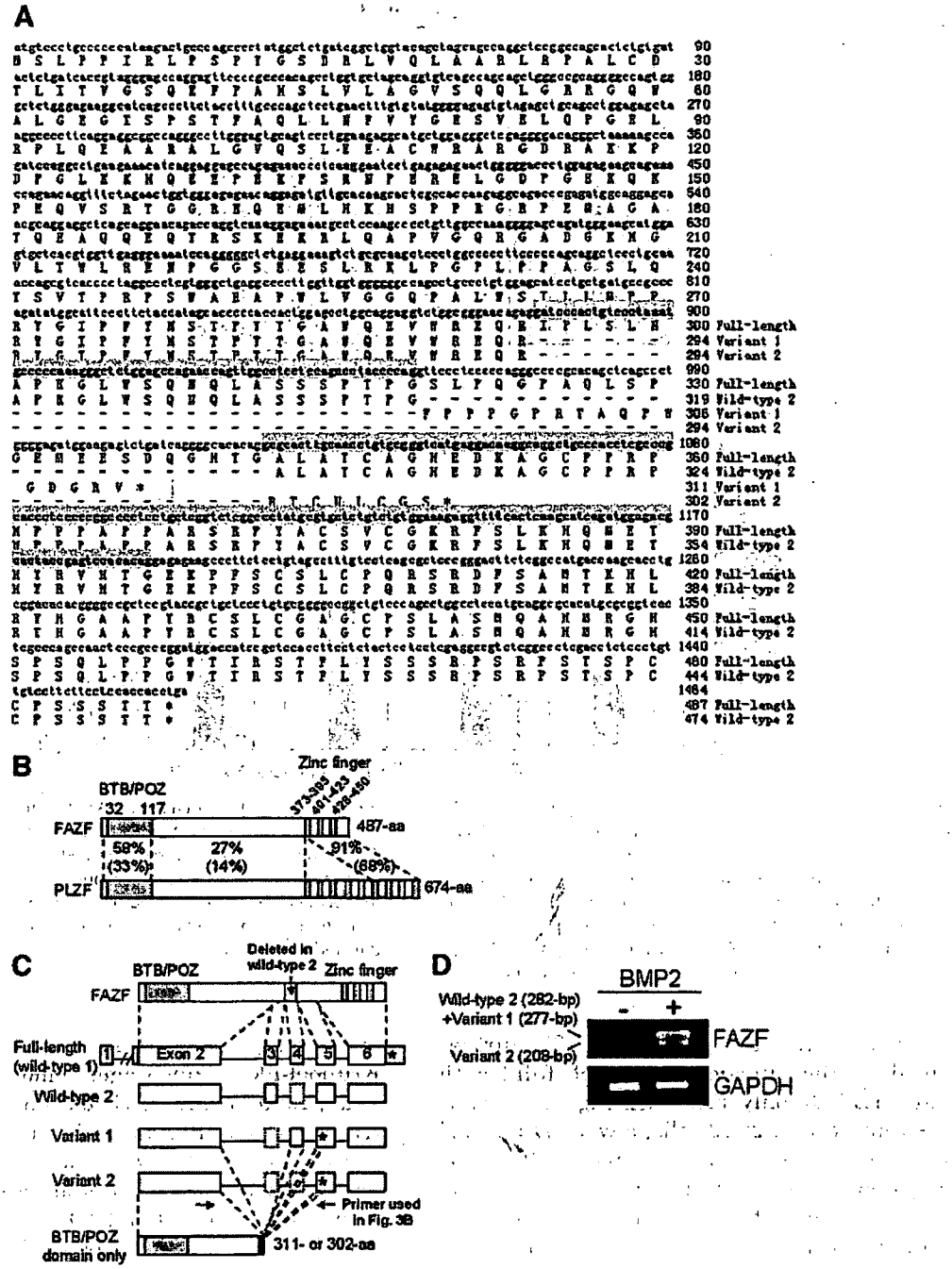

Fig. 3. Endogenous expression of full-length (wild-type) and C-terminal zinc finger motifs-deleted type (BTB/POZ-only) FAZF. **A**: Nucleotide sequences and the deduced amino acid sequences of human FAZF cDNA. The open reading frame (ORF) of full-length (wild-type 1), wild-type 2, variant 1 and 2 are shown in the one-letter amino acid code and aligned. To distinguish each exon (exons 2–6), the nucleotide sequences of exon 3 and 5 are indicated by the shaded boxes. Exon 1 is not included in the ORF. The asterisk indicates the stop codon. Nucleotide and amino acids numbers are shown on the right. **B**: Schematics of FAZF and PLZF proteins. The N-terminal BTB/POZ domain and the C-terminal three zinc finger motifs region (indicated by the gray box) are indicated. Amino acid homology (%) is shown for the different regions. The parentheses indicate amino acids similarities (%). **C**: Exon/intron structures and various isoforms of FAZF. Wild-type FAZF contains an N-terminal BTB/POZ domain and a C-terminal region containing three zinc finger motifs (indicated by the gray box). Exon 4 is deleted in-frame in FAZF wild-type 2. The full-length FAZF cDNA encodes a 487-amino acid protein encoded by exons 2–6. The BTB/POZ domain only FAZF proteins derived from variants 1 and 2, which are 311- and 302-amino acids, respectively, have different C-terminal tails (indicated by the black box). Variants 1 and 2 are shown where the boxes formed from dotted lines are spliced-out. The stop codon is indicated by an asterisk. The arrows indicate the position of RT-PCR primers used in Figure 3B. **D**: hMSCs were cultured in the presence or absence of 100 ng/ml of BMP2. The total RNA was extracted after 72 h of BMP2 treatment, and the expression patterns of wild-type or variant FAZF were verified by RT-PCR. PCR products were separated on 1.5% agarose gels and visualized using EtBr staining. The faster and slower migrating bands corresponded to variant 2 (208-bp) and wild-type 2 (282-bp)/variant 1 (277-bp), respectively. The GAPDH bands are shown as the loading control.

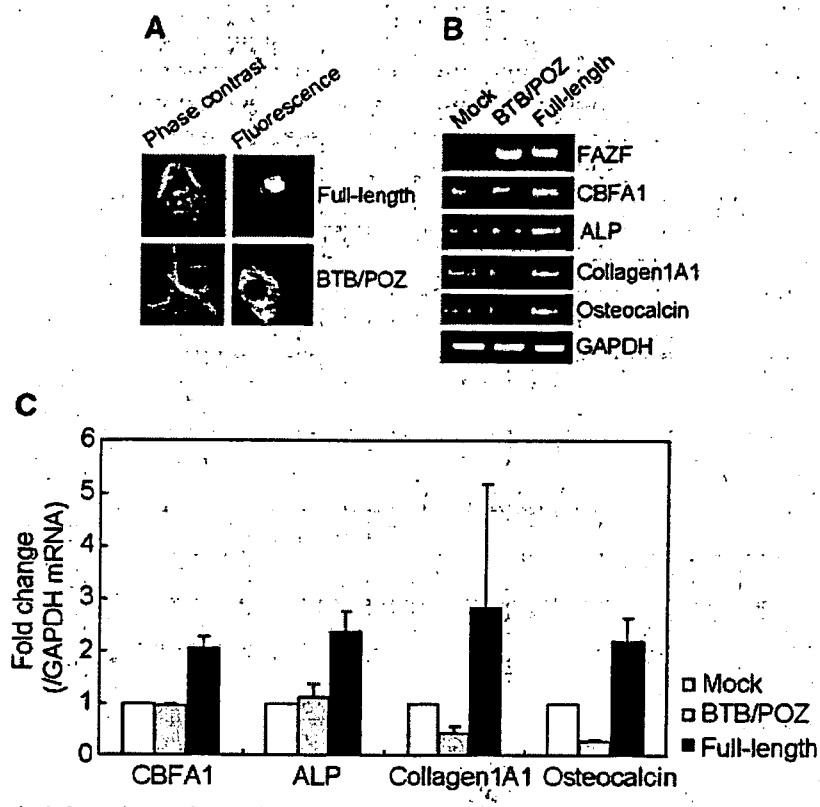

Fig. 4. BTB/POZ-only FAZF is distinct from full-length FAZF. A: Subcellular localization of FAZF. Full-length and BTB/POZ-only FAZF were transiently expressed in C2C12 cells, and the cells were immunostained. B: FAZF increased the expression of osteoblastic differentiation marker genes. C2C12 cells were transfected with a control vector (Mock) or an expression vector bearing full-length or BTB/POZ-only FAZF. After 48 h, RT-PCR was performed for the FAZF, CBFA1, ALP, collagen1A1, osteocalcin, and GAPDH mRNAs. C: Histogram analysis of expression changes of osteoblastic differentiation marker genes. The staining intensities were quantified using NIH image (http://www.scioncorp.com). Values represent the average of three independent experiments and are represented as relative-fold activation as compared to that of mock transfection taken as 1. Bars indicate the SD.

the transfected cells (Fig. 4A). To further elucidate the functional role of full-length and BTB/POZ-only FAZF during osteoblastic differentiation, C2C12 cells, a well-established mouse cell line capable of osteoblastic differentiation, were transfected with an expression vector bearing either the full-length or BTB/POZ-only FAZF cDNA, followed by RT-PCR. As shown in Figure 4B, the over-expression of full-length FAZF resulted in the elevated expression of CBFA1, ALP, collagen1A1, and osteocalcin, whereas BTB/POZ-only FAZF failed to induce the expression of osteoblastic differentiation markers. The GAPDH mRNA level was constant in each lane (Fig. 4B).

DISCUSSION

FAZF encodes a 487-amino acid protein containing a conserved N-terminal BTB/POZ interacting domain and three C-terminal Kruppel-like zinc-finger motifs [Hoatlin et al., 1999]. FAZF has been characterized as a transcriptional regulator that is important for the regulation of development, tissue-specific proliferation, and differentiation [Hoatlin et al., 1999; Dai et al., 2002]. In particular, FAZF has a high degree of similarity to PLZF, which was originally recognized as the fusion partner of the retinoic acid receptor α gene on chromosome 17 in a Chinese patient with acute promyelocytic leukemia and a translocation at t(11;17)(q23;21) [Chen et al., 1993]. More recently, PLZF was found to function upstream of CBFA1 in the context of osteoblastic differentiation [Ikeda et al., 2005]. In addition, PLZF nullizygous mice showed that PLZF is essential for axial skeleton patterning and normal limb development [Barna et al., 2005]. Because so little is known about the functions of FAZF, the

current work focused on the involvement of FAZF in pathways reminiscent of PLZF's unique effect on osteoblastic differentiation.

The present study revealed that FAZF is a BMP2-induced but not dexamethasone/ascorbic acid-2-phosphate/β-glycerophosphate (OS)-induced transcription factor during osteoblastic differentiation. During the osteogenic process, various extrinsic factors such as dexamethasone and cytokines regulate osteoblastic differentiation. Our results clearly suggest that the function of FAZF could be linked to a transcriptional regulation pathway involved in BMP2-regulated osteoblastic differentiation. In addition to PLZF, we demonstrated that FAZF is capable of inducing markers of gene expression that are associated with osteoblastic differentiation. Interestingly, the expression of FAZF, but not of PLZF, was increased by the addition of BMP2 to the culture medium of hMSCs. Conversely, an OS medium upregulated the PLZF, but not FAZF, mRNA expression in hMSCs. These results indicate that the differential involvement of PLZF and FAZF in the regulation of osteoblastic differentiation. From our results, distinct signaling pathways regulated by these two transcription factors during osteoblastic differentiation could be partially linked by CBFA1, and resulted in inducing the expression of osteoblastic differentiation marker genes.

