad vector should provide a useful method for safer gene therapy.

TLRs, which are crucial to the recognition of pathogen-associated molecular patterns, are expressed on various types of immune cells including macrophages, DC, B cells, splenic types of T cells, and even on nonimmune cells such as fibroblasts and epithelial cells (48). For example, HSV and CMV (dsDNA virus) activate inflammatory cytokines and type I IFN secretion by the stimulation of TLR9 (49–53). The innate immune receptor to the Ad has not yet been identified. It has not even been determined whether TLRs are involved in Ad-mediated innate immune response in vivo, although it has been reported that TLR signals are not involved in the DC maturation induced by the Ad vector (46). As shown in Fig. 3B, cytokine production against the Ad vector occurred mainly in conventional DC. It is noted that the TLR9-mediated innate immunity responses to DNA virus are cell type-specific and limited to plasmacytoid DC (50). The unidentified sensor receptor(s) for double-stranded Ad DNA or Ad capsid protein in conventional DC might play a critical role in the expression of inflammatory cytokines/chemokines and type I IFN. Although we have previously reported that large amounts of conventional Ad vector accumulate in nonparenchymal cells, including Kupffer cells and liver sinusoidal (endothelial) cells (22, 54), the expression of mRNA of cytokines, chemokines, and IFNs in the liver was weak after administration of the Ad vector (Fig. 3B). A lack of putative sensor receptor(s) against Ad or the inability of sensor receptor(s) to recognize Ad due to the specific cellular disposition of Ad in Kupffer cells might result in a reduced production of cytokines/chemokines/IFNs in the liver.

Another interesting finding is that the fiber-modified Ad vector containing the K7 peptide showed almost background levels of AST activity, which reflects liver toxicity (Fig. 2B). Histological analysis supported this finding (Fig. 2C). Because the K7-modified Ad vector showed higher transgene activity and a higher accumulation of viral DNA into the liver (Fig. 1), the transduction and distribution of the vector into the liver did not participate in liver toxicity. The cytokines/chemokines play a major causative role in liver damage associated with systemic Ad infusion as well as in the induction of an antiviral immune response (2). Ad-induced cytokines/chemokines recruit immune effector cells (neutrophils, monocyte/macrophages, and NK cells) to Ad-transduced cells (mainly liver), resulting in acute hepatic toxicity. Shayakhmetov et al. (35) have reported that hepatocytes and Kupffer cells trigger IL-1 transcription in liver tissue after i.v. administration of Ad vectors and that interference of IL-1-signaling reduces liver toxicity. We speculated that IL-6 could be the main mediator for hepatic toxicity because IL-6 is one of the main cytokines in the early stages of inflammation, IL-6 production by the fiber-modified Ad vector was much reduced (approximately a quarter) compared with that by the conventional Ad vector, and all of the cytokines/chemokines/IFNs we examined (including IL-6) were mainly produced by the spleen, not the liver. Treatment of the anti-IL-6R Ab decreased liver toxicity (Fig. 5), suggesting that IL-6 plays at least

some role in liver toxicity induced by systemic injection of the Ad vector. Because the AST levels were only partially reduced by the treatment with the anti-IL-6R Ab, another mechanism such as IL-1 signaling, rapid Kupffer cell death (55, 56), activation of the liver endothelium (55), or other factors might be involved in the liver toxicity. Nevertheless, it is attractive that the K7-modified Ad vector did not show liver toxicity despite the higher transduction efficiency and higher accumulation of the vector into the liver (probably Kupffer cells).

Our present study provides new insight into the cellular biological mechanism related to the innate immune response and liver toxicity against the systemically administered Ad vector. Modification of vector tropism should contribute to safe gene therapy procedures.

Acknowledgments

Disclosures

References

1. Muruve, D. A. 2004. The innate immune response to adenovirus vectors. Hum. Gene Ther. 15: 1157–1166.
2. Nazir, S. A., and J. P. Metcalf. 2005. Innate immune response to adenovirus. J. Investig. Med. 6: 292–304.
3. Yang, Y., H. C. Ertl, and J. M. Wilson. 1994. MHC class I-restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. Immunity 1: 433–442.
4. Yang, Y., F. A. Nunes, K. Berencsi, E. E. Furth, E. Gonczol, and J. M. Wilson. 1994. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. Proc. Natl. Acad. Sci. USA 91: 4407–4411.
5. Yang, Y., Q. Su, and J. M. Wilson. 1996. Role of viral antigens in destructive cellular immune responses to adenovirus vector-transduced cells in mouse lungs. J. Virol. 70: 7209–7212.
6. Morral, N., R. J. Parks, H. Zhou, L. C. G. Schiedner, J. Quinones, F. L. Graham, S. Kochanek, and A. L. Beaudet. 1998. High doses of a helper-dependent adenoviral vector yield supraphysiological levels of α_1-antitrypsin with negligible toxicity. Hum. Gene Ther. 9: 2709–2716.
7. Morsy, M. A., M. Gu, S. Motzel, J. Zhao, J. Lin, Q. Su, H. Allen, L. Franlin, R. J. Parks, F. L. Graham, S. Kochanek, A. J. Bett, and C. T. Caskey. 1998. An adenoviral vector deleted for all viral coding sequences results in enhanced safety and extended expression for a leptin transgene. Proc. Natl. Acad. Sci. USA 95: 7866–7871.
8. Schiedner, G., N. Morral, R. J. Parks, Y. Wu, S. C. Koopmans, C. Langston, F. L. Graham, A. L. Beaudet, and S. Kochanek. 1998. Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity. Nat. Genet. 18: 180–183.
9. Roberts, D. M., A. Nanda, M. J. Havenga, P. Abbink, D. M. Lynch, B. A. Ewald, J. Liu, A. R. Thorner, P. E. Swanson, D. A. Gorgone, et al. 2006. Hexon-chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity. Nature 441: 239–243.
10. Farina, S. F., G. P. Gao, Z. Q. Xiang, J. J. Rux, R. M. Burnett, M. R. Alvira, J. Marsh, H. C. Ertl, and J. M. Wilson. 2001. Replication-defective vector based on a chimpanzee adenovirus. J. Virol. 75: 11603–11613.
11. Sakurai, F., H. Mizuguchi, and T. Hayakawa. 2003. Efficient gene transfer into human CD34+ cells by an adenovirus type 35 vector. Gene Ther. 10: 1041–1048.
12. Vogels, R., D. Zuijdgeest, R. van Rijnsoever, E. Hartkoorn, I. Damen, M. P. de Bethune, S. Kostense, G. Penders, N. Helmus, W. Koudstaal, et al. 2003. Replication-deficient human adenovirus type 35 vectors for gene transfer and vaccination: efficient human cell infection and bypass of preexisting adenovirus immunity. J. Virol. 77: 8263–8271.
13. Holterman, L., R. Vogels, R. van der Vlugt, M. Sieuwerts, J. Grimbergen, J. Kaspers, E. Geelen, E. van der Helm, A. Lemckert, G. Gillissen, et al. 2004. Novel replication-incompetent vector derived from adenovirus type 11 (Ad11) for vaccination and gene therapy: low seroprevalence and non-cross-reactivity with Ad5. J. Virol. 78: 13207–13215.
14. Liu, Q., and D. A. Muruve. 2003. Molecular basis of the inflammatory response to adenovirus vectors. Gene Ther. 10: 935–940.
15. Zhang, Y., N. Chirmule, G. P. Gao, R. Qian, M. Croyle, B. Joshi, J. Tazelaar, and J. M. Wilson. 2001. Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages. Mol. Ther. 3: 697–707.
16. Clesham, G. J., P. J. Adam, D. Proudfoot, P. D. Flynn, S. Efstathiou, and P. L. Weissberg. 1998. High adenoviral loads stimulate NF-κB-dependent gene expression in human vascular smooth muscle cells. Gene Ther. 5: 174–180.
17. Lieber, A., C. Y. He, L. Meuse, D. Schowalter, I. Kirillova, B. Winther, and M. A. Kay. 1997. The role of Kupffer cell activation and viral gene expression in early liver toxicity after infusion of recombinant adenovirus vectors. J. Virol. 71: 8798–8807.

18. O'Riordan, C. R., A. Lachapelle, C. Delgado, V. Parkes, S. C. Wadsworth, A. E. Smith, and G. E. Francis. 1999. PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo. *Hum. Gene Ther.* 10: 1349–1358.

19. Croyle, M. A., Q. C. Yu, and J. M. Wilson. 2000. Development of a rapid method for the PEGylation of adenoviruses with enhanced transduction and improved stability under harsh storage conditions. *Hum. Gene Ther.* 11: 1713–1722.

20. Croyle, M. A., H. T. Le, K. D. Linse, V. Cerullo, G. Toietta, A. Beaudet, and L. Pastore. 2005. PEGylated helper-dependent adenoviral vectors: highly efficient vectors with an enhanced safety profile. *Gene Ther.* 12: 579–587.

21. Mok, H., D. J. Palmer, P. Ng, and M. A. Barry. 2005. Evaluation of polyethylene glycol modification of first-generation and helper-dependent adenoviral vectors to reduce innate immune responses. *Mol. Ther.* 11: 66–79.

22. Koizumi, N., K. Kawabata, F. Sakurai, Y. Watanabe, T. Hayakawa, and H. Mizuguchi. 2006. Modified adenoviral vectors ablated for coxsackievirus-adenovirus receptor, α_v integrin, and heparan sulfate binding reduce in vivo tissue transduction and toxicity. *Hum. Gene Ther.* 17: 264–279.

23. Wickham, T. J., E. Tzeng, L. L. Shears II, P. W. Roelvink, Y. Li, G. M. Lee, D. E. Brough, A. Lizonova, and I. Kovesdi. 1997. Increased in vitro and in vivo gene transfer by adenovirus vectors containing chimeric fiber proteins. *J. Virol.* 11: 8221–8229.

