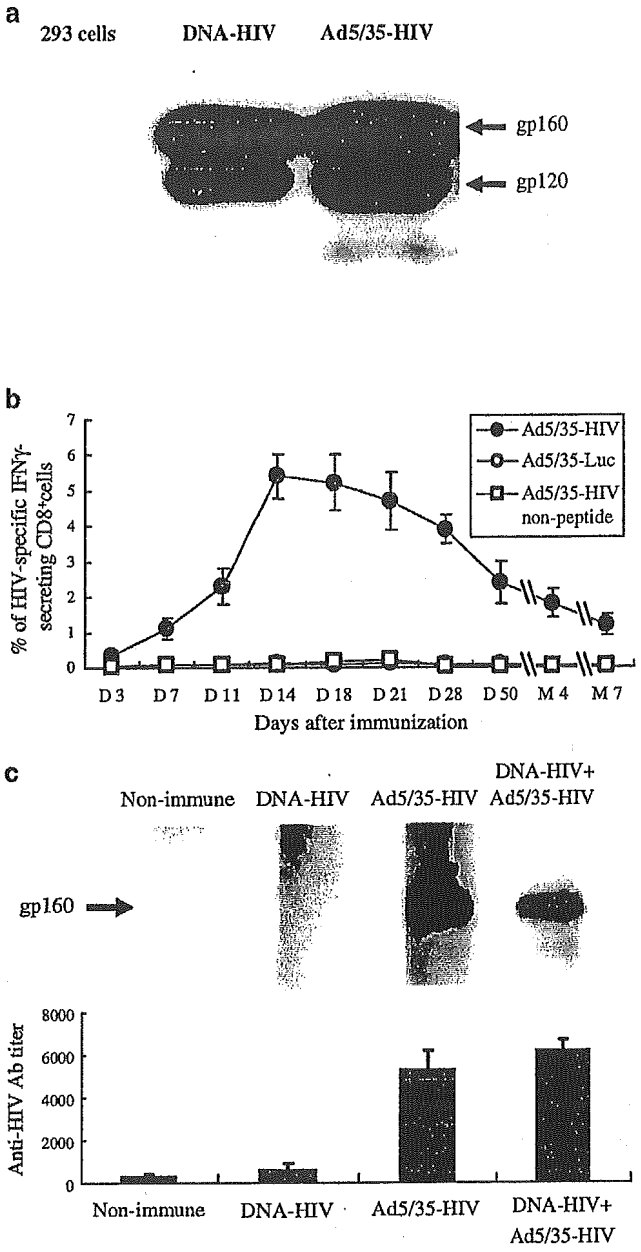

Figure 2 Time course of HIV-specific immune responses in mice. (a) HIV Env protein expression of DNA-HIV vaccine and Ad5/35-HIV on HEK293 cells was confirmed by Western blotting using an HIV Env-specific mAb. (b) Time-course study of cellular immune responses after a single i.m. injection of 10^{10} vp of Ad5/35-HIV vector (3 mice/time point). D: day; M: month. (c) HIV-specific Ab was detected by Western blotting using 100-fold diluted antisera (serum pool of 10 mice/group) (upper panel) and ELISA (10 mice/group) (bottom left panel).

vaccinated mice (1:186) and DNA prime/Ad5/35-HIV boosted mice (1:206).

Immune responses and challenge in mice 2 weeks after vaccination. There is growing evidence that cellular immunity contributes to protecting the host against HIV infection.[3,4,30,31] The ability of the Ad5/35 vector to trigger the activation and proliferation of antigen-specific T cells was monitored. Vaccination with the DNA-HIV vaccine induced the number of HIV-specific IFN-γ-secreting $CD8^+$ T cells to increase from background levels (<0.2–0.7%) ($P<0.05$) (Table 1). This was significantly less than the effect of vaccination with the Ad5/35-HIV vector (10^{10} vp/mouse) that increased the IFN-γ-secreting $CD8^+$ T cells to 5.5% ($P<0.05$). Priming with the DNA-HIV vaccine followed by an Ad5/35-HIV vector boost led to a further three-fold increase in the number of IFN-γ-secreting $CD8^+$ T cells ($P<0.05$).

A tetramer-binding assay was used to verify that the IFN-γ-secreting cells were MHC class I-restricted HIV-specific $CD8^+$ T cells.[32] A single immunization with Ad5/35-HIV vector elicited a significant increase in the number of tetramer-binding $CD8^+$ T cells (Table 1). When compared with DNA-HIV vaccination alone, immunization with the Ad5/35-HIV vector yielded five-fold more HIV-specific $CD8^+$ T cells ($P<0.05$). Priming with the DNA-HIV vaccine, followed by Ad5/35-HIV boosting, further increased the tetramer binding ($P<0.05$).

To examine the protective activity of the Ad5/35-HIV vector, immunized mice were challenged with 10^8 plaque forming units (PFU) of vPE16 2 weeks after final immunization. The animals that were vaccinated with the Ad5/35 vector alone or in combination with the DNA-HIV vaccine were completely protected from infection (Table 1); however, the DNA-HIV vaccination alone had little impact on the susceptibility to infection by vPE16.

Long-term cell-medicated immune responses and challenge in mice. The durability of these vaccine regimens was explored. HIV-specific cellular immune responses persisted through 7 months after final immunization (Table 1 and Figure 2b). To determine whether this immune response was protective, vaccinated mice were challenged with vPE16 (10^8 PFU/mouse) 7 weeks after final immunization. The viral load of Ad5/35-HIV-immunized mice was reduced by 10^5 as compared with that of the control mice ($P<0.05$). DNA-HIV vaccination by itself was not protective, but the combination of DNA-HIV priming and Ad5/35-HIV boosting yielded a prolonged and complete protection (Table 1).

Biodistribution of Ad in rhesus macaques

To study the biodistribution of Ad in monkeys, 10^{11} vp of Ad5-Luc and Ad5/35-Luc vectors was injected i.m. into two rhesus monkeys for each vector. The luciferase activity in the tissues was detected 3 days after administration, because high luciferase activity in the mouse liver was observed at that time point. Liver infection with Ad5 vector was 20- to 40-fold stronger than that with Ad5/35 vector (Figure 3a). It is important to note that the luciferase activity of the cerebellum and the posterior cerebrum in the monkeys that received the Ad5-Luc vector was two- and four-fold higher, respectively, than that of the monkeys that received the Ad5/35-Luc vector.

Immune response in rhesus monkeys after vaccination

To explore the immunogenicity of the Ad5/35-HIV vector in monkeys, two rhesus macaques were immunized i.m. with 10^{11} vp of Ad5/35-HIV vector. A detectable HIV-specific serum Ab response developed

Table 1 HIV-specific cell-mediated immune responses and virus challenge after vaccination

	Week 2			Week 7			Month 4	Month 7
	ICS (%)	*Tetramer (%)*	*Ovary viral titer*	*ICS (%)*	*Tetramer (%)*	*Ovary viral titer*	*Tetramer (%)*	*Tetramer (%)*
Nonimmune	0.1±0.1	0.1±0.1	$8\times10^8\pm35$	0.1±0.1	0.1±0.1	$1\times10^9\pm65$	0.0±0.0	0.0±0.0
DNA-Empty	0.1±0.1	0.1±0.1	$2\times10^9\pm45$	0.0±0.0	0.0±0.0	$8\times10^8\pm32$	0.0±0.0	0.0±0.0
Ad5/35-Luc	0.2±0.1	0.2±0.2	$2\times10^9\pm25$	0.0±0.0	0.0±0.0	$4\times10^8\pm46$	0.0±0.0	0.0±0.0
DNA-HIV	0.7±0.1	1.0±0.3	$6\times10^6\pm42$	0.4±0.2	0.6±0.1	$5\times10^7\pm51$	0.3±0.1	0.1±0.1
Ad5/35-HIV	5.5±0.3	5.2±0.3	ND	2.5±0.8	3.1±0.2	$2\times10^3\pm34$	2.5±0.5	1.2±0.4
DNA-HIV+Ad5/35-HIV	17.2±0.8	19.4±2.1	ND	8.2±1.2	8.9±0.8	ND	7.1±0.6	4.1±0.3

Mice were immunized with DNA plasmid or Ad5/35 vector, either alone or in combination. At 2 weeks, 7 weeks, 4 months, and 7 months after final immunization, HIV-specific cellular immune responses were detected by ICS assay and tetramer assay. The data represent the percentage of IFN-γ- or tetramer-positive $CD8^+$ T cells (5–10 mice/group). The backgrounds were less than 0.1% IFN-γ-secreting $CD8^+$ T cells when cells were stimulated with control peptide (influenza NP peptide, TYQRTRALV). The vaccinated mice (10 mice/group) were challenged with vaccinia virus vPE16 2 or 7 weeks after final immunization. At 6 days after the challenge, the vPE16 titer in mouse ovaries was measured. ND, not detectable.

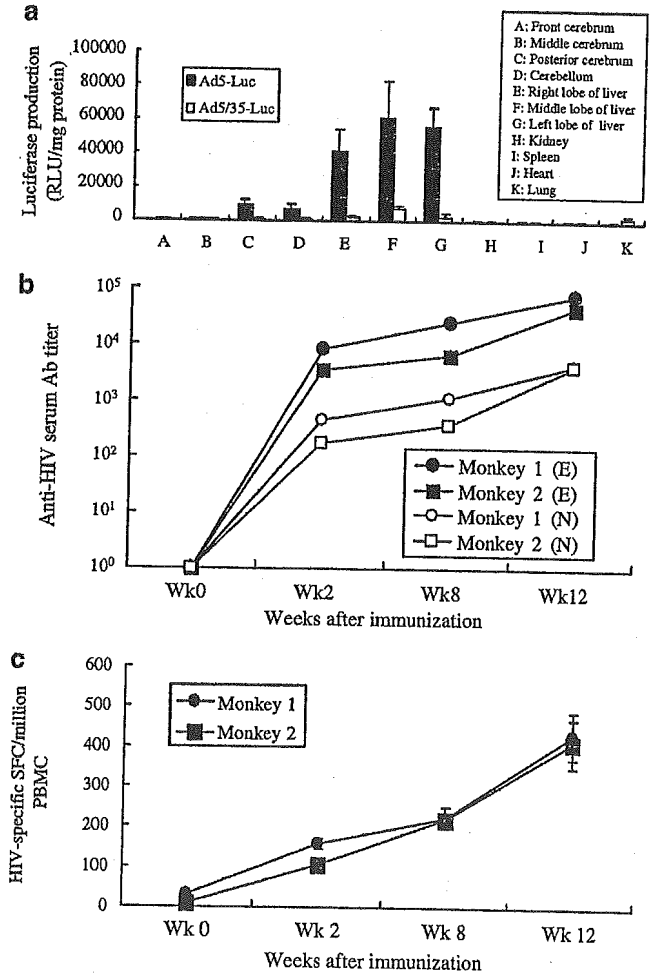

Figure 3 Biodistribution and HIV-specific immune responses in rhesus monkeys. Rhesus monkeys (2 monkeys/group) were administered i.m. 10^{11} vp of Ad5-Luc or Ad5/35-Luc vector. The luciferase activity in the organs of the monkey (expressed in RLU) was examined 3 days after administration (a). Rhesus monkeys were immunized i.m. with 10^{11} vp of Ad5/35-HIV vector at 0 and 8 weeks. PBMCs were isolated at weeks 0, 2, 8, and 12. HIV-specific Ab titers were measured in triplicate by ELISA (E) (●, ■) and neutralizing assay (N) (○, □) (b). The detecting limitation of the neutralizing assay was 100 ND_{50}/ml. PBMCs were stimulated with HIV Env gp120 protein, and the number of cells activated to secrete IFN-γ was determined in triple wells by ELISPOT (c). SFC: spot-forming cells.

within 2 weeks of immunization (Figure 3b). The animals were boosted at 8 weeks. After 4 weeks, titers in excess of 1:50 000 were achieved. Similar results were observed in neutralizing Ab. A increase in the number of HIV-specific IFN-γ-secreting T cells was also detected in the peripheral blood mononuclear cells (PBMCs) (Figure 3c). Boosting with Ad5/35-HIV vector further increased this T-cell response.

Effect of pre-existing immunity on vaccination

To evaluate the effect of the anti-Ad5 neutralization Ab (found in 60% of the adult human population)[17] on the Ad5/35 vector, the infectivity of the vector was examined after incubation with serially diluted serum from subjects with high titers of anti-Ad5 Abs (anti-Ad5 neutralizing titer = 1:64). As shown in Figure 4, the human antisera had 1:8 anti-Ad5/35 neutralizing titer and normal human sera against either Ad5 or Ad5/35 vector was less than 1:4. The sera derived from Ad5/35-HIV-immunized monkeys showed two-fold higher neutralizing Ab titer against Ad5/35 vector than Ad5 vector.

To examine the effect of pre-existing anti-Ad5 immunity on the activity of the Ad5/35 vector *in vivo*, mice were injected i.m. with 10^{10} or 10^{11} vp of Ad5-Luc. After 8 weeks, these animals were immunized with 10^{10} vp of Ad5-HIV or Ad5/35-HIV. The HIV-specific responses were detected by the tetramer assay 2 weeks after immunization. Although pre-existing immunity to Ad5 reduced the immune response elicited by both vectors, Ad5/35-HIV was significantly more immunogenic than Ad5-HIV ($P<0.05$; Figure 4).

Discussion

This study demonstrates that an Ad5/35-HIV vector vaccine induces strong cellular and humoral immune responses with minimal toxicity in mice and rhesus macaques. A prime–boost strategy involving the DNA-HIV vaccine and the Ad5/35-HIV vector generated protective immunity against viral infection in mice.

A widely used HIV vaccine should have high immunogenicity, low cost of production, and low or no pathogenicity. Replication-defective Ad5 is one of the best vectors for HIV vaccine development. Vaccination

with recombinant Ad5 has achieved great success in inducing protection against virus infection in several animal models.[4,15,33] Ad5 is well characterized, and its subclinical disease association in humans is well known.[34,35] However, a majority of the human population (more than 60%) is infected with the Ad5 virus.[17,36,37] The neutralizing Ab and the cellular immune responses against the Ad5 fiber and capsid may reduce the efficacy of the Ad5 vector when it is used in a clinical trial.[37,38] The switching of the Ad serotypes[37,39] and the use of animal Ads[40–44] enables the partial bypass of the pre-existing immune responses to Ad5 viruses. However, there are a few drawbacks: lack of knowledge regarding the biology of these viruses, including tropism on human cells; potential difficulties in manufacturing; and the possibility of *in vivo* recombination with other human viruses leading to unknown diseases. Animal Ad vectors may induce the antigen-specific responses as strongly as Ad5 in animal models.[43] However, their immunogenicity in humans is still unknown. This study used a chimera Ad5 vector with Ad35 fiber, which relates with cell tropism. The Ad5/35, similar to Ad5, has a high productive titer in tissue culture cells, because it is commonly known that human subgroup B Ads, such as Ad5, have a considerably higher titer as compared with other subgroup viruses, including Ad35. Nevertheless, the virus displayed the cell tropism of Ad35. We explored the immunogenicity of the Ad5/35 vector encoding HIV Env gene in both mice and non-human primates. The results indicate that the Ad5/35-HIV vector elicited strong HIV-specific humoral and cellular immune responses that conferred protective immunity (Table 1 and Figure 3b and c). Coupled with the evidence that an Ad5/35 vector transduces human dendritic cells more efficiently as compared with an Ad5 vector,[18,21,26,27] these findings suggest that the Ad5/35-HIV vector is a promising candidate for human trials.

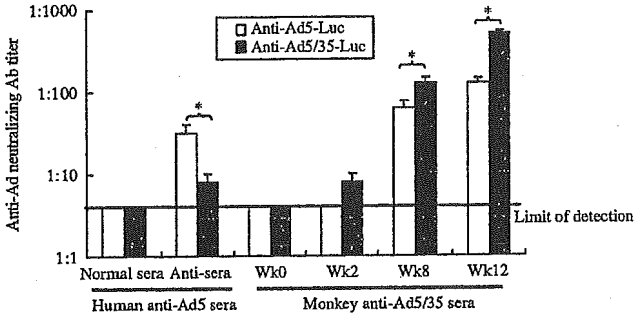

*Figure 4 Effect of pre-existing antiviral immunity. Ad5-Luc and Ad5/35-Luc vectors were incubated with an equal volume of serially diluted normal human sera (No. 2, anti-Ad5 neutralization titer <1:4), human antisera (No. 2, anti-Ad5 neutralization titer = 64), or monkey antisera from Figure 3b and c (No. 2) in triplicate and were subsequently added to infected Vero cells in a 96-well plate at 10^7 vp/well. The luciferase activity was measured 48 h after infection. The neutralizing titer was calculated with limited serum dilution when the luciferase activity in the Ad-infected cells was equal with the background. Average and standard deviations for three independent experiments are shown. *Mean values are significantly different between groups.*

Another concern regarding the use of the Ad5 vector in clinical trials is its strong tropism to hepatocytes that is caused by the high expression of CAR in the hepatocytes. Our experiments showed a high expression of the Ad5 vector in the liver in both mice and non-human primates after i.m. administration, but not of the Ad5/35 vector (Figure 1). In contrast to Ad5 vector, Ad/35 vector did not elevate the levels of serum markers (GOT/GPT) of hepatotoxicity and key proinflammatory cytokines (IFN-γ and IL-6) in mice (Figure 1b and c). These results demonstrate that, as a vaccine vector, Ad5/35 vector is safer than Ad5 vector. However, low expression of Ad5/35 vector in monkey liver was still detected after i.m. administration of Ad5/35-Luc vector to monkeys (Figure 3a). It may have resulted from low capacity of Ad5/35 to infect liver nonparenchymal cells, but not liver parencymal cells.[20] Interestingly, we found a certain magnitude of Ad5 vector expression in the posterior cerebrum and cerebellum of monkeys; however, the Ad5/35 vector was not expressed (Figure 4a). Nevertheless, in the present experiment, we could not precisely define the location of the Ad5-infected cells or determine whether the infection potentially causes local inflammation or toxicity. However, potential brain infection after Ad5 vector administration is a safety concern because intranasal administration of the Ad5 vector has been reported to result in the infection of the central nervous system.[45]

In this study, the effect of pre-existing anti-Ad5 immunity on the Ad5/35 vector was explored along with several immunization protocols as follows. (1) Both *in vitro* and *in vivo* studies demonstrate that the Ad5/35 vector is significantly less susceptible to neutralization by anti-Ad5 Abs as compared with a conventional Ad5 vector (Table 2 and Figure 4). The administration or infection of Ad can induce immune responses against the Ad hexon, penton, and fiber antigens. The exchange of fiber can partially reduce the inhibition of the

Table 2 Effect of pre-existing antiviral immunity

	Prime	*Anti-Ad5 neutralizing Ab titer*	*Boost*	*Tetramer assay (%)*
Control	Non	<1:4	Ad5-HIV (10^{10} vp/mouse)	4.8±0.2
			Ad5/35-HIV (10^{10} vp/mouse)	5.1±0.2
Low dose	Ad5-Luc (10^{10} vp/mouse)	1:102	Ad5-HIV (10^{10} vp/mouse)	2.3±0.4 *
			Ad5/35-HIV (10^{10} vp/mouse)	4.6±0.6
High dose	Ad5-Luc (10^{11} vp/mouse)	1:248	Ad5-HIV (10^{10} vp/mouse)	0.5±0.1 *
			Ad5/35-HIV (10^{10} vp/mouse)	2.6±0.4

After 8 weeks, naive mice or mice pretreated with 10^{10} or 10^{11} vp of Ad5-Luc vector (6 mice/group) were immunized with 10^{10} vp of Ad5-HIV or Ad5/35-HIV vector. At the time of vaccination, anti-Ad5 neutralizing titers were measured in Ad5-Luc-treated mice. At 2 weeks after vaccination, the HIV-specific responses were detected by an HIV-specific tetramer assay. *Mean values are significantly different between the groups.

pre-existing immunity against the parent Ads (Table 2 and Figure 4). The exchange of other genes, including hexon and penton, may further reduce the inhibition of pre-existing anti-Ad5 immunity. (2) We also explored the immune responses by using the same vector for prime/boost. When the mice were immunized i.m. with 10^{10} vp of Ad5/35-HIV 1–3 times at 4-week intervals, the HIV-specific cell-mediated immune responses were detected by the tetramer assay. An increased response was observed after the second immunization but not after the third immunization (data not shown). High anti-Ad5/35 neutralizing Ab after second immunization may block the Ad5/35-HIV infection (Figure 4). These results are in agreement with the data from our study on monkeys (Figure 3b and c) and with that from studies by other groups,[33,43] and other virus such as MVA vector may be applicable for further boost if high immune responses are required. The DNA-HIV vaccine prime/Ad5/35-HIV vector boost regimen greatly increased HIV-specific cell-mediated immune responses (Table 1 and Figure 3b and c), but not the humoral immune response (Figure 2c). This suggests that DNA vaccination enhances adenoviral recombinant-induced cell-mediated immunity rather than humoral immunity, as described by other groups.[4,38] Furthermore, DNA vaccine priming can reduce the humoral response to the adenoviral antigens and can counterbalance the impaired B-cell response to the antigen expressed by the adenoviral recombinant in mice that are preimmune to Ad.[46] This DNA prime/Ad5/35 boost regimen might be highly suitable for use in humans with previous exposure to the Ad5 virus. (3) To examine the immunogenicity and protective immunity of the Ad5/35 vector, we used HIV-1 IIIB in this study, because the strain has been well studied and we can compare our data from the new vector with that from other studies. For the clinical trail, we have constructed DNA vaccine and Ad5/5 vector expressing ENV and GAG of a Clade C HIV-1 isolate and similar results with HIV-1 IIIB strain were obtained in a mouse model (manuscript in preparation).

Considered together, using Ad5/35 vector, we developed an HIV vaccine with a higher immunogenicity and low pathogenicity. The Ad5/35-HIV vaccine induced strong HIV-specific immune responses in both BALB/c mice and rhesus monkeys. Priming with a DNA-HIV vaccine followed by Ad5/35-HIV boosting yielded protection against viral infection in mice. The Ad5/35 vector may be a promising vaccine for human clinical trial.

Materials and methods

Recombinant vectors

E1,E3-deletion, replication-defective recombinant viruses were constructed with an Ad generation kit (Avior Therapeutics Inc., Seattle, WA, USA).[21] Briefly, a 5.2k bp *Sal*I/*Pst*I fragment containing CAG promoter-HIV_{IIIB} rev/env gp160-polyA was isolated from pCAGrev/env.[28] A shuttle plasmid (pLHSP) containing Ad5 positions 22–342, Ad5 3523–5790, *Escherichia coli ori*, and ampicillin-resistant gene was obtained from Avior Therapeutics Inc. (Seattle, WA, USA). The 5.2k bp blunted fragment was subcloned into blunted *Eco*RI site of pLHSP plasmid vector to generate pLHSP-HIV shuttle plasmid. The pLHSP-HIV shuttle plasmid was linearized with *Pac*I and transfected with E1,E3-deletion, chimeric Ad5 or 5/35 genome to human embryonic kidney (HEK293) cells using calcium precipitation method to generate recombinant virus, Ad5-HIV and Ad5/35-HIV, respectively. The recombinant virus (Ad5/35-HIV, Ad5-HIV) was propagated in HEK293 cells and purified by two repetitions of the CsCl methods described elsewhere.[47] The total concentration of virions in each preparation was calculated from the optical density at 260 nm (OD_{260}), using the formula 1 $OD_{260} = 1 \times 10^{12}$ vp/ml. The HIV_{BH8} gp160-expressing replication-competent vaccinia virus (WR strain, vPE16; HIV_{BH8} gp160 has 97.32% amino-acid homology with HIV_{IIIB} gp160) was obtained from the AIDS Research and Reagent Program, National Institutes of Health, Rockville, MD, USA (Cat. No. 362). The vPE16 vectors were propagated in CV1 cells. The Ad5-Luc and Ad5/35-Luc vectors expressing luciferase coding gene were described previously.[26] The DNA-HIV vaccine (pCAGrev/env) containing HIV_{IIIB} *rev* and *env* genes has been previously reported.[28]

Biodistribution of Ad5 and Ad5/35 vectors in vivo

The experiment was performed as previously described.[48,49] In brief, the Ad5-Luc or Ad5/35-Luc vectors (10^{11} vp/mouse) were injected i.m. into BALB/c mice. On days 3 and 10, the mice were anesthetized with a 2% isofluorane/air mixture, and a single dose of 150 mg/kg luciferin in normal saline was administered intraperitoneally. The CCD images were obtained using a cooled *in vivo* imaging system (IVIS) CCD camera (Xenogen, Alameda, CA, USA) and analyzed. To study the viral biodistribution in primates, two rhesus monkeys (2 years old, male) were administered i.m. 10^{11} vp of Ad5-Luc or Ad5/35-Luc. At 3 days after administration, the luciferase activity was detected in the monkey organs (brain, liver, kidney, spleen, heart, and lung) using the Luciferase Assay Systems (Promega, Madison, WI, USA). Serum GOT and serum GPT were measured in mouse or monkey sera at the Kitayama-Rabesu Institute (Ina, Nagano, Japan). The concentration of serum IFN-γ and IL-6 was measured using the IFN-γ and IL-6 ELISA kits (Biosource, Camarillo, CA, USA), respectively, according to the manufacturer's protocol.

Animal immunization

Female BALB/c mice (8-week-old; H-$2D^d$) were purchased from Japan SLC Inc., Shizuoka, Shizuoka-ken, Japan. The mice were immunized with an i.m. injection of 100 μg of pCAGrev/env or pCAGempty plasmid DNA in phosphate-buffered saline (PBS) at 0, 1, and 2 weeks and were boosted with 10^{10} vp of Ad5/35-HIV or Ad5/35-Luc vector at week 3. For the time-course study, the mice were administered a single i.m. injection of 10^{10} vp of Ad5/35-HIV vector per mouse. To study the effect of pre-existing antiviral immunity on vaccination, the mice were injected i.m. with 10^{10} or 10^{11} vp of Ad5-Luc vector and then immunized 8 weeks later with 10^{10} vp of Ad5-HIV vector or Ad5/35-HIV vector. For rhesus monkey immunization, 10^{11} vp of Ad5/35-HIV vector was injected i.m. into two rhesus monkeys (2 years old, male) at weeks 0 and 8.

Intracellular cytokine staining assay

IFN-γ-secreting $CD8^+$ T cells were detected by the protocol recommended by the manufacturer (Cytofix/CytoPerm Plus kit, PharMingen, San Diego, CA, USA). In brief, lymphocytes were isolated from the mouse spleen. A single cell suspension was incubated with 10 μg/ml of the HIV V3 peptide (NNTRKRIQRGP GRAFVTIGKIGN) for 24 h at 37°C. At 2 h before the end of incubation, 1 μg/ml of GolgiPlug was added. The cells were washed with staining buffer (3% fetal calf serum (FCS), 0.1% sodium azide (NaN_3) in PBS), blocked with 4% normal mouse sera, and stained with phycoerythrin (PE)-conjugated anti-mouse CD8 Ab (Ly-2, PharMingen). The cells were then suspended in 250 μl of Cytofix/Cytoperm solution at 4°C for 20 min, washed with Perm/Wash solution, and stained with anti-mouse IFN-γ Ab conjugated with fluorescein isothiocyanate (FITC) (PharMingen) at 4°C for 30 min, followed by flow cytometric analysis.

Tetramer assay

The tetramer assay used a PE-conjugated $H\text{-}2D^d$/p18 tetramer (RGPGRAFVTI), as previously described.[28] In brief, splenocytes were isolated from mice and incubated for 30 min at 4°C with 4% normal mouse serum in PBS. Cells were stained with FITC-conjugated anti-mouse CD8 Ab (Ly-2, PharMingen) for 30 min at 4°C. After washing twice with the staining buffer (3% FCS, 0.1% NaN_3 in PBS), the cells were incubated with the tetramer reagent for 15 min at 37°C, followed by flow cytometric analysis (Becton Dickinson).

Recombinant vaccinia virus used for the challenge study

Using vPE16 vaccinia virus, the virus challenge experiment was performed as described previously.[28] Vaccinated female mice were intraperitoneally challenged with 10^8 PFU of vaccinia virus vPE16 at 2 or 7 weeks after the final immunization. At 6 days after challenge, the mice were killed, their ovaries were sonicated, and the vPE16 titer was determined by serial 10-fold dilution on a plate of CV1 cells. Infected cells were detected by staining with crystal violet and plaques were counted at each dilution.

Detection of HIV-1-specific Ab

The HIV-1-specific Ab was detected by the Western blotting method and the enzyme-linked immunosorbent assay (ELISA). By Western blotting method, the HIV envelope glycoprotein gp160-coated membrane from the New Lav Blot 1 kit (Bio-Rad, Marnes-la-Coquette, France) was incubated with a 100-fold dilution of mouse serum followed by an affinity-purified horseradish peroxidase (HRP)-labeled anti-mouse immunoglobulin (ICN Pharmaceuticals Inc., OH, USA). HIV gp160 protein was detected using the ECL Plus Western Blotting Detection System (Amersham Pharmacia Biotech).

ELISA was performed as described elsewhere.[10] To summarize, 96-well microtiter plates were coated with 10 μg/ml of HIV_{IIIB} gp120 protein (donated by AIDS Research and Reference Reagent Program, National Institutes of Health) and incubated overnight at 4°C. The wells were blocked with PBS containing 1% bovine serum albumin (BSA) for 2 h at room temperature. They were then treated with 100 μl of serially diluted antisera and incubated for an additional 2 h at 37°C. The bound immunoglobulin was quantified using an affinity-purified HRP-labeled anti-mouse Ab or anti-monkey Ab (both from Sigma). The mean Ab titer was expressed as the reciprocal of the serial serum dilution that exceeded the assay background by 2 s.d.

The HIV-specific neutralizing titer of immune mice or monkeys was also measured. The serially diluted antisera were incubated with 200–300 blue spot-forming units (BFU) of $HIV\text{-}1_{LAI}$ at 37°C for 1 h. The mixture was incubated with confluent MAGIC5 cells (from Dr Tatsumi, National Institute of Infectious Diseases, Tokyo, Japan),[50,51] Dulbecco's modified Eagle's medium (DMEM) with 10% FCS and 0.2 mg/ml of G418 in a 96-well plate at 37°C for 2 days. The cells were fixed with fixing solution (1% formaldehyde, 0.2% glutaraldehyde in PBS) for 5 min and stained with staining solution (4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM magnesium chloride, 0.4 mg/ml X-gal in PBS) at 37°C for 18–24 h. The staining was stopped by removing the staining solution and the cells were washed twice with PBS. The blue spot in each well was counted after the staining, and the neutralizing titer was calculated as $(1-(\%\text{ infection}/\%\text{ infection of control wells}))\times 100$. The 50% neutralization dose (ND_{50}) is defined as the concentration of the Ab that reduced the number of infected cells by 50%. The detecting limitation of the assay was 100 ND_{50}/ml.

ELISPOT assay

The frequency of HIV-specific IFN-γ-secreting cells in monkeys was determined using an ELISPOT assay kit (U-Cytech, Utrech, The Netherlands) according to the manufacturer's manual. In brief, 2×10^5 monkey PBMCs were stimulated in triplicate wells with 1 μg/ml of the HIV_{IIIB} gp120 protein for 16 h at 37°C. Nonstimulated cells were used to assess the background. The cells were transferred to an anti-IFN-γ Ab-coated 96-well plate and incubated for 5 h at 37°C. The cells were removed and 200 μl/well of ice-cold deionized water was added to lyse the remaining PBMCs. Subsequently, the plate was washed with PBS containing 0.05% Tween 20 (PBS-T) and incubated with biotinylated anti-IFN-γ Ab for 1 h at 37°C. After 10 washings with PBS-T, 50 μl of gold-labeled anti-biotin Ab was added and incubated for 1 h at 37°C. The plate was washed 10 times with PBS-T, and 30 ml of activator solutions was added. The plate was incubated in the dark for 30 min at room temperature to develop spot formations. After 30 min incubation, the plate was washed with deionized water and air-dried; spots were counted by a computer-assisted video image analysis. The results were expressed as spot-forming cells (SFC) per million cells.

Ad-specific neutralizing assay

Ad5-Luc or Ad5/35-Luc vector (10^7 vp) was incubated with an equal volume of serially diluted normal human sera (anti-Ad5 neutralizing titer $<1{:}4$), human antisera (anti-Ad5 neutralizing titer $=1{:}64$), or monkey sera (at weeks 0, 2, 8, and 12 after immunization with Ad5/35-HIV vector) at 37°C for 2 h. The mixture was incubated with confluent Vero cells in a 96-well plate at 37°C for an additional 48 h. The luciferase activity was detected by Luciferase Assay Systems (Promega, Madison, WI, USA).

The neutralizing titer was calculated with limited serum dilution when the luciferase activity in the Ad-infected cells was equal with the background.

Data analysis

All values were expressed as means ± standard error (s.e.). Statistical analysis of the experimental data and controls was conducted with one-way factorial analysis of variance. Significance was defined at $P<0.05$ in the statistical analysis.

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高齢社会にあって禁煙は歯科の重要テーマ

福岡歯科大学口腔保健学教授　埴岡隆

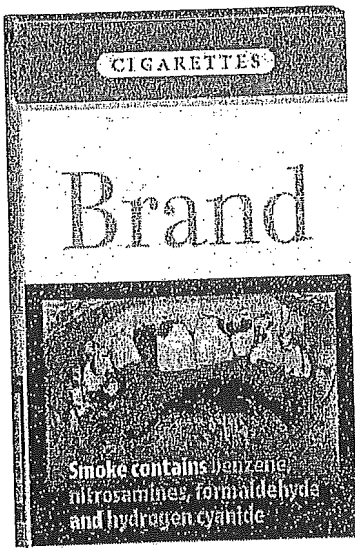
喫煙は8020運動の大きな壁

1. 家族で守る口の健康

全身の健康は口の健康から―歯科の最近の話題に、口腔と全身の健康がある。高齢社会が到来して、口の健康の大切さが高齢者に接する医療者に再認識されるようになった。口腔の不潔は、抵抗力が低下している高齢者の健康には脅威である。

口腔と直結する肺では、口の細菌が原因で炎症が起こり、生命が脅かされる。そして、咀嚼や嚥下といった日常生活に不可欠な機能の維持も高齢者のQOLには欠かせない。適切な口腔ケアにより口臭が消え、施設の臭いがなくなり、口腔の重要性が実感されるようになった。

高齢者の例とは別に、最近になって歯周病が、糖尿病、心疾患、呼吸器疾患、妊娠出産異常、骨粗鬆症といった全身性の疾患と関係があることがわかってきた。歯周ポケットの慢性炎症病巣から細菌や炎症性物質が、血流により全身に、しかも持続的に運ばれるからである。



EU諸国でタバコ箱に採用される口腔画像警告表示
(この画像は無煙タバコの使用頻度が高い東南アジア諸国でも採用)

口腔は身近な体の一部

口腔は日常生活で最も身近に感じられる体の一部分である。口の健康は歯を磨くことから始まる。ほとんど誰でも歯が生え始めるとブラッシングの真似事を始める。歯が生えることは、本人はもとより、親、親戚の共通の関心事であり、歯が生えるたびに歯を大切にしようと健康感を再認識させられる。

歯を磨く健康習慣は親子の協力で始まり、成長してからも家族が同じ場所で行う健康習慣である。また、甘味食品の摂取(間食)も齲蝕の予防に欠かせない習慣であり、間食も家族が気遣う生活の場面である。

そして、喫煙が歯周病の原因であることが最近わかった。歯周病は歯が抜けることにつながるので、「8020運動」にも大きな壁となる。喫煙は全身の健康に脅威であるが、口腔の健康も脅かすので禁煙は歯科の重要なテーマになってきた。

歯や歯茎の着色、舌の苔・口臭

2. 口の健康はタバコの煙に敏感

口は異物の体内侵入に敏感である。おおよそのタバコの煙は口から入る。したがって、口の健康はタバコの煙に敏感である、といっても過言でないだろう。

歯の着色はもっともわかりやすい。前歯の詰め物があれば、その周りが黒くなる。歯茎も喫煙で着色することがわかってきた。歯とは違って、煙草の煙が沈着するのではない。歯肉の色素細胞が喫煙の刺激でメラニン色素という茶褐色の色素を生成して、歯茎が黒っぽくなる。禁煙すると3年程度で非喫煙者のレベルにもどる。

また、舌に苔がつくことがある。この苔は口臭の原因になるが、喫煙者には特有の口臭がある。生命に直結する口腔癌は喫煙が原因である。アルコール摂取と同時に喫煙をすると、発癌物質が溶解して粘膜に吸収されやすくなり、口腔癌の危険性が高まる。

そこで、米国では、毎日鏡で粘膜をセルフチェックすることが勧められている。口腔癌は、舌、頬の粘膜、歯肉などの粘膜が周囲と違って白色が増すと要注意である。

ところが、口腔への影響は医師・保健師・養護教諭などの歯科以外の保健専門家からも注目されはじめている。というのは、口腔は目に見えるからである。

喫煙は身体に様々な影響を及ぼす。が、残念ながら、喫煙者や医療者本人にはその影響を見たり見せたりする機会は少ない。そして、若い喫煙者が自覚できるものも少ない。口は見ることができ、そして、同時に、自覚できるという特徴がある。

親の喫煙による影響

受動喫煙も口の健康に影響する。米国の大規模な研究では受動喫煙と歯周病に関連があり、子どもの齲蝕との間にも関連があることが発表された。厚生労働省科学研究班は、子どもの歯茎の色素沈着は、親の喫煙によって増加していることを明らかにした。このようにタバコの煙は口の健康に大きな悪影響をもたらす。

喫煙は口腔の健康を脅かすだけでなく歯科治療の効果を少なくする。歯周病の治療の効果を少なくしたり、インプラントの成功率が低下したりする。また、歯を抜いた後の傷の治りが悪く、ドライソケットの状態になり痛みが続く。

喫煙すると歯が抜け易くなることから、せっかく補綴や充填治療をしても、その歯自体が長持ちしない。義歯は残った歯に負担をかけているので、義歯を支える歯が長持ちしなくなると、作りなおさないといけなくなる。歯が全部なくなって総義歯になった人でも、喫煙は関係する。タバコのヤニが義歯に沈着し口臭の原因になる。

このように、歯の治療にとっても、喫煙は大きな負担になる。

口腔の健康を通して全身の健康づくり

3. 健康な身体(からだ)は口腔ケアと禁煙

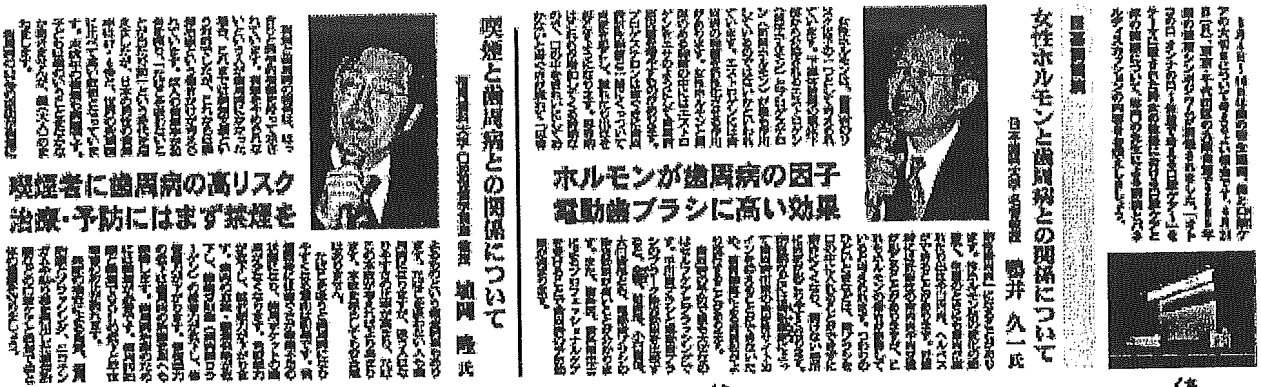
喫煙は口腔の健康を蝕み、口腔の健康は全身の健康と関係があり、口腔と関連する全身の健康は喫煙と関連がある。喫煙は、口腔の健康を通じてQOL・全身の健康を脅かす。そして、口腔の健康は、本人が見ることができ、専門家が見ることができる。口腔の健康を通じて、健康を自覚してもらうことができる。

さらに、誰もが人生の早い時期から口腔の保健行動を日常的に行っている。口腔ケアと禁煙による口腔の健康づくりは、全身の健康づくりへと発展させることができる。鏡の前で十分な明かりの下で口腔を眺めてみるのも良いだろう。

本来ならこの記事はここで終わるのだが、他に気を配らないといけないことがひとつある。日本ではまだ馴染みがないが、無煙タバコ(煙の出ないタバコ)の一種であるガムタバコが世界で初めて販売された。無煙タバコは口腔がんの原因になるニトロソアミンを含んでいる。

日本では、健康増進法により、喫煙場所が制限され、そして、タバコ規制条約の発効に伴って、喫煙の規制が格段に進む。その過程で煙の出ないチューイングタバコが発売された。ガムの形状をしているため、子どもによる使用は要注意であるが、インターネットで販売されている。

無煙タバコ使用は、煙が出ないので学校現場では発見されない。米国では大リーガーの選手が広告塔になって子どもにあっという間に広がってしまった。今、日本の関係者の取組みの動向が世界から注目されている。



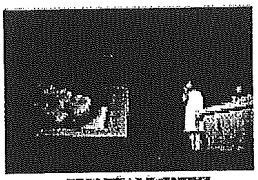
2005年
歯の健康シンポジウム

オトコの口、オンナの口

～性差で考える口腔ケア～

紙上採録

主催：日本歯科医師会、朝日新聞社　後援：厚生労働省、日本医師会、東京都歯科医師会、財団法人8020推進財団、日本口腔衛生学会、日本歯周病学会



喫煙と歯周病との関係について

喫煙者に歯周病の高リスク
治療・予防にはまず禁煙を

女性ホルモンと歯周病との関係について

ホルモンが歯周病の因子
電動歯ブラシに高い効果

ホルモンの変化により、口腔内も変化し続ける

子どもに歯の大切さを教えていきたいです

口と体の健康には、大きな関わりがありますね

朝日新聞　意見広告　松下電工ほか
2005年6月4日

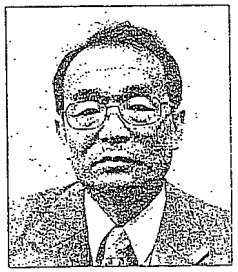
9学会で作成

日本初の禁煙ガイドライン 医療従事者から患者へ

喫煙対策関連9学会が合同で作成した禁煙ガイドラインがほぼ完成した。ここでは、GL合同研究班の藤原久義班長（日本循環器学会、岐阜大大学院教授）、班員の堺岡隆氏（日本口腔衛生学会、福岡歯科大教授）に禁煙GLの目的や意義、各学会の取り組みなどを聞いた。

疾患・背景別に禁煙治療法を提示

禁煙GL9学会合同研究班　班長・藤原久義氏



喫煙関連9学会が合同で作成した「禁煙ガイドライン」（以下GL）が11月に公表される。ここでは、禁煙GL9学会合同研究班班長の藤原久義班長（岐阜大大学院医学研究科教授）に、GL作成の背景や、目的、効果、今後の普及策などについて聞いた。

―GL作成の背景および目的、期待される効果は何でしょうか。

藤原氏　このGLは、医師・歯科医師・看護師・保健師・歯科衛生士・薬剤師などの医療関係者を対象にしたもので、医療関係者が患者に禁煙指導をどう行うかを示したものです。これまで日本では、医療関係者・学会の禁煙に対する取り組みが遅れていました。適切な禁煙GLもありませんでした。そこで、患者にどう禁煙指導するかを明記したGLを9学会合同で策定することになりました。

禁煙は容易にできるものではなく、個人で行おうとしても、100人中、90～95人が失敗します。禁煙指導を受けても2分の1以上が失敗します。しかも日本では、医師による禁煙指導はあまり行われていません。

日本では、今でも男性の喫煙率が約50％と、国際水準からみると圧倒的に高い状況です。喫煙率は、医師など医療関係者においても高く、まず、足もとから禁煙を始める必要があります。

そこでこのGLが、多くの医療関係者に配られ、まず医療関係者が禁煙することが大事です。そして、各医療関係者がこのGLにそって、まず患者に喫煙状況を聞き、喫煙者に積極的に禁煙指導をすることが望まれます。これにより、喫煙率の減少および禁煙率の上昇が期待できます。そして、国民の健康がさらに増進されることを期待しています。

―GLの特徴は何でしょうか。

藤原氏　日本での禁煙に対する取り組みの遅れは、この3～4年でようやく盛り返していますが、9学会がGL作成に着手した2003年には、どの学会も対応が遅れていることを自覚し、危機感をいだいていました。

このため、通常、日本の医学界では異なる領域の学会の横のつながりが密ではありませんが、今回は、各学会の危機感で合同のGL作成に成功したと思います。

またGLでは、喫煙者は喫煙病（依存症＋喫煙関連疾患）という全身疾患を持ち、積極的治療を必要とする患者として認識しています。喫煙は能動喫煙者に循環器、呼吸器、口腔組織のみならず、全身の臓器にさまざまな疾患を引き起こします。また受動喫煙にさらされる乳幼児や、妊婦を介した胎児など周囲の非喫煙者にも悪い影響を与えます。従って、禁煙GLの作成は、こうした喫煙に関係するさまざまな学会の参加が不可欠で、今回は実際にこうした9学会が合同でGLを策定しました。

2つ目の特徴は、喫煙者一般の禁煙治療と、それぞれ異なる個々の疾患や背景を持っている喫煙患者の禁煙治療を分けて述べている点です。

前者は第1章の総論で、後者は第2章の各論で記しています。第二章・各論では、循環器疾患、呼吸器疾患、女性と妊産婦、小児・育少年、歯科・口腔外科疾患、術前・外科疾患の6項目に分けて、このように異なる疾患や背景を持つ喫煙患者それぞれに対する禁煙治療の方法を解説しています。このため、例えば肺がん患者の禁煙指導の場合は呼吸器疾患の項目を、妊産婦の禁煙指導では女性と妊産婦の項目を読めば、それぞれの喫煙患者に応じた禁煙治療方法が分かります。このように疾患・背景別に、それに応じた各禁煙治療方法を詳細に記したGLは世界でも珍しいと思います。

3つ目の特徴は、未成年者の喫煙防止と禁煙推進、禁煙を推進するための社会制度の制定・政策の必要性など緊急課題を取り上げていることです。実際に、9学会は受動喫煙防止を求めて、全国のJR6社に新幹線・特急列車などの全面禁煙の要望書を2度にわたり、提出しました。また、ニコチン代替療法を含む禁煙治療の保険適用を求める要望書も厚生労働省に提出しました。

―GLの普及策については、いかがでしょうか。

藤原氏　日本循環器学会の学会誌（11月号）への掲載を皮切りに、他の8学会も各会員にこのGLを届けます。これだけでも医師の数は相当なものになります。さらに、日本循環器学会では、私が会長を務める来年3月の学会総会で市民公開講座などを開きます。各学会がこうした取り組みを積極的に行うことで、普及が図られると期待しています。

導入部などで幅広く活用を

日本口腔衛生学会　禁煙推進委員会副委員長・堺岡隆氏

日本口腔衛生学会は2002年に禁煙宣言「たばこのない世界を目指して」を公表するなど、早くから喫煙対策に取り組んできた。禁煙ガイドライン（GL）を合同で作成した同学会の堺岡隆・禁煙推進委員会副委員長（福岡歯科大口腔保健学講座教授）は、「歯科は喫煙リスクを目に見える形で訴えることができる」とし、禁煙指導の導入部などで幅広くGLを活用してもらいたい考えを示した。同学会としても、歯科に特化したGLの作成を検討していくという。

―喫煙の悪影響を教えてください。

堺岡氏　たばこの煙が最初に入るのは口です。毒性が強い部分は唾液（だえき）に溶けます。特に飲酒時の喫煙は、アルコールに溶けたニコチンが粘膜から吸収されて発がんリスクになることが米国で指摘されるなど、口腔内への悪影響は早くから指摘されてきました。

喫煙と歯周病との関係をめぐる研究成果も増えていますし、国と日本歯科医師会が推進する8020（ハチマルニイマル）運動との関係でいえば、喫煙者の方が歯を失う時期が早いという意味で、早期喪失のリスクが高いことも分かってきました。

歯の変色や歯茎への色素の沈着など、喫煙リスクを目に見える形で、性別や年齢を問わず幅広い層の患者さんに訴えられるのが歯科の特徴です。そういう点に医科も注目しており、禁煙指導の導入部で今回のGLを活用してもらえればと思っています。

―今回のGLで歯科領域はどのような形で取り上げられていますか。

堺岡氏　各論に当たる第2章「歯科・口腔外科疾患」で、歯科領域とのかかわりを取り上げています。歯科との関係でいえば、歯を磨く、甘いモノを食べない、ブラッシングの指導といったちょっとしたアドバイスを、治療の一環として行いやすい環境があります。

歯科医師が行うと指導的な性格が強くなりがちなため、歯科衛生士がフォローするといったチームアプローチも行いやすい。1診療所当たり1人の割合で禁煙を支援することができれば、約6万人の禁煙者が生まれます。そうした禁煙指導に、今回のGLを活用してもらえればと思っています。

また、禁煙指導といっても、アドバイスから手厚いサポートまでさまざまなレベルがあります。かかりつけ歯科医機能を重視した診療所では、より意図的な禁煙指導を行うことも必要ではないかと考えています。

―今後の活動を教えてください。

堺岡氏　GLの普及に向けた方策としては、歯周病の克服を掲げる日本歯周病学会との連携が必要です。04年に禁煙宣言を公表するなど喫煙対策には積極的で歯科衛生士も多い。GLの周知を通じて、禁煙推進につなげたいと考えています。日本口腔衛生学会としても、歯科に特化した禁煙GLを作成する予定です。

また、これは個人的な意見ですが、禁煙治療を医療保険の適用にする必要があると考えています。

10月には東京で日本口腔衛生学会・総会が開催されます。6日の自由集会では、歯科関係の6団体（注）からパネリストが個人の資格で参加し、各団体が禁煙治療の制度化に貢献できる可能性を議論します。私も、歯科界のネットワークを構築する必要性を訴えたいと思っています。

（注）日本歯科医学会、日本口腔衛生学会、日本口腔外科学会、日本歯周病学会、日本歯科医師会、歯科喫煙問題研究会

一丸の指導を