sampling time, but we did not notice a rapid disease progression in these subjects compared with the other subjects tested.

We investigated the distribution of the HIV-1 envelope subtype circulating in the metropolitan area of Tokyo, Japan. The subtype determination of 89 HIV-1 strains was performed by the subtype-specific PCR distinguishing envelope subtypes A, B, C, and CRF01_AE. All the samples were amplifiable by PCR using a uniquely designed subtype-independent primer mixture. Eighty-six strains (97%) were unequivocally subtyped by the subtype-specific PCR. Thirty-four of these specimens were sequenced and analyzed phylogenetically. The subtype results completely agreed with those by subtype-specific PCR.

The most accurate HIV-1 subtyping is phylogenetic analysis of the nucleotide sequence in several distinct regions of the viral genome. However, this procedure is expensive and time consuming. In Japan, nearly all circulating strains belong to subtype B or CRF01_AE as shown in this study. Thus, we propose the following scheme for subtyping: all samples are subjected to a simple screening method such as our subtype-specific PCR to identify subtype B and CRF01_AE, and then samples judged to be other subtypes or indeterminate are further characterized by phylogenetic analysis. Based on our results, this strategy will provide a rapid, efficient, and inexpensive measure for HIV-1 subtyping in Japan for the time being.

Intersubtype recombinant forms of HIV-1 are spread in different populations and geographic areas. Because the methods used for HIV-1 subtyping in this study rely on only the *env* C2V3C3 sequence, we cannot distinguish CRFs except CRF01_AE from pure subtypes (CRF01_AE has a unique *env* sequence.). The only exception was the Pt5 sequence. This sequence was involved in the cluster of CRF02_AG reference sequences that was located close to that of subtype A reference sequences (data not shown). Thus, we determined the *pol* sequence of the Pt5 strain and analyzed it phylogenetically; it also showed coclustering with CRF02_AG references (data not shown). This strain may belong to CRF02_AG, although the full-length genome sequence is not investigated. Subject Pt5 reported that one of the possible transmissions occurred in Europe. This may be the first report of CRF02_AG in Japan.

The V3 loop contains major epitopes of neutralizing antibodies[9] and cytotoxic T lymphocytes.[8] Therefore, elucidation of the predominant V3 sequence in a particular area is important for effective vaccine development. Yamanaka *et al.*[2] reported the V3 consensus sequence derived from 69 Japanese hemophiliacs, all of whom had been infected with subtype B. Their consensus sequence is identical to that obtained from non-hemophiliac subjects in this study except one amino acid at position 27; threonine and alanine are found in 57% and 41% in their study and 38% and 54% in this study, respectively ($p = 0.12$, Fisher's exact probability test). At the tip of the V3 loop, eight different sequences were found. GPGR is predominant (73%) in the subtype B strains and GPGQ is most common in the other subtypes. Novel tip motifs GPRQ and APGQ were found for subtype B and GQGQ for CRF01_AE.

Subtype distribution is characteristically different in two major Japanese risk groups: subtype B is exclusively predominant in the risk group of homosexual contact; subtypes B and CRF01_AE are evenly predominant in the risk group of heterosexual contact. These findings agree with those by Kitsutani *et al.*,[13] although their sample number is about one-third of ours. Imai *et al.*[14] reported the gag subtype distribution in Japan and suggested that the predominate subtype in Japanese male heterosexuals had switched from B to CRF01_AE since 1994 based on the results that subtype B and CRF01_AE were found, respectively, in 7 and 1 of 8 samples collected during 1991–1993 but in 1 and 10 of 11 samples collected during 1994–1996. Their results contrasted significantly with ours (11 B of 24 samples collected during 1998–2002). This discrepancy may be attributed to differences in infection time or sampling area of the subject groups. To elucidate the exact trend of subtype distribution in Japan, molecular epidemiological studies using systematic sampling strategy are required.

Analysis of HIV/AIDS surveillance data has suggested a unique HIV-1 epidemic in Japan, characterized by a relatively low prevalence, a negligible transmission through injecting drug use, copredominance of transmissions through homosexual contact and heterosexual contact, and few signs of a growing epidemic among Japanese commercial sex workers (CSWs).[3] It is also argued that the chance of heterosexual transmission from homosexual/bisexual men is limited and that transmission has probably most often occurred between middle-aged Japanese males and non-Japanese female CSWs.[15] Our results suggest that the sexual activity of homosexual men is limited in closed circles and that transmission associated with commercial sex linked to Southeast Asia is an important factor in heterosexual transmission in Japan. These may be useful in designing strategies to prevent an HIV-1 epidemic in this nation.

GenBank accession numbers for the new sequences described in this study are AY253944–AY253980.

ACKNOWLEDGMENTS

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Structure and expression of the human oocyte-specific histone H1 gene elucidated by direct RT-nested PCR of a single oocyte

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Abstract

Oocyte-specific histone H1 is expressed during oogenesis and early embryogenesis. It has been described in mice and some nonmammalian species, but not in humans. Here, we identified the cDNA in unfertilized human oocytes using direct RT-nested PCR of a single cell. Sequencing of this cDNA indicated an open reading frame encoding a 347-amino acid protein. Expression was oocyte-specific. Homology was closest with the corresponding gene of mouse (H1oo; 42.3%), and, to lesser extent, with that of *Xenopus laevis* (B4; 25.0%). The gene, named osH1, included five exons as predicted by the NCBI annotation project of the human genome, although the actual splicing site at the 3′ end of exon 3 was different by 48 nucleotides from the prediction. The presence of polyadenylation signals and successful amplification of cDNA by RT-PCR using an oligo(dT) primer suggested that the osH1 mRNA is polyadenylated unlike somatic H1 mRNA. Our technique and findings should facilitate investigation of human fertilization and embryogenesis.

Keywords: Human; Oocyte; Oocyte-specific histone H1

Histones are the major protein constituents of chromatin in eukaryotic cell nuclei. Two types of histone proteins exist: the core histones H2A, H2B, H3, and H4 and the linker histone H1. An octamer of the core histones intertwined with 146 bp of DNA forms a component of the nucleosome core particle. The linker histone H1 is involved in sealing DNA joining adjacent nucleosome core particles to maintain higher-order packaging of chromatin. In mammals, each of these classes except for H4 is subdivided into several subtypes [1]. The most divergent class of histones is the H1 protein family, which consists of eight subtypes in mammals: the somatic variants H1a, H1b, H1c, H1d, H1e, and H1^0; the testis-specific variant H1t; and the oocyte-specific variant H1oo [2–6].