We identified alternative splicing variants of FAZF that lacked the C-terminal zinc finger motifs. These types of transcripts were found to exert a dominant negative effect because the forced expression of C-terminal truncated BTB/POZ-only FAZF failed to induce the expression of marker genes of osteoblastic differentiation, including CBFA1, ALP, collagen 1A1, and osteocalcin in C2C12 cells. An immunofluoresence analysis of FAZF expression in this study indicated that full-length FAZF was expressed in the nuclear compartment, as previously reported [Hoatlin et al., 1999]; however, BTB/POZ-only FAZF was detected in the cytoplasmic compartment, suggesting that this form could associate with full-length FAZF or other BTB/POZ-containing proteins as a heterodimer and inhibit them from being tethered into the nucleus, where they normally function.

The diversity of FAZF proteins resulting from alternative splicing can be explained by the relatively short introns in the FAZF gene. For example, intron 1 is 1,489-bp, intron 2 is 249-bp, intron 3 is 108-bp, intron 4 is 125-bp, and intron 5 is 180-bp. More interestingly, the FAZF gene is closely linked to the MLL4 gene on 19q13.1, and this is reminiscent of the fact that the PLZF and MLL genes have both been mapped to 11q23 [Baysal et al., 1997]. Therefore, the two paralogous BTB/POZ domains and zinc finger motif-containing sequences may have arisen from a syntenic duplication during evolution [Zhang et al., 1999], suggesting that the two proteins not only have unique functional roles, but also share fundamental roles in biological processes, such as the regulation of gene expression.

FAZF mRNA and protein are detected in primary hematopoietic $CD34^+$ progenitor cells and increase during early proliferation; they are then downregulated during terminal differentiation in both erythroid and myeloid lineages [Dai et al., 2002]. These results, together with the present findings, suggest that FAZF influences the proliferation/differentiation status of specific cells. In conclusion, we showed that FAZF was upregulated in response to BMP2 in hMSCs and that it plays an important role in the upregulation of osteoblastic differentiation markers. BTB/POZ-only FAZF failed to induce osteoblastic differentiation markers. The biological impacts of FAZF in the context of osteoblastic differentiation should be addressed in the near future by means of RNA interference or DNA microarray-based global gene expression analyses.

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REFERENCES

Barna M, Pandolfi PP, Niswander L. 2005. Gli3 and Plzf cooperate in proximal limb patterning at early stages of limb development. Nature 436:277–281.

Baysal BE, van Schothorst EM, Farr JE, James MR, Devilee P, Richard CWr. 1997. A high-resolution STS, EST, and gene-based physical map of the hereditary paraganglioma region on chromosome 11q23. Genomics 44:214–221.

Chen SJ, Zelent A, Tong JH, Yu HQ, Wang ZY, Derre J, Berger R, Waxman S, Chen Z. 1993. Rearrangements of the retinoic acid receptor alpha and promyelocytic leukemia zinc finger genes resulting from t(11;17)(q23;q21) in a patient with acute promyelocytic leukemia. J Clin Invest 91:2260–2267.

Chen D, Zhao M, Mundy GR. 2004. Bone morphogenetic proteins. Growth Factor 22:233–241.

Dai MS, Chevallier N, Stone S, Heinrich MC, McConnell M, Reuter T, Broxmeyer HE, Licht JD, Lu L, Hoatlin ME. 2002. The effects of the Fanconi anemia zinc finger (FAZF) on cell cycle, apoptosis, and proliferation are differentiation stage-specific. J Biol Chem 277:26327–26334.

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. 1997. Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation. Cell 89:747–754.

Hoatlin ME, Zhi Y, Ball H, Silvey K, Melnick A, Stone S, Arai S, Hawe N, Owen G, Zelent A, Licht JD. 1999. A novel BTB/POZ transcriptional repressor protein interacts with the Fanconi anemia group C protein and PLZF. Blood 94:3737–3747.

Ikeda R, Yoshida K, Tsukahara S, Sakamoto Y, Tanaka H, Furukawa K, Inoue I. 2005. The promyelotic leukemia zinc finger promotes osteoblastic differentiation of human mesenchymal stem cells as an upstream regulator of CBFA1. J Biol Chem 280:8523–8530.

Johnson RL, Tabin CJ. 1997. Molecular models for vertebrate limb development. Cell 90:979–990.

Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, Rosen V, Wozney JM, Fujisawa-Sehara A, Suda T. 1994. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol 127:1755–1766.

Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, Komori T, Wozney JM, Kim EG, Choi JY, Ryoo HM, Bae SC. 2000. Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. Mol Cell Biol 20:8783–8792.

Lin W, Lai CH, Tang CJ, Huang CJ, Tang TK. 1999. Identification and gene structure of a novel human PLZF-related transcription factor gene, TZFP. Biochem Biophys Res Commun 264:789–795.

Nakashima M, Reddi AH. 2003. The application of bone morphogenetic proteins to dental tissue engineering. Nat Biotechnol 21:1025–1032.

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B. 2002. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108:17–29.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.

Stogios PJ, Downs GS, Jauhal JJ, Nandra SK, Prive GG. 2005. Sequence and structural analysis of BTB domain proteins. Genome Biol 6:R82.

Tang CJ, Chuang CK, Hu HM, Tang TK. 2001. The zinc finger domain of Tzfp binds to the tbs motif located at the upstream flanking region of the Aie1 (aurora-C) kinase gene. J Biol Chem 276:19631–19639.

Yamaguchi A, Katagiri T, Ikeda T, Wozney JM, Rosen V, Wang EA, Kahn AJ, Suda T, Yoshiki S. 1991. Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro. J Cell Biol 113:681–687.

Zhang T, Xiong H, Kan LX, Zhang CK, Jiao XF, Fu G, Zhang QH, Lu L, Tong JH, Gu BW, Yu M, Liu JX, Licht J, Waxman S, Zelent A, Chen E, Chen SJ. 1999. Genomic sequence, structural organization, molecular evolution, and aberrant rearrangement of promyelocytic leukemia zinc finger gene. Proc Natl Acad Sci USA 96:11422–11427.

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Mutational analysis of *TP53* and *p21* in familial and sporadic ovarian cancer in Japan

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Abstract

Objective. To investigate whether somatic mutations in cell cycle checkpoint genes, *TP53* and *p21*, are involved in the development of ovarian cancer with or without *BRCA1* germline mutation.

Methods. We analyzed somatic genetic alterations of *TP53* and *p21* in 46 ovarian cancer patients with *BRCA1* germline mutations and 93 sporadic patients, using direct sequencing for the entire coding sequences in *TP53* and *p21*.

Results. *TP53* somatic mutations were detected in 25 of the 46 *BRCA1* cases and 40 of the 93 sporadic cases (54.3% vs. 43.0%). In contrast, *p21* somatic mutations were detected in 1 of the 46 *BRCA1* cases and 2 of the 93 sporadic cases (2.2% vs. 2.2%). *TP53* mutations in sporadic cases more frequently occurred in exons 6–11 than those in cases with germline *BRCA1* mutations (84.4% vs. 56.3%: $P = 0.013$). The proportion of sporadic cases with *TP53* mutations in non-serous tumors (e.g. endometrioid, clear cell, or mucinous) was significantly lower than that in serous tumors (18.5% vs. 53.0%: $P = 0.0038$). However, there was no significant difference between the proportion of *BRCA1* cases with *TP53* mutation in non-serous and in serous tumors (37.5% vs. 57.9%).

Conclusions. Our results suggest that somatic mutation of *TP53* plays less of a role in the carcinogenesis of sporadic non-serous tumors than in that of sporadic serous tumors or *BRCA1*-related tumors. Furthermore, *p21* somatic mutation appears to be less involved in the development of ovarian cancer than *TP53* somatic mutation.
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Keywords: *BRCA1*; *p53*; *p21*; Ovarian cancer

Introduction

Ovarian cancer is the most lethal form of gynecological malignancy. Approximately 5–10% of cases are thought to have a hereditary basis [1], and germline mutations of *BRCA1* are estimated to be responsible for about 45% of breast cancer families and 80% of breast–ovarian cancer families [2,3].

Many reports suggest that p53 and BRCA1 protein interact directly and may play an important role in the DNA repair process [4,5]. In breast cancer, *BRCA1*-associated tumors exhibit a higher frequency of *TP53* expression and gene mutation than sporadic tumors. Crook et al. [6] detected *TP53* mutations in 19 of 28 (68%) *BRCA*-associated breast tumors but in only 7 of 20 (35%) sporadic tumors matched for grade and histological subtype. Phillips et al. [7] reported similar mutation frequencies in *BRCA*-associated breast tumors and sporadic tumors, 84% and 30% respectively. In ovarian cancer, there are few reports of *TP53* mutations in *BRCA1*-associated ovarian cancers [8]. These reports have limitations, such as evaluating only Caucasian populations and small numbers of cases. Mutations of genes acting upstream or downstream of *TP53* on the same control pathway might have a similar oncogenic effect. One such candidate gene is *p21*, which encodes a cyclin-dependent kinase inhibitor. Indeed, it has been shown that *p21* is up-regulated by *TP53* and mediates p53-induced cell cycle G1 arrest in response to DNA damage [9]. Moreover, the introduction of *p21* cDNA in a variety of human tumor cell lines inhibits cell growth in vitro and in vivo, suggesting that *p21* itself has a tumor-suppressive activity [10]. The *p21* expression is commonly altered in human malignancies, although it has not been as thoroughly studied in ovarian carcinoma as has *TP53* [11].

We previously reported that 45 out of 82 ovarian cancer families were found to carry *BRCA1* or *BRCA2* germline mutations (40 with *BRCA1* and 5 with *BRCA2*) in Japanese populations [12]. In the present study, we analyzed genetic alterations of *TP53* and *p21* in ovarian cancers from Japanese patients with and without *BRCA1* germline mutations, and the clinical characteristics of *BRCA1*-associated and sporadic cases were compared with regard to *TP53* or *p21* somatic mutations.

Materials and methods

Patients

We identified epithelial ovarian cancer patients with *BRCA1* mutations by direct sequencing. DNA was obtained from patients in ovarian cancer families, and the entire *BRCA1* coding region, 23 exons plus the intronic boundary regions, was sequenced in both forward and reverse directions to detect germline mutations. Patients who were shown to have germline *BRCA1* mutations were designated the *BRCA1* group. The sporadic group, on the other hand, was defined as epithelial ovarian cancer patients without family or past history of breast or ovarian cancers in relatives of second degree or closer [13]. We ascertained 103 sporadic cases from Niigata university hospital in Japan from 1996 to 2000. As 10 cases among them were considered as borderline malignant tumors, we used the other 93 cancers as control group. We performed mutational analysis of *TP53* and *p21* in epithelial ovarian cancers from 139 patients: 46 patients in 40 ovarian cancer families with *BRCA1* mutation (*BRCA1* group) and 93 patients in the sporadic group. All experiments were performed under informed consent.

Table 1 summarizes the characteristics of each group with respect to histological subtypes, age at diagnosis, and FIGO stage. The major histological type in both groups was serous adenocarcinoma, constituting 82.6% and 71.0% of *BRCA1*-related and sporadic tumors respectively, however, there was no significant difference of the proportion of tumors with each histological types between the two groups. The mean age at diagnosis of the *BRCA1* group, 50.6 years old, was younger than that for the sporadic group, 51.2 years old, however, the difference was not statistically significant. No difference was seen in the stage distribution between two groups.

Mutational analysis of TP53 and p21

Genomic and tumor DNA was prepared from lymphocytes and paraffin-embedded blocks using the standard phenol/chloroform methods. In this study, we did not perform microdissection method in DNA preparation, however, the tumors contained predominately tumor cells, approximately 90% or larger of all cells. The entire coding region, 11 exons in *TP53* and 3 exons

Table 1
Clinical and pathological characteristics of ovary

	BRCA1 group (n = 46)		sporadic group (n = 93)	
pathology				
serous	38(82.6%)	a	66(71.0%)	a
mucinous	0(0.0%)		6(6.5%)	
endometrioid	7(15.2%)		13(14.0%)	
clear cell	1(2.2%)		8(8.6%)	
age(mean ± SD)	50.6±8.6	b	51.2±13.4	b
FIGO stage				
I	5(10.9%)	c	24(25.8%)	c
II	6(13.0%)		15(16.1%)	
III	28(60.9%)		44(47.3%)	
IV	7(15.2%)		10(10.8%)	

a, b, c: all differences are not significant.

in *p21*, and the intronic boundary regions were sequenced in both forward and reverse directions to detect germline mutations. The non-coding intronic regions that were analyzed did not extend more than 20 base pairs proximal to the 5′ end and 10 base pairs distal to the 3′ end of each exon. These regions were amplified by two rounds of PCR using nested primers. The first round used as template 30 ng of genomic and tumor DNA (35 cycles). Next, the products of the first PCR were used as template for the second round (25 cycles). The products of the second PCR were sequenced by the dideoxy method using the BigDye terminator Cycle Sequencing kits Version 2.0 (Applied Biosystems) and a non-labeled primer. The sequence products were analyzed using an automated DNA sequencer (Applied Biosystems 3100 Genetic Analyzer). Sequencing results were compared with sequence data located at the National Center for Biotechnology Information (NCBI) web site to identify mutations of *TP53* and *p21*.

Statistical analysis

Clinical characteristics among ovarian cancer patients were tested by unpaired *t* test (two-sided), Chi-square analysis (two-sided), and Fisher's Exact Test.

Results

We analyzed 139 patients with epithelial ovarian cancer for mutations in *TP53* and *p21*: 46 patients (from 40 families) in the *BRCA1* group and 93 patients in the sporadic group. The patients with *TP53* or *p21* somatic mutations are listed in Tables 2 and 3. *TP53* somatic mutations were detected in 25 of the 46 *BRCA1* cases and 40 of the 93 sporadic cases (54.3% vs. 43.0%). In contrast, *p21* somatic mutations were detected in 1 of the 46 *BRCA1* cases and 2 of the 93 sporadic cases (2.2% vs. 2.2%). As regards the types of mutations in *TP53*, 23 of 25 cases in the *BRCA1* group were single base substitutions and the other 2 were deletions, while 37 of 40 sporadic cases were single base substitutions, one was an insertion, and two were deletions. On the other hand, all three mutations of *p21* were single base substitutions. All three tumors with *p21* somatic mutation were serous adenocarcinomas and also had *TP53* mutations.

The distribution of *TP53* mutations by exon is shown in Fig. 1. *TP53* mutations in sporadic ovarian cancers were found in all exons except 3. Most of the mutations were concentrated in exons 6–8 (33/45 = 73.3%). In contrast, *TP53* mutations found in individuals carrying germline *BRCA1* mutations were

Table 2
Familial ovarian cancer patients with *TP53* or *p21* mutation

No.	*TP53* mutation	*TP53* exon	*TP53* mutation database	*p21* mutation	*p21* mutation database	Pathology	Age	FIGO stage	*BRCA1* mutation
1	R110L	4	Yes	ND	–	Serous	51	3	L63X
2	R110P	4	Yes	ND	–	Serous	54	1c	L63X
3	859delG	8	Yes	ND	–	Serous	51	3c	L63X
4	V173M	5	Yes	ND	–	Serous	49	3c	L63X
5	Y220C	6	Yes	ND	–	Serous	59	3c	L63X
6	P190R	6	Yes	ND	–	Serous	39	2c	L63X
7	W91X, R175H, D392H	4, 5, 11	Yes, yes, no	ND	–	Serous	50	3c	Q934X
8	P278R	8	Yes	ND	–	Serous	42	4	Q934X
9	G245V	7	Yes	ND	–	Serous	44	3c	Q934X
10	L194H	6	Yes	ND	–	Serous	64	3c	Q934X
11	C141Y	5	Yes	ND	–	Serous	49	3	2080delA
12	S313R	9	Yes	ND	–	Serous	57	3c	241delA
13	Y205H	6	Yes	ND	–	Serous	40	4	3226–3231delTTAAAG
14	Y234C	7	Yes	ND	–	Serous	46	3	3376–337insT
15	P151H, L194R	5, 6	Yes	R83W	No	Serous	44	3c	3516–3517delTT
16	R273H	8	Yes	ND	–	Serous	59	3	4046–4049delTACA
17	R175H	5	Yes	ND	–	Serous	59	3c	5326delT
18	E11Q, V272L	2, 8	Yes	ND	–	Serous	53	3c	C61G
19	R175H	5	Yes	ND	–	Serous	44	3c	M1R
20	IVS8 + 1G > T	8	No	ND	–	Serous	47	3c	Q169X
21	R248W	7	Yes	ND	–	Serous	73	3c	3532delG
22	P92S, Q136E	4, 5	No, yes	ND	–	Serous	28	1c	Q934X
23	P190R	6	Yes	ND	–	Endometrioid	49	2c	L63X
24	R248W	7	Yes	ND	–	Endometrioid	53	3c	Q934X
25	150delT, R156L, D207N	4, 5, 6	No, yes, no	ND	–	Endometrioid	44	1a	Q934X

ND: not detected.

Table 3
Sporadic ovarian cancer patients with *TP53* or *p21* mutation

No.	*TP53* mutation	*TP53* exon	*TP53* mutation database	*P21* mutation	*p21* mutation database	Age	Pathology	FIGO stage
1	1176–1177insT	11	No	G135E	No	45	Serous	1c
2	S6L, P190S	2, 6	No, yes	T118I	No	38	Serous	1c
3	Y220C	6	Yes	ND	–	57	Serous	1c
4	E298X	8	Yes	ND	–	50	Serous	1c
5	T230I	7	Yes	ND	–	38	Serous	2c
6	G245S	7	Yes	ND	–	67	Serous	2c
7	R273C, A365V	8, 10	Yes, no	ND	–	45	Serous	2c
8	P151R, R364K	5, 10	Yes, no	ND	–	57	Serous	2c
9	E221D	6	Yes	ND	–	54	Serous	3c
10	I195N	6	Yes	ND	–	68	Serous	3c
11	R196X	6	Yes	ND	–	56	Serous	3c
12	R273C	8	Yes	ND	–	44	Serous	3c
13	R273H	8	Yes	ND	–	46	Serous	3c
14	D281H	8	Yes	ND	–	74	Serous	3c
15	859–860delGA	8	Yes	ND	–	52	Serous	3c
16	H214R	6	Yes	ND	–	80	Serous	3c
17	R196X	6	Yes	ND	–	57	Serous	3c
18	G245V	7	Yes	ND	–	44	Serous	3c
19	G245D	7	Yes	ND	–	51	Serous	3c
20	L201X	6	Yes	ND	–	52	Serous	3c
21	R213X	6	Yes	ND	–	70	Serous	3c
22	C238F	7	Yes	ND	–	55	Serous	3c
23	C277F	8	Yes	ND	–	45	Serous	3c
24	S215I	6	Yes	ND	–	70	Serous	3c
25	D207N, G245C	6, 7	No, yes	ND	–	45	Serous	3c
26	R248P	7	Yes	ND	–	61	Serous	3c
27	H193R	6	Yes	ND	–	72	Serous	3c
28	Q165X	5	Yes	ND	–	55	Serous	3c
29	580–582delCTT	6	No	ND	–	72	Serous	3c
30	E271D	8	Yes	ND	–	46	Serous	3c
31	E62X	4	Yes	ND	–	47	Serous	4
32	R248W	7	Yes	ND	–	52	Serous	4
33	F54Y	4	Yes	ND	–	58	Serous	4
34	P98L	4	Yes	ND	–	43	Serous	4
35	V217M, N263H	6, 8	Yes, yes	ND	–	56	Serous	4
36	G361R	10	Yes	ND	–	19	mucinous	1c
37	E285K	8	Yes	ND	–	21	mucinous	1c
38	T329S	9	No	ND	–	38	Endometrioid	1c
39	S90Y	4	No	ND	–	52	Endometrioid	3c
40	R306X	8	Yes	ND	–	46	Endometrioid	4

ND: not detected.

more uniformly distributed throughout exons 4–9. The proportion of mutations in the distal part of *TP53* coding region (exons 6–11) was higher in the sporadic group than in the *BRCA1* group (84.4% vs. 56.3%: $P = 0.013$).

Table 4 demonstrates the proportion of cases with *TP53* or *p21* somatic mutation in the *BRCA1* and sporadic groups by histological subtypes. *TP53* mutations were found in 54.3% (25/46) of *BRCA1*-associated tumors but only 43.0% (40/93) of sporadic tumors, however, the difference was not statistically significant. Within the sporadic group, the incidence of *TP53* mutations was significantly lower in non-serous (e.g., endometrioid, clear cell, or mucinous) than serous tumors (5/27 = 18.5% vs. 35/66 = 53.0%: $P = 0.0038$). On the other hand, there was no significant difference in the incidence of *TP53* mutations between non-serous and serous tumors within the *BRCA1* group (3/8 = 37.5% vs. 22/38 = 57.9%). Among non-serous tumors, *TP53* mutations were more common in *BRCA1* cases than sporadic cases, however, the difference was not statistically significant (3/8 = 37.5% vs. 5/27 = 18.5%).

Table 5 shows clinical and pathological characteristics of *BRCA1*-associated and sporadic cases with or without *TP53* somatic mutation. There was no significant difference between the mean age at diagnosis for cases with and without *TP53* somatic mutations. In regard to clinical stage, the presence of *TP53* mutations was significantly correlated with higher FIGO stages ($P = 0.024$) in sporadic tumors, however, no such association was observed in the *BRCA1* group.

Discussion

Several lines of evidence suggest a potentially important interaction between *BRCA1* and *TP53* [5]. Both proteins play a similar role in regulating cellular proliferation and have been implicated in DNA damage surveillance [14]. To our knowledge, there are only five other studies of *TP53* mutations in

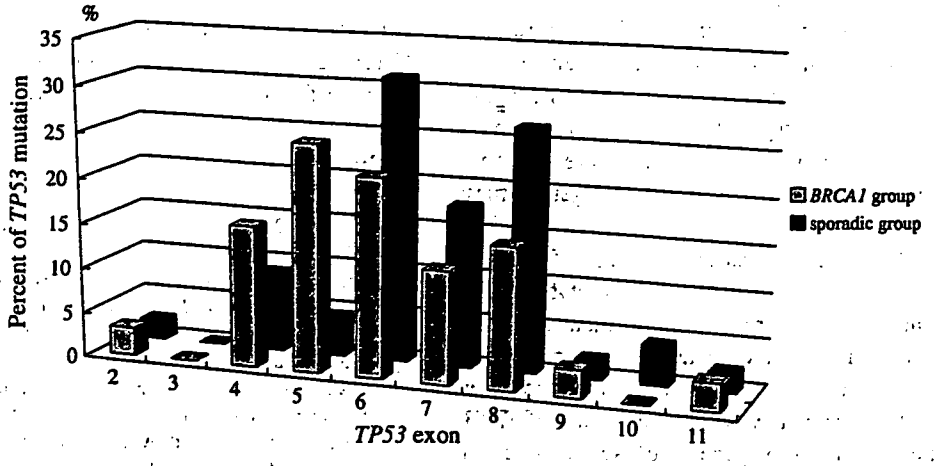

Fig. 1. Distribution of *TP53* somatic mutation by exon for *BRCA1* group ($n = 46$) and sporadic group ($n = 93$). The proportion of mutations in the distal part of the *TP53* coding region (exons 6–11) was higher in the sporadic group than in the *BRCA1* group (84.4% vs. 56.3%: $P = 0.013$; gray: *BRCA1* group, black: sporadic group).

BRCA1-related ovarian or peritoneal cancers, all in Caucasian populations [3,8,15–17]. This study is the first report of *TP53* mutations in *BRCA1*-related ovarian cancers in Asian populations. The previous reports show that 60% or more of *BRCA1*-associated tumors carry *TP53* mutations, a proportion which is significantly higher than that in sporadic tumors. These results suggest that *BRCA1* tumors may develop through a specific pathway of carcinogenesis which is distinct from that of sporadic tumors. A similar conclusion has been drawn from cytogenetic studies, which identified particular genetic and mRNA expression changes characteristic of *BRCA1* tumors [18]. In fact, loss of BRCA1 function is reported to cause genomic instability due to lack of DNA damage surveillance [19]. Therefore, *BRCA1*-related tumors are predicted to arise only after a specific cell cycle checkpoint gene is inactivated to allow cell proliferation. It has been suggested that alterations in other components in the *TP53* pathway either upstream or downstream of *TP53* may be analogous to inactivation of *TP53* itself.

The *TP53* pathway and *BRCA* seem to be functionally interrelated since the p53 and BRCA1 proteins are physically associated and cooperatively involved in *p21* transcription, and presumably they collectively contribute to apoptosis of cancer cells. In mice, *BRCA1* is essential for cellular proliferation and *BRCA1* knockout is embryonically lethal, with an increased activity of p53 and p21 proteins also detected in these same embryonal tissues [20]. In *BRCA1* knockout mice, a germline mutation in either *TP53* or *p21* can prolong survival of the embryos, although not completely rescue them [21]. Moreover, *BRCA1* can activate *p21* in a *TP53*-independent way [22]. Therefore, we investigated whether alterations of *p21* could substitute for *TP53* somatic mutations in ovarian cancer with or without *BRCA1* germline mutations.

In the course of our screen for *p21* somatic mutations, we found only three tumors with candidate variants, and we further found that these three tumors also contained *TP53* somatic mutations. In contrast to *TP53*, which is frequently mutated in human cancer, *p21* mutations seem to be rare in a broad range of human malignancies, among them ovarian cancer [23]. In fact, with the exception of prostate cancer and bladder cancer, no somatic mutation of this gene has been described in any tumor type [24,25]. Taken together, these observations suggest that, in human cancers, including ovarian cancer, mutations of *p21* may not frequently contribute to tumor formation or progression.

Several studies suggest that serous ovarian tumors are more genomically unstable than other histological subtypes. In serous ovarian tumors, loss of heterozygosity for *TP53* is

Table 4
Somatic mutation of *TP53* and *p21* in *BRCA1* group and sporadic group

	BRCA1 group		Sporadic group	
	Serous $n = 38$	Non-serous $n = 8$	Serous $n = 66$	Non-serous $n = 27$
TP53 mutation	22	3	35*	5*
p21 mutation	1	0	2	0

* $P = 0.0038$.

Table 5
Clinical and pathological characteristics of BRCA1 group an with or without TP53 somatic mutation

	BRCA1 group		sporadic group	
	TP53 mutation (+) n=25	TP53 mutation (-) n=21	TP53 mutation (+) n=40	TP53 mutation (-) n=53
pathology				
serous	22	16	35	31
mucinous	0 a	0 a	2 c	4 c
endometrioid	3	4	3	10
clear cell	0	1	0	8
age (mean ± SD)	50.2 ± 7.1	51.1 ± 7.9	50.3 ± 10.8	51.4 ± 14.1
FIGO stage				
I+III	5 b	6 b	11 d	28 d
II+IV	20	15	29	25

a, b: not significant c: $P = 0.0038$, d: $P = 0.024$.

more frequent than in non-serous tumors [19]. Moreover, Pieretti et al. [26] showed that serous ovarian cancers are affected most frequently by apparent chromosome loss. These results suggest that *TP53* somatic mutations in serous tumors may occur after tumor formation. However, there is some possibility that molecular alterations in some genes may influence a phenotypical classification, such as histological subtypes, in the early events of carcinogenesis of ovarian cancer. Some studies have shown that *PTEN* mutations are more frequent in mucinous ovarian cancers than in other histological subtypes, and beta-catenin and *PTEN* mutations are more frequent in endometrioid ovarian cancers than in other histological subtypes [27]. To verify whether mutation of *TP53* is implicated in the initial tumorigenesis of serous ovarian cancer, further investigation is required.

As our result of direct sequencing, LOH was detected in similar proportion of patients with *BRCA1* mutation and sporadic patients, 84.0% and 82.5% respectively (data not shown). We did not perform detailed LOH analysis of *TP53* to determine whether both alleles were inactivated due to two-hit of the gene, however, we considered that the genetic variants of *TP53* which produce amino acid changes are missense mutations. The reason is as follows: (a) the most common *TP53* mutations are missense variants that alter the structure of the protein so that it is incapable of binding DNA and is therefore nonfunctional [28]. (b) A single mutant *TP53* allele is capable of inducing carcinogenesis in the presence of wild-type *TP53*, indicative of a dominant negative phenotype [29,30].

In this study, we found no difference in *BRCA1* and sporadic cancers with regard to *TP53* somatic mutations, however, we showed that *TP53* somatic mutations are less implicated in the carcinogenesis of non-serous sporadic tumors than in that of other ovarian cancers. Furthermore, *p21* somatic mutation appears to be less involved in the development of ovarian cancer than *TP53* somatic mutation. This result suggests that the remaining tumors with no detectable mutations in *TP53 or p21* may have mutations in some other gene in the *TP53* pathway, either upstream or downstream of *TP53*, involved in cell cycle checkpoint control.

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References

[1] Boyd J. Molecular genetics of hereditary ovarian cancer. Oncology (Huntingt) 1998;12:399–406.
[2] Sobol H, Stoppa-Lyonnet D, Bressac-de Paillerets B, Peyrat JP, Guinebretiere J-M, Jacquemier J, et al. BRCA1–p53 relationship in hereditary breast cancer. Int J Oncol 1997;10:349–53.
[3] Rhei E, Bogomolniy F, Federici MG, Maresco DL, Offit K, Robson ME, et al. Molecular genetic characterization of BRCA1- and BRCA2-linked hereditary ovarian cancers. Cancer Res 1998;58:3193–6.
[4] Bertwistle D, Ashworth A. Functions of the BRCA1 and BRCA2 genes. Curr Opin Genet Dev 1998;8:14–20.
[5] Bernstein C, Bernstein H, Payne CM, Garewal H. DNA repair/pro-apoptotic dual-role proteins in five major DNA repair pathways: fail-safe protection against carcinogenesis. Mutat Res 2002;511:145–78.
[6] Crook T, Brooks LA, Crossland S, Osin P, Barker KT, Waller J, et al. p53 mutation with frequent novel condons but not a mutator phenotype in BRCA1- and BRCA2-associated breast tumours. Oncogene 1998;17:1681–9.
[7] Phillips KA, Nichol K, Ozcelik H, Knight J, Done SJ, Goodwin PJ, et al. Frequency of p53 mutations in breast carcinomas from Ashkenazi Jewish carriers of BRCA1 mutations. J Natl Cancer Inst 1999;91:469–73.
[8] Rose SL, Buller RE. The role of p53 mutation in BRCA1-associated ovarian cancer. Minerva Gynecol 2002;54:201–9.
[9] Agarwal ML, Taylor WR, Chernov MV, Chernova OB, Stark GR. The *p53* network. J Biol Chem 1998;273:1–4.
[10] Ramirez PT, Gershenson DM, Tortolero-Luna G, Ramondetta LM, Fightmaster D, Wharton JT, et al. Expression of cell-cycle mediators in ovarian cancer cells after transfection with p16INK4a, p21WAF1/Cip-1, and p53. Gynecol Oncol 2001;83:543–8.
[11] Elbendary AA, Cirisano FD, Evans AC, Davis PL, Iglehart JD, Marks JR, et al. Relationship between p21 expression and mutation of the p53 tumor suppressor gene in normal and malignant ovarian epithelial cells. Clin Cancer Res 1996;2:1571–5.
[12] Sekine M, Nagata H, Tsuji S, Hirai Y, Fujimoto S, Hatae M, et al. The Japanese Familial Ovarian Cancer Study Group: mutational analysis of BRCA1 and BRCA2 and clinicopathologic analysis of ovarian cancer in 82 ovarian cancer families: two common founder mutations of BRCA1 in Japanese population. Clin Cancer Res 2001;7:3144–50.
[13] Tong D, Stimpfl M, Reinthaller A, Vavra N, Mullauer-Ertl S, Leodolter S, et al. BRCA1 gene mutations in sporadic ovarian carcinomas: detection by PCR and reverse allele-specific oligonucleotide hybridization. Clin Chem 1999;45:976–81.
[14] Douglas A, Levine MD, Mark G, Federici MS, Victor E, Reuter MD, et al. Cell proliferation and apoptosis in BRCA-associated hereditary ovarian cancer. Gynecol Oncol 2002;85:431–4.
[15] Schorge JO, Muto MG, Lee SJ, Huang LW, Welch WR, Bell DA, et al. BRCA1-related papillary serous carcinoma of the peritoneum has a unique molecular pathogenesis. Cancer Res 2000;60:1361–4.
[16] Buller RE, Lallas TA, Shahin MS, Sood AK, Hatterman-Zogg M, Anderson B, et al. The p53 mutational spectrum associated with BRCA1 mutant ovarian cancer. Clin Cancer Res 2001;2001 7:831–8.
[17] Ramus SJ, Bobrow LG, Pharoah PD, Finnigan DS, Fishman A, Altaras M, et al. Increased frequency of TP53 mutations in BRCA1 and BRCA2 ovarian tumours. Genes Chromosomes Cancer 1999;25:91–6.
[18] Hilton JL, Geisler JP, Rathe JA, Hattermann-Zogg MA, DeYoung B, Buller RE. Inactivation of BRCA1 and BRCA2 in ovarian cancer. J Natl Cancer Inst 2002;94:1396–406.
[19] Quezado MM, Moskaluk CA, Bryant B, Mills SE, Merino MJ. Incidence of loss of heterozygosity at p53 and BRCA1 loci in serous surface carcinoma. Hum Pathol 1999;30:203–7.
[20] Hakem R, de la Pompa JL, Elia A, Potter J, Mak TW. Partial rescue of

BRCA1 (5–6) early embryonic lethality by p53 or p21 null mutation. Nat Genet 1997;16:298–302.
[21] Hakem R, de la Pompa JL, Sirard C, Mo R, Woo M, Hakem A, et al. The tumor suppressor gene BRCA1 is required for embryonic cellular proliferation in the mouse. Cell 1996;85:1009–23.
[22] Somasundaram K. Breast cancer gene 1 (BRCA1): role in cell cycle regulation and DNA repair—Perhaps through transcription. J Cell Biochem 2003;88:1084–91.
[23] Terry LA, Boyd J, Alcorta D, Lyon T, Solomon G, Hannon G, et al. Mutation analysis of the p21?WAF1/CIP1/SDI1 cording region in human tumor cell lines. Mol Carcinog 1996;16:221–8.
[24] Konishi R, Sakatani S, Kiyokane K, Suzuki K. Polymorphisms of p21 cyclin-dependent kinase inhibitor and malignant skin tumors. J Dermatol Sci 2000;24:177–83.
[25] Martinez LA, Yang J, Vazquez ES, Rodriguez-Vargas Mdel C, Olive M, Hsieh JT, et al. p21 modulates threshold of apoptosis induced by DNA-damage and growth factor withdrawal in prostate cancer cells. Carcinogenesis 2002;23:1289–96.
[26] Pieretti M, Hopenhayn-Rich C, Khattar NH, Cao Y, Huang B, Tucker TC. Heterogeneity of ovarian cancer: relationships among histological group, stage of disease, tumor markers, patient characteristics, and survival. Cancer Invest 2002;20:11–23.
[27] Sato N, Tsunoda H, Nishida M, Morishita Y, Takimoto Y, Kubo T, et al. Loss of heterozygosity on 10q23.3 and mutation of the tumor suppressor gene PTEN in benign endometrial cyst of the ovary: possible sequence progression from benign endometrial cyst to endometrioid carcinoma and clear cell carcinoma of the ovary. Cancer Res 2000;60:7052–6.
[28] Cho Y, Gorina S, Jeffrey PD, Pavletich NP. Crystal structure of a p53 tumor suppressor–DNA complex: understanding tumorigenic mutations. Science 1994;265:346–55.
[29] Harvey M, Vogel H, Morris D, Bradley A, Bernstein A, Donehower LA. A mutant p53 transgene accelerates tumour development in heterozygous but not nullizygous p53-deficient mice. Nat Genet 1995;9:305–11.
[30] Liu G, McDonnell TJ, Montes de Oca Luna R, Kapoor M, Mims B, El-Naggar AK, et al. High metastatic potential in mice inheriting a targeted p53 missense mutation. Proc Natl Acad Sci U S A 2000;97:4174–9.

Alteration of TH1 and TH2 cells by intracellular cytokine detection in patients with unexplained recurrent abortion before and after immunotherapy with the husband's mononuclear cells

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Objective: To elucidate the possible mechanisms of immunotherapy for unexplained recurrent aborters using their husband's mononuclear cells.
Design: Prospective clinical study.
Setting: Institutional practice at the Outpatient Clinic for Infertility, Niigata University Medical Hospital.
Patient(s): Fifty-two unexplained recurrent aborters were chosen as an experimental group.
Intervention(s): Each patient was injected with her husband's mononuclear cells as immunotherapy. Peripheral blood was obtained from the patients.
Main Outcome Measure(s): The percentage of CD4-positive cells, TH1 cells, TH2 cells, and the TH1/TH2 ratio were analyzed in the patients before and after immunotherapy. The same analyses were performed in the successful and the unsuccessful group.
Result(s): To date, 42 of the 52 patients have become newly pregnant. Of the 42, 34 patients have already delivered (successful group) and 3 are now pregnant, while the remaining 8 cases experienced repeated abortion (unsuccessful group). The percentage of TH2 cells significantly increased in the total patient population, while the TH1/TH2 ratio significantly decreased in the total patient population and in the successful group.
Conclusion(s): These findings suggest that immunotherapy with the husband's mononuclear cells for unexplained recurrent abortion induces a dominant state of TH2 cells in the patients. (Fertil Steril® 2006;85:1452–8. ©2006 by American Society for Reproductive Medicine.)
Key Words: Unexplained recurrent abortion, TH1, TH2, immunotherapy, mechanisms

The observation that the human fetus or feto-placental unit is a semiallograft and thus antigenically foreign to the mother, yet does not undergo maternal immune rejection in normal pregnancy, was first made just a half century ago (1). However, successful human pregnancy remains an immunologic enigma, and our understanding of its pathologic manifestations is limited.

It has recently been found that the production of a diversity of cytokines by maternal immune-competent cells in the decidual tissues promotes the growth of trophoblastic cells, and a shift to TH2-related humoral immunity from TH1-driven, cell-mediated immunity was suggested to be beneficial for immunologically successful continuation of pregnancy (2–9).

On the contrary, the lack of such an appropriate immune reaction is considered to cause recurrent spontaneous abortion, especially unexplained recurrent spontaneous abortion (10, 11).

Some investigators, including ourselves, demonstrated the possible efficacy of immunotherapy for unexplained recurrent aborters using paternal mononuclear cells (12–19).

The immunotherapy mechanisms have not yet been fully elucidated, but it is possible that immunotherapy will give rise to a TH1/TH2 balance in patients with unexplained recurrent abortion who undergo immunotherapy.

To date, however, the association between immunotherapy and the TH1/TH2 balance has not yet been fully elucidated. In this context, we analyzed the alteration in the TH1/TH2 balance in a patient population of unexplained

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recurrent aborters who underwent immunotherapy to elucidate the possible immunotherapy mechanisms.

MATERIALS AND METHODS

Fifty-two unexplained recurrent aborters who had sustained three or more consecutive first-trimester spontaneous abortions were chosen as an experimental group. The age of the patients ranged from 23 to 45 with a mean of 32.0 years old.

All of them had experienced three or more consecutive confirmed first trimester spontaneous abortions with one partner. None of the participants had any genetic impairment, Mullerian anomaly, hormonal deficiency, infectious disease, metabolic disorder, or autoimmune abnormalities, such as positive antiphospholipid antibodies or lupus anticoagulant, in our systemic work-up.

In addition, tests for thrombophilic status, such as protein C activity, protein S activity, and thrombin-anti-thrombin III complex, were routinely performed for all patients.

All patients were healthy except for their history of recurrent abortions, and were negative for blocking antibodies, identified by a one-way mixed lymphocyte culture reaction (responder: patient; stimulator: husband), in their sera. Each patient was injected with her husband's mononuclear cells with informed consent.

The percentage of CD4-positive cells, TH1 cells, TH2 cells, and the TH1/TH2 ratio was compared before and after immunotherapy in the total patient populations, in the patients whose pregnancies continued successfully after immunotherapy (successful group), and in the patients whose pregnancies had resulted in repeated abortion (unsuccessful group). Institutional Review Board approval was obtained before the studies.

Immunotherapy Procedure Using the Husband's Mononuclear Cells

Details of the immunotherapy procedure were described elsewhere (15, 16, 18, 20). Mononuclear cells from about 100 mL of heparinized peripheral blood of the husband, irradiated with 30 Grey of X-rays to prevent any graft-versus-host (GVH) reaction, were suspended in approximately 1 mL of normal physiological saline solution. This cell suspension was intradermally injected into the patients after obtaining informed consent. After the appearance of mixed lymphocyte culture reaction-blocking antibodies (MLR-BAbs) in patients' sera following a series of two or more injections 1 month apart, the patients were allowed to become pregnant.

To date, a significant level of MLR-BAbs has been detected after a series of the injections in all patients. Analyses of CD4 positive cells, TH1 cells, and TH2 cells were performed just after the last abortion as preimmunotherapy tests, and were also performed about 4 weeks after the last injection as postimmunotherapy tests.

Mixed Lymphocyte Culture Reaction-Blocking Assay

The blocking effect of sera was investigated in a one-way MLR between spouses. Lymphocytes were collected from heparinized blood via Ficoll-Hypaque gradient centrifugation. Mixed culturing of mitomycin C-treated stimulator cells of the husband and responder cells of the patient was performed in a microtiter plate in Roswell Park Memorial Institute (RPMI) 1640 containing either pooled human AB (blood type of AB) serum or tested serum for 6 days. The cultured cells were harvested onto a glass fiber filter after a pulse time of 18 hours with ^{3}H-thymidine. The DNA synthesis was evaluated by liquid scintillation counting, and the blocking effect (BE) was calculated by the formula:

$$BE = (1 - \text{mean cpm of culture in tested serum} / \text{mean cpm of culture in AB serum}) \times 100\ (\%)$$

The significant level of the MLR-blocking effect was determined to be more than 22%, which was designated as positive for MLR-BAbs, as previously reported (15, 16, 18, 20).

Analyses of CD4-Positive Cells

One hundred microliters of whole blood collected from patients were incubated with 10 μL of appropriately titered fluorescein isothiocyanate (FITC)-conjugated anti-CD4 antibody (NU-TH/I-FITC, Nichirei, Japan) in an ice bath for 30 minutes, then treated with 2 mL of a lysing agent (0.83% ammonium chloride) for 10 minutes at room temperature.

The pellet was washed once in phosphate-buffered saline (PBS), and the cells were then diluted to a final volume of 2 mL in PBS. The antibody-reacted cells were analyzed with a Flowcytometer (Ortho Clinical Diagnostics, Raritan, NJ).

Analyses of the TH1 and TH2 Cells

Cells with TH1 and TH2 were determined by detecting the intracellular interferon (IFN)-gamma and IL-4 production (21–23).

Peripheral heparinized venous blood cells were washed three times in Hanks' balanced salt solution and resuspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 50 U/mL of penicillin, and 50 μg/mL of streptomycin. After 2 hours' cultivation in a culture dish, nonadherent cells were collected and stimulated with 25 ng/mL of phorbol-12-myristate-13-acetate and 1 μmol/l of ionomysin in the presence of 10 μg/mL of brefeldin A (Sigma, St. Louis, MO) for 4 hours at 37°C with 7% CO_2 in RPMI 1640 supplemented with FCS.

Peridininchlorophyll protein (PerCP)-conjugated anti-CD4 and PerCP-conjugated antimouse immunoglobulin G1 (IgG1) were used to analyze cell surface antigens. The FITC-conjugated IFN-gamma and phycoerythrin (PE)-conjugated anti-IL-4 (Becton Dickinson Immunocytometry Systems [BDIS], Mountain View, CA) were used to analyze

intracellular cytokines. The FITC-conjugated IgG2a and PE-conjugated IgG1 antibodies were used as control antibodies.

An anti-CD4-PerCP antibody was added to the lymphocytes and they were incubated for 15 minutes at room temperature. Then, the cells were washed with PBS with 0.1% bovine serum albumin. The cell pellet was fixed with lysing solution (BDIS) and permeability was achieved with a permeabilizing solution (BDIS) according to the manufacturer's instructions. Anti-IFN-gamma FITC and anti-IL-4 PE were added, and incubation was performed for 30 minutes at room temperature. For control samples, FITC-conjugated IgG2a and PE-conjugated IgG1 antibodies were used in the same reaction.

The samples were analyzed on a FACScan (BDIS) using Cell Quest Software (BDIS). Dead cells and monocytes were excluded from lymphocytes initially by side scatter gating and then by forward scatter gating. Cell populations were defined as follows: TH1: IFN-gamma-positive and IL-4-negative; TH2: IFN-gamma negative and IL-4 positive.

STATISTICAL ANALYSES

A paired *t*-test was used to analyze the significance of the difference in the percentage of CD4-positive cells, TH1 cells, TH2 cells, and the TH1/TH2 ratio before and after immunotherapy.

RESULTS

The mean percentage of CD4-positive cells in the total patient population with unexplained recurrent abortion before immunotherapy was 44.6% ± 7.29%, and the mean percentage after immunotherapy was 43.7% ± 7.70%. The mean percentage of TH1 cells in all patients with unexplained recurrent abortion before immunotherapy was 21.4% ± 7.88%, and the mean percentage after immunotherapy was 21.0% ± 5.97%.

Thus, the percentages of CD4-positive cells and TH1 cells were not significantly different before and after immunotherapy in this population.

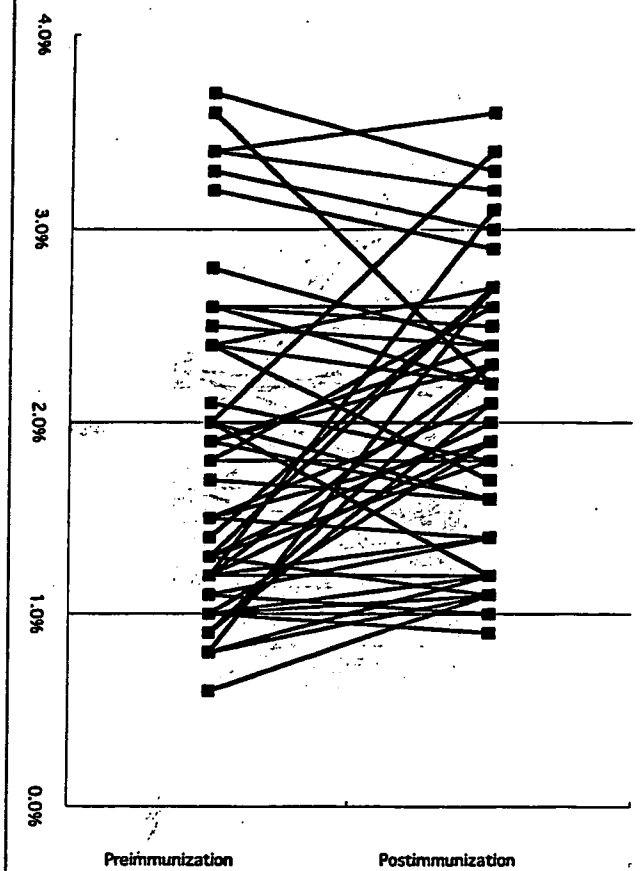
The mean percentage of TH2 cells in patients with unexplained recurrent abortion before immunotherapy was 1.78% ± 0.82%, and the mean percentage after immunotherapy was 2.03% ± 0.71% (Fig. 1). Thus, the percentage of TH2 cells significantly increased after immunotherapy compared with that before immunotherapy (P=.012, paired *t*-test).

The mean of TH1/TH2 ratio in patients before immunotherapy was 14.0 ± 7.21, and that after immunotherapy was 11.7 ± 5.26 (Fig. 2); therefore, the mean TH1/TH2 ratio significantly decreased after immunotherapy compared with that before immunotherapy (P=.002, paired *t*-test).

To date, 42 of the 52 patients have become newly pregnant. Of the 42, the pregnancy continued in 34 patients (81.0%), while the remaining 8 cases experienced repeated abortion (unsuccessful group). Of the 34 patients in whom

FIGURE 1

The change in the mean percentage of TH2 cells in the total patient population before immunotherapy (1.78% ± 0.82%) and after immunotherapy (2.03% ± 0.71%). The percentage of TH2 cells significantly increased after immunotherapy compared with that before immunotherapy (P=.012, paired *t*-test).



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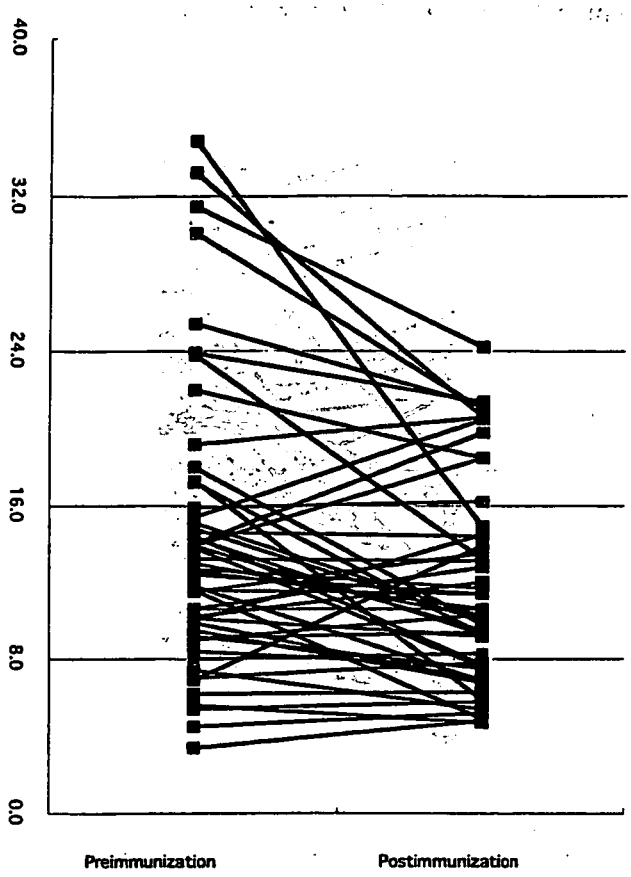
the pregnancy continued, 31 have already delivered babies, and the remaining 3 are now pregnant (their gestational weeks [Gw] are 29, 22, and 17, respectively).

Twenty-eight patients of 31 experienced term delivery, and 3 experienced preterm delivery (1 of them was singleton delivery at the 36th Gw, 1 was a twin delivery at the 36th Gw, and the remaining 1 was a singleton delivery at the 34th Gw). All infants born to the patients displayed an uneventful neonatal course. We named the patients who had already experienced delivery the "successful group" (n = 31).

The mean percentage of CD4-positive cells in the successful group before immunotherapy was 42.6% ± 6.13%, and the mean percentage after immunotherapy was 42.6% ± 7.00%, whereas the mean percentage of TH1 cells in the successful group before immunotherapy was 23.1% ± 7.70%, and the mean percentage after immunotherapy was 22.1% ± 5.15%.

FIGURE 2

The change in the TH1/TH2 ratio in the total patient population before immunotherapy (14.0 ± 7.21) and after immunotherapy (11.7 ± 5.26). The mean TH1/TH2 ratio significantly decreased after immunotherapy compared with that before the immunotherapy (P=.002, paired t-test).



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The mean percentage of TH2 cells in the successful group before immunotherapy was 1.81 ± 0.75%, and the mean percentage of TH2 cells after immunotherapy was 2.01 ± 0.75% (Fig. 3). Thus, the percentages of CD4 positive cells, TH1 cells, and TH2 cells were not significantly different between before and after immunotherapy in the successful group.

The mean TH1/TH2 ratio in the successful group before immunotherapy was 14.5 ± 6.74, and the mean TH1/TH2 ratio after immunotherapy was 12.6 ± 5.57 (Fig. 4). Thus, the mean TH1/TH2 ratio significantly decreased after immunotherapy compared with that before immunotherapy (P =.018, paired t-test).

The mean percentage of CD4-positive cells in the unsuccessful group before immunotherapy was 46.7 ± 7.11%, and the mean percentage of CD4-positive cells after immunotherapy was 44.0 ± 5.34%. The mean percentage of TH1 cells in the unsuccessful group before immunotherapy was 17.5 ± 7.24%, and the mean percentage of TH1 cells after immunotherapy was 18.4 ± 7.82%.

The mean percentage of TH2 cells in the unsuccessful group before immunotherapy was 1.46 ± 0.99%, and the mean percentage of TH2 cells after immunotherapy was 1.76 ± 0.82%. The mean TH1/TH2 ratio in the unsuccessful group before immunotherapy was 16.0 ± 11.0, and the mean TH1/TH2 ratio after immunotherapy was 11.5 ± 4.9 (Fig. 5).

The percentages of CD4-positive cells, TH1 cells, TH2 cells, and the TH1/TH2 ratio were not significantly different between before and after immunotherapy in the unsuccessful group (paired t-test).

DISCUSSION

In this study, we analyzed the changes in the percentage of CD4-positive cells, TH1 cells, TH2 cells, and the TH1/TH2

FIGURE 3

The change in the percentage of TH2 cells in successful group before immunotherapy (1.81% ± 0.75%) and after immunotherapy (2.01% ± 0.75%). No significant difference was observed between before and after immunotherapy.

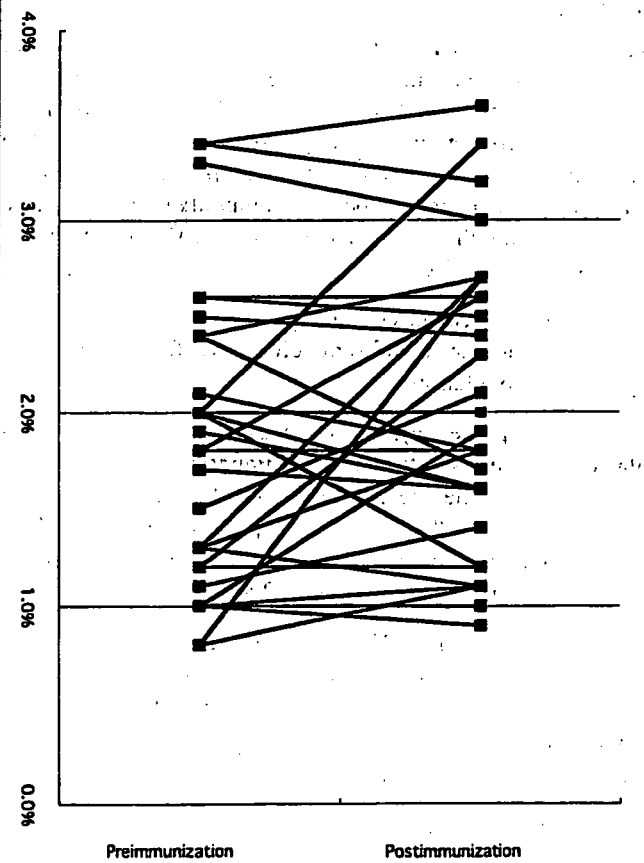

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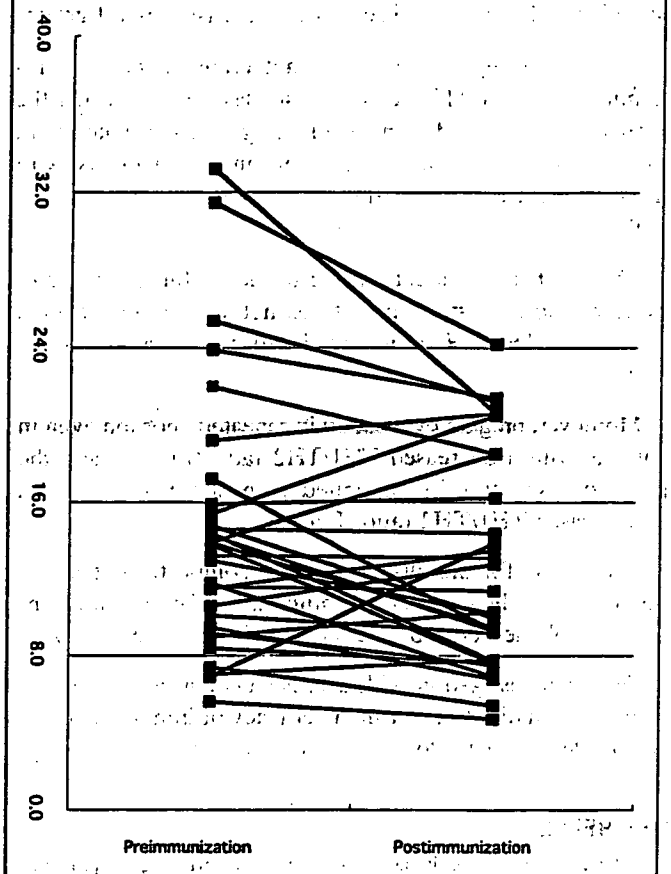
FIGURE 4

The change in the TH1/TH2 ratio in successful group before immunotherapy (14.5 ± 6.74) and after immunotherapy (12.6 ± 5.57). The mean TH1/TH2 ratio in the successful group significantly decreased after immunotherapy compared with that before immunotherapy ($P = .018$, paired t-test).



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ratio in patients with unexplained recurrent abortions before and after immunotherapy with the husband's mononuclear cells. The changes were also analyzed in a successfully immunized group and an unsuccessful group.

It was observed that the percentage of TH2 cells significantly increased with immunotherapy in the total patient population, and that the TH1/TH2 ratio significantly decreased with immunotherapy in the total patient population and in the successful group, although no significant change was observed in the unsuccessful group.

As antigens expressed on the surface of fetal or placental tissues possibly induce the alloimmune response of the mother, there appear to be certain immunologic mechanisms that sustain the continuation of normal pregnancy.

Progress in understanding the immunologic mechanisms for continuation of pregnancy has been made in studies of women with unexplained recurrent abortion over the past three decades. That is, several investigators have reported the existence of immunologically explainable recurrent spontaneous aborters, and immunotherapy for these patients using their husbands' or a third party's leukocytes has been reported by several authors, including ourselves (12–19).

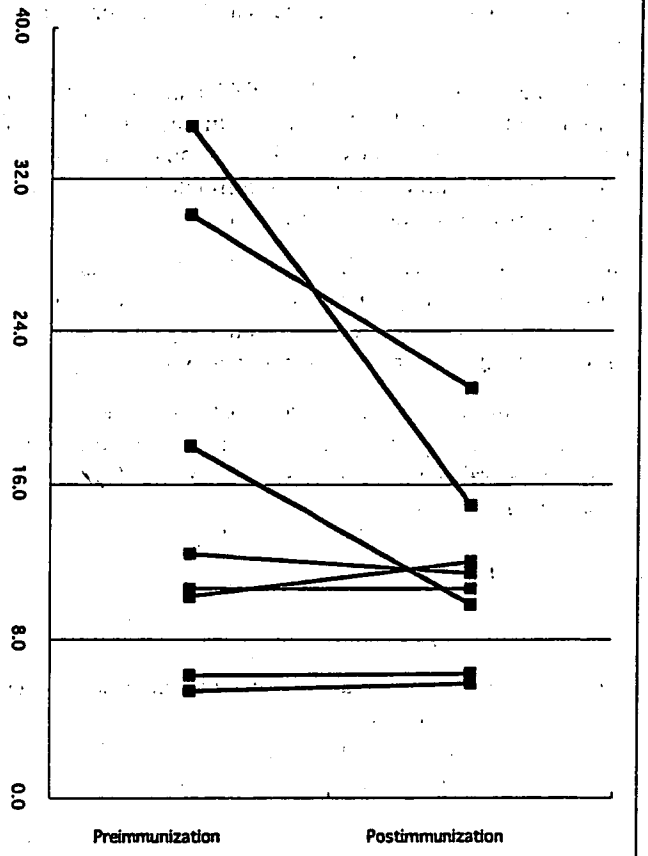
The results of some case-controlled immunotherapy studies on recurrent spontaneous abortions indicate that the outcome of subsequent pregnancies is significantly improved by injection of paternal lymphocytes, as compared with that after injection of autologous cells (12, 13, 19), although Ober et al. reported the ineffectiveness of this treatment (24).

A worldwide meta-analysis study has concluded that immunization may be highly effective, although only for a small number of patients who have the indication (25).

Although immunotherapy is considered possibly efficacious, the underlying mechanisms have not yet been fully

FIGURE 5

The change in the TH1/TH2 ratio in the unsuccessful group before immunotherapy (16.0 ± 11.0) and that after immunotherapy (11.5 ± 4.9). No significant difference was observed between before and after immunotherapy.



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elucidated. Several investigators reported the production of blocking antibodies in a patient's serum, which inhibited the response of autologous lymphocytes to transfused paternal lymphocytes (14, 17, 19).

We reported that patients with negative MLR-BAbs benefited from immunotherapy with the husband's lymphocytes in both unexplained primary recurrent abortions (15) and secondary recurrent abortions (16), and also that significant MLR-BAbs were induced in almost all patients who underwent immunotherapy with their husband's lymphocytes (15, 16, 18, 20).

Concerning the changes in cellular immunity before and after immunotherapy, we previously reported that a significant decrease in the CD4/CD8 ratio was observed in immunized patients mainly because of an increase in the CD8 subpopulation (26).

In this study, we analyzed the alterations in the CD4 cell population, and no significant change was observed in immunized patients, which is the same tendency as the previous report. On the other hand, Miki et al. reported that immunotherapy had no influence on natural killer receptor status (27).

As one of the important mechanisms which immunologically sustain pregnancy, Wegmann et al. proposed immunotrophic theory (2, 3), whereby some cytokines produced by maternal cells, which recognize fetal antigens, promote the proliferation of trophoblastic cells and sustain pregnancy continuation.

Following this theory, the "TH1/TH2 paradigms" theory was proposed. TH1 and TH2 cells are the major subsets of fully differentiated CD4-positive T cells, and their distinctive functions in immune responses correlate with their distinctive cytokine secretion patterns (28).

In this theory, a TH2 cell bias against TH1 cells is important for normal pregnancy, indicating the crucial role of the activation of maternal humoral immunity following recognition of fetal antigens during pregnancy (4–9).

Regarding the TH1/TH2 balance in patients with recurrent abortion, Hill first reported increased TH1 cytokine production by peripheral lymphocytes exposed to JEG3 choriocarcinoma stimulation (10). Lim et al. reported that levels of TH1 cytokines were significantly higher in women with recurrent abortions compared with normal controls (11).

In this context, it is possible that immunotherapy affects the TH1 cell and TH2 cell balance in immunized patients. Hayakawa et al. reported that a significant decrease in TH1 cells, a significant increase in TH2 cells, and a significant decrease in the TH1/TH2 ratio was observed in 12 patients with recurrent abortion after immunotherapy (29).

In this study, we increased the number of patients with unexplained recurrent abortion and analyzed the alterations in CD 4-positive cells, TH1 cells, and TH2 cells.

A significant increase in TH2 cells and a significant decrease in the TH1/TH2 ratio were observed in the total immunized population, although no significant change could be observed concerning TH1 cells.

Moreover, we analyzed the alterations in TH1 cells, TH2 cells, and the TH1/TH2 ratio both in a successful group and an unsuccessful group; a significant decrease in the TH1/TH2 ratio was observed in the successful group, while no significant change was observed in the unsuccessful group.

These data suggest that immunotherapy induces a predominant state of TH2 cells against TH1 cells, and that the induction of this predominant state might be correlated with the successful continuation of pregnancy in patients with unexplained recurrent abortion who undergo immunotherapy with their husband's lymphocytes.

The number of patients in the unsuccessful group, however, was low ($n = 8$), and it has not yet been confirmed whether the TH1/TH2 ratio was significantly changed in this group.

Moreover, pregnancy resulted in repeated abortion even in patients with a decreased TH1/TH2 ratio (Fig. 5), and the pregnancy successfully continued even in the patients with an increased TH1/TH2 ratio (Fig. 4).

The reason for this discrepancy is thought to be that the immunologically successful continuation of pregnancy cannot be explained only by the TH1/TH2 paradigm theory.

Further investigation with a larger patient population, as well as the studies to confirm the efficacy of immunotherapy, is considered crucial to reach a definite conclusion.

REFERENCES

1. Medawar PB. Some immunological and endocrinological problems raised by evolution of viviparity in vertebrates. Symp Soc Exp Biol 1953;7:320–38.
2. Wegmann TG. Placental immunotrophism: maternal T cell enhance placental growth and function. Am J Reprod Immunol Microbiol 1987; 156:67–9.
3. Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunol Today 1993;14:353–6.
4. Lin H, Mosmann TR, Guilbert L, Tuntipopipat S, Wegmann TG. Synthesis of T helper 2-type cytokines at the maternal-fetal interface. J Immunol 1993;151:4562–73.
5. Marzi M, Vigano A, Trabattoni D, Villa ML, Salvaggio A, Clerici E, et al. Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy. Clin Exp Immunol 1996; 106:127–33.
6. Raghupathy R. Th1-type immunity is incompatible with successful pregnancy. Immunol Today 1997;18:478–2.
7. Guilbert L. There is a bias against type 1 (inflammatory) cytokine expression and function in pregnancy. J Reprod Immunol 1996;32:105–10.
8. Raghupathy R, Makhseed M, Azizieh F, Hassan N, Al-Azemi M, Al-Shamali E. Maternal Th1- and Th2-type reactivity to placental antigens in normal human pregnancy and unexplained recurrent spontaneous abortions. Cell Immunol 1999;196:122–30.
9. Chaouat G, Zourbas S, Ostogic S, Lappree-Delage G, Dubanchet S, Ledee N, et al. A brief review of recent data on some cytokine

expression at the materno-foetal interface which might challenge the classical Th1/Th2 dichotomy. J Reprod Immunol 2002;53:241–56.
10. Hill JA. T helper 1 immunity to trophoblast: evidence for a new immunological mechanism for recurrent abortion in women. Hum Reprod 1995;10(suppl 2):114–20.
11. Lim KJH, Odukoya OA, Ajjan RA, Li T, Weetman AP, Cooke ID. The role of T-helper cytokines in human reproduction. Fertil Steril 2000; 73:136–42.
12. Mowbray JF, Gibbings C, Liddell H, Reginald PW, Underwood JL, Beard RW. Controlled trial of treatment of recurrent spontaneous abortion by immunisation with paternal cells. Lancet 1985;i:941–3.
13. Gatenby PA, Cameron K, Simes RJ, Adelstein S, Bennett MJ, Jansen RPS, et al. Treatment of recurrent spontaneous abortion by immunization with paternal lymphocytes: results of a controlled trial. Am J Reprod Immunol 1993;29:88–94.
14. Unander AM, Lindholm A. Transfusion of leucocyte-rich erythrocyte concentrates: a successful treatment in selected cases of habitual abortion. Am J Obstet Gynecol 1986;154:516–20.
15. Takakuwa K, Goto S, Hasegawa I, Ueda H, Kanazawa K, Takeuchi S, et al. Result of immunotherapy on patients with unexplained recurrent abortion: a beneficial treatment for patients with negative blocking antibodies. Am J Reprod Immunol 1990;23:37–41.
16. Takakuwa K, Higashino M, Yasuda M, Ishii S, Ueda H, Asano K, et al. Is an additional vaccination necessary for a successful second pregnancy in unexplained recurrent aborters who were successfully immunized with their husband's lymphocytes before the first pregnancy? Am J Reprod Immunol 1993;29:39–44.
17. Park MI, Edwin SS, Scott JR, Branch DW. Interpretation of blocking activity in maternal serum depends on the equation used for calculation of mixed lymphocyte culture results. Clin Exp Immunol 1990;82: 363–8.
18. Adachi H, Takakuwa K, Mitsui T, Ishii K, Tamura M, Tanaka K. Results of immunotherapy for patients with unexplained secondary recurrent abortions. Clin Immunol 2003;106:175–80.
19. Pandey MK, Agrawal S. Induction of MLR-Bf and protection of fetal loss: a current double blind randomized trial of paternal lymphocyte immunization for women with recurrent spontaneous abortion. Int Immunopharmacol 2004;4:289–98.
20. Takakuwa K, Kanazawa K, Takeuchi S. Production of blocking antibodies by vaccination with husband's lymphocytes in unexplained recurrent aborters: the role in successful pregnancies. AM J Reprod Immunol Microbiol 1986;10:1–9.
21. Jung T, Shauer U, Heussern C, Neumann C, Rieger C. Detection of intracellular cytokines by flow cytometry. J Immunol Methods 1993; 159:179–207.
22. Picker LJ, Singh MK, Zdraveski Z, Treer JR, Waldrop SL, Bergstresser PR. Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. Blood 1995;86: 1408–19.
23. Aoki Y, Tsuneki I, Sasaki M, Watanabe M, Sato T, Aida H, et al. Analysis of TH1 and TH2 cells by intracellular cytokine detection with flow cytometry in patients with ovarian cancer. Gynecol Obstet Invest 2000;50:207–11.
24. Ober C, Karrison T, Odem RR, Barnes RB, Branch DW, Stephenson MD, et al. Mononuclear-cell immunization in prevention of recurrent miscarriages: A randomised trial. Lancet 1999;354:365–9.
25. Recurrent Miscarriage Immunotherapy Trialists Group. Worldwide collaborative observational study and meta-analysis on allogeneic leucocyte immunotherapy for recurrent spontaneous abortions. Am J Reprod Immunol 1994;32:55–72.
26. Takakuwa K, Ueda H, Goto S, Hasegawa I, Yamada K, Kazama Y, et al. Influence of immunotherapy on the cellular immunity of unexplained recurrent aborters. J Reprod Immunol 1991;20:153–63.
27. Miki A, Fujii T, Ishikawa Y, Hamai Y, Yamashita T, Tadokoro K, et al. Immunotherapy prevents recurrent abortion without influencing natural killer receptor status. Am J Reprod Immunol 2000;43:98–106.
28. Seder RA, Mosmann TM. Differentiation of effector phenotypes of CD4+ and CD8+ T cells. In: Paul WE, ed. Fundamental Immunology. 4th ed. Philadelphia: Lippincott-Raven Publishers, 1999:879–908.
29. Hayakawa S, Karasaki-Suzuki M, Itoh T, Ishii M, Kanaeda T, Nagai N, et al. Effects of paternal lymphocyte immunization on peripheral Th1/Th2 balance and TCR Vβ and Vγ repertoire usage of patients with recurrent spontaneous abortions. Am J Reprod Immunol 2000;43;107–15.

Complete removal of HIV-1 RNA and proviral DNA from semen by the swim-up method: assisted reproduction technique using spermatozoa free from HIV-1

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Background: Use of antiretroviral drugs has reduced the mortality rate for HIV infection and many HIV-discordant couples wish to have children. It is possible for an HIV-infected man to father children without risk of HIV transmission if HIV-free spermatozoa can be obtained from his semen.

Methods: An improved swim-up method was used to collect HIV-free spermatozoa from the semen of HIV-positive males. Diluted semen was layered over a Percoll solution with a continuous density gradient of 30–98%, and then centrifuged. The bottom layer was collected by cutting the end from the tube and the sperm suspension was collected using the swim-up method. Spermatozoa were tested by nested polymerase chain reaction (PCR) for HIV-1 RNA and DNA, with a detection limit of one copy. Spermatozoa were used for assisted reproduction in 43 couples.

Results: HIV-1 RNA and proviral DNA were not detected by nested-PCR assay in all 73 of the collected spermatozoa samples from 52 patients. The HIV-1-negative sperm was used for *in vitro* fertilization in 12 couples and for intracytoplasmic sperm injection in 31 couples. No detection of HIV-1 RNA or proviral DNA in the culture medium of the fertilized eggs was confirmed again before embryo transfer. Of the 43 female partners, 20 conceived and 27 babies were born. HIV antibodies, HIV RNA and proviral DNA were negative in all of the females and babies.

Conclusions: HIV-negative spermatozoa could be obtained from semen of HIV-positive men. The method involves no risk of HIV transmission to female partners and their children.
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Keywords: assisted reproductive techniques, HIV-1, sperm, HIV-free spermatozoa

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Introduction

Since the mid-1990s, the use of HAART has spread, and the AIDS mortality rate has decreased by more than 80% in the industrialized world [1]. HIV infection/AIDS is becoming a controllable chronic infection and HIV-infected individuals are now living longer. Many HIV-positive people are getting married and wishing to have children.

Semprini *et al.* [2] reported that they had conducted artificial inseminations in more than 2000 HIV-discordant couples (HIV-positive male and HIV-negative female) using their swim-up method, and that no HIV transmission was observed. However, their method may be suboptimal because it has not been proven to remove HIV RNA completely, and they did not measure proviral DNA in infected cells in the semen. Zhang *et al.* [3] reported that HIV may be present as proviral DNA in seminal cells in HIV-infected men who have achieved undetectable levels of viral RNA in plasma with HAART, and this HIV could be capable of sexual transmission. It has not been determined whether HIV is attached to spermatozoa or whether spermatozoa can be infected with HIV [4,5]. Therefore, contraception is recommended for HIV-discordant couples, even if HIV RNA is undetectable in plasma [3].

Authorities in different countries have different opinions concerning the use of assisted reproductive technology using spermatozoa collected by the swim-up method [6–8]. However, it would be possible for an HIV-infected man to father children without risk of HIV transmission if HIV-free spermatozoa can be obtained from his semen. This study examines an improved swim-up method for isolating HIV-free sperm and its use in assisted reproductive methods.

Methods

This clinical study was approved by the ethics committees of Niigata University, Ogikubo Hospital, Keio University and Kyorin University. All of the couples visited the Hematology Department of Ogikubo Hospital and received counselling and explanations of the clinical study. Informed consent was obtained from all participating couples. Semen was obtained by masturbation, and then tested for sperm concentration, motility and deformity.

Percoll preparation

An isotonic solution of Percoll (Amersham Life Science, Tokyo, Japan) was made by dissolving 980 g Percoll in 10.0 ml 2.0 mol/l Hepes-NaOH, pH 7.4, 10.0 ml human serum albumin (25%w/v), 0.05 g fosfomycin and 0.05 g cefarotin. The resulting 98% Percoll solution was sterilized with a Millipore filter (0.45 μm pore size).

Semen pretreatments

The procedure is shown in Fig. 1. Ejaculates were diluted twice with Hanks solution, followed by standing in a test tube for 10 min to precipitate filterable micro-calculus, then filtered through an ART filter (20 μm clearance; ART filter, Nipro, Osaka, Japan) to remove fibers, micro-calculus and mucinous debris. The upper phase of sperm suspension was loaded onto 6 ml Percoll linear gradient from 98% to 30% in a separable fine-neck tube (Nipro) and centrifuged at 400 × *g* for 30 min. The separable fine-neck tube was made of glass, and its bottom was squeezed to minimize the volume of sediment. To recover the sperm precipitated in the bottom tip, the top of the tube was plugged with a rubber cap, and the middle of the squeezed bottom was snapped off with an ampoule cutter.

Motile sperm were separated by the modified swim-up method. A fine glass capillary was inserted in 2 ml of the medium in a vial, then a needle tip was introduced to the bottom through the inner capillary. The motile sperm were allowed to swim up at 37°C in an incubator with 5% CO_2–air. After 60 min, 1 ml of upper layer was collected, containing the sperm that had swum up.

The sperm suspension was divided into two portions. One was used for HIV assessment, and the other was cryopreserved with KS-II medium [9] in a liquid nitrogen container.

Standard HIV-1 materials

MOLT-4 cells infected with HIV_{LAI} and its culture supernatant were used as standards for HIV-1-infected cells and virus stock, respectively. RNA purified from virus stock and the pNL4-3 plasmid [10] were the standards for HIV-1 RNA and DNA, respectively. The concentrations of the standard HIV-1 DNA and RNA were determined by spectrophotometry and the null-class equation of the Poisson distribution of the reverse transcriptase (RT)-nested polymerase chain reaction (PCR). Cells were counted using a Burker–Turk hemocytometer (Emergo, