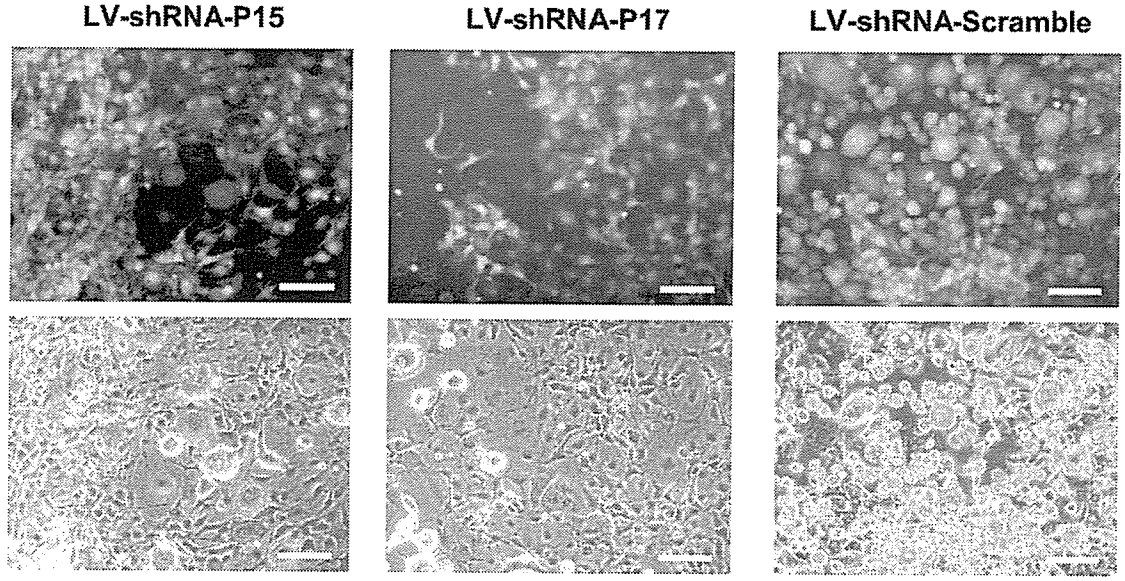

Fig. 5. Identification of the 3T3-L1 cells transduced with LV-shRNA. Brightfield and fluorescent microscopy images collected from the same field. The LV-shRNA-infected cells, which expressed EGFP, were detected as green fluorescence (upper panels) and morphologically identified mature adipocyte with a voluminous spherical shape and a large accumulation of intracytoplasmic lipid vesicles (lower panels). Bars represent 100 μm.

References

[1] Kubota, N. et al. (1999) Mol. Cell 4, 597–609.
[2] Su, C.G. et al. (1999) J. Clin. Invest. 104, 383–389.
[3] Desreumaux, P. et al. (2001) J. Exp. Med. 193, 827–838.
[4] Nakajima, A. et al. (2001) Gastroenterology 120, 460–469.
[5] Katayama, K. et al. (2003) Gastroenterology 124, 1315–1324.
[6] Schlezinger, J.J., Jensen, B.A., Mann, K.K., Ryu, H.Y. and Sherr, D.H. (2002) J. Immunol. 169, 6831–6841.
[7] Wang, Y.L., Frauwirth, K.A., Rangwala, S.M., Lazar, M.A. and Thompson, C.B. (2002) J. Biol. Chem. 277, 31781–31788.
[8] Brummelkamp, T.R., Bernards, R. and Agami, R. (2002) Science 296, 550–553.
[9] Abbas-Terki, T., Blanco-Bose, W., Deglon, N., Pralong, W. and Aebischer, P. (2002) Hum. Gene Ther. 13, 2197–2201.
[10] Hasuwa, H., Kaseda, K., Einarsdottir, T. and Okabe, M. (2002) FEBS Lett. 532, 227–230.
[11] Kunath, T., Gish, G., Lickert, H., Jones, N., Pawson, T. and Rossant, J. (2003) Nat. Biotechnol. 21, 559–561.
[12] Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F.H., Verma, I.M. and Trono, D. (1996) Science 272, 263–267.
[13] Kafri, T., Blomer, U., Peterson, D.A., Gage, F.H. and Verma, I.M. (1997) Nat. Genet. 17, 314–317.
[14] Takahashi, M., Miyoshi, H., Verma, I.M. and Gage, F.H. (1999) J. Virol. 73, 7812–7816.
[15] Miyoshi, H., Smith, K.A., Mosier, D.E., Verma, I.M. and Torbett, B.E. (1999) Science 283, 682–686.
[16] Pfeifer, A., Kessler, T., Yang, M., Baranov, E., Kootstra, N., Cheresh, D.A., Hoffman, R.M. and Verma, I.M. (2001) Mol. Ther. 3, 319–322.
[17] Brummelkamp, T.R., Bernards, R. and Agami, R. (2002) Cancer Cell 2, 243–247.
[18] van de Wetering, M. et al. (2003) EMBO Rep. 4, 609–615.
[19] Mizuguchi, H. and Kay, M.A. (1999) Hum. Gene Ther. 10, 2013–2017.
[20] Tahara-Hanaoka, S., Sudo, K., Ema, H., Miyoshi, H. and Nakauchi, H. (2002) Exp. Hematol. 30, 11–17.
[21] Miyoshi, H., Blomer, U., Takahashi, M., Gage, F.H. and Verma, I.M. (1998) J. Virol. 72, 8150–8157.
[22] Tontonoz, P., Hu, E., Graves, R.A., Budavari, A.I. and Spiegelman, B.M. (1994) Genes Dev. 8, 1224–1234.
[23] Gimble, J.M., Robinson, C.E., Wu, X., Kelly, K.A., Rodriguez, B.R., Kliewer, S.A., Lehmann, J.M. and Morris, D.C. (1996) Mol. Pharmacol. 50, 1087–1094.
[24] Baer, M., Nilsen, T.W., Costigan, C. and Altman, S. (1990) Nucleic Acids Res. 18, 97–103.
[25] Chawla, A., Schwarz, E.J., Dimaculangan, D.D. and Lazar, M.A. (1994) Endocrinology 135, 798–800.
[26] Kliewer, S.A., Forman, B.M., Blumberg, B., Ong, E.S., Borgmeyer, U., Mangelsdorf, D.J., Umesono, K. and Evans, R.M. (1994) Proc. Natl. Acad. Sci. USA 91, 7355–7359.
[27] Tontonoz, P., Hu, E. and Spiegelman, B.M. (1994) Cell 79, 1147–1156.
[28] Ramirez-Zacarias, J.L., Castro-Munozledo, F. and Kuri-Harcuch, W. (1992) Histochemistry 97, 493–497.
[29] Green, H. and Kehinde, O. (1975) Cell 5, 19–27.
[30] Wise, L.S. and Green, H. (1979) J. Biol. Chem. 254, 273–275.
[31] Doench, J.G., Petersen, C.P. and Sharp, P.A. (2003) Genes Dev. 17, 438–442.

Gene Therapy (2004) 11, 187–193

www.nature.com/gt

RESEARCH ARTICLE

Dominant-negative mutant of c-Jun gene transfer: a novel therapeutic strategy for colorectal cancer

R Suto[1], K Tominaga[1], H Mizuguchi[2], E Sasaki[1], K Higuchi[1], S Kim[3], H Iwao[3] and T Arakawa[1]
[1]Department of Gastroenterology, Graduate School of Medicine, Osaka City University Medical School, Abeno-ku, Osaka, Japan; [2]Division of Cellular and Gene Therapy Products, National Institute of Health Sciences, Tokyo, Japan; and [3]Department of Pharmacology, Graduate School of Medicine, Osaka City University Medical School, Abeno-ku, Osaka, Japan

Activator protein-1 (AP-1), a transcription factor, is activated through many oncogenic signals. However, its biological role in colorectal cancer has not been fully elucidated. To investigate the role of AP-1 in colorectal cancer, we constructed an adenovirus-expressing TAM67, a dominant-negative mutant of c-Jun lacking the transactivation domain of wild c-Jun (DN-c-Jun), to inhibit endogenous AP-1. AP-1 DNA-binding activity was increased in colon cancer cells (HT-29 cells) by serum stimulation, followed by an increase in both [^{3}H]thymidine incorporation and cell number. Transfection of Ad-DN-c-Jun to HT-29 cells significantly inhibited serum-induced cell proliferation in vitro. As shown by flow cytometric analysis, DN-c-Jun significantly inhibited entrance into S phase after serum stimulation, thereby leading to G_1 arrest. In vivo transfection of Ad-DN-c-Jun into xenografted HT-29 cell tumors in nude mice significantly decreased tumor volume on day 21 after treatment. A change was associated with decrease in Ki-67 labeling index. These observations together showed that AP-1 is a critical modulator for proliferation and cell cycle of HT-29 cells. We obtained the first evidence that DN-c-Jun gene transfer exerted a significant antitumor effect on colon cancer both in vitro and in vivo. DN-c-Jun gene transfer may be a new candidate for treatment of colorectal cancer.
Gene Therapy (2004) **11**, 187–193. doi:10.1038/sj.gt.3302158

Keywords: *activator protein-1; c-Jun; colorectal cancer; gene therapy*

Introduction

Colorectal cancer, a significant cause of morbidity and mortality throughout the world, is due to the pathologic transformation of normal colonic epithelium to an adenomatous polyp and ultimately an invasive cancer. This multistep progression is associated with a number of recently characterized genetic alterations, including mutations of tumor-suppressor genes and proto-oncogenes, such as APC, K-ras, p53 or DCC.[1]

Tumor-suppressor gene replacement therapy with wild-type p53 has been developed as a mechanism-based therapy.[2,3] This therapy yielded antitumor effects in animal and cell culture experiments, but only partial effects in clinical trials. Other gene therapies for human colorectal cancer, such as enzyme/prodrug systems (HSVtk/ganciclovir; CD/5-fluorocytosine) and immune-gene therapy based on cytokine or tumor antigen expression to induce tumor immunity (eg CEA), have not yet improved prognosis.[4,5–7] Elucidation of the basic molecular mechanisms of colorectal cancer and determination of a novel target for gene therapy are thus required.

Activator protein-1 (AP-1) is one of the transcriptional factors related to multiple gene transcription of mitogenic signal cascades. AP-1 is expressed in most types of cells and is composed of hetero- and homodimers of the Jun and Fos families. As recently reviewed, AP-1 exerts diverse biological effects on proliferation, transformation, differentiation and apoptosis, depending on cell type and surrounding environment.[8] AP-1 proteins, mostly those belonging to the Jun group, have also been suggested to control cell life and death through their ability to regulate the expression and function of cell cycle regulators, such as Cyclin D1, p53, p21(cip1/waf1), p19(ARF) and p16.[8]

Although relatively little is known about the role of AP-1 activation in colorectal cancer growth, there are some reports indicating that AP-1 activation is related to colorectal cancer growth.[9–11] In contrast, other reports suggest that AP-1 is important for differentiation, apoptosis or resistance to therapy. Thus, the role of AP-1 in colorectal cancer has been controversial, and it remains to be determined whether AP-1 is essential for the multiplication of colorectal cancer.

In the present study, we constructed a recombinant adenovirus containing the dominant-negative mutant of c-Jun (Ad-DN-c-Jun) and infected it into colon cancer cells (HT-29) *in vitro* and *in vivo* to elucidate the roles of AP-1. We demonstrated that AP-1 was essential for colon cancer growth both *in vitro* and *in vivo*, and an important modulator of the cell cycle in HT-29 cells. We obtained the first evidence that DN-c-Jun gene transfer exhibited a significant antitumor effect on colon cancer both *in vitro* and *in vivo*, and demonstrated the possibility of AP-1-

Correspondence: Dr R Suto, Department of Gastroenterology, Graduate School of Medicine, Osaka City University Medical School, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan
Received 04 April 2003; accepted 02 September 2003

based gene therapy as a novel treatment of colorectal cancer.

Results

Induction of AP-1 DNA-binding activity in HT-29 cells

Electrophoretic mobility shift assay (EMSA) in Figure 1 shows that AP-1 DNA-binding activity in HT-29 cells increased the 1% serum stimulation in a time-dependent manner, and peaked 6 h later.

Efficiency of transfection of adenovirus in HT-29 cells

After infection at multiplicities of infection (MOI) of 3, 30 or 100 with Ad-LacZ, 30, 70 (Figure 2) or approximately 100% of the cells expressed LacZ gene (β-galactosidase-positive cells). These findings indicated that the number of cells expressing LacZ gene was dependent on MOI of Ad-LacZ, and that infection of MOI of 100 with Ad-LacZ was enough to transduce the gene to HT-29 cells.

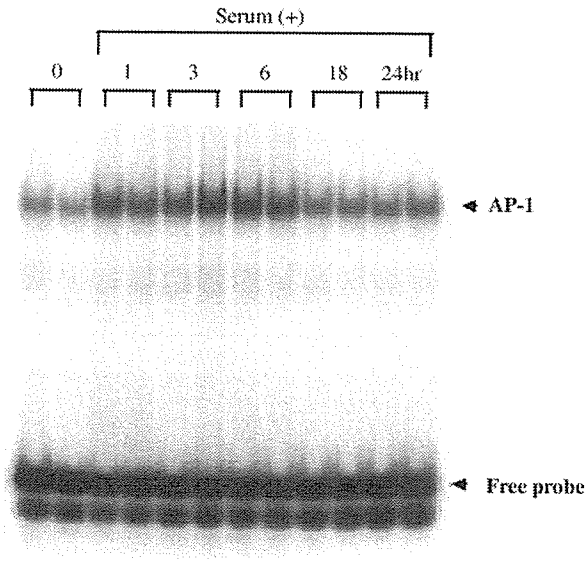

Figure 1 Time dependency of AP-1 DNA-binding activity in HT-29 cells stimulated by FBS (1%). FBS (1%) was added to culture medium for 0, 1, 3, 6, 18 or 24 h. EMSA was performed with double-stranded consensus oligonucleotide AP-1, as described in Materials and methods. Representative autoradiograms of AP-1-binding activitiy are shown.

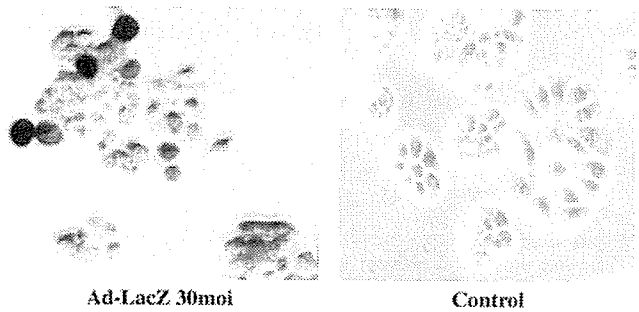

Figure 2 Histological estimation of transduction efficiency of adenovirus in HT-29 cells. HT-29 cells were seeded on six-well cultured plates and infected with Ad-LacZ at 30 MOI. At 48 h after infection, the cells were fixed and stained with X-gal. Blue staining of cell nuclei identified LacZ expression. At an MOI of 30, the percentage of cells expressing LacZ gene reached 70%.

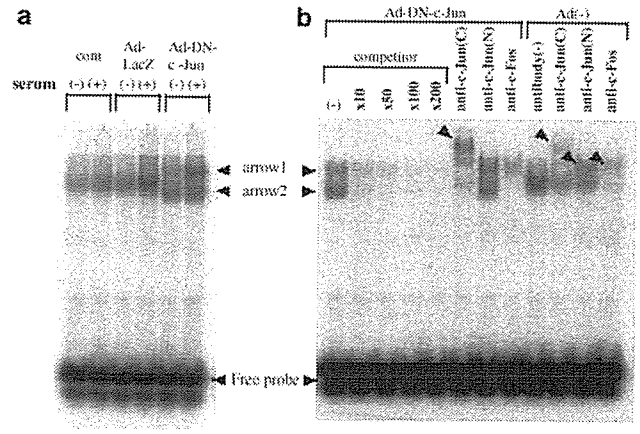

Figure 3 Characteristics of AP-1 DNA-binding activity in HT-29 cells infected with Ad-DN-c-Jun. (a) HT-29 cells were infected with Ad-DN-c-Jun or Ad-LacZ, and 48 h later, were stimulated with FBS (1%) for 6 h. Nuclear protein extracts were prepared from HT-29 cells, and then EMSA was performed. Representative autoradiograms of AP-1-binding activity are shown. (b) Competition assay for AP-1 was carried out in the presence of 10-, 50-, 100- or 200-fold molar excess of unlabeled AP-1 oligonucleotide (competitor). Super-shift assay was performed with rabbit polyclonal anti-c-Fos IgG (2 μg) (anti-c-Fos), anti-c-Jun IgG (sc-822X, 2 μg) (anti-c-Jun(N)) or anti-c-Jun IgG (sc-44X, 2 μg) (anti-c-Jun(C)). Open arrows indicate the AP-1 DNA-binding band due to Ad-DN-c-Jun; closed arrowheads indicate the super-shifted band.

Characteristics of AP-1 DNA-binding activity in HT-29 cells induced by Ad-DN-c-Jun infection

As shown in Figure 3a, infection with Ad-DN-c-Jun produced a new AP-1 DNA-binding activity (arrow 1) whose position was upper to that of the endogenous AP-1 band (arrow 2). On the other hand, control adenovirus did not change the pattern of AP-1 DNA-binding activity. As shown in Figure 3b, the bands due to Ad-DN-c-Jun (arrow 1) and endogenous AP-1 band (arrow 2) were found to indicate specific binding for AP-1, since the addition of unlabeled AP-1 oligonucleotide resulted in a decrease in the formation of AP-1 complex in a dose-dependent manner. As shown by supershift analysis in Figure 3b, endogenous AP-1 (arrow 2) was supershifted with anti-c-Jun (sc-822X) antibody recognizing the transactivation domain of wild c-Jun, anti-c-Jun antibody (sc-44X) recognizing the conserved DNA-binding domain of wild c-Jun, or anti-c-Fos antibody. On the other hand, Ad-DN-c-Jun-induced AP-1 DNA-binding activity (arrow 1) was supershifted with anti-c-Jun antibody (sc-44X) but not with anti-c-Fos antibody or anti-c-Jun antibody (sc-822X). Previous studies have already shown that DN-c-Jun gene transfer effectively inhibited the transcriptional activity of AP-1 via the increase in DN-c-Jun-induced AP-1 DNA-binding activity.[12–14]

Effect of Ad-DN-c-Jun on HT-29 cell proliferation in vitro

Infection with Ad-DN-c-Jun at an MOI of 30 100, or 200 to HT-29 cells diminished serum-induced increase in [^{3}H]thymidine incorporation in a dose-dependent manner, while Ad-LacZ at none of the tested MOI affected it (Figure 4a). Ad-DN-c-Jun at an MOI of 100 also inhibited the serum-induced increase in cell number on days 3 and 5.

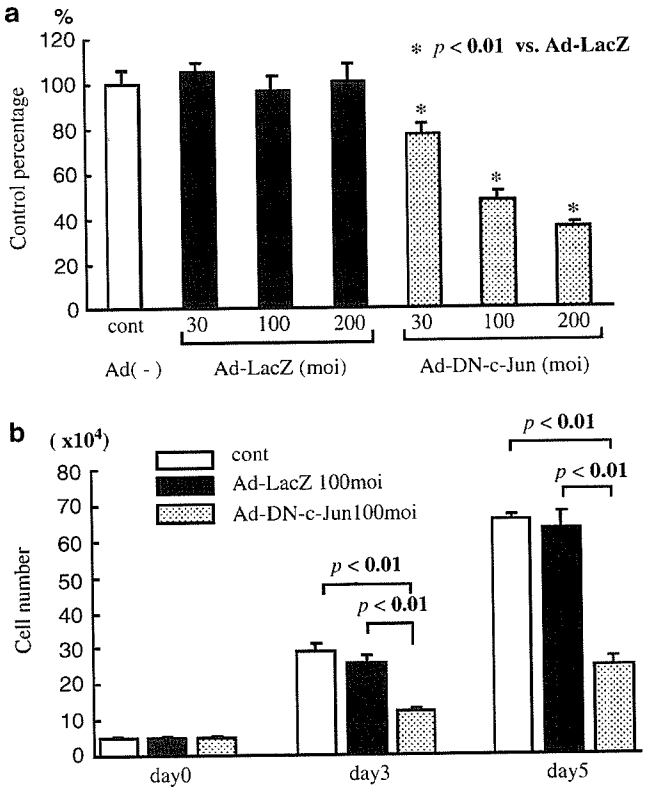

*Figure 4 Effects of Ad-DN-c-Jun on serum-induced increase in [³H]thymidine incorporation (a) and cell number (b). (a) Quiescent cells, infected with Ad-DN-c-Jun (hatched column) or Ad-LacZ (closed column) at MOI of 30, 100 or 200, were stimulated with FBS (1%, 19 h) and further incubated with [³H]thymidine (0.5 μCi/ml) for 5 h. Each value represents the mean±s.e.m. (n=6). *P<0.01 versus Ad-LacZ. (b) HT-29 cells, infected with Ad-DN-c-Jun (hatched column) or Ad-LacZ (closed column) at 100 MOI, were treated with 1% FBS for 3 or 5 days and trypsinized. Viable cell number was counted by a hemocytometer. Each value represents the mean±s.e.m. (n=6). *P<0.01 versus PBS or Ad-LacZ.*

Ad-DN-c-Jun inhibited serum-induced HT-29 cell proliferation through G1 arrest

A flow cytometric analysis was performed to evaluate the underlying mechanism of inhibition by Ad-DN-c-Jun of HT-29 cell proliferation. HT-29 cells infected with each adenovirus at an MOI of 100 were stained with propidium iodide (PI). As shown in Figure 5a, Ad-DN-c-Jun significantly inhibited serum-induced S-phase entrance by cells, and increased the percentage of cells in G_0/G_1 phase after serum stimulation, whereas Ad-LacZ did not affect it (Figure 5b).

In vivo gene transfer into xenografted tumors and inhibition of tumor growth

After direct injection of Ad-DN-c-Jun into xenografted tumors *in vivo*, the expression of the transferred mutant c-Jun was demonstrated by EMSA and Western blot analysis. As shown in Figure 6a and b, Ad-DN-c-Jun infection *in vivo* generated a significant amount of AP-1-binding activity with a higher position than that of the endogenous AP-1 band, as shown by EMSA, and produced a significant amount of DN-c-Jun protein, as shown by Western blot analysis.

To assess the effect of Ad-DN-c-Jun on xenografted tumor growth, Ad-DN-c-Jun (3×10^8 plaque-forming

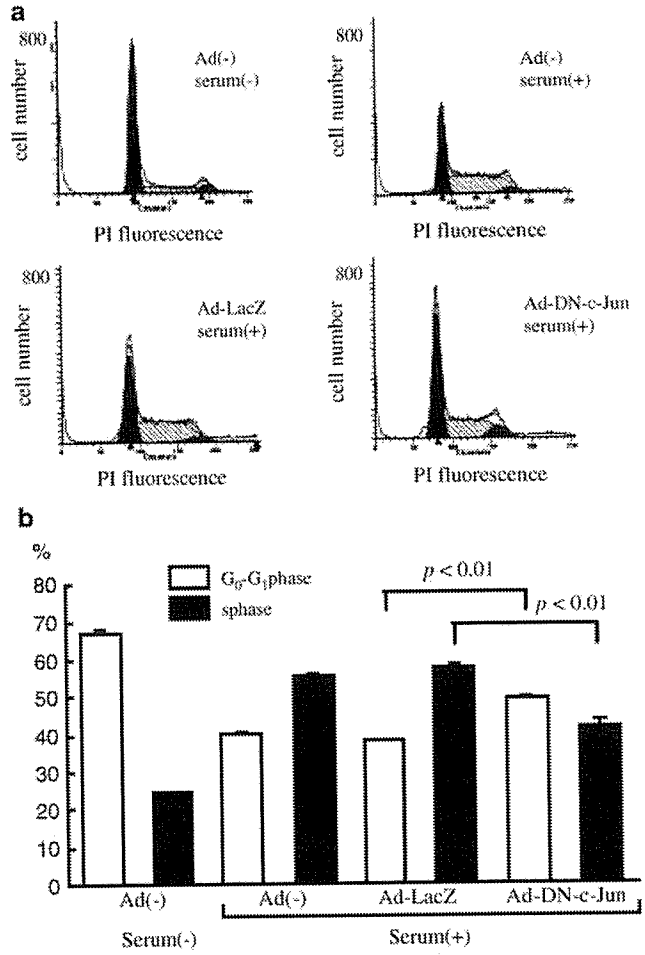

*Figure 5 Effects of Ad-DN-c-Jun on cell cycle of HT-29 cells. (a) Representative DNA histogram of PI fluorescence in cells, as assessed by FACS flow cytometry. (b) Ratio of cells in S phase (closed column) or G_0–G_1 phase (open column) of the cell cycle was measured by an FACS flow cytometer, and analyzed by ModFitLT software. Each value represents the mean±s.e.m. (n=6). *P<0.01 versus Ad-LacZ.*

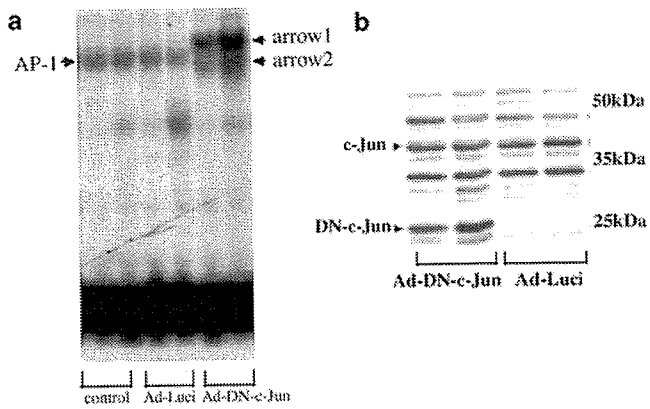

Figure 6 In vivo expression of increased AP-1-binding activity (a) and DN-c-Jun protein (b) induced by subcutaneous Ad-DN-c-Jun injection into xenografted HT-29 cell tumors. Ad-DN-c-Jun and Ad-Luci were injected into xenografted HT-29 cell tumors in nude mice three times (3×10^8 p.f.u./injection). At 48 h after final injection, nuclear protein extracts were prepared for EMSA assay (a) Arrow 1 indicate the AP-1 DNA-binding band due to Ad-DN-c-Jun; arrow 2 indicate the AP-1 DNA-binding band due to endogenous AP-1. In vivo *expression of c-Jun protein and DN-c-Jun protein due to Ad-DN-c-Jun infection in xenografted tumors was determined by immnoblot analysis (b).*

unit (pfu)), Ad-Luciferase (Ad-Luci) or phosphate-buffered saline (PBS) (vehicle: as a control) was subcutaneously injected into established xenografted tumors. On day 21, the volume of tumors treated with Ad-DN-c-Jun was smaller than that of those treated with Ad-Luci or PBS (535 ± 26 *versus* 990 ± 265 or 1296 ± 101 mm^3; $P<0.05$). Although there was a slight tendency of reduction of tumor volume by control adenovirus, no statistical difference in the tumor volume was found between both groups treated with control adenovirus and PBS. Thus, Ad-DN-c-Jun significantly inhibited the growth of xenografted HT-29 tumors (Figure 7). In this *in vivo* study, the administration of DN-c-Jun did not affect animal mortality, body weight or appetite (data not shown).

Evaluation of Ki-67 immunostaining of HT-29 cells in xenografted tumors

Immunoreactive Ki-67 cells were found in the xenografted tumors, and Ki-67 labeling index was measured by histological findings according to the described methods (Figure 8). Ad-DN-c-Jun significantly decreased the labeling index compared with Ad-Luci (38 ± 1.0 *versus* $50.6 \pm 1.5\%$; $P<0.01$) (Figure 9).

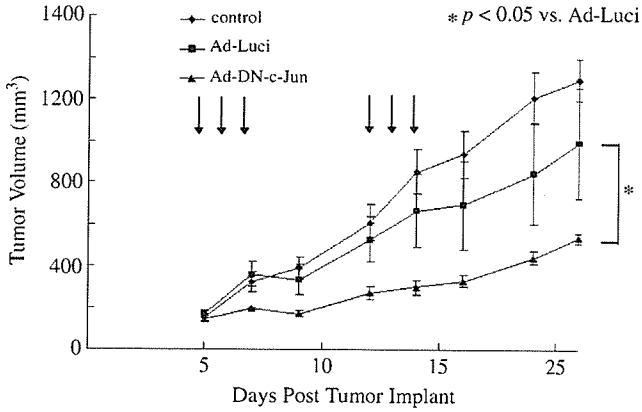

*Figure 7 Effect of subcutaneous injection of Ad-DN-c-Jun on volume of xenografted HT-29 cell tumors. Established tumors (150 mm^3) were randomized into three groups in a blind manner to be treated with Ad-DN-c-Jun (3×10^8 p.f.u.), Ad-Luci (3×10^8 p.f.u.) or PBS (vehicle control). Each treatment was performed from days 5 to 7 and from days 12 to 14. Tumor size was measured in two dimensions and tumor volumes were calculated as described above. Each value represents the mean ± s.e.m. ($n=6$). *$P<0.05$ versus Ad-Luci.*

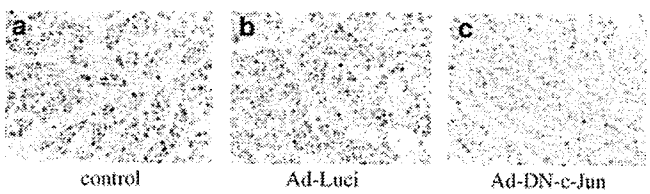

Figure 8 Immunostaining for Ki-67 in cancer cells of xenografted HT-29 cell tumors. Representative sections of xenografted tumors treated with PBS (a), Ad-Luci (b) or Ad-DN-c-Jun (c). Immnoreactivity for Ki-67 of cancer cells in xenografted tumors treated with Ad-DN-c-Jun (c) was weaker than that in those treated with PBS (a) or Ad-Luci (b).

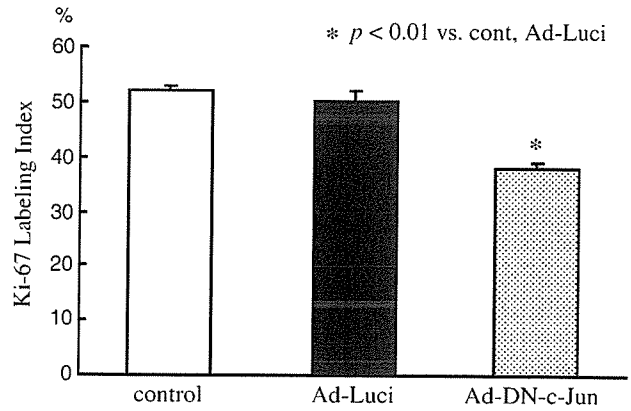

*Figure 9 Effects of Ad-DN-c-Jun on Ki-67 labeling index in xenografted HT-29 cell tumors. Xenografted tumors were treated with PBS (a), Ad-Luci (b), or Ad-DN-c-Jun (c). Assessment of positive cells was performed using light microscopy at × 400 magnification, and the positive ratio was calculated as the percentage with >2000 cancer cells per tumor. Each value represents the mean ± s.e.m. ($n=6$). *$P<0.01$ versus PBS or Ad-Luci.*

Discussion

AP-1, a nuclear protein, is an important transcription factor regulating DNA transcription and gene expression. The mammalian AP-1 proteins are homo- and heterodimers composed of basic region-leucine zipper proteins including those of the Jun families (c-Jun, JunB and JunD) and Fos families (c-Fos, FosB, Fra-1 and Fra-2).[8] A variety of stimuli, including serum, growth factors and cytokines, induce AP-1 activity, and AP-1 activation is also reported to occur in cancer cells with Ras mutation.[15] Accumulating evidence suggests that AP-1 activity plays a role in cancer growth control.[16] Many reports suggest that elevated concentrations of fecal bile acids are associated with the promotion of colon cancer.[17–19] In these reports, Hirano *et al*[9] demonstrated that AP-1 is associated with the promotion of colon cancer induced by bile acids. Interestingly, however, other transcription factors such as NF-kappa B, Sp1 or ATF/CREB was not affected by bile acid. Thus, they suggested that AP-1 but not other transcription factors may be specific for colon cancer induction by bile acids. Glinghammar *et al*[10] also suggested that diluted lipid extracts from fecal water samples significantly induced AP-1-dependent gene transcription and induce cell proliferation. On the other hand, it has been proposed that AP-1 affects differentiation rather than multiplication, since AP-1 is highly activated during late progression of colon cancer.[20] Mandal *et al*[21] suggested that apoptosis triggered by butyric acid involves transcriptional stimulation of the Bax gene via the activation of the JNK/AP-1 pathway in colonic epithelial cells. Furthermore, Yao *et al*[22] reported the induction of AP-1 activity in human HT29 cells exposed to hypoxic conditions that was considered to cause the resistance to anticancer drugs. However, the biological and physiological functions of AP-1 in relation to the growth of cancer, in particular that of colorectal cancer, are not fully understood, and there is no report concerning direct blockade of AP-1 in colorectal cancer since no specific and potent pharmacological inhibitor of AP-1 is available.

Recently, Liu *et al*[14] tested the effects of the same DN-c-Jun as we employed here on the growth of MCF7 breast cancer cells, using cells stably transfected with DN-c-Jun under the control of a doxycycline-inducible promoter, and found that growth of MCF7 cells injected into nude mice was suppressed by AP-1 blockade with DN-c-Jun. However, in their study, the effect of *in vivo* gene transfer of DN-c-Jun on breast cancer cells has not been investigated. These findings encouraged us to examine the effect of direct *in vivo* AP-1 blockade on colorectal cancer growth with adenoviral vector containing DN-c-Jun.

In the present *in vitro* study, AP-1-binding activity in HT-29 cells was increased by serum stimulation. DN-c-Jun gene transfer, which was effectively transferred into HT-29 cells and markedly suppressed endogenous AP-1-binding activity, inhibited the proliferation of HT-29 cells *in vitro*, as shown by decrease in not only [^{3}H]thymidine incorporation but also cell number. These results showed that AP-1 played a role in colon cancer proliferation *in vitro*. Recent reports indicate that c-Jun also plays a role in controlling the cell cycle.[23–25] Therefore, in this study, we also evaluated the effect of DN-c-Jun on the cell cycle of HT-29 cells, using flow cytometric analysis. Ad-DN-c-Jun, but not Ad-LacZ, significantly suppressed entry into S phase and caused arrest in early G_1 phase. Thus, our study showed that inhibition of HT-29 cell growth by DN-c-Jun is mediated by suppression of S-phase entry.

To examine whether the above-mentioned antiproliferative effect of DN-c-Jun on HT-29 cells *in vitro* occurs *in vivo*, we further examined the efficacy of DN-c-Jun gene transfer on colon cancer *in vivo*. In this experiment, with adenoviral vector, we could successfully express DN-c-Jun protein in xenografted tumors of HT-29 cells, and repeated treatment with Ad-DN-c-Jun of xenografted tumors of HT-29 cells significantly decreased tumor volume compared with controls. Furthermore, this inhibition of tumor growth by DN-c-Jun was associated with decrease in Ki-67 labeling index. However, there was a tendency of reduction of tumor volume by control adenovirus. We performed the same experiments three times and obtained the reproducible results. Many reports using adenovirus for *in vivo* studies suggested that adenovirus itself has a faint nonspecific cytotoxic effect.[26,27] Importantly, however, no statistically significant decrease was found in the growth curves of control adenovirus compared to that with PBS in the present study. In addition, a significant effect by Ad-DN-c-Jun on the growth curves was found compared to control adenovirus. All these findings suggest that the observed results of control adenovirus for tumor volume reduction must be due to the nonspecific responses derived from Ad vector.

It is also unclear whether c-Jun has the relation to the cell proliferation of normal colon cells in this study. However, the activation of AP-1 and expression of c-Jun are more increased in colon cancer cells than in normal cells.[28] Inhibition of c-Jun by Ad-DN-c-Jun should be more effective for proliferation of colon cancer cells than nonmalignant cells. On the other hand, adverse side effects of Ad-DN-c-Jun infection such as mortality, body weight loss and appetite loss were not apparent in the present study. These observations suggest that DN-c-Jun is a promising target for gene therapy of colon cancer. Generally, it is important that more effective local–regional delivery system of target gene should be established not only to enhance the efficiency for tumor reduction, but also to avoid the nonspecific responses. Further investigation will be needed.

In conclusion, we obtained the first evidence that specific blockade of AP-1 by a dominant-negative mutant of c-Jun gene transfer prevented colon cancer growth both *in vitro* and *in vivo*. Our study provides new insights into the molecular mechanisms of colorectal cancer. AP-1 appears to be a novel target for gene therapy of colorectal cancer.

Materials and methods

Cells and culture conditions

The human colon adenocarcinoma cell line HT-29 was purchased from American Type Culture Collection. Cells were constantly grown as monolayers in McCoy's 5A medium, supplemented with 10% heat-inactivate fetal bovine serum (FBS) in a 5% CO_2 and 95% incubator atmosphere at 37°C and subcultured every 7 days.

Construction of recombinant adenovirus containing the dominant-negative mutant of c-Jun (DN-c-Jun)

The dominant-negative mutant of c-Jun (DN-c-Jun), TAM67, was generated by removal of the transactivational domain of amino acids from 3 to 122 of wild-type c-Jun by polymerase chain reaction.[29] DN-c-Jun has the DNA-binding domain of wild-type c-Jun. Recombinant adenovirus containing DN-c-Jun was prepared as described previously. Recombinant adenoviruses containing the bacterial β-galactosidase gene (Ad-LacZ) and luciferase gene (Ad-Luci) were also constructed as a negative control for Ad-DN-c-Jun. The titer of the virus was determined by limiting dilution in 293 cells and expressed in pfu's.

Adenovirus-mediated gene transfer to HT-29 cells in vitro

In vitro gene transfer to HT-29 cells was carried out by incubation with the adenoviral vector with MOI of 30–200 in McCoy's 5A medium for 1 h, followed by addition of 1% FBS overnight at 37°C in a 5% CO_2 and 95% atmosphere. HT-29 cells were then made quiescent for 24 h before the experiments.

LacZ gene transfer by Ad-LacZ in vitro

We evaluated LacZ gene expression by the X-gal staining method as described previously.[30] Briefly, 48 h after infection, HT-29 cells were washed with PBS and fixed in 2% (vol/vol) glutaraldehyde in PBS, pH 7.4 for 5 min at 4°C. The cells were then washed and stained with X-gal solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-b-gal-actopyranoside, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ in PBS, pH6.5) for 12–18 h at 37°C. Blue staining of cell nuclei identified β-galactosidase expression.

Measurement of DNA synthesis and cell proliferation

HT-29 cells in six-well plates were stimulated by 1% serum for 19 h and further incubated with [^{3}H]thymidine (0.5 μCi/ml) for 5 h. After washing with PBS three times, the cells were detached from culture plates by trypsin/EDTA. Then radioactivities of each sample were

measured by a MicroBeta® TriLux liquid scintillation counter (Amersham Pharmacia Biotech, UK). For assay of cell growth, HT-29 cells in six-well cultured plates were stimulated by 1% FBS for 3 or 5 days and harvested by trypsin/EDTA. Number of viable cell number was counted by a hemocytometer.

Western blot analysis

Western blot analysis was carried out as described in detail.[31] *Ex vivo* tissue samples were immediately frozen at −80°C after dissection. Frozen tissue was homogenized by thawing the samples in an appropriate volume of the lysis buffer, followed by disruption with a polytron homogenizer. After centrifugation of the sample, supernatants were assessed for protein expression of c-Jun and DN-c-Jun. Antibodies to c-Jun (sc-44; Santa Cruz, CA, USA) were used for the detection of DN-c-Jun protein.

Electrophoretic mobility shift assay

To detect DNA protein-binding reaction, EMSA) was performed as described in detail.[32] The double-stranded consensus oligonucleotide of the AP-1-binding probe, 5′-CGCTTGATGAGTCAGCCGGAA-3′, was synthesized and end labeled with [^{32}P]dATP. Nuclear protein extracts were prepared from HT-29 cells with or without serum stimulation (1%), infected with Ad-lacZ or Ad-DN-c-Jun for 48 h before serum stimulation. Supershift assay was performed with rabbit polyclonal anti-c-Fos IgG (anti-c-Fos), anti-c-Jun IgG (sc-44X) (anti-c-Jun (C)) recognizing the conserved DNA-binding domain of wild-type c-Jun, or anti-c-Jun IgG (sc-822X) (anti-c-Jun (N)) recognizing the amino acids from 56 to 69 (transactivation domain) of wild-type c-Jun (all from Santa Cruz)

Flow cytometric analysis

Cells were trypsinized, washed twice with PBS, and fixed in 70% ethanol at −20°C. Before measurement, fixed cells were centrifuged at 3000 r.p.m. for 5 min and resuspended in dye combination containing 50 μg/ml RNase and 25 μg/ml PI (Sigma, St Louis, MO, USA) for DNA staining. Cellular DNA content was assessed by an FACS flow cytometer (Becton Dickinson, Mountain View, CA, USA). DNA histograms of cell distribution were obtained using ModFitLT software (Verita).

Production of xenografted tumor of HT-29 cells and its treatment with DN-c-Jun

HT-29 cells (1×10^6 cells) were inoculated subcutaneously into the hind paws of BALB/cA Jcl nu/nu female mice (4–5 weeks old; CLEA Japan, Inc., Osaka, Japan). After tumor size reached a mean volume of 150 mm^3, tumors were randomized into three groups in a blind manner; group 1, Ad-DN-c-Jun (3×10^8 p.f.u.); group 2, Ad-Luciferase (Ad-Luci) (3×10^8 p.f.u.); group 3, control vehicle (PBS) (n=6). Direct injection was performed three times a week, with a total of six sessions of treatment. Each injection of purified virus was diluted in a total volume of 30 μl PBS and administered in a single pass with a 30-gauge needle, using gentle, constant infusion pressure with diethylether. Tumors were measured three times a week and immediately before each treatment, and tumor size was estimated using the following formula: length (mm) × width (mm) × width (mm)/2. All experimental procedures were approved by the Animal Care Committee of Osaka City University Medical School.

Immunohistochemical staining

Staining for Ki-67 was performed using rabbit polyclonal antihuman Ki-67 antigen (Dako Japan Co., Kyoto, Japan). A measure of 6-μm-thick serial cryostat sections were mounted on sialynized slide (Dako Japan Co). The sections were heated at a temperature of 95°C for 45 min with a citrate acid buffer (pH 6.0) for the purpose of antigen retrieval. The primary anti-Ki-67 antibody was used at a dilution of 1:1500 and the slides were incubated in a humidified chamber at room temperature for overnight. Immunohistochemical staining was performed with the streptavidin–biotin peroxidase method (LSAB2 Kit, Dako Japan Co). Counterstaining was performed with hematoxylin. Assessment of Ki-67-positive cells was performed using light microscopy at × 400 magnification, and labeling index was defined as the Ki-67-positive ratio determined by calculating the percentage with >2000 cancer cells per tumor.

Statistical analysis

All values are presented as means ± s.e.m. Statistical significance was determined with one-way analysis of variance and Duncan multiple range test. Differences were considered statistically significant at a value of $P < 0.05$.

References

1 Gryfe R *et al*. Molecular biology of colorectal cancer. *Curr Probl Cancer* 1997; **21**: 233–300.
2 Baker SJ *et al*. Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science* 1990; **249**: 912–915.
3 Harris MP *et al*. Adenovirus-mediated p53 gene transfer inhibits growth of human tumor cells expressing mutant p53 protein. *Cancer Gene Ther* 1996; **3**: 121–130.
4 Zwacka RM, Dunlop MG. Gene therapy for colon cancer. *Hematol Oncol Clin N Am* 1998; **12**: 595–615.
5 Greco O, Dachs GU. Gene directed enzyme/prodrug therapy of cancer: historical appraisal and future prospectives. *J Cell Physiol* 2001; **187**: 22–36.
6 Palmer DH, Chen MJ, Kerr DJ. Gene therapy for colorectal cancer. *Br Med Bull* 2002; **64**: 201–225.
7 Fillat C, Carrio M, Cascante A, Sangro B. Suicide gene therapy mediated by the herpes simplex virus thymidine kinase gene/ganciclovir system: fifteen years of application. *Curr Gene Ther* 2003; **3**: 13–26.
8 Shaulian E, Karin M. AP-1 in cell proliferation and survival. *Oncogene* 2001; **20**: 2390–2400.
9 Hirano F *et al*. Induction of the transcription factor AP-1 in cultured human colon adenocarcinoma cells following exposure to bile acids. *Carcinogenesis* 1996; **17**: 427–433.
10 Glinghammar B, Holmberg K, Rafter J. Effects of colonic lumenal components on AP-1-dependent gene transcription in cultured human colon carcinoma cells. *Carcinogenesis* 1999; **20**: 969–976.
11 Guo YS, Hellmich MR, Wen XD, Townsend Jr CM. Activator protein-1 transcription factor mediates bombesin-stimulated cyclooxygenase-2 expression in intestinal epithelial cells. *J Biol Chem* 2001; **276**: 22941–22947.
12 Yasumoto H *et al*. Dominant negative c-jun gene transfer inhibits vascular smooth muscle cell proliferation and neointimal hyperplasia in rats. *Gene Ther* 2001; **8**: 1682–1689.

13 Zhan Y *et al.* Effects of dominant-negative c-Jun on platelet-derived growth factor-induced vascular smooth muscle cell proliferation. *Arterioscler Thromb Vasc Biol* 2002; **22**: 82–88.
14 Liu Y *et al.* Inhibition of AP-1 transcription factor causes blockade of multiple signal transduction pathways and inhibits breast cancer growth. *Oncogene* 2002; **21**: 7680–7689.
15 Miyanishi K *et al.* Glutathione *S*-transferase-pi overexpression is closely associated with K-ras mutation during human colon carcinogenesis. *Gastroenterology* 2001; **121**: 865–874.
16 Angel P, Karin M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim Biophys Acta* 1991; **1072**: 129–157.
17 Ochsenkuhn T *et al.* Colonic mucosal proliferation is related to serum deoxycholic acid levels. *Cancer* 1999; **85**: 1664–1669.
18 Qiao D, Chen W, Stratagoules ED, Martinez JD. Bile acid-induced activation of activator protein-1 requires both extracellular signal-regulated kinase and protein kinase C signaling. *J Biol Chem* 2000; **275**: 15090–15098.
19 Milovic V *et al.* Deoxycholic acid stimulates migration in colon cancer cells. *Eur J Gastroenterol Hepatol* 2001; **13**: 945–949.
20 Licato LL *et al. In vivo* activation of mitogen-activated protein kinases in rat intestinal neoplasia. *Gastroenterology* 1997; **113**: 1589–1598.
21 Mandal M *et al.* Butyric acid induces apoptosis by up-regulating Bax expression via stimulation of the c-Jun N-terminal kinase/activation protein-1 pathway in human colon cancer cells. *Gastroenterology* 2001; **120**: 71–78.
22 Yao KS, Xanthoudakis S, Curran T, O'Dwyer PJ. Activation of AP-1 and of a nuclear redox factor, Ref-1, in the response of HT29 colon cancer cells to hypoxia. *Mol Cell Biol* 1994; **14**: 5997–6003.
23 Shaulian E *et al.* The mammalian UV response: c-Jun induction is required for exit from p53-imposed growth arrest. *Cell* 2000; **103**: 897–907.
24 Schreiber M *et al.* Control of cell cycle progression by c-Jun is p53 dependent. *Genes Dev* 1999; **13**: 607–619.
25 Smith MJ, Prochownik EV. Inhibition of c-jun causes reversible proliferative arrest and withdrawal from the cell cycle. *Blood* 1992; **79**: 2107–2115.
26 Yang Y, Su Q, Wilson JM. Role of viral antigens in destructive cellular immune responses to adenovirus vector-transduced cells in mouse lungs. *J Virol* 1996; **70**: 7209–7212.
27 Lusky M *et al. In vitro* and *in vivo* biology of recombinant adenovirus vectors with E1, E1/E2A, or E1/E4 deleted. *J Virol* 1998; **72**: 2022–2032.
28 Magrisso IJ *et al.* Immunohistochemical detection of RAS, JUN, FOS, and p53 oncoprotein expression in human colorectal adenomas and carcinomas. *Lab Invest* 1993; **69**: 674–681.
29 Brown PH, Chen TK, Birrer MJ. Mechanism of action of a dominant-negative mutant of c-Jun. *Oncogene* 1994; **9**: 791–799.
30 Kanegae Y *et al.* Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. *Nucleic Acids Res* 1995; **23**: 3816–3821.
31 Kim S *et al.* Angiotensin blockade inhibits activation of mitogen-activated protein kinases in rat balloon-injured artery. *Circulation* 1998; **97**: 1731–1737.
32 Yano M *et al.* Differential activation of cardiac c-jun amino-terminal kinase and extracellular signal-regulated kinase in angiotensin II-mediated hypertension. *Circ Res* 1998; **83**: 752–760.

—Reviews—

免疫細胞の体内動態制御に基づいた癌免疫療法の最適化

岡田直貴,* 中川晋作

Optimization of Cancer Immunotherapy by Controlling Immune Cell Trafficking and Biodistribution

Naoki OKADA,* and Shinsaku NAKAGAWA
Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita City 565-0871, Japan

(Received May 31, 2006)

An immunosurveillance system for tumor-associated antigens (TAAs) plays an important role in the elimination of cancer cells during the initial stage. Although cancer immunotherapy targeting TAAs has progressed steadily with the development of various vaccine strategies, excellent therapeutic efficacy, as evidenced by marked tumor regression and complete response, has not been reported in a clinical setting to date. To improve the therapeutic effects of cancer immunotherapy, we are attempting to establish an innovative concept, the "cell delivery system," capable of better controlling the trafficking and biodistribution of immune cells by applying chemokine-chemokine receptor coupling, which regulates leukocytic migration and infiltration of local sites in the living body. This review introduces our approaches that employ an Arg-Gly-Asp (RGD) fiber-mutant adenovirus vector encoding the chemokine or chemokine receptor gene in cancer immunotherapy.

Key words——cell delivery system; chemokine-chemokine receptor coupling; cancer immunotherapy; adenovirus vector; tumor-infiltrating immune cell; dendritic cell

1. はじめに

腫瘍免疫学の進展によって，遺伝子変異を蓄積した癌細胞が正常細胞とは質的・量的に異なる腫瘍関連抗原（TAA）と呼ばれる分子を発現しており，TAA に対する免疫監視機構が初期癌細胞の排除という生体の恒常性維持に重要な役割を果たしていることが明らかとされた．これに伴って，TAA を標的とした免疫療法が次世代の癌治療戦略として世界的に活発に研究されており，基礎研究と臨床研究の連携によって着実な進歩を遂げている．しかしながら，腫瘍の完全退縮あるいは病状の寛解といった顕著な治療効果を報告した臨床研究はほとんど認められないのが現状であり，この一因として，従来の癌免疫療法研究の多くが腫瘍免疫の存在実証とその効率的な誘導法の探索を中心に進められ，効果的な治療を達成する上で必要な免疫エフェクター細胞の腫瘍組織への集積性改善という側面がいまだ十分に検討されていないことが挙げられる．つまり，癌細胞を殺傷する能力を有する免疫エフェクター細胞が例え患者体内に誘導されたとしても，それらが十分に腫瘍組織に移行・浸潤して癌細胞と接触できなければ，癌免疫療法の有効性は大きく制限されてしまうと考えられる．

また近年，免疫系を構成する細胞の機能解明が急速に進んだことによって，樹状細胞（DC）が T 細胞依存性の獲得免疫応答の始動及び増幅，さらには自然免疫応答の制御をも含めて，免疫監視機構を多方面から統御する抗原提示細胞であることが明らかとされた．このような免疫学的特性に基づいて，TAA を導入した DC を "nature's adjuvant" として利用する細胞免疫療法が有望な新規癌治療戦略として精力的に研究されており，DC の腫瘍免疫誘導能を最大限に発揮させる方法論の探索が進められている．[1] 生体に投与した TAA 導入 DC ワクチンは，投与部位から所属リンパ組織へ移行することによっ

大阪大学大学院薬学研究科薬剤学分野（〒565-0871 吹田市山田丘 1-6)
*e-mail: okada@phs.osaka-u.ac.jp
本総説は，日本薬学会第 126 年会シンポジウム S33 で発表したものを中心に記述したものである．

て始めてT細胞への抗原提示・感作を行い一連の初期免疫応答を惹起する．すなわち，患者に投与したTAA導入DCワクチンのリンパ組織集積性が，DC免疫療法の治療効果を規定する要因の1つとして考えられる．しかし現在のDC免疫療法では，投与部位からリンパ組織に移行させるための最適なDCコンディショニングについて十分な検討がなされていないため，投与DCワクチンのうちリンパ組織に集積するものはわずか0.1—1%程度とされている.[2—4] したがって，TAA導入DCワクチンに高いリンパ組織移行能を付与することができれば，リンパ組織における免疫エフェクター細胞の活性化を増強することが可能であり，ひいてはDC免疫療法の有効性を飛躍的に改善できるものと期待される．

以上のような観点から筆者らは，癌免疫療法の有効性改善に貢献するアプローチとして，“Cell Delivery System”ともいうべき新たなコンセプトに基づいた免疫細胞の体内動態制御法の構築を試みている．本稿では，ケモカイン—ケモカインレセプター連関を応用した1）免疫エフェクター細胞の腫瘍集積性，並びに2）DCワクチンのリンパ組織集積性，を増強し得る方法論を紹介するとともに，これらCell Delivery Systemの癌免疫療法における有用性について概説する．

2. ケモカイン—ケモカインレセプター連関

癌免疫療法の有効性及び安全性を確保・向上するためには，生体内の免疫細胞あるいは細胞医薬として生体に投与（移入）する免疫細胞を「必要なときに，必要な場所に，必要な量」送達できるCell Delivery Systemの確立が必要とされる．しかし，生体を構成する約60兆個の細胞の秩序を保った移動・分布・配列等を制御する巧妙な仕組みに関する知見はいまだ乏しく，Cell Delivery Systemの構築へと展開できる基礎情報並びに基盤技術は限られている．現時点でCell Delivery Systemへの応用に最も利用価値の高い生命現象は，ケモカイン-ケモカインレセプター連関に基づいた免疫細胞の局所への遊走・浸潤制御機構であろう．

ケモカインは8—14 kDa程度の塩基性・ヘパリン結合性分泌タンパク群であり，種々の細胞接着分子と協調して炎症反応やリンパ球のホーミングを制御している（Fig. 1）.[5] 現在，ヒトでは約50種類のケモカインが同定されており，それらは保存された4つのシステイン残基のうちN末端の2個のシステインの位置からCケモカイン，CCケモカイン，CXCケモカイン，及びCX_3Cケモカインの4つのサブグループに分類されるスーパーファミリーを形成している.[6] また，すべてのケモカインは7回膜貫通Gタンパク質共役型レセプターを介して作用

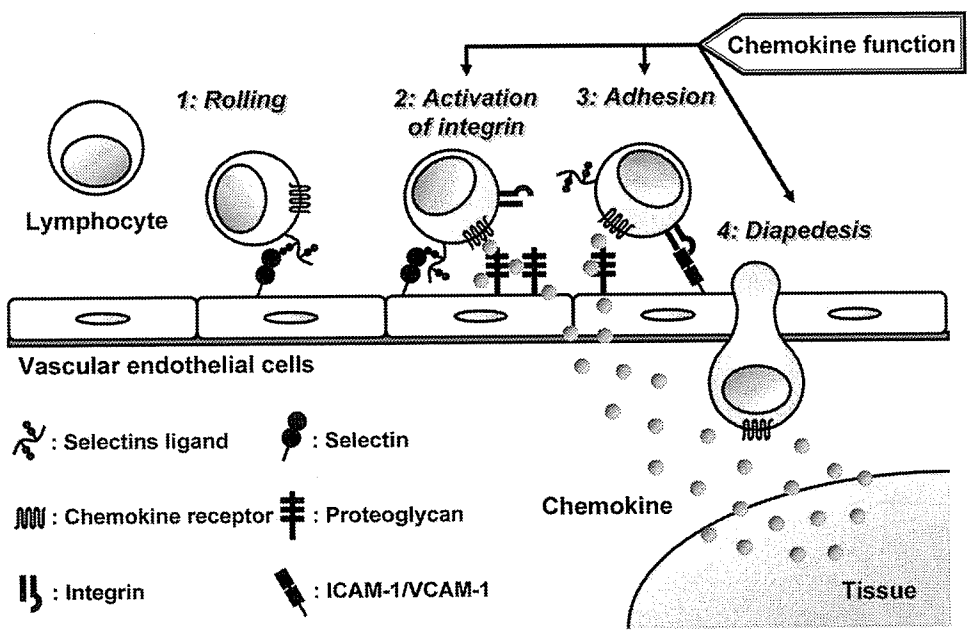

Fig. 1. Schematic Representation of Chemokine Functions in Tissue Infiltration of Lymphocyte

し，同定されている約20種類のケモカインレセプターもスーパーファミリーを構築している.[7,8] ケモカインは当初，好中球や単球を遊走させるサイトカインの一群として発見され，主に炎症での役割が研究されてきた．これら炎症性ケモカインに対して，1990年代後半より，バイオインフォマティクスを駆使してESTデータベースを検索するという手法によって新しいケモカインが次々と発見され，リンパ球やDCなどを標的細胞とする免疫系ケモカインの存在が明らかとなった（Table 1）．これによって，免疫細胞の生体内での移動や局在の制御機構に関する理解が急速に進展するとともに，ケモカイン—ケモカインレセプター連関を応用することによって免疫細胞の体内動態・体内分布を制御し得る新たな癌免疫療法の開発に足掛かりができた.

3. RGDファイバーミュータントアデノウイルス（AdRGD）ベクター

ケモカイン—ケモカインレセプター連関を利用したCell Delivery Systemを実現するためには，生体内局所あるいは生体に投与する細胞医薬にケモカインやケモカインレセプターを豊富かつ持続的に発現させる必要があり，遺伝子導入技術はそれらを満足する有力な手段である．アデノウイルス（Ad）ベクターは，現存する遺伝子導入ベクターの中で最も高い遺伝子導入効率を誇り，かつ高力価のベクター調製が容易であることから *in vivo* 直接投与への応

Table 1. Chemokine-Chemokine Receptor Coupling in Immune Cell Trafficking

Immune cell subset	Chemokine receptor expression	Corresponding chemokines	Immune cell subset	Chemokine receptor expression	Corresponding chemokines
Naive T cell	CCR7	CCL19, 21	NK cell	CCR1	CCL3, 5, 7
	CXCR4	CXCL12		CCR2	CCL2, 7, 13
Th1	CCR2	CCL2, 7, 13		CCR4	CCL17, 22
	CCR5	CCL3, 4, 5		CCR5	CCL3, 4, 5
	CXCR3	CXCL9, 10, 11		CCR7	CCL19, 21
	CXCR6	CXCL16		CCR8	CCL1
	CX_3CR1	CX_3CL1		CXCR1	CXCL6, 8
Th2	CCR2	CCL2, 7, 13		CXCR2	CXCL1, 2, 3, 5, 6, 7, 8
	CCR3	CCL5, 7, 8, 11, 13, 24, 26		CXCR3	CXCL9, 10, 11
	CCR4	CCL17, 22		CXCR4	CXCL12
	CCR8	CCL1		CXCR6	CXCL16
CTL	CCR5	CCL3, 4, 5		XCR1	XCL1
	CXCR1	CXCL6, 8		CX_3CR1	CX_3CL1
	CXCR2	CXCL1, 2, 3, 5, 6, 7, 8	Monocyte	CCR1	CCL3, 5, 7
	CXCR3	CXCL9, 10, 11		CCR2	CCL2, 7, 13
	CXCR6	CXCL16		CCR5	CCL3, 4, 5
	CX_3CR1	CX_3CL1		CCR8	CCL1
Memory T cell	CCR1	CCL3, 5, 7		CXCR2	CXCL1, 2, 3, 5, 6, 7, 8
	CCR2	CCL2, 7, 13		CXCR4	CXCL12
	CCR4	CCL17, 22		CX_3CR1	CX_3CL1
	CCR6	CCL20	Immature DC	CCR1	CCL3, 5, 7
	CCR7	CCL19, 21		CCR2	CCL2, 7, 13
	CCR10	CCL27		CCR3	CCL5, 7, 8, 11, 13, 24, 26
	CXCR1	CXCL6, 8		CCR5	CCL3, 4, 5
	CXCR2	CXCL1, 2, 3, 5, 6, 7, 8		CCR6	CCL20
	CXCR4	CXCL12		CCR9	CCL25
B cell	CCR6	CCL20		CXCR4	CXCL12
	CCR7	CCL19, 21	Mature DC	CCR7	CCL19, 21
	CXCR3	CXCL9, 10, 11		CCR9	CCL25
	CXCR4	CXCL12		CXCR4	CXCL12
	CXCR5	CXCL15			

用も図り易いという利点を有している．しかし，Adベクターが標的細胞内に侵入するには，ファーストステップとして細胞表面上の感染受容体（coxsackievirus-adenovirus receptor; CAR）に結合する必要があり，[9,10] 一部の癌細胞，血球系細胞及び幹細胞においてはCARの発現が乏しいあるいは欠損しているために，Adベクター介在性遺伝子導入に抵抗性を示すことが知られている．この問題点を克服した次世代型ベクターシステムの1つがAdRGDベクターであり，α_v-integrinに親和性を有するRGD（Arg-Gly-Asp）配列をAdベクターのカプシドタンパク質（ファイバー領域）に表現させることによって感染域の拡大を図り，CAR低発現の細胞・組織にも極めて効率よく遺伝子導入することが可能となった（Fig. 2）．[11] 実際に，CAR低発現のマウスB16BL6メラノーマ細胞並びにマウス骨髄由来DCへのAdRGDベクターによる遺伝子導入効率を従来型Adと比較してみると，Fig. 3に示すように従来型Adベクターでは両細胞への遺伝子導入効率は20%にも満たないレベルであったのに対して，AdRGDベクターでは90%以上もの効率で外来遺伝子を発現させることができた．[12—14] そこで，種々のケモカイン遺伝子あるいはケモカインレセプター遺伝子の発現カセットを搭載したAdRGDベクターを構築し，これらを用いた免疫細胞の体内動態制御法の確立と癌免疫療法への応用を試みた．

4. 免疫エフェクター細胞の腫瘍集積性の増強

免疫細胞の腫瘍組織内浸潤率と癌患者の予後を調査した結果から，原発腫瘍に多くの免疫細胞が集積している症例では，治療後の再発あるいは転移が高率に抑制されるという相関が報告されており，[15—17] 免疫系による腫瘍細胞認識を促進させる方法論の開発が癌の治癒率の向上にいかに重要であるかをうかがわせる．また，T細胞，特に細胞傷害性T細胞（CTL），が腫瘍免疫における最も強力なエフェクター細胞であると広く考えられており，[18—20] これまでの癌免疫療法研究によって腫瘍特異的CTLを効率よく誘導できる様々なワクチン戦略が提案されている．しかし，腫瘍細胞のケモカイン産生レベルは正常細胞よりも低いことや，[21] 腫瘍組織に新生された血管内皮細胞には接着分子がほとんど発現していない[22]などの理由から，癌免疫療法によって活性化された免疫エフェクター細胞は一般的に腫瘍組織内に十分に集積することができない．したがって，免疫細胞の局所への遊走・浸潤を制御するケモカインを利用することによって腫瘍組織内への免疫細胞の集積を増強しようとする試みは，効果的な癌免疫療法の開発において非常に魅力的な手段であると言

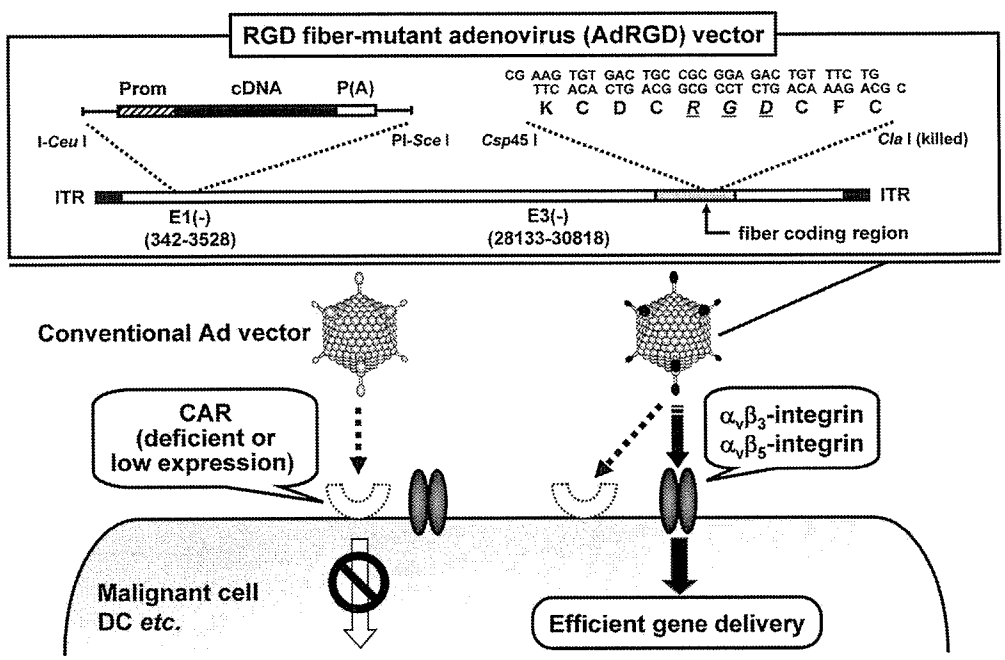

Fig. 2. Construct and Tropism of RGD Fiber-mutant Adenovirus (AdRGD) Vector
ITR: inverted terminal repeat, Prom: promoter, P(A): polyadenylation signal, CAR: coxsackievirus-adenovirus receptor.

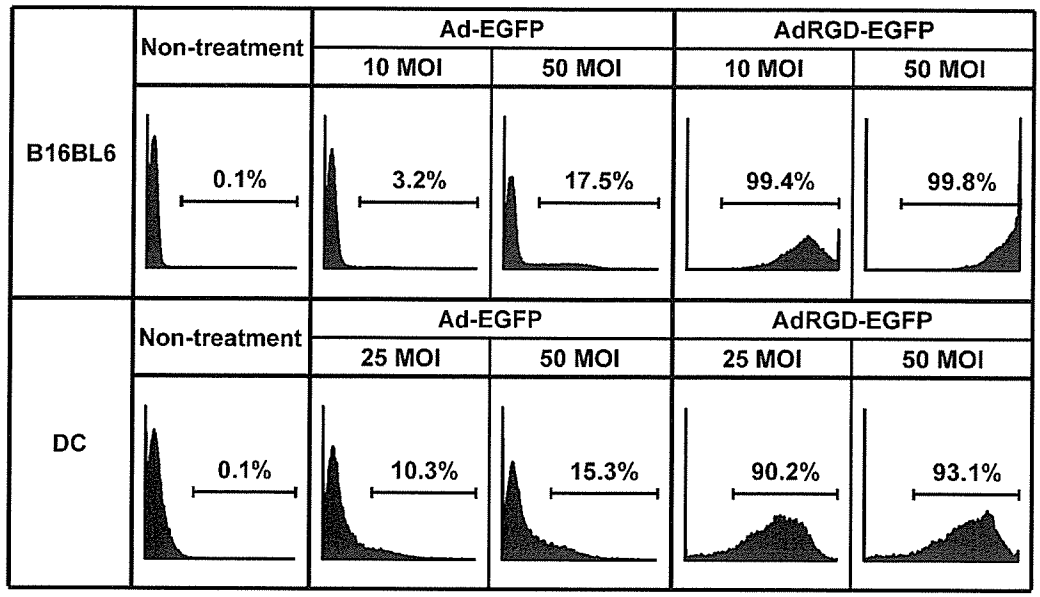

Fig. 3. EGFP Expression in Murine B16BL6 Cells and DCs Transduced with EGFP cDNA by AdRGD or Conventional Ad Vector
B16BL6 cells and DCs were transfected for 2 h with Ad-EGFP or AdRGD-EGFP at the indicated MOI (multiplicity of infection). Two days later, EGFP expression in cells was evaluated by flow cytometric analysis. The % value expresses the percentage of EGFP-positive cells.

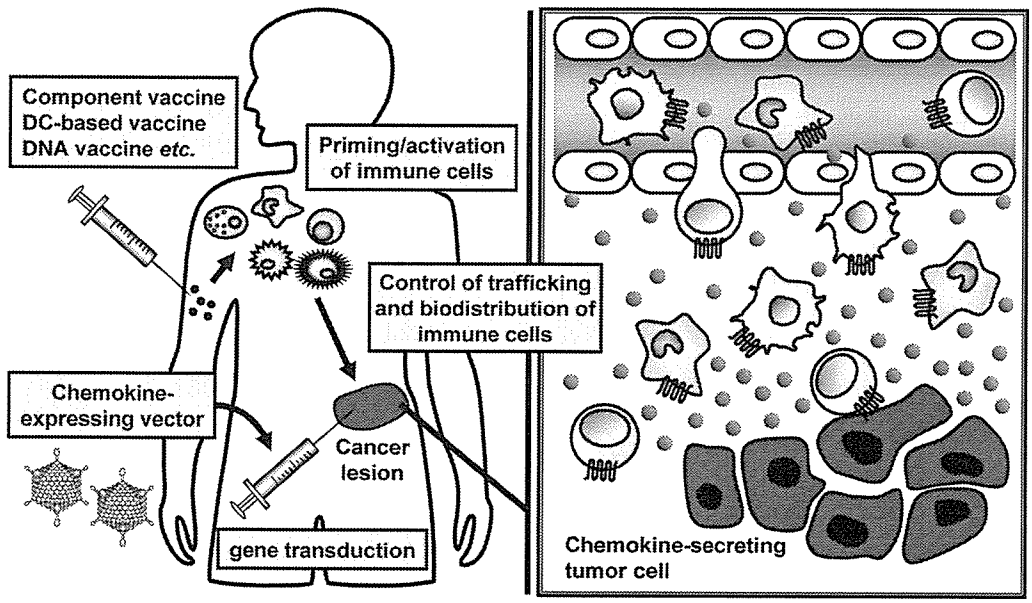

Fig. 4. Conceptual Representation of *In vivo* Chemokine Gene Transduction to Manipulate Immune Cell Trafficking and Biodistribution in Cancer Immunotherapy

えよう（Fig. 4）．そこで筆者らは，目的遺伝子の搭載が簡便であるとともに，広範な種類の腫瘍細胞に対して非常に効率よく遺伝子導入することができるAdRGDベクターを駆使することによって，種々の免疫系ケモカインを発現させた腫瘍の免疫細胞浸潤度と治療効果との連関を網羅的に解析した．[23—27] それらの中から本稿では，マウスB16BL6腫瘍モデルにおいてCCL17及びCCL19を発現するAdRGDベクター（AdRGD-CCL17, AdRGD-CCL19）を用いた検討結果について紹介する．

まず，マウスの腹部皮内に生着させたB16BL6腫瘍にAdRGD-CCL17あるいはAdRGD-CCL19を投与し，2日後における免疫細胞の腫瘍実質内浸潤度を免疫組織染色により解析したところ（Fig. 5

(A))，AdRGD-CCL19 投与腫瘍においては，コントロールベクター（ルシフェラーゼ発現 AdRGD ベクター；AdRGD-Luc）を投与した腫瘍と比較して CD3$^+$ T 細胞の著しい浸潤増加が観察された．また，これら T 細胞のサブセット解析の結果から，AdRGD-CCL19 の投与によって腫瘍内には CD4$^+$ ヘルパー T 細胞と CD8$^+$ CTL がともに集積していることも判明した．一方，AdRGD-CCL17 を投与した腫瘍ではコントロール群と比較して有意な T 細胞数の増加を認めなかった．このように腫瘍組織への T 細胞動員能が大きく異なる CCL17 と CCL19 ではあるが，Fig. 5(B) に示すように AdRGD-CCL17 あるいは AdRGD-CCL19 の投与は B16BL6 腫瘍の増殖をコントロール群と比較してわずかに遅延させたのみであり，ケモカイン両群間の抗腫瘍効果に明らかな差は認められなかった．筆者らはこの一見矛盾する結果に対して，腫瘍組織に発現させた CCL19 によって動員される T 細胞が腫瘍細胞を排除する活性に乏しい未感作（naive）な状態であったために，効果的な腫瘍退縮が観察されなかったのではないかと考えた．事実，CCL19 に対応するケモカインレセプターである CCR7 は，T 細胞サブセットの中でも主に naive T 細胞に発現することが知られており（Table 1），また活性化 T 細胞の細胞傷害分子の 1 つであるパーフォリンに対する免疫組織染色において，CCL19 により腫瘍内に動員された T 細胞の大半がパーフォリン陰性であることを確認している．したがって，ケモカイン遺伝子の腫瘍内導入に基づいた免疫細胞の腫瘍内浸潤増強（Cell Delivery System）を癌免疫療法の開発に活かすためには，免疫細胞を腫瘍特異的に活性化し得るワクチン戦略との併用によってこそ真価が発揮されるであろうと考えられた．

そこで，B16BL6 腫瘍の TAA の 1 つである gp100 の遺伝子を導入した DC ワクチン（gp100/DC）を免疫投与することによって，gp100 特異的 CTL が感作・活性化された状態の担癌マウスを作成し，その後ケモカイン発現 AdRGD ベクターを腫瘍内投与した際の治療効果とそのメカニズムを解析した．Figure 6(A) に示すように，B16BL6 腫瘍を接種したマウスに gp100/DC を皮内投与し，その後 PBS あるいはコントロールベクターを腫瘍内投与した群では効果的な腫瘍退縮は認められず，この結果はひとたび増殖を始めた B16BL6 腫瘍を

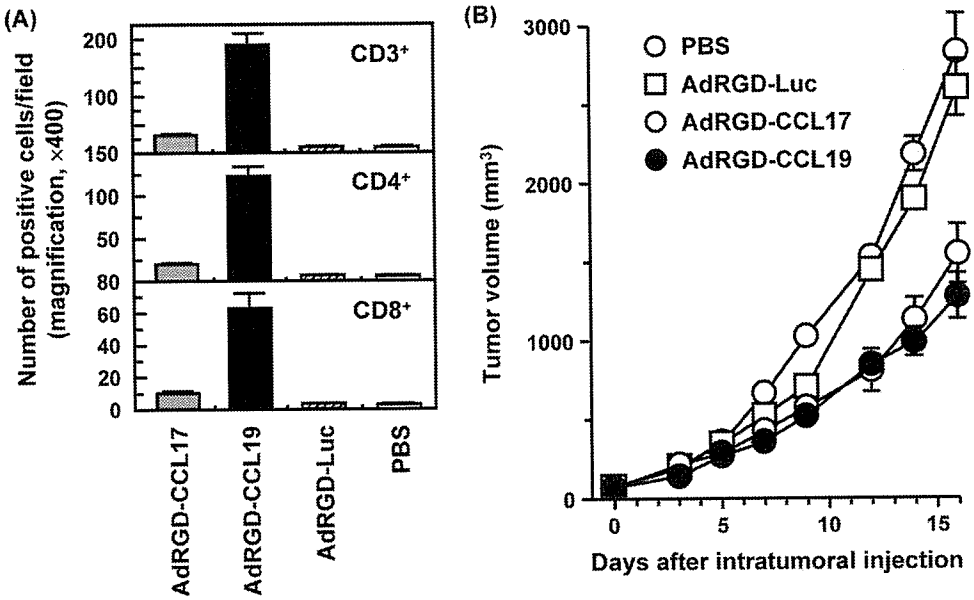

Fig. 5. T Cell Infiltration (A) and Growth Suppression (B) in B16BL6 Tumors Injected with AdRGD-CCL17 or AdRGD-CCL19

B16BL6 cells were intradermally inoculated into the right flank of C57BL/6 mice at 4×10^5 cells/mouse. One week later, tumors (5—7 mm in diameter) were injected with AdRGD-CCL17, AdRGD-CCL19, or AdRGD-Luc (control vector) at 3×10^8 PFU (plaque-forming unit). PBS was administered to control tumors. (A): On day 2 after intratumoral injection, immunohistochemical staining against CD3 (pan-T marker), CD4 (helper T marker), and CD8 (CTL marker) was performed using frozen tumor sections. Then, the number of positive cells in tumor parenchyma was assessed by counting six fields per specimen under ×400 magnification. (B): The tumor sizes were assessed using microcalipers, and the tumor volume was calculated using the following formula: (tumor volume [mm^3]) = (major axis [mm]) × (minor axis [mm])^2×0.5236.

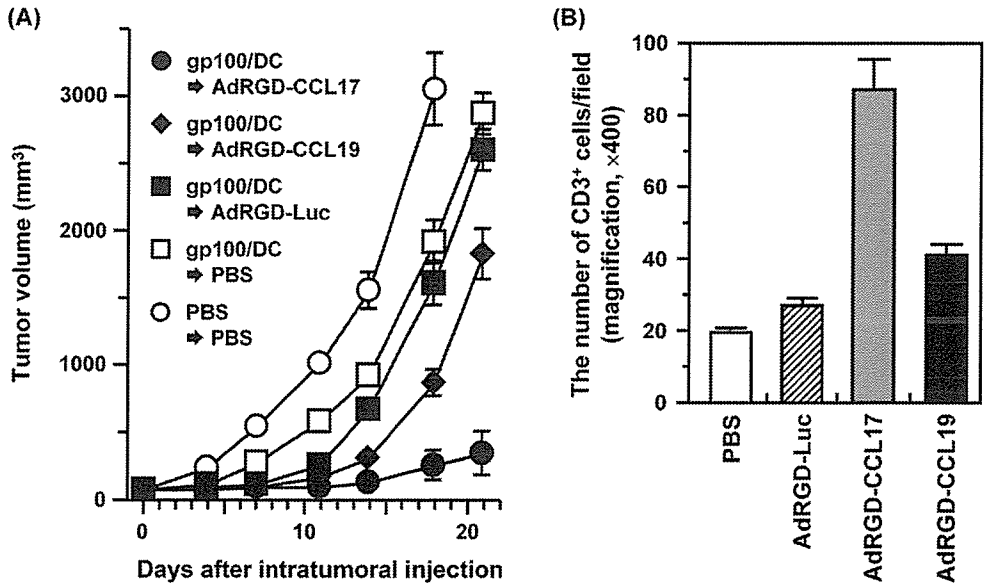

Fig. 6. Growth Suppression (A) and T Cell Infiltration (B) in B16BL6 Tumors of Mice Treated with the Combination of gp100/DC-immunization and Intratumoral Injection of Chemokine-expressing AdRGD Vector

B16BL6 cells were intradermally inoculated into the right flank of C57BL/6 mice at 4×10^5 cells/mouse. One day later, the mice were intradermally immunized in the left flank with 10^6 DCs transduced with AdRGD-gp100 at 25 MOI. Then, the tumors (5—7 mm in diameter) were injected with AdRGD-CCL17, AdRGD-CCL19, or AdRGD-Luc (control vector) at 3×10^8 PFU. PBS was administered to control tumors of mice immunized with gp100/DCs or PBS. (A): The tumor volume was assessed as described in the legend to Fig. 5. (B): In mice immunized with gp100/DCs, immunohistochemical staining against CD3 to determine T cells was performed with frozen tumor sections on day 2 after intratumoral injection with each AdRGD vector or PBS. The number of CD3-positive cells in tumor parenchyma was assessed by counting six fields per specimen under ×400 magnification.

gp100/DC の単回免疫投与だけで抑制することは非常に困難であることを示している．これに対して，gp100/DC 免疫後に AdRGD-CCL17 を腫瘍内投与した場合には，極めて強力な腫瘍増殖抑制効果が観察され，AdRGD-CCL17 投与 2 日後における腫瘍組織にはコントロール群と比較して顕著な $CD3^+$ T 細胞の浸潤増加が検出された（Fig. 6(B)）．一方，gp100/DC を免疫投与した担癌マウスに AdRGD-CCL19 を腫瘍内投与した場合には，腫瘍増殖のわずかな遅延が観察されたのみであり，腫瘍浸潤 T 細胞数もコントロール群と比較して若干の増加を認めるのみであった．これらの結果は，Fig. 5 に示したケモカイン発現 AdRGD ベクターの腫瘍内投与だけを施した際の抗腫瘍効果及び腫瘍浸潤 T 細胞数の結果とは全く異なる傾向を示し，免疫細胞のケモカイン応答性（ケモカインレセプターの発現パターン）が自身の分化・活性化とともに変化することを考え合わせると，有効な治療プロトコールを確立するためには腫瘍内に発現させるケモカインの種類を併用する癌免疫療法の特性（宿主免疫細胞の活性化状態）を考慮して選択する必要があることを示唆している．また，ケモカイン遺伝子を導入した腫瘍内へ集積する免疫細胞のサブセット並びに細胞数を，これまでに報告されている *in vitro* 解析に基づいた特定のケモカインと標的細胞との対応関係から予測することは極めて困難であり，これらは，発現したケモカインの作用・腫瘍組織の環境要因・集積した免疫細胞による二次的な作用・宿主免疫系の活性化状態などを包含した複合的イベントとして決定されると考えられた．したがって，ケモカインを応用した有効な癌免疫療法を開発するためには，腫瘍組織内への免疫細胞の集積性増強ばかりでなく，腫瘍局所に集積した免疫細胞を腫瘍特異的に活性化するためのサイトカイン療法や細胞療法の併用をも視野に入れた取り組みが必要であろうと思われる．

以上に紹介した筆者らの研究成果は，ケモカインを利用した腫瘍浸潤免疫細胞の増強（Cell Delivery System）が癌免疫療法の有効性改善に大いに貢献できるアプローチであることを実証するものであり，今後，免疫系におけるケモカイン—ケモカインレセプター連関のより詳細な解明に伴って，免疫エフェクター細胞の緻密な体内動態制御を導入した有効かつ安全な癌免疫療法の開発に結びついていくものと期待している．

5. DCワクチンのリンパ組織集積性の増強

骨髄幹細胞に由来するDCは生体内に広く分布しており，外来性の病原体や内在性の変異細胞（癌細胞・ウイルス感染細胞）断片を捕食すると，それらに含まれるタンパク抗原をペプチドにまでプロセッシングして細胞表面の主要組織適合遺伝子複合体（MHC）分子上に提示する．さらに，輸入リンパ管を介して最寄りのリンパ組織へと遊走し，リンパ組織に存在するT細胞を抗原特異的に感作・活性化することにより病原体や変異細胞の排除に働くCTLを増幅する.[28,29] DCをワクチン担体として利用する癌免疫療法は，患者から単離したDC前駆細胞を *in vitro* 培養系においてDCへと増殖・分化させ，TAA導入処理を施したDCを患者に投与することによって，DCが誘発する一連の免疫応答の活性化に基づいた癌治療を達成しようとするものである（Fig. 7）．本療法の一部のプロトコールについては既に臨床試験も開始されているが，残念ながら劇的な有効性は報告されておらず，現在，DCワクチンの免疫誘導能を最大限に発揮させる調製法の探索が筆者らを含めて多くの研究グループによって進められている.[30–35] 前述の通りAdRGDベクターは，DCに対しても他のベクターシステムと比較して圧倒的に優れる遺伝子導入効率を有することから，筆者らは，AdRGDベクターを応用してTAA遺伝子を効率よく導入したDCワクチンの創製が，これまでDCに対する低いTAA導入効率によって制限されていたDC癌免疫療法の有効性を飛躍的に改善できることを報告してきた.[12,32,33] また，AdRGDベクターはDCへの抗原遺伝子デリバリーのみならず，従来の遺伝子導入法では困難とされてきた種々の機能修飾を目的とする遺伝子改変DCワクチンの創製をも可能とする.[34] したがって，AdRGDベクターを用いたDCへの効率的な遺伝子導入は，臨床的に最適なDCワクチンを設計・開発するためのブレークスルー的な手法になり得ると期待される．このコンセプトに基づいて筆者らは，ケモカインレセプター遺伝子を搭載したAdRGDベクターを応用することにより，生体に投与するDCワクチンの体内動態を操作し，より効果的なDC癌免疫療法の開発へと発展させるという独自のアイデアを実践している.[36] その一例として，CCケモカインレセプター7（CCR7）遺伝子を導入することでリンパ組織移行性を増強したDCワクチンの創製とその癌免疫療法における有用性を紹介する．

生体内に存在するDCの末梢組織からリンパ組織への遊走は，抗原認識・捕捉や炎症反応に伴って成熟分化したDCにCCR7が一過性に発現し，リンパ組織から構成的に産生・分泌されているCCL21に応答することによって誘発されるというコンセン

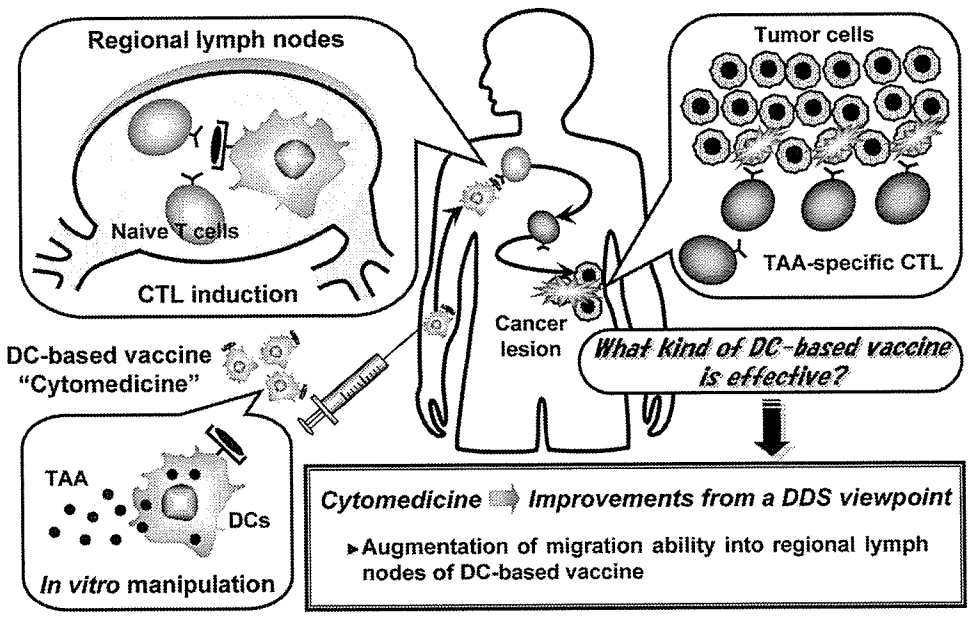

Fig. 7. Schematic Representation of DC-based Cancer Immunotherapy

サスが得られている.[37,38] したがって，抗原導入とともにCCR7を十分に発現させたDCワクチンは，生体に投与したあとに積極的にリンパ組織へと移行して免疫系を効率よく活性化できることが強く予測され，この“リンパ組織指向性DC”ともいうべき新たなワクチンの創製手段としてDCへのCCR7遺伝子導入が挙げられる（Fig. 8）．そこでまず，CCR7遺伝子搭載AdRGDベクター（AdRGD-CCR7）を用いて遺伝子導入したDC（CCR7/DC）におけるCCR7発現レベルをflow cytometryにより解析したところ，90%以上のDCで細胞表面に豊富なCCR7タンパク発現が確認された（Fig. 9(A)）．また，*in vitro* chemotaxis assayにおいてCCR7/DCはCCL21の濃度に依存した遊走活性の上昇を示したことから，遺伝子導入によって細胞膜上に強制発現させたCCR7タンパクは，CCL21濃度を感知してDCに遊走刺激を伝える本来の生物活性を保持したケモカインレセプターであることも判明した（Fig. 9(B)）．さらに，CCR7/DCをマウスの側腹部皮内に投与し，48時間後に投与部位から所属リンパ節（鼠径部リンパ節）へと遊走したDC数を解析したところ，コントロールDCと比較して5—15倍高いリンパ節への集積が認められた（Fig. 9(C)）．これらの結果は，AdRGDベクターを応用したDCへのケモカインレセプター遺伝子の導入によって，DCのケモカイン応答性並びに生体に投与した際の体内挙動を改変できることを示すとともに，CCR7/DCが，DC免疫療法における効率のよい免疫エフェクター細胞の活性化と全身への迅速なエフェクター細胞の供給という観点から，非常に優れたワクチン担体として機能することを示唆するものである．

そこで，CCR7/DCの優れたリンパ組織集積性とDC免疫療法の有効性改善との関連性を検証するために，メラノーマ関連抗原（gp100）遺伝子とCCR7遺伝子とを共導入したDC（gp100＋CCR7/DC）を調製し，マウスB16BL6メラノーマモデルにおけるワクチン機能を解析した（Fig. 10(A)）．Mock DCあるいはCCR7/DCを投与した群と比較して，gp100遺伝子のみを導入したDC（gp100/DC）をワクチン投与したマウスにおいては攻撃接種したB16BL6腫瘍の顕著な増殖遅延が観察され，さらにgp100＋CCR7/DCを免疫した群においてはより強力な腫瘍増殖抑制効果が認められた．また，これらの遺伝子導入DCワクチンを投与したマウスにおけるCTL活性を比較したところ，gp100

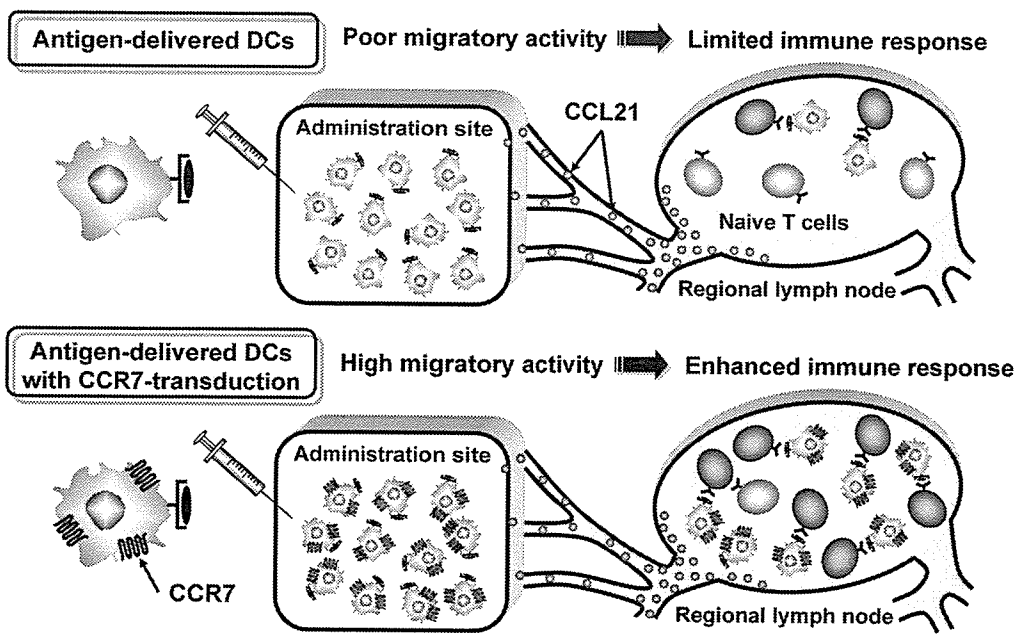

Fig. 8. Conceptual Representation of “Lymphoid Tissue-directivity DC” Vaccine Created by CCR7 Gene-transduction

The migration of DCs from administration site to regional lymph nodes is critical for the priming of T cells in DC-based immunotherapy. However, the poor migratory ability of antigen-delivered DCs limits the induction of a potent immune response. On the other hand, DCs transduced efficiently with the CCR7 gene may adequately respond to CCL21, which is constitutively released from lymphoid tissue, and acquire migratory ability to lymph nodes. Consequently, antigen-delivered DCs with CCR7 transduction can enhance the initiation and amplification of the T cell-dependent immune response.

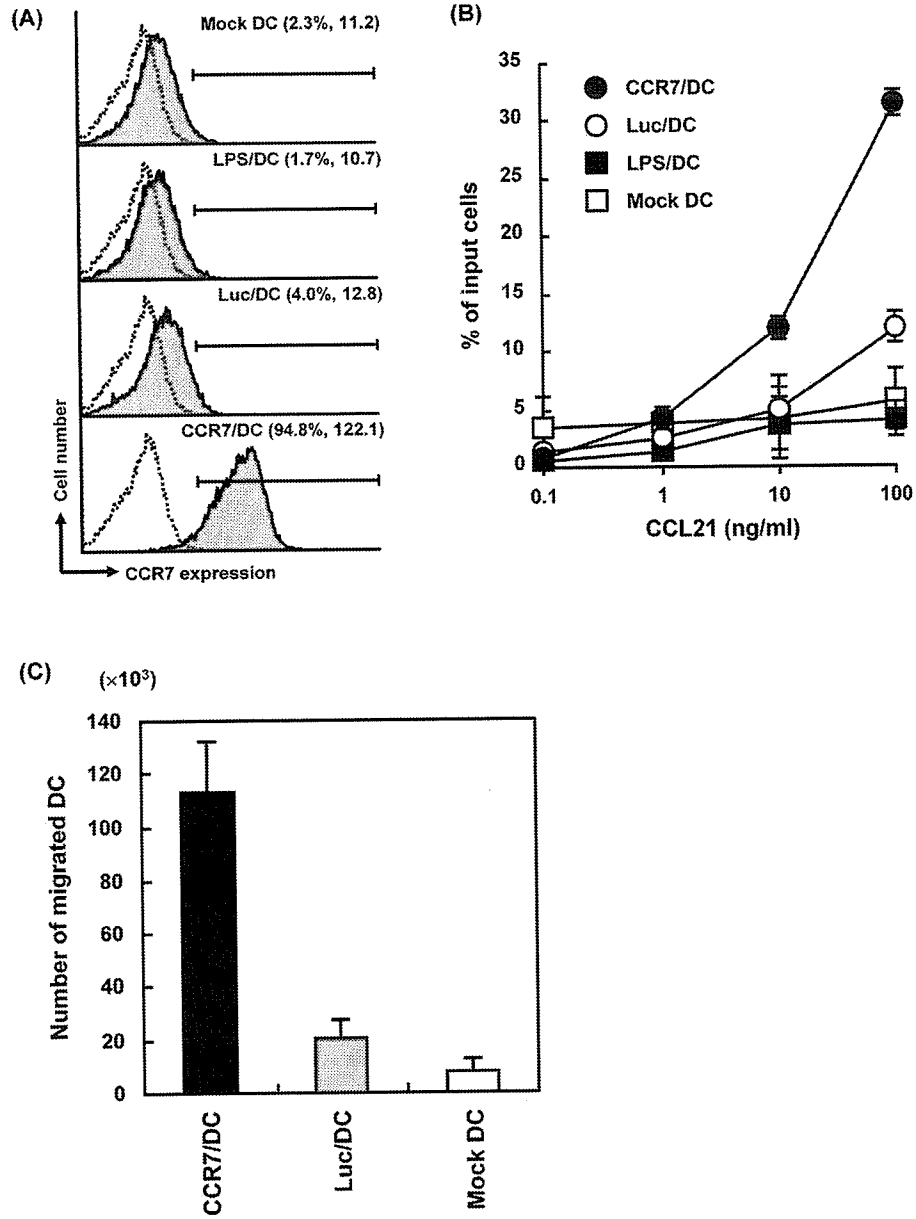

Fig. 9. Analysis for CCR7 Expression Levels (A), *In vitro* Chemotaxis (B), and *In vivo* Migration (C) of CCR7/DCs

DCs were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI. These transduced cells (CCR7/DC and Luc/DC), LPS/DCs stimulated with 1 μg/ml lipopolysaccharide, and mock DCs were cultured for 24 h. (A): Flow cytometric analysis was performed by using anti-mouse CCR7 antibody. Negative control (dotted line) represents mock DCs stained by second antibody alone. The % value and the numerical value indicated in the upper part of each panel express % of gated cells and mean fluorescence intensity (MFI), respectively. (B): *In vitro* chemotaxis assay was performed by a Chemotaxicell-24 installed on 24-well culture plate. CCL21 solution was added in the lower compartment at the indicated concentration, and DCs were placed in the upper chamber at 10^6 cells. After 4 h-incubation, the number of cells that migrated to the lower compartment was counted. The chemotactic activity was expressed in terms of the percentage of the input cells calculated by the following formula: (% of input cells) = (the number of migrated cells) / (the number of cells placed in Chemotaxicell-24 [10^6 cells]) × 100. (C): DCs derived from EGFP-transgenic mice were transduced with AdRGD-CCR7 or AdRGD-Luc at 50 MOI, and then were intradermally injected into the left flank of C57BL/6 mice at 2×10^6 cells/50 μl. Two days later, the draining inguinal lymph nodes were collected from these mice, and a single cell suspension was prepared. The abundance of EGFP-positive DCs was assessed by flow cytometric analysis acquiring 500000 events. The number of DCs that had migrated into draining lymph nodes was calculated by multiplying the EGFP-positive DC-frequency by the total number of isolated lymph node cells.

＋CCR7/DC を投与したマウスの脾細胞中には gp100/DC 投与群を上回る B16BL6 特異的細胞傷害活性が検出された (Fig. 10(B))．これらの結果は，CCR7-CCL21 連関を利用した DC ワクチンへの積極的なリンパ組織移行能の付与（Cell Delivery System）が，細胞医薬としての DC ワクチンの生物学的利用能を向上させることを意味しており，DC 免疫療法の臨床応用実現に貢献する有益な基礎情報を提供できるものと考えている．

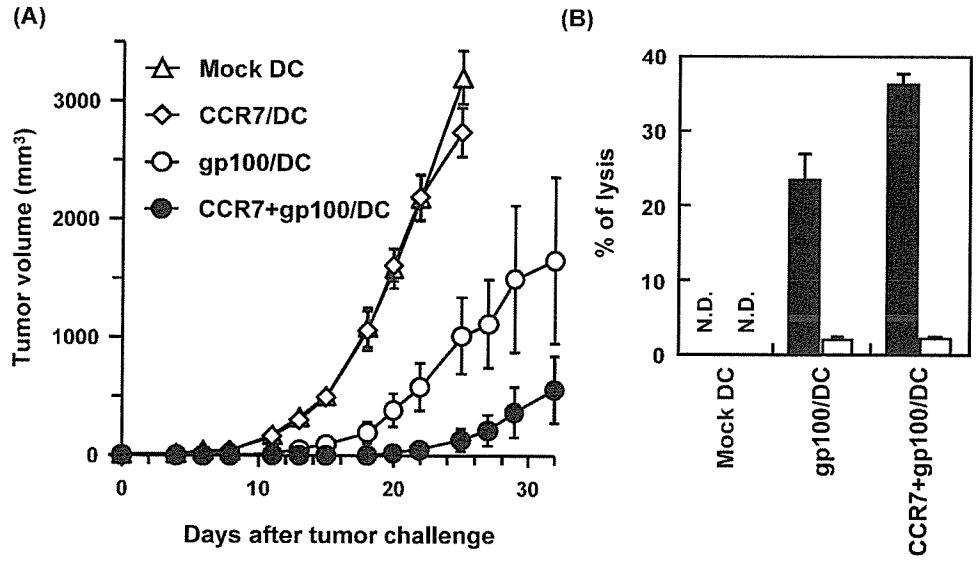

Fig. 10. Anti-B16BL6 Tumor Efficacy (A) and CTL Activity (B) in Mice Immunized with DCs Cotransduced with CCR7 and gp100 Gene by AdRGD

CCR7/DCs, gp100/DCs, and gp100+CCR7/DCs were prepared using corresponding AdRGD vectors at 25 MOI, and then cultured for 24 h. C57BL/6 mice were immunized by intradermal injection of 5×10^5 transduced DCs or mock DCs into the left flank. (A): One week later, 4×10^5 B16BL6 cells were inoculated into the right flank of the mice, and then the tumor volume was assessed as described in the legend to Fig. 5. (B): At 1 week after immunization, non-adherent splenocytes were prepared from these mice, and then were re-stimulated *in vitro* for 5 days with IFN-γ-stimulated and mitomycin C-inactivated B16BL6 cells. A cytolytic assay using the re-stimulated splenocytes (effector cells) was performed against IFN-γ-stimulated B16BL6 (closed column) or EL4 cells (open column) at effector/target ratio at 25. N.D.: not detectable.

6. おわりに

近年の免疫学及び分子生物学の著しい進展に伴い，免疫系を構築する細胞や分子の情報ネットワークが詳細に解明されつつあり，さらにDCやCTLなど免疫機能細胞を医薬品として捉えた細胞療法の研究が精力的に推し進められている．しかし，これら細胞医薬自身の体内動態制御あるいは細胞医薬により活性化された免疫細胞の体内動態制御によって治療効果の改善を目指した研究は，いまだ緒に就いたばかりであり，癌免疫療法へと展開するために必要とされる基礎的な情報さえ不足しているのが現状である．筆者らは，本稿で紹介したケモカイン—ケモカインレセプター連関を利用したCell Delivery Systemのみならず，アポトーシス抑制機構を利用した細胞医薬の生体内安定性（生存性）の向上，抗原—抗体反応を利用した細胞医薬への標的指向性の付与，といった様々なCell Delivery Systemの開発を試みており，これらが免疫療法に留まらず，細胞機能によって疾病治療を達成しようとする次世代医療（再生療法，*ex vivo* 遺伝子治療など）の現在の治療限界を切り崩す有望な方法論となるものと考えている（Fig. 11）．これら次世代医療の臨床応用に向けてはまだまだ長い道のりが待っており，治療法の理論的根拠，有効性・安全性の評価基準，遺伝子医薬や細胞医薬の性能・品質管理など，従来の低分子有機化合物を用いた薬物治療がなし遂げてきたような厳しい基準をクリアしていくことが要求される．現在はこれら多岐に渡る検討課題を個々に克服している段階であるが，筆者らのCell Delivery Systemがより確かな次世代医療を実現するための一助となることを期待する．

謝辞　本研究の遂行に有益な御助言及び多大な御支援を賜りました水口裕之先生（医薬基盤研究所基盤研究部遺伝子導入制御プロジェクトプロジェクトリーダー，大阪大学大学院薬学研究科招聘助教授），山本　昌先生（京都薬科大学薬剤学教室教授），並びに藤田卓也先生（京都薬科大学薬剤学教室助教授）を始めとする共同研究者の先生方，及び実験に御協力頂きました大学院生・学生諸氏に厚く御礼申し上げます．なお本研究の一部は，文部科学省科学研究費補助金，厚生労働省科学研究費補助金，創薬等ヒューマンサイエンス総合研究事業，薬学研究奨励財団研究助成金，佐川がん研究助成振興財団研究助成，千里ライフサイエンス振興財団奨励研究助成の援助の下に行われたことを記して感謝申

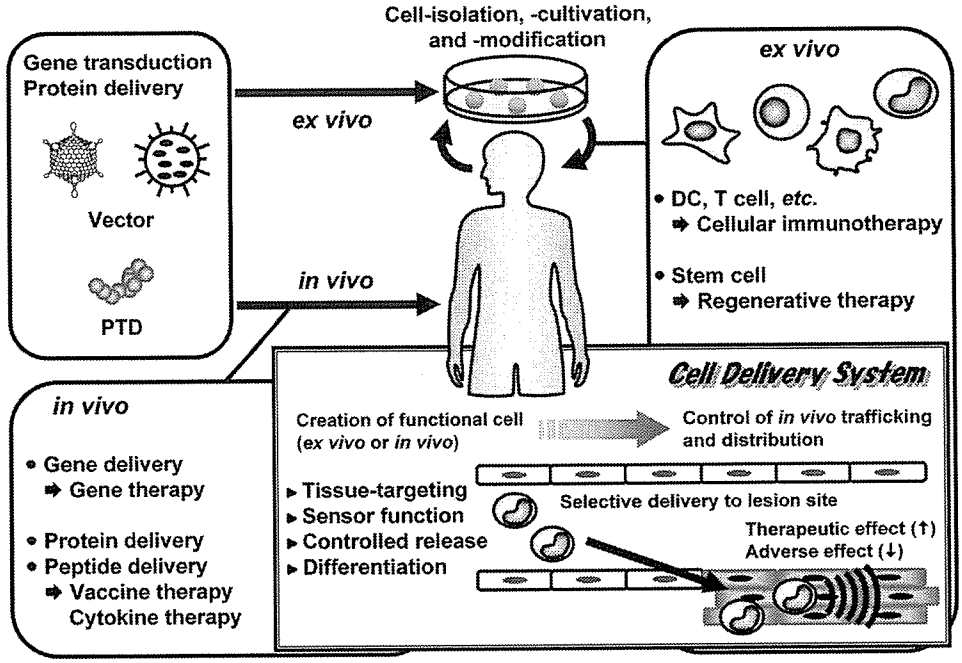

Fig. 11. Conceptual Illustration of Cell Delivery System in Next Generation Medical Treatment

し上げます.

REFERENCES

1) Esche C., Shurin M. R., Lotze M. T., *Curr. Opin. Mol. Ther.*, **1**, 72–81 (1999).
2) Kupiec-Weglinski J. W., Austyn J. M., Morris P. J., *J. Exp. Med.*, **167**, 632–645 (1988).
3) Lappin M. B., Weiss J. M., Delattre V., Mai B., Dittmar H., Maier C., Manke K., Grabbe S., Martin S., Simon J. C., *Immunology*, **98**, 181–188 (1999).
4) Martin-Fontecha A., Sebastiani S., Hopken U. E., Uguccioni M., Lipp M., Lanzavecchia A., Sallusto F., *J. Exp. Med.*, **198**, 615–621 (2003).
5) Yoshie O., Imai T., Nomiyama H., *Adv. Immunol.*, **78**, 57–110 (2001).
6) Zlotnik A., Yoshie O., *Immunity*, **12**, 121–127 (2000).
7) Bokoch G. M., *Blood*, **86**, 1649–1660 (1995).
8) Murphy P. M., *Annu. Rev. Immunol.*, **12**, 593–633 (1994).
9) Bergelson J. M., Cunningham J. A., Droguett G., Kurt-Jones E. A., Krithivas A., Hong J. S., Horwitz M. S., Crowell R. L., Finberg R. W., *Science*, **275**, 1320–1323 (1997).
10) Wickham T. J., Mathias P., Cheresh D. A., Nemerow G. R., *Cell*, **73**, 309–319 (1993).
11) Mizuguchi H., Kay M. A., *Hum. Gene Ther.*, **10**, 2013–2017 (1999).
12) Okada N., Tsukada Y., Nakagawa S., Mizuguchi H., Mori K., Saito T., Fujita T., Yamamoto A., Hayakawa T., Mayumi T., *Biochem. Biophys. Res. Commun.*, **282**, 173–179 (2001).
13) Okada N., Masunaga Y., Okada Y., Iiyama S., Mori N., Tsuda T., Matsubara A., Mizuguchi H., Hayakawa T., Fujita T., Yamamoto A., *Cancer Gene Ther.*, **10**, 421–431 (2003).
14) Okada Y., Okada N., Nakagawa S., Mizuguchi H., Takahashi K., Mizuno N., Fujita T., Yamamoto A., Hayakawa T., Mayumi T., *Jpn. J. Cancer Res.*, **93**, 436–444 (2002).
15) Eerola A. K., Soini Y., Paakko P., *Clin. Cancer Res.*, **6**, 1875–1881 (2000).
16) Yin X. Y., Lu M. D., Lai Y. R., Liang L. J., Huang J. F., *Hepatogastroenterology*, **50**, 1281–1284 (2003).
17) Fukunaga A., Miyamoto M., Cho Y., Murakami S., Kawarada Y., Oshikiri T., Kato K., Kurokawa T., Suzuoki M., Nakakubo Y., Hiraoka K., Itoh T., Morikawa T., Okushiba S., Kondo S., Katoh H., *Pancreas*, **28**, e26–e31 (2004).
18) Naito Y., Saito K., Shiiba K., Ohuchi A.,