24. Bouri, K., W. G. Feero, M. M. Myerburg, T. J. Wickham, I. Kovesdi, E. P. Hoffman, and P. R. Clemens. 1999. Polylysine modification of adenoviral fiber protein enhances muscle cell transduction. *Hum. Gene Ther.* 10: 1633–1640.

25. Koizumi, N., H. Mizuguchi, N. Utoguchi, Y. Watanabe, and T. Hayakawa. 2003. Generation of fiber-modified adenovirus vectors containing heterologous peptides in both the HI loop and C terminus of the fiber knob. *J. Gene Med.* 5: 267–276.

26. Mizuguchi, H., N. Koizumi, T. Hosono, N. Utoguchi, Y. Watanabe, M. A. Kay, and T. Hayakawa. 2001. A simplified system for constructing recombinant adenoviral vectors containing heterologous peptides in the HI loop of their fiber knob. *Gene Ther.* 8: 730–735.

27. Maizel, J. V., D. O. White, and M. D. Scharff. 1968. The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology* 36: 115–125.

28. Xu, Z.-L., H. Mizuguchi, A. Ishii-Watabe, E. Uchida, T. Mayumi, and T. Hayakawa. 2001. Optimization of transcriptional regulatory elements for constructing plasmid vectors. *Gene* 272: 149–156.

29. Huard, J., H. Lochmuller, G. Acsadi, A. Jani, B. Massie, and G. Karpati. 1995. The route of administration is a major determinant of the transduction efficiency of rat tissues by adenoviral recombinants. *Gene Ther.* 2: 107–115.

30. Wood, M., P. Perrotte, E. Onishi, M. E. Harper, C. Dinney, L. Pagliaro, and D. R. Wilson. 1999. Biodistribution of an adenoviral vector carrying the luciferase reporter gene following intravesical or intravenous administration to a mouse. *Cancer Gene Ther.* 6: 367–372.

31. Worgall, S., G. Wolff, E. Falck-Pedersen, and R. G. Crystal. 1997. Innate immune mechanisms dominate elimination of adenoviral vectors following in vivo administration. *Hum. Gene Ther.* 8: 37–44.

32. Liu, Q., A. K. Zaiss, P. Colarusso, K. Patel, G. Haljan, T. J. Wickham, and D. A. Muruve. 2003. The role of capsid-endothelial interactions in the innate immune response to adenovirus vectors. *Hum. Gene Ther.* 14: 627–643.

33. Schiedner, G., S. Hertel, M. Johnston, V. Dries, R. N. Van, and S. Kochanek. 2003. Selective depletion or blockade of Kupffer cells leads to enhanced and prolonged hepatic transgene expression using high-capacity adenoviral vectors. *Mol. Ther.* 7: 35–43.

34. Engler, H., T. Machemer, J. Philopena, S. F. Wen, E. Quijano, M. Ramachandra, V. Tsai, and R. Ralston. 2004. Acute hepatotoxicity of oncolytic adenoviruses in mouse models is associated with expression of wild-type E1a and induction of TNF-α. *Virology* 328: 52–61.

35. Shayakhmetov, D. M., Z. Y. Li, S. Ni, and A. Lieber. 2005. Interference with the IL-1-signaling pathway improves the toxicity profile of systemically applied adenovirus vectors. *J. Immunol.* 174: 7310–7319.

36. Castell, J. V., T. Geiger, V. Gross, T. Andus, E. Walter, T. Hirano, T. Kishimoto, and P. C. Heinrich. 1988. Plasma clearance, organ distribution and target cells of interleukin-6/hepatocyte-stimulating factor in the rat. *Eur. J. Biochem.* 177: 357–361.

37. Geiger, T., T. Andus, J. Klapproth, T. Hirano, T. Kishimoto, and P. C. Heinrich. 1988. Induction of rat acute-phase proteins by interleukin 6 in vivo. *Eur. J. Immunol.* 18: 717–721.

38. Vink, A., P. Coulie, G. Warnier, J. C. Renauld, M. Stevens, D. Donckers, and J. Van Snick. 1990. Mouse plasmacytoma growth in vivo: enhancement by interleukin 6 (IL-6) and inhibition by antibodies directed against IL-6 or its receptor. *J. Exp. Med.* 172: 997–1000.

39. Boulanger, M. J., D. C. Chow, E. E. Brevnova, and K. C. Garcia. 2003. Hexameric structure and assembly of the interleukin-6/IL-6 α-receptor/gp130 complex. *Science* 300: 2101–2104.

40. Shayakhmetov, D. M., Z. Y. Li, S. Ni, and A. Lieber. 2004. Analysis of adenovirus sequestration in the liver, transduction of hepatic cells, and innate toxicity after injection of fiber-modified vectors. *J. Virol.* 78: 5368–5381.

41. Smith, T. A., N. Idamakanti, M. L. Rollence, J. Marshall-Neff, J. Kim, K. Mulgrew, G. R. Nemerow, M. Kaleko, and S. C. Stevenson. 2003. Adenovirus serotype 5 fiber shaft influences in vivo gene transfer in mice. *Hum. Gene Ther.* 14: 777–787.

42. Smith, T. A., N. Idamakanti, J. Marshall-Neff, M. L. Rollence, P. Wright, M. Kaloss, L. King, C. Mech, L. Dinges, W. O. Iverson, et al. 2003. Receptor interactions involved in adenoviral-mediated gene delivery after systemic administration in non-human primates. *Hum. Gene Ther.* 14: 1595–1604.

43. Schnell, M. A., Y. Zhang, J. Tazelaar, G. P. Gao, Q. C. Yu, R. Qian, S. J. Chen, A. N. Varnavski, C. LeClair, S. E. Raper, and J. M. Wilson. 2001. Activation of innate immunity in nonhuman primates following intraportal administration of adenoviral vectors. *Mol. Ther.* 3: 708–722.

44. Morral, N., W. K. O'Neal, K. M. Rice, P. A. Piedra, E. Aguilar-Cordova, K. D. Carey, A. L. Beaudet, and C. Langston. 2002. Lethal toxicity, severe endothelial injury, and a threshold effect with high doses of an adenoviral vector in baboons. *Hum. Gene Ther.* 13: 143–154.

45. Reid, T., E. Galanis, J. Abbruzzese, D. Sze, L. M. Wein, J. Andrews, B. Randlev, C. Heise, M. Uprichard, M. Hatfield, et al. 2002. Hepatic arterial infusion of a replication-selective oncolytic adenovirus (dl1520): phase II viral, immunologic, and clinical endpoints. *Cancer Res.* 62: 6070–6079.

46. Philpott, N. J., M. Nociari, K. B. Elkon, and E. Falck-Pedersen. 2004. Adenovirus-induced maturation of dendritic cells through a PI3 kinase-mediated TNF-α induction pathway. *Proc. Natl. Acad. Sci. USA* 1001: 6200–6205.

47. Bart, D. G., S. Jan, V. L. Sophie, L. Joke, and C. Desire. 2005. Elimination of innate immune responses and liver inflammation by PEGylation of adenoviral vectors and methylprednisolone. *Hum. Gene Ther.* 16: 1439–1451.

48. Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell* 124: 783–801.

49. Lund, J., A. Sato, S. Akira, R. Medzhitov, and A. Iwasaki. 2003. Toll-like receptor 9-mediated recognition of herpes simplex virus-2 by plasmacytoid dendritic cells. *J. Exp. Med.* 198: 513–520.

50. Hochrein, H., B. Schlatter, M. O'Keeffe, C. Wagner, F. Schmitz, M. Schiemann, S. Bauer, M. Suter, and H. Wagner. 2004. Herpes simplex virus type-1 induces IFN-α production via Toll-like receptor 9-dependent and -independent pathways. *Proc. Natl. Acad. Sci. USA* 101: 11416–11421.

51. Krug, A., A. R. French, W. Barchet, J. A. Fischer, A. Dzionek, J. T. Pingel, M. M. Orihuela, S. Akira, W. M. Yokoyama, and M. Colonna. 2004. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. *Immunity* 21: 107–119.

52. Krug, A., G. D. Luker, W. Barchet, D. A. Leib, S. Akira, and M. Colonna. 2004. Herpes simplex virus type 1 activates murine natural interferon-producing cells through toll-like receptor 9. *Blood* 103: 1433–1437.

53. Tabeta, K., P. Georgel, E. Janssen, X. Du, K. Hoebe, K. Crozat, S. Mudd, L. Shamel, S. Sovath, J. Goode, et al. 2004. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc. Natl. Acad. Sci. USA* 101: 3516–3521.

54. Koizumi, N., H. Mizuguchi, F. Sakurai, T. Yamaguchi, Y. Watanabe, and T. Hayakawa. 2003. Reduction of natural adenovirus tropism to mouse liver by fiber-shaft exchange in combination with both CAR- and α_v integrin-binding ablation. *J. Virol.* 24: 13062–13072.

55. Schiedner, G., W. Bloch, S. Hertel, M. Johnston, A. Molojavyi, V. Dries, G. Varga, N. Van Rooijen, and S. Kochanek. 2003. A hemodynamic response to intravenous adenovirus vector particles is caused by systemic Kupffer cell-mediated activation of endothelial cells. *Hum. Gene Ther.* 14: 1631–1641.

56. Manickan, E., J. S. Smith, J. Tian, T. L. Eggerman, J. N. Lozier, J. Muller, and A. P. Byrnes. 2006. Rapid Kupffer cell death after intravenous injection of adenovirus vectors. *Mol. Ther.* 13: 108–117.

Post-transcriptional downregulation of sarcolipin mRNA by triiodothyronine in the atrial myocardium

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Abstract Thyroid hormone-mediated positive cardiotropic effects are differently regulated between the atria and ventricles. This regulation is, at least in part, dependent on sarcoplasmic reticulum (SR) proteins. Sarcolipin, a homologue of phospholamban, has been recently identified as an atrium-specific SR protein. The expression of sarcolipin mRNA was significantly decreased in the atria of mice with hyperthyroidism and in 3,5,3′-triiodo-L-thyronine-treated neonatal rat atrial myocytes. Promoter activity and mRNA stability analyses revealed that thyroid hormone post-transcriptionally downregulated the expression of sarcolipin mRNA. The atrium-specific effect of thyroid hormone may occur in part through the regulation of atrial sarcolipin gene expression.
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Keywords: Thyroid hormone; Calcium; Gene expression; Cardiomyocyte; Sarcoplasmic reticulum

1. Introduction

Thyroid hormone plays an important role in normal cardiovascular development and exerts positive inotropic, lusitropic, and chronotropic effects on the heart [1]. These effects are mediated in part by regulating the transcription of the genes encoding proteins involved in Ca^{2+} cycling, ion transport, β-

adrenergic signaling, and myofibrillar organization. In addition to its physiological role, the excess of thyroid hormone, or the state of "hyperthyroidism", often induces pathological changes in the heart such as cardiac hypertrophy, sinus tachycardia and atrial arrhythmias including atrial fibrillation. The prevalence of atrial arrhythmias is much higher than that of ventricular arrhythmias in patients with hyperthyroidism, indicating that the atrial myocardium is more susceptible to rhythm disturbance by the excess of thyroid hormone than the ventricular myocardium. In this regard, it has been known that the responses to thyroid hormone differ between the atria and ventricles [2–4]. Kaasik et al. demonstrated that thyroid hormone activated sarcoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA) more in the atria than in the ventricles [3]. Shenoy et al. showed that the Ca^{2+} transporter proteins were differently regulated in the atria and the ventricles [4]. Several recent studies have suggested that abnormal intracellular Ca^{2+} homeostasis and perturbations in Ca^{2+} cycling play an important role in atrial fibrillation-induced atrial remodeling [5,6]. Therefore, the differential effect of thyroid hormone on the proteins involved in Ca^{2+} cycling may account for the occurrence of atrial rather than ventricular arrhythmias.

We have found that sarcolipin (SLN), a homologue of phospholamban (PLN), is specifically expressed in the atrial myocardium and skeletal muscle, but not in the ventricular myocardium [7]. SLN interacts with SERCA and regulates excitation–contraction coupling in the atrial and skeletal muscles [8]. Accordingly, SLN may play an important role in characterizing the chamber-specific physiological properties of the atria. The atrial chamber-specific expression of SLN could be primarily regulated at the transcriptional level. Our previous studies have demonstrated that SLN mRNA expression is increased during development, and decreased by hypertrophic remodeling of the atria [7,9] as well as in patients with atrial fibrillation [10]. However, the effect of thyroid hormone on the transcriptional regulation of the SLN gene in the heart has not been investigated, although thyroid hormone is known to regulate the transcription of the SR genes such as PLN and cardiac SERCA2 isoform (SERCA2a) in the heart [11–13]. In the present study, we examined the effect of thyroid hormone on the transcription of SLN gene in the atrial tissue and myocytes.

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Abbreviations: ANF, atrial natriuretic factor; CSQ2, cardiac calsequestrin; EDD, end-diastolic left ventricular dimension; ESD, end-systolic left ventricular dimension; %FS, percent fractional shortening; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IVS, interventricular wall thickness; LVPW, left ventricular posterior wall thickness; MLC2a, atrial myosin light chain 2; NCX1, cardiac sodium calcium exchanger; PLN, phospholamban; RT-PCR, reverse transcription polymerase chain reaction; RyR2, cardiac ryanodine receptor; SERCA, SR Ca^{2+}-ATPase; SLN, sarcolipin; SR, sarcoplasmic reticulum; T3, 3,5, 3′-triiodo-L-thyronine; TR, thyroid hormone receptor; TRE, thyroid hormone response element

2. Materials and methods

2.1. Animals

All treatments were approved by the Animal Protocol Committee of Yokohama City University School of Medicine. Hyperthyroidism was induced in ddY mice (Nippon SLC Inc.) at 6.5 weeks of age ($n = 5$) by intraperitoneally injecting 3,5,3′-triiodo-L-thyronine (T3) at a dose of 2 μg/g body weight/day for 4 weeks. Hypothyroidism was induced in ddY mice at 3 weeks of age ($n = 5$) by feeding the animals with a 0.05% 5-propyl-2-thiouracil (PTU)-containing water for 8 weeks. Non-treated (euthyroid) mice ($n = 5$) were bred for the same period.

2.2. Transthoracic echocardiography

Transthoracic echocardiography was performed as previously described [14].

2.3. Cell culture and stimulation with T3

Neonatal rat atrial myocytes were prepared as the same method described previously for neonatal rat ventricular myocytes [15]. Cardioblast H9C2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 1% fetal calf serum (FCS) for 7 days after confluence. Neonatal rat atrial myocytes and H9C2 cells were treated with 1 nM of T3 for 24 h, and were then collected.

2.4. Northern blot and quantitative reverse transcription polymerase chain reaction (RT-PCR) analyses

Isolation of total RNA and Northern blot analysis were performed as previously described with modification [7,9,16]. Mouse cDNA probes including the SLN gene were used as reported previously [9]. The quantitative RT-PCR was performed by using SYBR Green PCR reagents (Applied Biosystems) as previously described [10]. The forward and reverse primer sequences of SLN were 5′-CTGAGG-TCCTTGGTAGCCTG-3′ and 5′-GGTGTGTCAGGCATTGTGAG-3′, respectively.

2.5. Cloning of mouse sarcolipin promoter

During the present study was prepared, the genomic sequence of mouse chromosome 9 including 5′ flanking sequence of the SLN gene has been published (NCBI Accession No.: NT_039474). The primers for PCR amplification of 5′ flanking sequence of the SLN gene were designed as based on the nucleotide sequence (forward: 5′-CAGCT-AACCAGGCACAACAA-3′, reverse: 5′-CACTCAGGCTACCAAG-GACC-3′). A 2299-bp fragment between −2237 of the 5′ flanking sequence and +62 of the non-coding exon 1 of the SLN gene was amplified and was subcloned into a pDrive vector (Qiagen). The nucleotide sequence was confirmed by direct DNA sequencing.

2.6. Promoter activity assay

The 2299-bp and fragments of the SLN promoter region was cloned into the promoter-less firefly luciferase expression vector, pGL3-Basic (Promega). This construct was named −2237/SLNluc. A shorter construct, −563/SLNluc, containing 563 upstream nucleotides from the putative transcriptional start site was made by self-ligation after digestion of MluI restriction enzyme. Neonatal rat atrial myocytes were incubated with serum-free medium for 24 h and then they were transiently co-transfected with 300 ng of each SLN promoter luciferase test plasmid and 75 ng of phRL-TK control plasmid (Promega), using Fu-Gene 6 (Roche). Three hours after the transfection, the myocytes were incubated with or without T3 (1 nM) for 24 h, and the luciferase activity was measured with Dual-Luciferase Reagents (Promega).

2.7. mRNA stability assay

To determine whether T3 alters SLN mRNA stability, H9C2 cells were treated with 5 μg/ml actinomycin D, a blocker for transcription, in the presence or absence of 1 nM T3 for up to 8 h. At the indicated times, total cellular RNA was prepared and analyzed by quantitative RT-PCR analysis.

2.8. SERCA activity

SERCA activity was measured as previously described [7].

2.9. Statistics

Data are expressed as means ± S.E.M. Differences were considered significant at $P < 0.05$ (one- or two-way ANOVA with Student–Newman–Kuel's post hoc test).

3. Results

3.1. The expression of SLN mRNA was decreased in the atria of hyperthyroid mice

Serum concentrations of free triiodothyronine were >32.6, 2.75 ± 0.17, and 1.71 ± 0.12 pg/ml in hyperthyroid, euthyroid and hypothyroid mice, respectively. Hyperthyroidism was associated with significant increases in the left ventricle/body weight ratio and the atria/body weight ratio when compared with euthyroid mice (Fig. 1A and B). Transthoracic echocardiography revealed that heart rate, LV chamber size and LV ventricular wall thickness were significantly increased in hyperthyroid mice when compared with those in euthyroid mice (Fig. 1C–E). However, these parameters except heart rate were not different between hypothyroid and euthyroid mice (Fig. 1). Percent fractional shortening (%FS) was not different among three groups (Fig. 1F).

The expression of SLN mRNA was significantly decreased in the atria of the hyperthyroid mice when compared with both euthyroid and hypothyroid mice (Fig. 2A and B). The expression of SLN mRNA was slightly higher in the hypothyroid atria than the euthyroid atria, although the difference did not reach statistical significance. No SLN transcript was detected in the ventricles of both hyperthyroid and hypothyroid mice (data not shown). The expression levels of PLN, SERCA2a, cardiac sodium calcium exchanger (NCX1), RyR2, CSQ2, ANF and atrial myosin light chain 2 (MLC2a) mRNAs were also examined in these atria. We found that the expression level of PLN (Fig. 2C) and MLC2a (data not shown) RNAs were significantly decreased in the hyperthyroid mice.

SERCA activity in the hyperthyroid atria ($n = 4$) was significantly higher than that in the euthyroid atria ($n = 4$) (590 ± 66 nmol/mg protein/min and 270 ± 66 nmol/mg protein/min, respectively). SR Ca^{2+} ATPase activity was much higher in the atria than that in the ventricles (hyperthyroid: 142 ± 25 nmol/mg protein/min; euthyroid: 56 ± 20 nmol/mg protein/min).

3.2. Thyroid hormone downregulated the expression of SLN in neonatal rat atrial myocytes

To avoid a secondary effect of thyroid state-mediated hemodynamics on the expression of SLN mRNA, we examined the effect of T3 in cultured neonatal rat atrial myocytes. T3 decreased the expression of SLN mRNA by 20% ($P < 0.05$) in neonatal rat atrial myocytes that did not exhibit a significant enlargement in size, whereas T3 increased the expression levels of SERCA2a and NCX1 mRNAs by 80% ($P < 0.001$) and 37% ($P < 0.01$), respectively (Fig. 3A and B). PLN mRNA was undetectable in neonatal rat atrial myocytes in our experiments.

3.3. Thyroid hormone treatment did not suppress the SLN transcriptional activity, but accelerated SLN mRNA degradation

Transfection with −2237/SLNluc or −563/SLNluc resulted in a 40-fold increase in transcriptional activity in neonatal
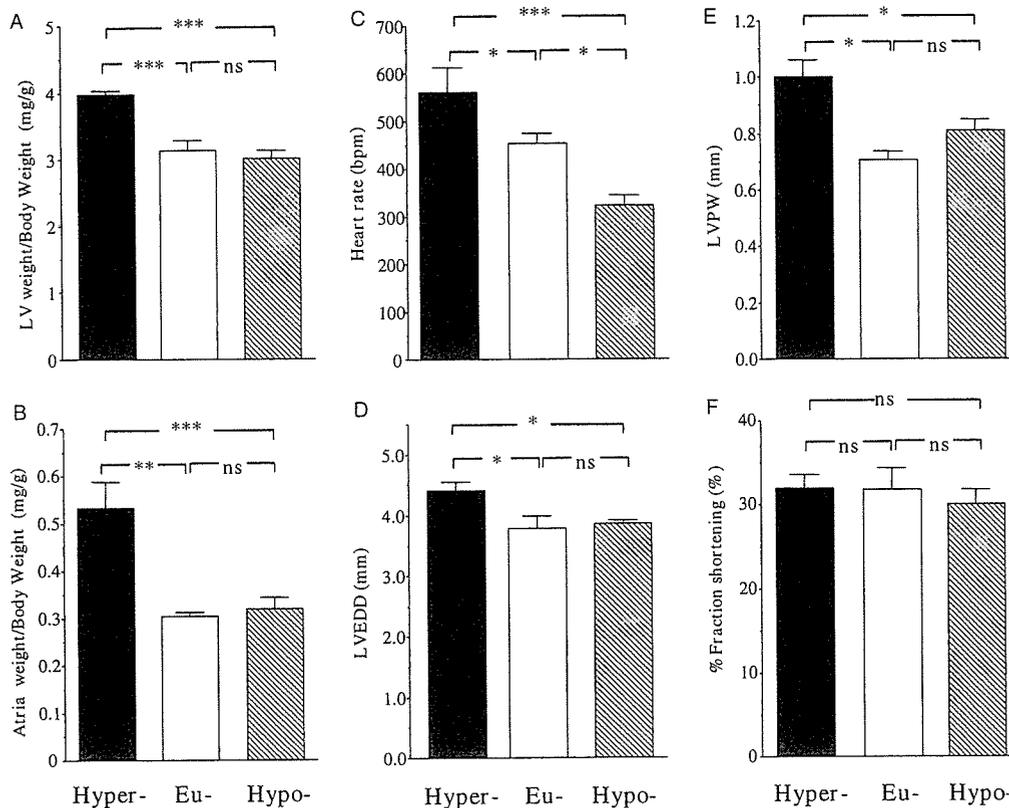
Fig. 1. Cardiac morphology and function in hyperthyroid and hypothyroid mice. (A) Left ventricle/body weight ratio; (B) atria/body weight ratio; (C) heart rate; (D) left ventricular end-diastolic diameter (EDD); (E) left ventricular posterior wall thickness (LVPW); (F) percent fractional shortening (%FS). The left ventricle/body weight ratio and the atria/body weight ratio were significantly increased in hyperthyroid mice. Transthoracic echocardiography revealed that heart rate, EDD and LVPW were significantly increased in hyperthyroid mice, whereas %FS was not different among three groups.

rat atrial myocytes when compared with an empty luciferase control plasmid. The transcriptional activity, however, was not changed in the presence or absence of T3 (Fig. 4A). We then examined the effect of T3 on SLN mRNA stability in H9C2 cells in the presence or absence of T3. The decay of SLN mRNA levels was greater in H9C2 cells in the presence of T3 for 4 and 8 h after actinomycin D treatment (Fig. 4B).

4. Discussion

The present in vivo and in vitro studies demonstrated that thyroid hormone decreased the expression of SLN mRNA in the atrial tissue and myocytes. To our knowledge, this is the first report showing that the expression of the SLN gene are regulated by thyroid hormone. Thyroid hormone binds to high-affinity nuclear receptors that consist of thyroid hormone response elements (TREs), resulting in activating or repressing gene transcription. The thyroid hormone repressed-genes generally contain one or more negative TREs. In the present study, we found four putative TREs in the SLN promoter region between −2237 of the 5′ flanking sequence and +62 of the partial non-coding exon 1 of the SLN gene. However, our data indicated that these TREs were not responsible for the suppressive SLN transcription by thyroid hormone. Instead, we found that thyroid hormone accelerated the decay of the expression of SLN mRNA, suggesting that thyroid hormone

altered SLN mRNA stability and thus increased its degradation. In this regard, a number of studies have demonstrated that thyroid hormone post-transcriptionally downregulates the expression of several genes [17,18]. The in vitro study described here indicated that T3 (1 nM) was sufficient to decrease the SLN transcripts in cultured neonatal rat atrial myocytes, where the stresses such as tachycardia and increased afterload due to ventricular hypertrophy were eliminated. Therefore, we think that the excess of thyroid hormone directly downregulates the expression of SLN mRNA. However, the depressing effect of T3 on SLN mRNA levels on in vitro neonatal atrial cardiomyocytes was much less pronounced than the in vivo atrial tissues. Accordingly, we have demonstrated that local pressure overload or atrial fibrillation decreases the expression of SLN mRNA in the atria [9,10]. Since the hyperthyroid mice investigated here displayed cardiac hypertrophy and sinus tachycardia, we postulated that these stresses augmented the decrease in the expression of SLN mRNA in the in vivo atria.

On the other hand, the present study demonstrated that the hypothyroid mice exhibited no change in cardiac morphology and function except slower heart rates. Accordingly, we found that the expression of SLN mRNA was not significantly increased in the atria of hypothyroid mice. We assume that the expression of SLN mRNA would reach the maximal level in the adult atria even in the euthyroid state and that a decrease in thyroid hormone does not enhance SLN mRNA stability. There is an alternative possibility that the intensity and dura-
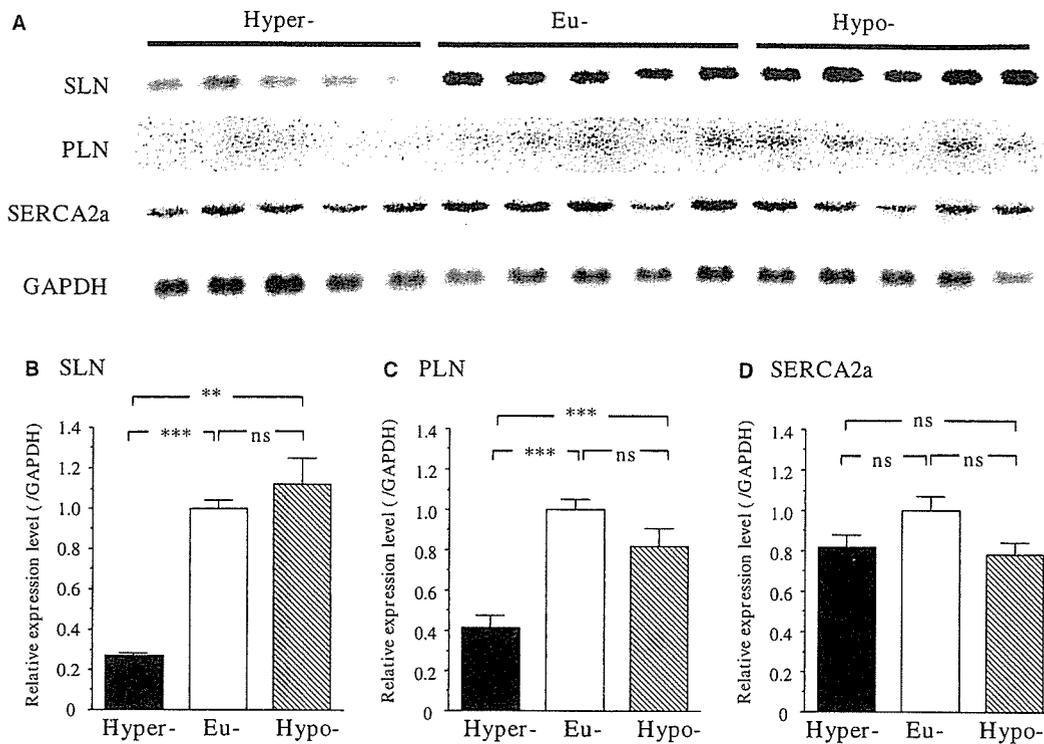
Fig. 2. The expression of SLN mRNA was decreased in the atrial myocardium of the hyperthyroid mice. (A) The raw data of the expression of SLN mRNA by Northern blot analysis. Ten μg of total RNA was loaded per lane. (B–D) The summary of Northern blot analyses. The expression levels of SLN and PLN mRNAs were significantly decreased in the atria of the hyperthyroid mice (Hyper-) when compared with euthyroid and hypothyroid mice (Eu- and Hypo-, respectively). The expression levels of SERCA2a mRNA were not different between three groups. Each signal intensity was standardized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as an internal control. The data are presented as means ± S.E.M. $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant.
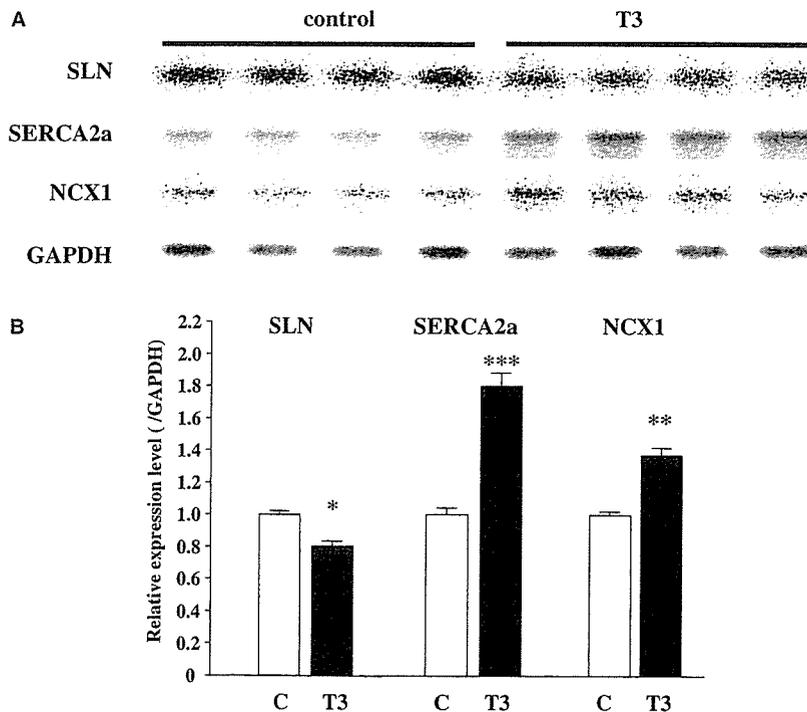

Fig. 3. Thyroid hormone treatment decreased the expression of SLN mRNA in neonatal rat atrial myocytes. (A) The raw data of the expression of SLN mRNA by Northern blot analysis. Ten μg of total RNA was loaded per lane. (B) The summary of Northern blot analyses. The expression level of SLN mRNA was significantly decreased in neonatal rat atrial myocytes in the presence of T3 (T3), whereas the expression levels of SERCA2a and NCX1 mRNAs were significantly increased by T3. Each signal intensity was standardized by GAPDH used as an internal control. The data are presented as means ± S.E.M. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (C) Control myocytes in the absence of T3.
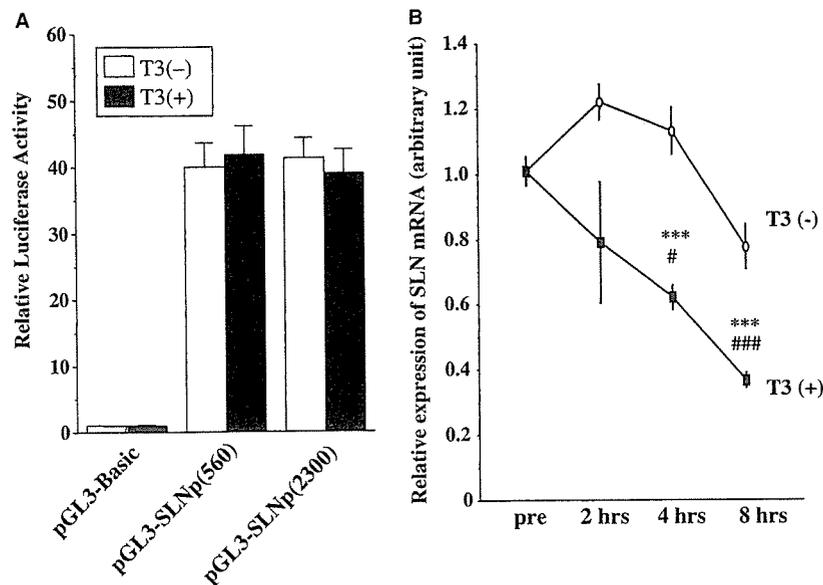
Fig. 4. Thyroid hormone treatment accelerated the decay of SLN mRNA. (A) The relative luciferase activity of the mouse SLN promoter in neonatal rat atrial myocytes. (B) The relative expression of SLN mRNA in H9C2 cells treated by actinomycin D. The luciferase activity was not changed in the presence or absence of T3. The decay of SLN mRNA levels was greater in H9C2 cells in the presence of T3 for 4 and 8 h after actinomycin D treatment. The data are presented as means ± S.E.M. $^{***}P < 0.001$ versus T3(−). # and ###, $P < 0.05$ and $P < 0.001$ versus pre, respectively.

tion of the hypothyroid state were not enough to increase the expression of SLN mRNA in the present study.

A large body of evidence has indicated that thyroid hormone plays an important role in the expression of the SR Ca^{2+} genes [19–21]. It has been known well that PLN is downregulated by thyroid hormone, which was confirmed in the atria by the present study. The downregulation of PLN mRNA contributes to the positive inotoropic effects mediated by thyroid hormone treatment. Like PLN, SLN also inhibits SERCA2a function and reduces SR Ca^{2+} stores [22]. Accordingly, the atrial tissues may be more sensitive to the stimulation of thyroid hormone than the ventricular tissues because of a decrease in SLN expression in addition to a decrease in PLN. It is of great interest that the downregulation of SLN mRNA by thyroid hormone would result in a decrease in SLN protein in the atria. In the present study, we could not show it, because it is difficult to obtain a SLN antibody probably due to the very short cytoplasmic region of the structure. Further investigation will be required to clarify whether thyroid hormone-mediated downregulation of SLN mRNA increases Ca^{2+} uptake in the atria and thus function.

In conclusion, the SLN transcripts in the atrial tissue and myocytes were post-transcriptionally downregulated by thyroid hormone. The atrium-specific effect of thyroid hormone may occur, at least in part, through the regulation of atrial SLN gene expression.

References

[1] Klein, I. and Ojamaa, K. (2001) Thyroid hormone and the cardiovascular system. N. Engl. J. Med. 344, 501–509.
[2] Izumo, S., Lompre, A.M., Matsuoka, R., Koren, G., Schwartz, K., Nadal-Ginard, B. and Mahdavi, V. (1987) Myosin heavy chain messenger RNA and protein isoform transitions during cardiac hypertrophy. Interaction between hemodynamic and thyroid hormone-induced signals. J. Clin. Invest. 79, 970–977.
[3] Kaasik, A., Minajeva, A., Paju, K., Eimre, M. and Seppet, E.K. (1997) Thyroid hormones differentially affect sarcoplasmic reticulum function in rat atria and ventricles. Mol. Cell Biochem. 176, 119–126.
[4] Shenoy, R., Klein, I. and Ojamaa, K. (2001) Differential regulation of SR calcium transporters by thyroid hormone in rat atria and ventricles. Am. J. Physiol. Heart Circ. Physiol. 281, H1690–H1696.
[5] Ohkusa, T., Ueyama, T., Yamada, J., Yano, M., Fujumura, Y., Esato, K. and Matsuzaki, M. (1999) Alterations in cardiac sarcoplasmic reticulum Ca^{2+} regulatory proteins in the atrial tissue of patients with chronic atrial fibrillation. J. Am. Coll. Cardiol. 34, 255–263.
[6] Van Gelder, I.C. et al. (1999) Alterations in gene expression of proteins involved in the calcium handling in patients with atrial fibrillation. J. Cardiovasc. Electrophysiol. 10, 552–560.
[7] Minamisawa, S., Wang, Y., Chen, J., Ishikawa, Y., Chien, K.R. and Matsuoka, R. (2003) Atrial chamber-specific expression of sarcolipin is regulated during development and hypertrophic remodeling. J. Biol. Chem. 278, 9570–9575.
[8] MacLennan, D.H., Asahi, M. and Tupling, A.R. (2003) The regulation of SERCA-type pumps by phospholamban and sarcolipin. Ann. N. Y. Acad. Sci. 986, 472–480.
[9] Shimura, M., Minamisawa, S., Yokoyama, U., Umemura, S. and Ishikawa, Y. (2005) Mechanical stress-dependent transcriptional regulation of sarcolipin gene in the rodent atrium. Biochem. Biophys. Res. Commun. 334, 861–866.
[10] Uemura, N. et al. (2004) Down-regulation of sarcolipin mRNA expression in chronic atrial fibrillation. Eur. J. Clin. Invest. 34, 723–730.
[11] Kiss, E., Jakab, G., Kranias, E.G. and Edes, I. (1994) Thyroid hormone-induced alterations in phospholamban protein expression. Regulatory effects on sarcoplasmic reticulum Ca^{2+} transport and myocardial relaxation. Circ. Res. 75, 245–251.

[12] Khoury, S.F., Hoit, B.D., Dave, V., Pawloski-Dahm, C.M., Shao, Y., Gabel, M., Periasamy, M. and Walsh, R.A. (1996) Effects of thyroid hormone on left ventricular performance and regulation of contractile and Ca(2+)-cycling proteins in the baboon. Implications for the force-frequency and relaxation-frequency relationships. Circ. Res. 79, 727–735.

[13] Holt, E., Sjaastad, I., Lunde, P.K., Christensen, G. and Sejersted, O.M. (1999) Thyroid hormone control of contraction and the Ca^{2+}-ATPase/phospholamban complex in adult rat ventricular myocytes. J. Mol. Cell Cardiol. 31, 645–656.

[14] Yoshida, M. et al. (2005) Impaired Ca2+ store functions in skeletal and cardiac muscle cells from sarcalumenin-deficient mice. J. Biol. Chem. 280, 3500–3506.

[15] Minamisawa, S., Sato, Y., Tatsuguchi, Y., Fujino, T., Imamura, S., Uetsuka, Y., Nakazawa, M. and Matsuoka, R. (2003) Mutation of the phospholamban promoter associated with hypertrophic cardiomyopathy. Biochem. Biophys. Res. Commun. 304, 1–4.

[16] Minamisawa, S., Gu, Y., Ross Jr., J., Chien, K.R. and Chen, J. (1999) A post-transcriptional compensatory pathway in heterozygous ventricular myosin light chain 2-deficient mice results in lack of gene dosage effect during normal cardiac growth or hypertrophy. J. Biol. Chem. 274, 10066–10070.

[17] Kenessey, A. and Ojamaa, K. (2005) Ligand-mediated decrease of thyroid hormone receptor-alpha1 in cardiomyocytes by proteosome-dependent degradation and altered mRNA stability. Am. J. Physiol. Heart Circ. Physiol. 288, H813–H821.

[18] Fukuyama, K. et al. (2003) Downregulation of vascular angiotensin II type 1 receptor by thyroid hormone. Hypertension 41, 598–603.

[19] Kolar, F., Seppet, E.K., Vetter, R., Prochazka, J., Grunermel, J., Zilmer, K. and Ostadal, B. (1992) Thyroid control of contractile function and calcium handling in neonatal rat heart. Pflugers Arch. 421, 26–31.

[20] Carr, A.N. and Kranias, E.G. (2002) Thyroid hormone regulation of calcium cycling proteins. Thyroid 12, 453–457.

[21] Dillmann, W.H. (2002) Cellular action of thyroid hormone on the heart. Thyroid 12, 447–452.

[22] Tupling, A.R., Asahi, M. and MacLennan, D.H. (2002) Sarcolipin overexpression in rat slow twitch muscle inhibits sarcoplasmic reticulum Ca^{2+} uptake and impairs contractile function. J. Biol. Chem. 277, 44740–44746, (Epub 2002 Sep 16).

話 題

ICH 遺伝子治療専門家会議 シカゴミーティングと今後の展望

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1. はじめに

ICH(日米 EU 医薬品規制調和国際会議)は医薬品の承認申請に関わる規制を調和し，申請に必要な様々なデータ等の作成における不必要な重複を避け，医薬品開発のグローバルな促進とよりよい医薬品を一刻も早く患者の元に届けることを目的として活動を行っている．2005 年11 月 7〜11 日にかけてシカゴ市で開催された ICH ミーティングでは，有効性，安全性，品質，及び境界領域の 17 課題に関し討議が行われた．本稿では，遺伝子治療専門家会議が ICH 公開ワークショップとして開催した「腫瘍溶解性ウイルスワークショップ」の成果と，「遺伝子治療薬の生殖細胞系列への伝達リスクの最小化に関する ICH 見解(案)」作成のための議論を中心に，その活動について紹介したい．

2. 遺伝子治療専門家会議の活動

遺伝子治療専門家会議(Gene Therapy Discussion Group；GT-DG)は，2002 年のワシントン D. C. 会議にて正式に発足した作業部会である．それ以前は，バイオテクノロジー医薬品専門家会議及び Ad hoc な遺伝子治療専門家会議として議論を重ねてきた．GT-DG では，周辺技術を含め急激に進歩する遺伝子治療薬をめぐる科学的な諸問題に柔軟に対処するために，公開ワークショップの開催や ICH ホームページ等を通じて得られた議論の成果を広く公開するとともに，新たな知見が得られた場合に迅速に対応していくというスタンスで活動を行っている．これまで GT-DG で取り上げた話題について表 1 にあげたが，非常に多岐にわたる科学的課題について議論を行ってきている．

シカゴ会議では腫瘍溶解性ウイルスの専門家を招聘し，公開ワークショップを開催した．本ワークショップでの発表及び議論に基づき，現時点での問題点の洗い出しを行った．また，「遺伝子治療薬の生殖細胞系列への伝達リスクの最小化に関する ICH 見解(案)」作成のための議論を行った．

表 1

ICH 遺伝子治療専門家会議で取り上げられた主なトピック

患者からの遺伝子治療用ベクターやウイルスの放出
遺伝子治療薬に含まれる増殖性ウイルスの検出とその手法(RCA or RCR)
遺伝子治療薬のためのウイルス参照品(Adenovirus type 5)
遺伝子治療薬の生殖細胞系列への伝達リスクを最小にするための方策
遺伝子治療用ベクターによる挿入変異の評価
腫瘍溶解性ウイルス(ワークショップ)
遺伝子治療を受けた患者の長期フォローアップ(FDA ガイドライン案)
遺伝子治療用レンチウイルスベクター(EMEA ガイドライン案)

3. 腫瘍溶解性ウイルス公開ワークショップ

腫瘍溶解性ウイルスの発見は非常に古く，悪性腫瘍患者がウイルス感染又は生ワクチンを接種された際に，腫瘍の縮小や寛解が認められたことから開発が始まった．腫瘍溶解性ウイルス開発は，腫瘍特異的に増殖する野生型ウイルスや弱毒化ウイルスを用いた研究から，遺伝子改変技術を用いた病原性の除去や腫瘍指向性をより高めた制限増殖性ウイルスベクターを用いるものへと移行しつつある．腫瘍溶解性ウイルスの開発はここ数年急速に進展しており，多くの総説も書かれている．しかし，腫瘍溶解性ウイルスの臨床適用は未知・未経験の要素も多い領域であり，基礎となる科学的知見も十分に集積されていないことから，品質確保の方策，安全性や有効性評価のための動物を用いた非臨床試験のあり方，さらには臨床研究における安全性・有効性評価等を議論するために，この分野の専門家を招き今回の公開ワークショップを開催することになった．

本ワークショップでは，①腫瘍溶解性ウイルスの選択・設計(野生型・弱毒型・遺伝子組換え型)，②動物やヒトで期待される効果の評価，③ウイルス複製の腫瘍選択性，④臨床上の安全性，⑤動物試験に用いる適切な動物モデル，⑥腫瘍溶解性ウイルスの体外放出の測定法と実データについて議論が行われた．

腫瘍溶解性ウイルスの設計及び特性解析

現在使用されている腫瘍溶解性ウイルス開発では，腫瘍細胞内で選択的に複製する非組換えウイルスを用いる場合と遺伝子組換え型ウイルスを用いる場合がある．通常の遺伝子治療では，ベクター中の増殖性ウイルス(RCV)の検出が品質・安全性の観点から重要であるが，腫瘍溶解性ウイルスは制限複製能を持つことから，RCV 検出よりも目的ウイルスの変化体をどのように検出するかについて議論が行われた．また品質の恒常性の観点から，ウイルス感染性力価ばかりでなく力価に対する粒子数の比を規格化することの必要性について，その重要度を含めた活発な議論が行われた．

非臨床試験

安全性評価や腫瘍溶解性ウイルスが目的とする効果が得られるか否かの評価に，動物モデルが有用であることは，多くの講演者の一致した意見であった．しかし，①腫瘍溶解性ウイルスの感染及び複製能に動物種特異性があること，②動物にヒト腫瘍(細胞)を移植した腫瘍モデル動物ではウイルスがヒト体内とは異なる指向性/分布を示すこと，③動物での免疫反応がヒトとは異なること等から，動物モデルの限界も指摘された．しかし，生体内分布や安全性/毒性の評価，臨床での投与経路や用法・用量の選択などに関して動物モデルが有用な情報を与え得るということについては，コンセンサスが得られた．腫瘍選択性に関しては，非腫瘍細胞培養株及び腫瘍細胞培養株を用いた試験又はヒト健康組織及びヒト腫瘍組織からの初代組織片培養を用いた試験の有用性について報告された．

臨床研究

腫瘍溶解性ウイルスの複雑な性質から，開発の基礎段階で十分に特性解析することが困難であり，また有用な動物モデルが必ずしも存在するわけではないことから，臨床研究の開始に当たっては多くの検討すべき課題があげられた．

今回のワークショップで取り上げられた臨床研究では，主に中等度以下(グレード 1・2)の有害事象が観察されたことが報告された．また，その多くはインフルエンザ様症状であり，また一過性の臨床検査値異常及び投与部位の局所反応も報告された．しかしこれらの有害事象が，

腫瘍溶解性ウイルス特有の有害事象と捉えるべきではないとの意見も出された.

臨床薬物動態

臨床薬物動態の解析手法として被験者のモニタリングには，PCR・感染性力価試験のいずれも用いられている．いくつかの腫瘍溶解性ウイルスの臨床研究において，血液中に検出されるウイルス量は投与直後と4～7日目にピークが認められた．このような2相性のピークは局所投与及び静注した場合のいずれでも観察されており，ウイルスの複製をモニターする手段となり得ることが指摘された.

用法・用量設定の必要性，腫瘍溶解性ウイルスに対する患者の中和抗体の影響などについて活発な議論が行われた．さらに，腫瘍溶解性ウイルスの体外放出に関する予防措置についても議論が行われた.

腫瘍溶解性ウイルス開発の今後の展望

腫瘍溶解性ウイルスの開発の新たな流れとして，化学療法又は放射線療法と腫瘍溶解性ウイルス療法を組み合わせる併用療法の有用性が示唆されており，今後このような併用療法の開発も進むものと考えられる．腫瘍溶解性ウイルスの設計改良のアプローチとしては，免疫反応を活性化する遺伝子のウイルスゲノムへの挿入や，腫瘍細胞へのウイルスの感染能の増強などが行われている．また，殺腫瘍効果の作用機序を解明できるデータを得るための非臨床試験及び臨床研究の取り組みも行われている．特に，様々な腫瘍溶解性ウイルスの臨床研究で具体的な進歩がみられていることは特筆すべき点である.

4. 遺伝子治療薬の生殖細胞系列への伝達リスクを最小にするための方策に関するICH「見解(第2次案)

今回のGT-DGでは，「遺伝子治療薬の生殖細胞系列への伝達リスクを最小にするための方策に関するICH見解(第2次案)」の議論が行われた．本見解案は，EMEAが1stラポーターとして第2次案までの取りまとめを行い，今回はこの第2次案について討議した．本見解案は，遺伝子治療薬の生殖細胞への伝達リスクを評価するための非臨床試験の実施スキームの提示を目的としている．図1は，どのような試験を行う必要があるのかを示したフロー図である．本見解案作成の2ndラポーターとして日本(筆者)が指名され，第3次案に反映すべき意見があれば，2006年1月までに2ndラポーターに送付することとされた．送付された意見に基づいて第3次案を作成し，2006年6月の横浜会議で詳細な議論を行い，遅くとも2007年初めに最終案を取りまとめICH運営委員会に報告する方針が確認された．また本見解案は，科学的な情報が十分に集積された段階でガイドライン作成につなげていくことも確認された.

5. GT-DGの今後の活動について

ICH GT-DG活動では，遺伝子治療をめぐって取り組むべき課題がより明確になりつつあることや，ここにきてICH各極で遺伝子治療医薬品の規制当局への承認申請が出されていることへの早急な対応もあり，ICH「見解」の作成やガイドライン策定を見据えた議論も行われるようになってきている．また，EMEAの「遺伝子治療用レンチウイルスベクター」ガイドライン案やFDAの「遺伝子治療を受けた患者の長期フォローアップ」ガイドライン案についても，GT-DGの場で活発な議論が行われてきており，これらの案の策定に当たっては，我々日本を含めた他極の意見が多く取り入れられている．我が国における遺伝子治療の臨床研究の数は欧米に比べて非常に少なく，臨床研究での情報は多くが海外に依存している現況であることは否めない．しかし，ここ数年は我が国においても遺伝子治療薬の開発が急速に進んでおり，
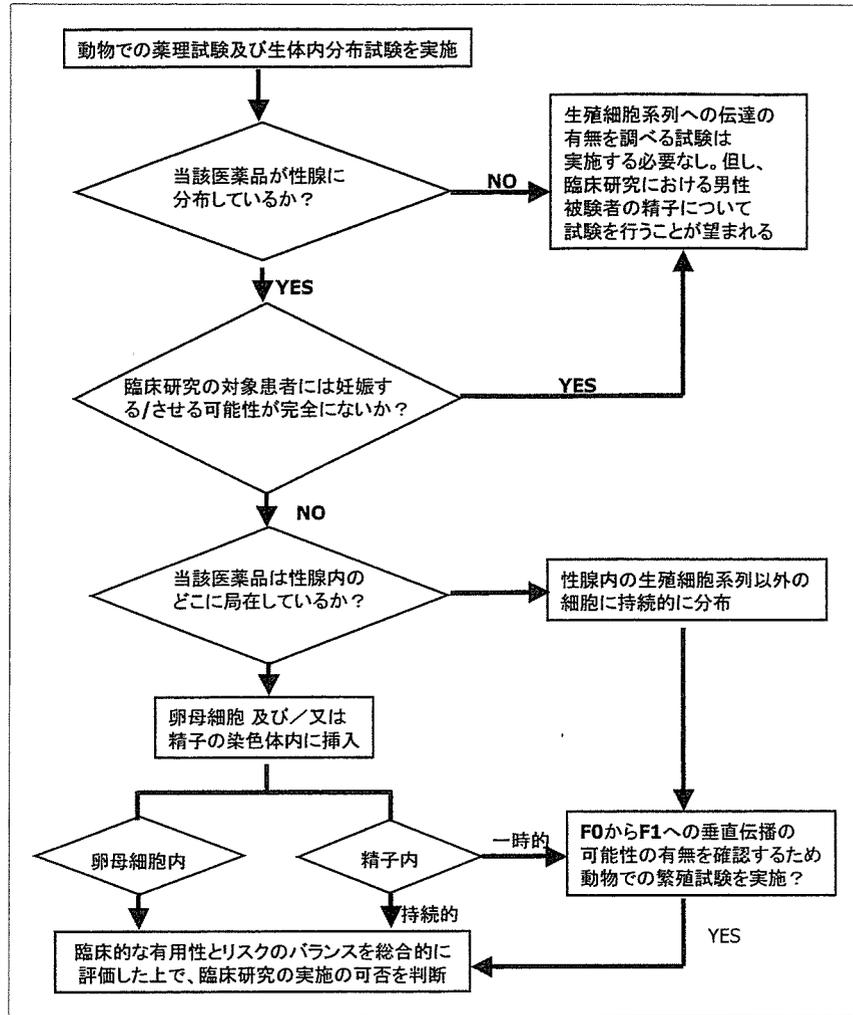
図1 遺伝子治療薬の生殖細胞系列への伝達の有無を調べるための動物試験のフロー（案）

遺伝子治療薬に関する ICH 見解やガイドラインの策定が，我が国の遺伝子治療薬の開発の促進につながっていくと期待される.

▶ 医薬品各条の改正点

生物薬品

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生物薬品の各条新規収載品目および改正品目

生物薬品委員会の医薬品各条関係では，たん白質性/ペプチド性および高分子多糖性の新規収載品目の審議および既収載品目についての改正作業を行ってきた．

第十四改正日本薬局方（十四局）第一追補，第二追補を経て，第十五改正日本薬局方（十五局）に至るまで，新規収載品目は8品目，改正は9品目であった（表1）．これらのうち，第十四改正日本薬局方第一追補（十四局第一追補）にはリゾチーム塩酸塩（十四局第一追補では塩化リゾチームとして収載）が新規収載された．第十四局改正日本薬局方第二追補（十四局第二追補）では，オキシトシン，セラペプターゼの新規収載が行われるとともに，オキシトシン注射液，カリジノゲナーゼ，バソプレシン注射液，ヘパリンナトリウム，ヘパリンナトリウム注射液，血清性性腺刺激ホルモン，注射用血清性性腺刺激ホルモン，絨毛性性腺刺激ホルモン（十四局第二追補では胎盤性性腺刺激ホルモンとして収載），注射用絨毛性性腺刺激ホルモン（十四局第二追補では注射用胎盤性性腺刺激ホルモンとして収載）の改正が行われた．

生物薬品委員会の局方収載における各条審議のポイントと基本方針

生物薬品委員会の局方収載における各条審議は，表2にあげた基本方針に沿って行った．これらの基本方針は，十四局収載における基本方針に準じている．

① 合理性に基づく規格および試験方法の設定

生物薬品の各条における試験項目や規格値の設定においては，①品質の恒常性維持，②科学の進歩に即応した品質確保，品質管理，③後続品に対する品質基準の規範となる規格・試験法の設定，④同一原理の試験項目を重複させないなど，設定する各条ごとに合理性に基づく試験項目の設定，⑤簡便で精度の高い試験法への変更，⑥実測値に基づいた規格値の設定などの観点から審議を行うこととした．

表1　生物薬品の各条新規収載品目および改正品目

新規収載品目	改正品目
リゾチーム塩酸塩	カリジノゲナーゼ
ゴナドレリン酢酸塩	オキシトシン注射液
セラペプターゼ	血清性性腺刺激ホルモン
パルナパリンナトリウム	注射用血清性性腺刺激ホルモン
オキシトシン	絨毛性性腺刺激ホルモン
セルモロイキン	注射用絨毛性性腺刺激ホルモン
テセロイキン	バソプレシン注射液
注射用テセロイキン	ヘパリンナトリウム
	ヘパリンナトリウム注射液

表2 生物薬品委員会の各条審議の基本方針

1) 各条全体としての合理性に基づく規格および試験方法の設定
2) 範囲のある含量規格の設定
3) 性状の溶解性試験の見直し
4) 試験動物の使用削減の観点からみた合理的試験法の設定
　① バイオアッセイ法から理化学的試験法や免疫化学的方法への移行
　② ヒスタミンおよびヒスタミン様物質，異常毒性否定試験などの設定の意義および必要性の見直し
　③ 毒性試験の設定の意義および必要性の見直し
　④ 発熱性物質試験のエンドトキシン試験への切り替え
5) 比活性の独立示性値としての設定
6) 純度試験などに液体クロマトグラフィー法の積極的活用，電気泳動法で試験を実施する際の検出感度の保証
7) 血液凝固性物質，血液型物質などの設定の意義および必要性の見直し
8) 同種同効医薬品の収載にあたっての調和

② 範囲のある含量規格の設定

酵素製剤などの原薬については，単位質量当たりの活性について，上限値と下限値を設定する．このことにより，品質の恒常性の確保を目指す．

③ 性状の溶解性試験の見直し

たん白質やペプチド医薬品の特性から，有機溶媒などへの溶解性に関して，意義の乏しいものは削除することとし，合理的な性状を規定する．

④ 試験動物の使用削減の観点からの合理的試験法の設定

動物福祉やより合理的な試験法の採用を行うとの観点から，*in vivo*アッセイ法から*in vitro*アッセイ法や理化学的試験法などへの移行を図る．ヒスタミンやヒスタミン様物質試験や異常毒性否定試験などは，設定の意義や必要性を見直すこととした．発熱性物質試験からエンドトキシン試験への切り替えを進める．

⑤ 比活性の独立示性値としての設定

比活性は製品の特性を示す値であることから，純度試験として設定するのではなく，独立した示性値として設定することにした．

⑥ 純度試験などに液体クロマトグラフィー法の積極的活用，電気泳動法で試験を実施する際の検出感度の保証

液体クロマトグラフィー法などの技術進歩が著しいことから，純度試験などに積極的に活用していくこととした．その際，検出感度を設定し，保証していくことにした．

⑦ 血液凝固性物質，血液型物質などの設定の意義および必要性の見直し

血液凝固物質や血液型物質の混入に関する試験項目については，起源からみて混入の可能性のないものについては見直すことにした．

⑧ 同種同効医薬品の収載にあたっての調和

インスリンや成長ホルモンなどの同種同効医薬品の収載にあたっては，試験項目や規格値について，合理的な場合に調和を図ることとした．

新規収載候補品目の策定

上記の十五局の収載方針の策定と並行して，継続審議中の各条審議に加えて，新規収載候補品目の策定を行った．候補品目の策定

にあたっては，保険医療上重要な医薬品としてオーファンドラッグの指定を受けた医薬品や市場で広く使用されている医薬品，さらには米国薬局方（USP）や欧州薬局方（EP）に掲載されているなど国際的にも広く使用されている医薬品から，候補とすべき品目を選定した．さらに，標準品の策定が可能な医薬品を候補品目とすることにし，20品目を新規収載候補品目とした．十五局では，このうちパルナパリンナトリウム，セルモロイキン，テセロイキンが収載されることになった．

収載品目別の概説と審議の経緯の要点

① リゾチーム塩酸塩

リゾチームはムコ多糖分解作用をもつ酵素であり，溶菌活性を示す．リゾチーム塩酸塩の本質はニワトリ卵白より精製される塩基性ポリペプチドである．消炎酵素剤として用いられ，単味あるいは感冒薬に配合して用いられている．さらに，歯科領域などの小手術の止血時，点眼剤として慢性結膜炎，外用剤として皮膚科医用などにも幅広く臨床使用されている．

リゾチーム塩酸塩は医療用，一般用として広く使用され，品目数としても多数に上っている．日本薬局方外医薬品規格（局外規）に収載されていた品目であり，第十四局第一追補に収載された．

基原，性状，確認試験，純度試験，乾燥減量，強熱残分，定量法を規定した．性状についてはエーテルへの溶解性の情報は意義が乏しいことから，水およびエタノールへの溶解性を記載することとした．確認試験では，ニンヒドリンによる呈色反応および参照スペクトルとの一致を規定した．純度試験として，局外規では，溶状，重金属，ヒ素，および窒素を規定していたが，製造工程からヒ素を規定する意義は乏しいとして，原案ではヒ素の規定を除くことが提案された．審議の結果，ヒ素を規定する必要はないとされた．一方，窒素については示性値として設定すべきとして，純度試験から独立した示性値として規定することとし，幅をもつ規格値とした．定量法としては，迅速分析法の記載に従い*Micrococcus luteus*を基質として，その溶菌活性で規定することとした．局外規では，同様に溶菌活性を指標とする定量法を採用していたが，反応停止法が規定されていなかった．そこで，定量法として反応停止を含めて規定することにより，より精度の高い試験法を採用することとした．保存条件としては，気密容器で保存可能であり，冷所などの温度規定は必要とされた．

② ゴナドレリン酢酸塩

ゴナドレリン酢酸塩は，ゴナドトロピン（LH，FSH）分泌促進作用をもつペプチドで，視床下部性性腺機能低下症，ゴナドトロピン分泌不全を伴う下垂体性小人症，視床下部器質性障害，ゴナドトロピン単独欠損症の治療や下垂体性LH分泌機能検査薬として用いられる．

基原，性状，確認試験，施光度，pH，構成アミノ酸，純度試験，水分，定量法を規定した．性状について，水，メタノール，酢酸，エタノールに対する溶解性を規定した．確認試験として，紫外可視吸収スペクトルと赤外吸収スペクトルを採用し，参照スペクトルとの同等性を確認することとした．また，本品は酢酸塩であることから，酢酸を含むことを確認することとした．液体クロマトグラフィー法を用いた構成アミノ酸分析法を採用し，アルギニンに対する各構成アミノ酸の比を規定した．純度試験として，溶状を紫外可

視吸光度測定法を用いた350nmにおける吸光度を限度値として規定した．また，液体クロマトグラフィー法を用いた類縁物質の限度値を規格した．定量法として，生物活性試験に替えて，液体クロマトグラフィー法を採用し，ゴナドレリン酢酸塩標準品を基準としてゴナドレリン量を求めることとした．ゴナドレリン酢酸塩は，吸湿性があり，また光感受性もあるため遮光して，冷所，密封容器に保存することとされた．

　一方，ゴナドレリン酢酸塩は現在のところ，製造しているのは1社のみであり，供給できる標準品が非常にわずかしかなく，受け入れ試験として吸湿性のある本品の水分含量の測定や他の項目を十分に試験するだけの量の確保が困難との報告があった．生物薬品では，各条に収載される製品で1社のみが製造しているものが増加してくることが予想され，標準品の策定においても特別の配慮が必要と考えられた．このため，標準品の試験として必ずしも必要と考えられない試験項目については，設定しないこととした．また使用量などが必要以上に多い項目については適切な量を用いるように変更することとした．確認試験として，質量分析法を用いた分子量の規定，およびゴナドレリン酢酸塩のペプチド配列を確認できるMS/MS分析（質量分析）を行うこととした．さらに，標準品の配布制限も行うこととした．

⚛ セラペプターゼ

　セラチア属菌から製造されたたん白質分解活性を有する酵素であり，抗炎症作用を有する．手術後や外傷時の抗炎症作用，副鼻腔炎や気管支炎の消炎，喀痰喀出不全に対する治療を目的として内服で用いる．

　基原，性状，確認試験，純度試験，乾燥減量，強熱残分，定量法を規定した．SDSポリ

アクリルアミド電気泳動や免疫化学的手法を用いた確認試験の設定を要望したところ，金属プロテアーゼの特性を利用した方法が原案作成者より提案された．検討の結果，提案は適切であるとされ，定量法を準用した方法を規定することとした．原案では，重金属およびヒ素に対して独立した試験が規定されていたが，これらは純度試験として規定するべきとされた．とくに重金属に関しては実測値に基づき，より適切な規格(50ppmから20ppm)にすることとされた．定量法として，乳性カゼインの分解活性をトリクロロ酢酸可溶性画分のチロジン残基の増加を指標とする試験法により設定した．貯法としては気密容器とした．

⚛ パルナパリンナトリウム

　ブタ腸粘膜由来のヘパリンを化学的に分解して得たヘパリンのナトリウム塩であり，血液中のアンチトロンビンⅢ(ATⅢ)を活性化することにより血液凝固阻止作用を発現する．血液透析や血液ろ過などの血液体外循環時の灌流血液の凝固防止を目的として使用されている．

　基原，性状，確認試験，pH，純度試験，乾燥減量，分子量，分子量分布，硫酸エステル化の度合，総窒素，抗第Ⅱa因子活性，抗第Ⅹa因子活性・抗第Ⅱa因子活性比，定量法を規定した．基原に関して，ダルテパリンナトリウムの整合を図り，参考情報の「日本薬局方の通則等に規定する動物由来医薬品起源としての動物に求められる要件」との対応から，原料を得る動物の表記に「健康な動物」と記載することとされた．原案では試験溶媒として用いていない，エタノール(95)およびエーテルへの溶解性について記載されていたが，意義は低いとして削除することとされ，エタノール(99.5)への溶解性を追加することとし

た．確認試験として，トルイジンブルーOの反応を用いた多糖類染色試験を設定するとともに，ナトリウム塩の定性反応を規定した．パルナパリンナトリウムは多くの分子量の異なるヘパリンナトリウムの集合体であることから，規格項目として分子量と分子量分布を設定し，分子量の項では平均分子量を規定することとした．分子量測定には「分子量測定用低分子量ヘパリン標準品」を用いることとした．ヘパリンナトリウムは，注射剤としても使用されることから「溶状」を設定した．硫酸エステル化は電位差滴定法を用いる試験法を採用した．原料のヘパリンナトリウムでは総窒素を規定していることから，総窒素の項を設定することとした．定量法は，標準品として低分子量ヘパリンを用い抗第Ⅹa因子活性を指標とする試験法を採用し，1mg中の低分子ヘパリン単位を求めることとした．また，別に抗Ⅱa因子活性の比活性を求め，抗第Ⅹa因子活性・抗Ⅱa因子活性の比を幅で規定することとした．貯法は密封容器とした．

⑤ オキシトシン

下垂体後葉から分泌されるペプチドホルモンで，化学合成により製造され，子宮収縮作用がある．子宮収縮の誘発，促進ならびに子宮出血の治療を目的とし，分娩誘発，微弱陣痛，弛緩出血などの治療に点滴静注あるいは，筋注により用いられる．オキシトシン注射液は既収載であったが，その原薬の新規収載を行った．

基原，性状，確認試験，構成アミノ酸，純度試験，水分，定量法を規定した．性状について，規格・試験法で使用する溶媒について調査し，水，エタノール（99.5），および塩酸試液への溶解性を規定した．確認試験として，吸収スペクトル法を採用し，参照スペクトルとの同等性を確認することとした．構成アミ

ノ酸を設定したが，血圧上昇成分であるバソプレシン様ペプチドが存在しないことを担保するために，オキシトシンに含まれないほかのアミノ酸の限度値を規定した．純度試験では，酢酸を幅規格で設定するとともに，類縁物質について規格値を設定した．定量法は，オキシトシン注射液で動物を用いた生物活性試験が規定されていたが，高速液クロマトグラフィー（HPLC）法に変更することとした．このために，オキシトシンの強制分解溶液を用いて，生物活性試験とHPLC法を比較し，両試験法に良好な相関関係が認められたことから，HPLC法の採用が妥当とされた．貯法温度は2〜8℃とし，吸湿性をもつことから気密容器とした．

⑥ セルモロイキン

セルモロイキンの本質は組換えDNA技術によって作られるインターロイキン（IL）-2であり，進行腎細胞がん，悪性黒色腫，血管肉腫に点滴静注ないしは皮下投与される．

基原，性状，確認試験，pH，純度試験，酢酸アンモニウム，エンドトキシン，無菌，定量法が設定された．確認試験では，フォリン試薬を用いたたん白質としての反応性の確認，アミノ酸分析による構成アミノ酸，SDSポリアクリルアミド電気泳動を用いた分子量，ペプチドマップ試験，IL-2依存性マウスナチュラルキラー細胞の増殖活性の抗IL抗体による阻害を指標とする試験を設定した．アミノ酸分析の試験法は，第十四局第二追補参考情報アミノ酸分析法に準じた設定を行った．ペプチドマップ試験法の規格として，ピーク位置の同等性のみならず量的な同等性を確認するために溶出ピークの高さの同等性についても確認することとした．純度試験として，酵素免疫手法による大腸菌由来たん白質，SDSポリアクリルアミド電気泳動を用いた重