Oocyte-specific H1 is characteristic in a number of ways. First, it is expressed only during oogenesis and early embryogenesis and then replaced by somatic H1 subtypes shortly after fertilization [6–8]. This replacement may loosen the condensed chromatin structure, resulting in a change in transcriptional pattern from the oocyte to the embryo [9–11]. Second, unlike most other subtypes, it contains both introns and a poly(A) tail, and lacks characteristic motifs in the promoter regions that are shared by the other H1 genes [12]. Finally, in comparison with other subtypes, sequence homology of oocyte-specific H1 genes between different organisms are relatively low.

All H1 subtypes except for oocyte-specific H1 have been characterized at the levels of protein and gene in both humans and mice [5,6]. Oocyte-specific H1 has been identified in four species; csH1 in the sea urchin [13], B4 in *Xenopus laevis* [7,14], H1M in the zebrafish [15], and H1oo in the mouse [6]; but it has not yet been identified in humans. Oocyte-specific H1 may play an important role in forming the male pronucleus during fertilization, since remodeling of sperm chromatin requires replacement of protamine by oocyte-specific H1. Thus, identification and characterization of the human

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oocyte-specific H1 gene would be prerequisite for elucidation of human infertility.

Both clinical and technical constraints limit genomic and protein investigation in human oocytes. Since oocytes usable for research are limited to those failing to initiate cleavage for more than 2 days after retrieval, the amount of available sample is absolutely insufficient for the conventional analysis of gene expression at the molecular level. Therefore, we developed a new method for investigation of gene expression in a single oocyte and used it to identify and characterize the human oocyte-specific histone H1 gene.

Materials and methods

All procedures were approved by the Ethics and Legal Committee of Keio University Hospital. Specimens used in our research were obtained from informed and consenting patients with written agreements.

Cells. Human oocytes were retrieved from infertile women by routine methods to carry out intracytoplasmic sperm injection (ICSI) according to the standard procedure at the Infertility Clinic of Keio University Hospital. After incubation for a few hours, a spermatozoon was injected into each oocyte. If cell cleavage was not initiated after at least 48 h, an oocyte was considered unfertilized and used in our program if the donor couple approved.

Specimens of normal human ovarian tissue were obtained from consenting women of reproductive age who were undergoing oophorectomy in the course of surgery for uterine cervical cancer or uterine leiomyoma at Keio University Hospital. Human ovarian granulosa cells were collected from aspirated follicular fluid during the oocyte retrieval procedure. Briefly, the fluid was centrifuged at 1500 rpm for 5 min. The cell pellet was washed twice by suspension and centrifugation.

MOLT-4 cells (a human T-cell line) were maintained at 37 °C in RPMI 1640 medium supplemented with 10% fetal bovine serum, 0.1 M L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin in an atmosphere containing 5% CO_2.

Human semen was obtained with consent from healthy men who had come to the outpatient clinic to have their sperm examined. The semen was processed using polyvinylpyrrolidone-coated colloid media for purification of sperm.

RNA preparation. RNA in a single human oocyte was analyzed directly by our newly established method. An unfertilized oocyte selected from sperm-injected oocytes by aspiration was transferred into 1.0 µl of 0.2% sarcosyl solution to give a final volume of 2.0 µl. This concentration of sarcosyl was enough to break down cell membrane of oocytes without inhibiting reverse transcription after a 10-fold dilution. After suspending and agitation, the cell lysate was immediately frozen in liquid nitrogen and then stored at −28 °C in a deeper freezer until use. The lysate was directly subjected to RT-nested PCR as described below. Total RNA was isolated from human ovarian tissue, human ovarian granulosa cells, MOLT-4 cells, and sperm using the RNAeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Isolated RNAs were stored at −28 °C. RNAs from other human tissues (breast, colon, kidney, liver, lung, skeletal muscle, and spleen) were purchased from Stratagene (CA, USA).

RT-nested PCR. Sixteen primers were designed according to the genomic sequence [AC080007; *Homo sapiens* 3q BAC RP11-529F4 (Roswell Park Cancer Institute Human BAC Library)] and the mRNA sequence [XM_067747; the gene similar to the H1 histone family (oocyte specific)] predicted by the NCBI (National Center for Biotechnology Information) annotation system (Table 1). The RNA was converted to cDNA using ThermoScript reverse transcriptase (Invitrogen, CA, USA) and RNAsin (Promega, WI, USA) according to the manufacturer's instructions. The cDNA was then amplified by nested PCR using AmpliTaq (Applied BioSystems, NJ, USA) and PCRx Enhancer System (Invitrogen) to attain efficient amplification of GC-rich sequences according to the manufacturer's instructions. The amplification steps involved 30 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min. PCR products were analyzed by electrophoresis in 1.5% agarose gels. All the primers listed in Table 1 were used for each oocyte. For RNAs of ovarian granulosa cells, sperm, MOLT-4, and human tissues, primers used were GM1D for RT; GM1D and MR3U in first-round PCR; and GM2D and MR4U in second-round PCR. As a control, RT-PCR of β-actin was performed with primers, 5′-CACTCTTCCAGCCTTCC TTCC-3′ and 5′-CGGACTCGTCATACTCCTGCTT-3′, which were designed according to the previously reported data [16]. The second-round PCR was not performed for the β-actin controls.

Table 1
Sequences of primers designed for RT-nested PCR

Primers	Sequence
MR3U	5′-GGTGAGGGGTCTGCTGGCTG-3′
MR4U	5′-GTCTGCTGGCTGCACCTGTC-3′
GM2U	5′-GGAGGCCCGAGCCACAGCAG-3′
GM2BU	5′-TCCCGGTGGGACGCCGCCAC-3′
GM2D	5′-CGAGGAGGCCACGGCGCATG-3′
GM1D[a]	5′-CAGTGGCCCCCCTGGCTTTG-3′
MR5U	5′-AATCCAGCCCAGGAAGATGG-3′
MR6U	5′-CTCCCAGGAGAGCGGGTGAG-3′
MR4D	5′-CACCTTGCCCACGTTGGGAG-3′
MR3D[a]	5′-CTTTGCTGGCCTCTTGGCTG-3′
MR7U	5′-GGCAGGGGCCAAACACCAAG-3′
MR8U	5′-TGCTCCTGCTAAGGGCAGTG-3′
MR2D	5′-GATGAGGCCTTGATGGGCAG-3′
MR1D[a]	5′-CTTCAGCCCTCTGGCTGGAC-3′
MR8D	5′-TAGGGCAGAGGCTCGGTCTC-3′
MR7D[a]	5′-AGAGCAGTGGTTAGTTGAAG-3′

[a] Primers GM2D, MR1D, MR3D, and MR7D were used for reverse transcription.

Sequencing. PCR products were sequenced by Takara (Shiga, Japan) using a DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, NJ, USA).

Accession Number. AY158091 for the oocyte-specific histone H1 gene.

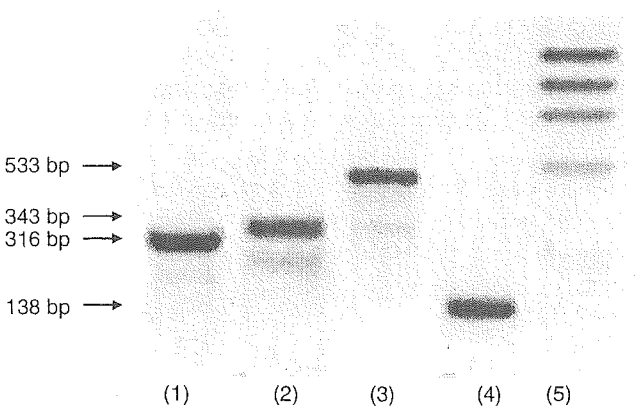

Fig. 1. Products of RT-nested PCR of a single human oocyte. Primer pairs used (Table 1) are: (1) MR3U/GM1D, MR4U/GM2D; (2) GM2U/MR3D, GM2BU/MR4D; (3) MR5U/MR1D, MR6U/MR2D; and (4) MR7U/MR7D, MR8U/MR8D. An *Hae*III digest of ΦX DNA is used for molecular weight markers (5). A contig of cDNA sequence was established from the DNA sequences of the four products.

Results

Identification of H1 mRNA from a single human oocyte

Since few human oocytes are available for our experiments, we chose RT-nested PCR rather than Northern blotting in order to identify oocyte-specific histone mRNA. The lysate of a single oocyte was directly subjected to RT-nested PCR using four sets of primers, whose sequences were determined based on a putative human oocyte-specific gene (Table 1). Discrete bands with expected lengths were detected from any sets of primers (Fig. 1). When either of sequence-specific primer MR7U and oligo(dT) primer was used for reverse transcription, the primer set of MR7D/MR7U and MR8D/MR8U produced an identical cDNA sequence (data not shown). The DNA bands obtained were considered to have been derived from mRNA but not from genomic DNA because each of a primer set is located in each of an exon pair flanking a long intron. A contig was constructed from DNA sequences of the PCR products, giving a 1067-bp molecular sequence with a 1041-bp open reading frame (Fig. 2). We designated this gene as osH1. The initiation codon was assigned as shown in Fig. 2 because it is the first ATG downstream of the TATA box on the putative genomic DNA. The osH1 gene encoded a 35.8-kDa protein of 347 amino acids. According to the GenBank genomic sequence data, the gene includes five exons and is located on 3q21-22 in contrast with the somatic H1 genes which are located in a large cluster on the short arm of chromosome 6.

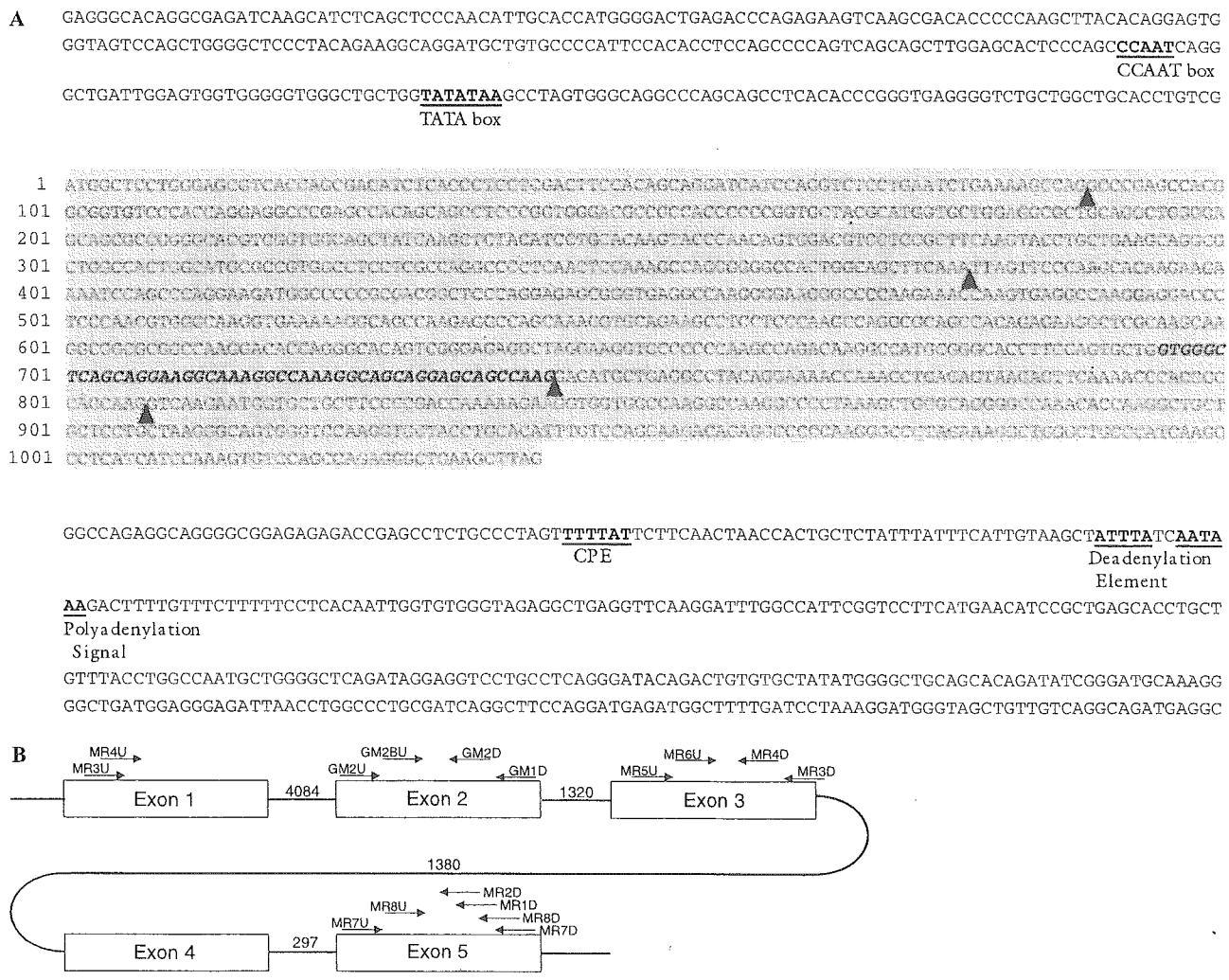

Fig. 2. The osH1 gene. (A) Sequence of the osH1 cDNA. Numbering starts from the initiation codon. The gray-shaded sequence is the open reading frame. The unshaded sequences are the UTR at both ends and the promoter region. Splicing sites are shown in triangles. A sequence of 48 nucleotides not present in the NCB-predicted version is shown in bold italics. The CCAAT box, the TATA box, the cytoplasmic polyadenylation element (CPE), the deadenylation element, and the polyadenylation signal are indicated as bold letters with underlines. (B) Schematic presentation of exons and introns of osH1 in the genomic sequence. Exons are indicated as open boxes. Numbers between exons indicate length of introns. Locations of all primers employed for RT-nested PCR are indicated.

Homology with oocyte-specific H1 of other species

Alignment of amino acid sequences of oocyte-specific H1 of human and other species was performed with the assistance of CLUSTAL W software (version 1.8) [17] (Fig. 3). The osH1 gene exhibited a 42.3% identity score with H1oo (mouse), 25.0% with B4 (*X. laevis*), 25.1% with H1M (zebrafish), and 22.5% with csH1 (sea urchin). In contrast, homology of osH1 with H1b (somatic subtype) and H1t (testis-specific subtype) was only 18.5% and 15.3%, respectively (Table 2). In general, linker histones of the H1 variety possess a three-domain structure including a central globular domain (consisting of Helix I, II, and III) flanked by amino- and carboxy-terminal domains. Sequence homology was greatest in the central globular domain, consistent with the observation that linker DNA interacts with the central globular domain of H1 protein [5,6,12]. When homology analysis was limited to the globular domain, osH1 shared 74.3%, 57.1%, 45.1%, and 38.9% identity with the corresponding regions of H1oo, B4, H1M, and csH1, respectively.

Oocyte-specific expression of osH1

RT-nested PCR was performed for human ovarian tissue and ovarian granulosa cells. In the ovarian tissue, osH1 cDNA was detectable up to a 25-fold dilution, while β-actin was detectable up to a 5^6-fold dilution. In ovarian granulosa cells, however, osH1 cDNA was not

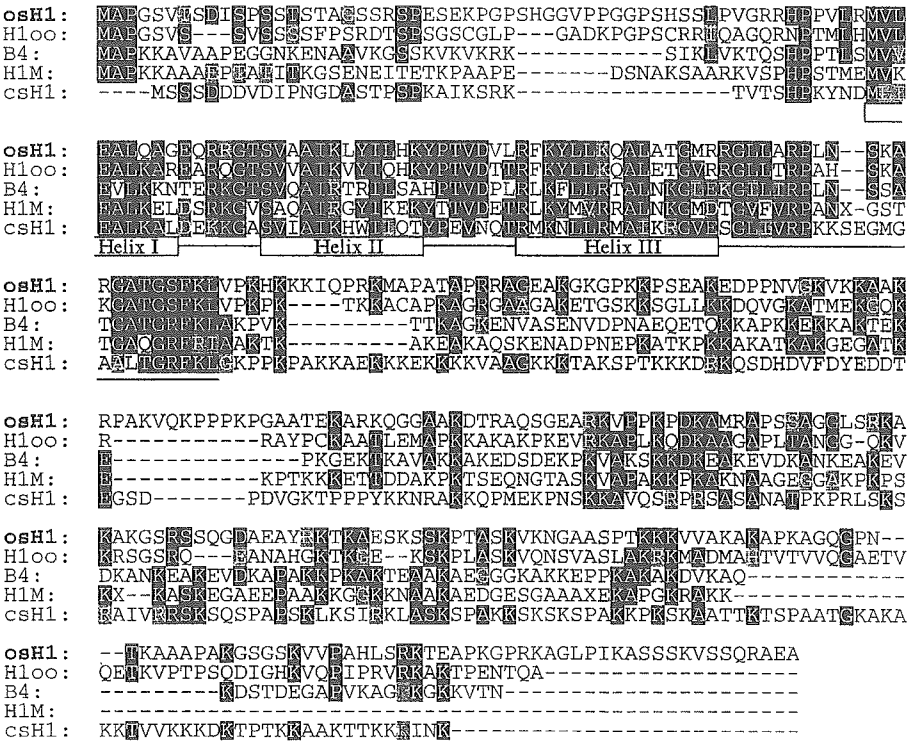

Fig. 3. Amino acid sequence alignment of osH1 with other oocyte-specific H1s. The amino acid sequences of other oocyte-specific H1s were extracted from the GenBank database (for Accession number, see Table 2). Identical amino acid residues are shaded black; similar amino acids are shaded bright gray; and unrelated residues are unshaded. The globular domains are underlined (Helix I, II, and III).

Table 2
Sequence similarity of osH1 protein to oocyte-specific H1 of other species and other subtypes of human H1

Protein	Sequence identity (%)	Gaps	Amino acid overlap
H1oo (mouse)	42.3	47	148
H1M (zebrafish)	25.1	87	85
B4 (*Xenopus laevis*)	25:0	74	87
csH1 (sea urchin)	22.5	47	78
H1t (human)	15.3	129	52
H1b (human)	18.5	127	64
$H1^0$ (human)	19.6	116	68

Alignment was performed with the assistance of CLUSTAL W software (version 1.8). Accession numbers for each proteins are: AY007195 (H1oo), X13855 (B4), AF499607 (H1M), AAB48830 (csH1), NP_005314 (H1t), and NP_005312 (H1b).

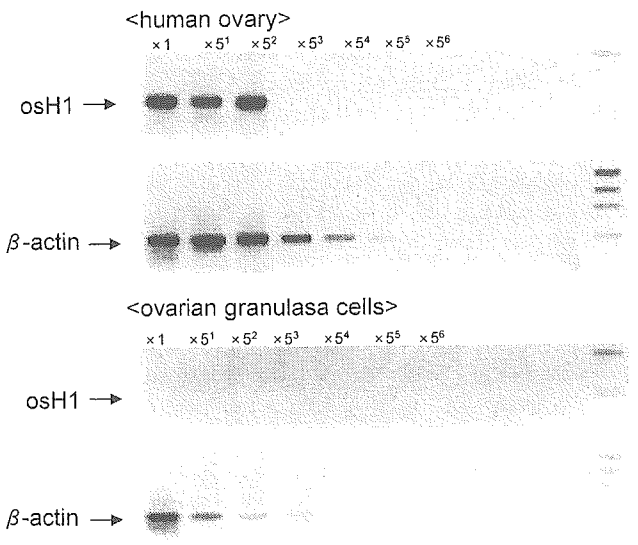

Fig. 4. Expression of osH1 and β-actin mRNA in human ovary and ovarian granulosa cells. RT-nested PCR for osH1 was performed using the primer pairs GM1D for reverse transcription, GM1D/MR1U for the first-round PCR, and GM2D/MR2U for the second-round PCR. RT-PCR for β-actin mRNA was performed as described in Materials and methods.

detected even in the undiluted RNA sample, while β-actin was detectable up to a 125-fold dilution (Fig. 4). The sequence of the cDNA product was identical to that of osH1 (data not shown).

No osH1 mRNA was detected by RT-nested PCR in 10 μg of total RNAs from any human tissues tested (breast, colon, kidney, liver, lung, skeletal muscle, spleen, lymphocytes, and sperm). In contrast, β-actin mRNA was detected up to a 125- or 625-fold dilution of 10 μg of total RNA by RT-PCR (Fig. 5). It must be noted that the second-round PCR was performed for osH1 but not for β-actin; therefore, the former assay was much more sensitive than the latter.

Discussion

Heterogeneity of histone H1 was first described in 1966 [18]. At the present time, eight subtypes of histone H1 have been identified in mammals. Five of these are collectively called the somatic types, which are designated H1a, H1b, H1c, H1d, and H1e [2–6]. Subtypes from H1b to H1e are present in all somatic cells, while H1a is restricted to the thymus, testis, spleen, and possibly lymphocytes and neurons [19]. They share a highly conserved central globular domain and exhibit variation in their amino- and carboxy-terminal domains [20]. The sixth subtype, known as $H1^0$ or H5, is differentiation stage-specific and has a divergent sequence in the globular domain and the tails compared with somatic subtypes [20]. The remaining two subtypes are known as tissue-specific; testis-specific H1 (H1t) and oocyte-specific H1 (H1oo in mouse) [6,21,22]. H1oo shows little homology with other somatic subtypes over the full length, while H1t exhibits some homology in the globular domain [23].

To date, the oocyte-specific histone H1 gene has been reported in four species of organisms; csH1 in the sea urchin, B4 in *X. laevis*, H1M in the zebrafish, and H1oo in the mouse [6,7,13,14]. Presence of a human homo-

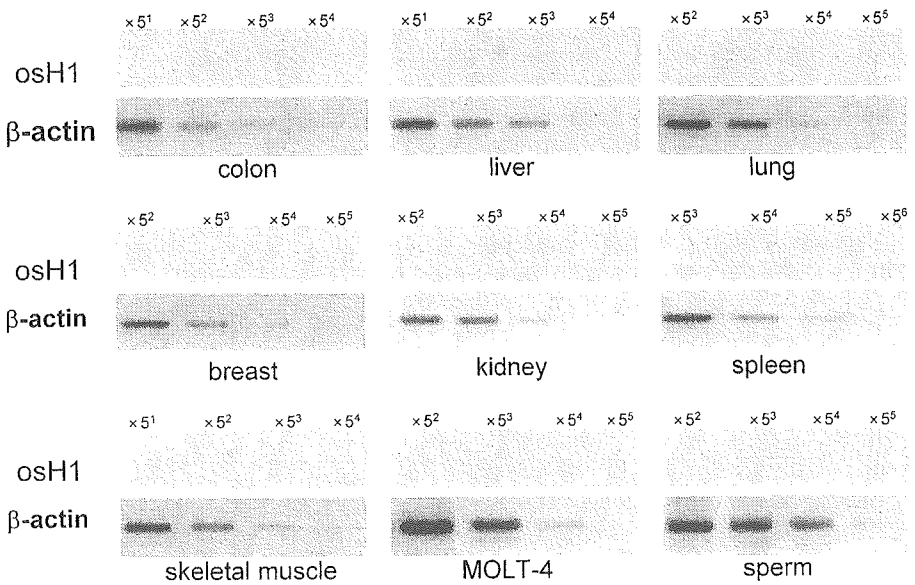

Fig. 5. Tissue localization of osH1 transcripts studied by semi-quantitative RT-nested PCR. Total RNAs from human colon, liver, lung, breast, kidney, spleen, skeletal muscle, MOLT-4 cells, and sperm were diluted fivefold sequentially. All original solutions contained 10 μg of total RNA. β-Actin was detectable up to a 5^4 dilution in colon, 5^4 in liver, 5^5 in lung, 5^5 in breast, 5^5 in kidney, 5^6 in spleen, 5^4 in skeletal muscle, 5^4 in MOLT-4 cells, and 5^4 in sperm. osH1 was undetectable in any of these samples.

logue of the mouse oocyte-specific H1 gene was recently predicted by the NCBI annotation system, which had not been proved in vivo, however. In the present study, using our method for single-cell analysis, we found transcripts of the predicted gene encoding an oocyte-specific linker histone H1 (osH1), as a 347-amino acid protein with a calculated molecular mass of 35.8 kDa. We believe that the osH1 gene belongs to the gene family of oocyte-specific histone H1 for the following reasons. (1) The mRNA was expressed only in the human oocyte and ovary. (2) The encoded protein had the closest homology with H1oo in the mouse, and to lesser extent, with B4 in *X. laevis*. The oocyte-specific H1s are the longest H1 histones described to date [6]; even among these, osH1 appeared exceptionally long (cf. H1oo, 304 amino acids; B4, 273; and csH1, 299).

We identified cDNA of the human oocyte-specific H1 gene with PCR primers designed according to the gene structure that had been predicted by the NCBI annotation project from the human genomic sequence through a gene prediction algorithm, GenomeScan. The cDNA that we obtained was longer than the predicted cDNA by 48 nucleotides at the 3′ end of exon 3 (Fig. 2A). In other words, the actual 5′ splice site of intron 3 is located 48 nucleotides downstream of the predicted one. GenomeScan was developed in 2001; its sensitivity and specificity both have been estimated to exceed 80%, which are better than those of any other gene prediction programs [24,25]. Howsoever the accuracy of programs, our result indicated that exact sequence of a computer-predicted gene should be determined by experiments at the molecular level.

Generally, tissue-specific H1 subtypes are less conserved among organisms than somatic H1 subtypes. For instance, in comparisons between the human and mouse genes, H1b (somatic subtype) and H1t (testis-specific subtype) exhibited homologies of 70.1% and 59.7%, respectively [21,22]. Even when considering these scores, the homology score of 42.3% between mouse and human oocyte-specific H1 (osH1 and H1oo) determined in this study was remarkably low. Oocyte-specific H1 is thought to bind less tightly to linker DNA than the other H1 subtypes because its net charge is less basic [12,14]. Stronger binding of oocyte-specific H1 with linker DNA could interfere with its replacement by somatic H1 during embryogenesis. This need for less stringent interaction may allow oocyte-specific H1 to acquire greater variation.

The cDNA sequence of oocyte-specific H1 involved only 6 and 21 bp in the 5′ and 3′ untranslated regions (UTR), respectively. The remainder of both the UTR and the promoter region, however, can be deduced from the genomic sequence in GenBank. Somatic H1 genes generally harbor TATA boxes, CCAAT boxes, H1-boxes (AAACACA), and TG boxes (TGTGT/CTA) in the promoter [26,27]. Although the oocyte-specific H1 genes of nonhuman species lack such promoter sequences [12], the 5′ flanking region of the osH1 gene contains two of these promoter elements, the TATA box at position −67 and the CCAAT box at −108, with position 1 corresponding to the initiation of translation. On the other hand, the 3′ UTR of both osH1 and H1oo contain the consensus polyadenylation signal (AAUAAA), the cytoplasmic polyadenylation element (UUUUAU), and the deadenylation element (AUUUA) (Fig. 2A). In our analysis, osH1 cDNA was detected by RT-nested PCR using an oligo(dT) primer for reverse transcription. These findings indicated that the osH1 mRNA is polyadenylated like other oocyte-specific H1 mRNAs, in contrast with a lack of polyadenylation in somatic H1 mRNAs.

In the process of normal fertilization, the condensed state of the sperm chromatin is loosened by replacement of protamine by oocyte-specific H1, followed by formation of the male pronucleus, which eventually leads to cleavage [28]. In the clinical practice of in vitro fertilization for infertile couples, some oocytes do not initiate cleavage even after intracytoplasmic sperm injection, thus remaining unfertilized. Morphological studies have shown that some of the unfertilized oocytes terminate the fertilization process at the stage of chromatin decondensation of sperm, suggesting that inadequate replacement of protamine by osH1 is a cause of fertilization failure [29]. Accordingly, further investigation of osH1 may provide new understanding of human fertilization and embryogenesis.

In conclusion, cDNA encoding a human oocyte-specific linker histone H1, osH1, was identified for the first time in human oocytes using a novel method for single-cell analysis of gene expression that was developed in the present work.

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Studies on the IVF-ET for Discordant Couples Where the Man is HIV Positive and the Woman is Negative Using Sperm Washing Technique and Highly Sensitive PCR Method

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Summary

Thirteen discordant couples, where the man is HIV positive and the woman is negative, were enrolled into this study. The spermatozoa were separated from semen samples from HIV-positive husband by discontinuous Percoll gradient centrifugation and the swim up method. The HIV-1 RNA and proviral DNA were measured using a highly sensitive PCR twice, i.e., before the fertilization and before the transfer of fertilized eggs to the wives, and confirmed the almost complete elimination of HIV from the samples. So far, 10 women underwent 10 times of embro-transfer. All of 10 women conceived (100%), and all pregnancy continued without early abortion, and all of them gave birth to HIV-negative infants. All of the wives remained HIV-negative through the study period. Such data strongly indicates that the technology may allow discordant couples, where the man is HIV positive and the woman is negative, to conceive more safely.

Introduction

Recently, people infected with human immunodeficiency virus (HIV)

are living longer and experiencing improved health, especially, the application of highly active antiretrovial therapy (HAART) has greatly improved survival in HIV-infected patients (1, 2). It is said that patients infected with HIV who has access to the treatment can expect to live for at least 20 years from the time of diagnosis. Consequently an increasing number of couples discordant with respect to HIV-infection are seeking safe ways to have children. In this study, we applied IVF-ET technique for discordant couples where the man is HIV positive and the woman is negative, using sperm washing technique and a highly sensitive PCR method.

Materials and Methods

Information of this modality to the patient couples: Firstly, the patients visited to Ogikubo Hospital and the condition of HIV infection of the husband was assessed, and were also informed the detail of this treatment by one of the doctors in this study group (H.H) and a counsellor. After the patients couple revealed the first desire for this treatment, they visited to Niigata University Hospital, and they again were informed the detail of this treatment by another doctor in this study group (Ko.T.) and a counsellor. The explanation consisted of (1) the detail of the procedure of the ovulation induction, the oocyte retrieval, and the embryo transfer as well as the risks of these procedures, (2) the protocol for confirming the elimination of HIV virus from husband's semen. The risk of the secondary HIV infection to both mother and fetus, if the wife would conceive, was also sufficiently announced. After the patients revealed the final decision undergoing this treatment and gave the consent to this treatment, the treatment started. The approval of ethical committee of Niigata University School of Medicine was obtained.

Patient Couples: Thirteen couples, who were discordant couples where the man is HIV positive and the woman is negative, were enrolled into this study. The age of the wives ranged from 21 to 40 with the mean of 29.5 years old.

The protocol for IVF-ET using sperm washing technique and a highly sensitive PCR method. (Figure 1): The spermatozoa were separated from semen samples from HIV-positive husband by discontinuous percoll gradient centrifugation and the swim up method. The HIV-1 RNA and proviral DNA were measured by a highly sensitive PCR which was developed by Kato et al. (3). The oocytes obtained from the HIV-negative wife were fertilized in vitro with the spermatozoa of her husband, after confirming that the HIV-1 could not be detected in the washed semen samples by the highly sensitive PCR. The fertilized eggs were cultured for two days, and transferred in utero after the negative

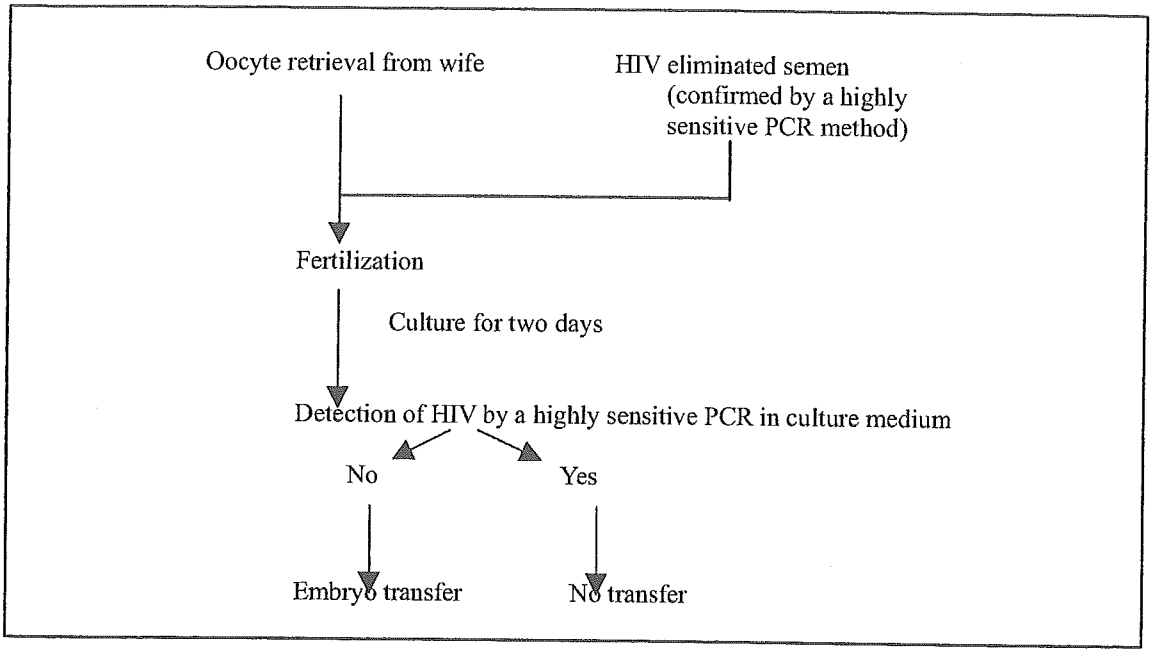

Figure 1 Protocol for the IVF-ET for Discordant Couples Where the Man is HIV Positive and the Woman is Negative Using Sperm Washing Technique and Highly Sensitive PCR Method

result was obtained by the highly PCR procedure in the medium for fertilized eggs.

Follow-up of wives: The serum samples for HIV DNA and antibody were obtained serially after the embryo transfer for three months. If the women became pregnant, the usual prenatal care was offered.

Results

Thirteen couples were enrolled into this study, and 12 women underwent 15 times of ovulation induction. Of 12 patients, one patient underwent twice of ovulation induction, and another one patient underwent three times of ovulation induction. Remaining 10 patients underwent once of induction. So far, the fertilized eggs were obtained in 10 women and embryo transfer was performed in all 10 women after confirming that the HIV-1 could not be detected in the culture medium of fertilized eggs. All of these10 women conceived (100%), and all pregnancy continued without early abortion, and all of them gave birth to HIV-negative infants. All of the women remained HIV-negative through the study period.

Conclusion

Heterosexual transmission of the virus is not so high, but a risk does

exist. Areneta et al. reported that the risk of transmission with insemination using non-washed semen from an infected man is 3.52% (95% confidence interval, 1.55-7.41%) (4). European Study Group on Heterosexual Transmission of HIV reported the seroconversion rate of 4.8 per 100 person-years among couples who used condoms inconsistently (5). For HIV discordant couples where the man is positive and the woman is negative, a technique known as "sperm washing" is a way to further reduce the risk of transmission. Semprini et al. reported that the sperm can be combined with ova from the female partner using in vitro fertilisation (IVF) techniques or direct injection of the sperm into a selected oocyte-intracytoplasmic sperm injection (ICSI) after washing of semen (6). They also reported that three hundred children have been born without seroconversions in children or uninfected partners since the technique has been offered (7). And now, sperm washing with IVF or ICSI is routinely offered by centers in Italy and Spain, and a protocol for the treatment of HIV discordant couples using their own gametes has now been developed in Australia (8).

However, the procedure, which confirmed the complete elimination of HIV from the semen samples obtained from HIV-positive man and the medium of fertilized eggs, was not applied in these protocols, and the safer protocol needs to be established. In fact, it is not readily available in the United States, because a woman seroconverted after being inseminated with her husband's sperm. The US Center for Disease Control has been reluctant to support future trials until the procedure can be proved safe, although it has not been proven that it was the insemination techniques that caused the infection in the woman (9, 10). In this context, we adopted the highly sensitive PCR method in two occasion during the treatment, i.e., before the fertilization and before the transfer of fertilized eggs to the wives. And we confirmed the almost complete elimination of HIV from the both samples (washed semen samples and culture medium of fertiliezed eggs). We consider that HIV discordant couples should be given access to "sperm washing" and IVF technique, if they desire safer pregnancy. And the technology employed in this study is considered to offer the most promissing results to the discordant couples, who wish to conceive more safely.

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Ⅱ．感染症

4．HIV

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はじめに

1982年，米国のCDCにより，AIDS（後天性免疫不全症候群）の症例が報告されて以来，アフリカ，アジアなどを中心に世界的な広がりを見せ，2000年における死亡者数は300万人にのぼるといわれている．わが国においては，AIDS患者およびAIDSの原因ウイルスであるHIV（Human Immunodeficiency Virus，ヒト免疫不全ウイルス）感染患者の絶対数は諸外国に比べ多くないものの，患者数は漸増傾向にあり，2010年には5万人を超えると推計されている．産婦人科領域においては，各種性感染症患者に対するHIV検査，妊婦健診時の検査としてのHIV抗体検査が推奨されている．とくに後者については，出生する児への垂直感染を防ぐという意味からも重要なものである．本稿においては，HIV感染症とHIV関連検査の実際について解説する．

HIV感染症の経過とHIV抗体スクリーニング検査の重要性

HIV感染症の臨床経過は，以下のような3期に分類され，進行していく[1]．

1．感染初期（急性期）

HIVに感染後，体内で急速に増殖する．感染機会があった6～8週間後に70～80％の患者には発熱，倦怠感，筋肉痛といったインフルエンザ類似の症状が認められる．このような症状は一過性で消失する．

2．無症候期（無症候性キャリア期）

急性症状が消失した後，ウイルスは増殖を繰り返す．宿主の免疫的な抵抗性により症状のない期間が長期にわたり続くことが多い（長期の潜伏期）．しかし，この無症候性キャリアの期間でも，HIVは1日100億個前後という割合で増殖し，CD4陽性Tリンパ球はHIVに感染し，その平均寿命は2.2日とされる．

3．ARCあるいはAIDS発症期

無治療では感染後5～10年の経過で，ウイルスの増殖能が宿主の免疫能に優るようになり，血中ウイルス量（HIV-RNA量）の増加に伴い，CD4陽性リンパ球数が減少する．やがて，全身のリンパ節腫張，下痢，発熱，体重減少，倦怠感，口腔カンジダ症などを伴う，いわゆる「エイズ関連症候群（AIDS-related complex；ARC）」の状態となる．さらに免疫能が高度に障害された場合，免疫不全状態となって，カリニ肺炎などの日和見感染症，カポジ肉腫などの悪性腫瘍，HIV脳症などを発症してくる．この病態がエイズ（Acquired Immunodeficiency Syndrome；AIDS）であり，生命に危険を及ぼすようになる．

以上がHIV感染後の経過であるが，上記の経過のなかで，臨床症状からHIV感染を診断する（あるいは疑う）ことのできる時期は感染初期と，ARCになってからであり，このことが臨床における大きな問題である．しかも感染

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初期には抗体検査により陽性を示さないことがあり，また，単にインフルエンザ様の症状では，HIV 抗体検査を行わない可能性もある．

HIV 感染の可能性のある患者が一般病院を受診する契機を進行期別に表 1 に示した[2]．産婦人科領域では，クラミジア，淋菌，梅毒，外陰部ヘルペス，治療抵抗性を示すカンジダ感染などを診断することは多く，これらの患者に対し，積極的に HIV 抗体検査を実施することが重要であるが，その理由として以下のようなことがあげられる[3]．

HIV 抗体検査により，HIV 感染が判明した場合，各種抗 HIV 薬の服用により，無症候キャリアの状態をより長期にわたり保つことが可能となる．また，性感染症でもある HIV 感染は，1 人の HIV 感染者の発見により，そのパートナーの感染の有無を確認することが可能であり，陰性であれば，コンドームの使用などの「安全なセックス」の指導により二次感染を未然に防ぐことが可能である．

また，妊娠初期の感染症検査の一つとして，HIV 抗体検査を行うことも重要である．HIV 抗体陽性妊婦については，妊娠期間中抗 HIV 薬を服用し，陣痛発来前に帝王切開術を行うことにより，児への垂直感染を 2％以下に低下させることが可能であることが示されている[4]．HIV 感染妊婦に対し対策の講じられていないアフリカ諸国での垂直感染の割合は 20 ～ 40％といわれており，妊娠初期に HIV 抗体検査を行うことの重要性を示している．

われわれが，産婦人科診療所施設を対象として行ったアンケート調査では，妊娠初期における HIV 抗体検査の実施率はいまだ低いものであり，今後，実施率を増加させるような方策が必要であるものと判断される[4][5]．

ただし，HIV 抗体のスクリーニング検査に当たっては，十分な説明を行い，同意を得てから実施する必要があることは当然のことである．

表 1　HIV 感染者が一般病院を受診する契機となる症状

初感染期	発熱，全身倦怠感，インフルエンザ様症状
無症候キャリア期	一般的な各種性感染症（クラジミア，淋菌，梅毒，外陰ヘルペス），反復性のカンジダ症，帯状疱疹，急性 B 型肝炎，A 型肝炎，赤痢アメーバ，結核，口腔カンジダ症，伝染性軟属腫など
エイズ（ARC を含む）	上記に加え カリニ肺炎，トキソプラズマ脳症，クリプトコッカス髄膜炎，食道カンジダ症 原因不明の発熱，下痢，体重減少 他エイズ指標疾患

HIV 感染の診断

HIV 感染の診断は最初に HIV 抗体検査により行われるが，酵素抗体法（ELISA 法）あるいは粒子凝集法（PA 法）により行われる．これらが陰性であれば，HIV 感染は否定されるが，感染のリスクが高く，感染初期の可能性が考えられる場合には，数週間後に再度の抗体検査が必要である．

ELISA 法または PA 法による HIV 抗体検査が陽性の場合にはウェスタンブロット法（WB 法），核酸増幅法（polymerase chain reaction ; PCR 法）を実施する．HIV には HIV-1 と HIV-2 の 2 種類があり，HIV-1 感染が主であるが，一般には両者同時の抗体検査が行われる．

より鋭敏な抗体検査が WB 法であるが，HIV-1 の WB 法では，HIV 抗原蛋白質の gag（p 55, p 25, p 18），pol（p 68, p 52, p 34），env（gp 160, gp 110, gp 41）に対する特異抗体が検出されるが，2 本以上の env バンドが検出された場合に陽性と判断される．WB 法の判定基準を表 2 に示した．

HIV の抗原検査は核酸増幅法（PCR 法）に

より行われる．血液中のHIVはRT-PCR法で検査され，HIV-RNAを逆転写酵素（RT）によりDNAに転写し測定し，その量はコピー数/mlで表わされる．この検査は確認検査としても行われるが，HIV感染者の病態の進展，治療開始時期の決定および治療の経過観察などに用いられる．核酸増幅の定量法には通常の定量法と高感度定量法があり，通常の定量法では測定範囲は $4.0 \times 10^2 \sim 7.5 \times 10^5$ コピー/mlであり，高感度定量法では $5.0 \times 10^1 \sim 1.0 \times 10^5$ コピー/mlである．HIV感染者の病勢把握においては，通常の定量法で 4.0×10^2 コピー/ml未満となった場合，高感度定量法を実施することが臨床的に有用とされている．

確認検査の判定であるが，WB法が陽性である場合，核酸増幅法が陽性である場合はもちろん，検出感度未満であってもHIV感染と判定される．WB法が判定保留あるいは陰性であって核酸増幅法が陽性の場合には，感染初期の可能性が高いので，数週間後に再度検査を行い，WB法を再検査することが必要である．WB法が判定保留または陰性で，核酸増幅法が検出感度未満の場合には陰性と判定されるが，感染のリスクが高く，感染初期の可能性が考えられる場合には，数週間後の再検査が望ましい．

HIVはリンパ球中のCD4陽性細胞（ヘルパー-インデューサーT細胞）に感染し，その機能を破壊する．そこで，HIV感染者に対する治療の選択に当たっては，CD4陽性細胞数

表2　HIV-1に関するウェスタンブロット（WB法）の解釈（WHO判定基準）

陽性：	envバンドが2本以上検出された場合
陰性：	HIV抗原に対するバンドが検出されない場合
判定保留：	HIV抗原に対するバンドが検出されるが，陽性の判定基準に一致しない場合（HIV感染の疑いがある場合には数週間後の再検査が必要）

表3　未治療患者に対する抗HIV療法の開始基準（推奨）

臨床症状*がある場合		
CD4陽性リンパ球数・血中ウイルスの数値にかかわらず		治療開始
臨床症状*がない場合		
CD4陽性リンパ球数（/mm³）	治療開始に際し考慮すべき項目	
≦200		治療開始
200～350	CD4陽性リンパ球数の減少速度が速い場合***	治療開始を考慮**
	血中ウイルス量が高い場合***	治療開始を考慮**
＞350		
		血中ウイルス量が低ければ*** 3～4カ月に1回程度の検査で経過観察を行う
		血中ウイルス量が高ければ*** 検査を頻回（1～2カ月に1回程度）行う

* ：AIDSおよびAIDSに関連する重篤な症状．

** ：患者の状態，服薬アドヒアランスへの意識・理解度，副作用および薬物相互作用なども考慮する．

*** ：CD4陽性リンパ球数の減少速度：＞100/mm³/年の場合を速いと考える．
血中ウイルス量：5万～10万コピー/ml以下を低い，それ以上を高いと考える．

（文献1より引用）

が重要である．CD4陽性細胞数は，抗CD4抗体を用いたフローサイトメトリーにより測定され，リンパ球に対する百分率で表される．したがって，白血球数，リンパ球分画の割合，CD4陽性細胞の割合により，CD4陽性細胞数が算出される．CD4陽性細胞はHIV感染者の治療開始基準として重要であり，その基準を表3に示した[1]．

おわりに

わが国におけるHIV感染は増加傾向にあり，感染爆発が危惧される状況である．性感染症を管理する機会の多い産婦人科領域におけるHIV抗体のスクリーニング検査が今後重要性を増すものと考えられる．

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49. 精液中HIVの完全除去法

49. Semen processing method for complete elimination of HIV-1

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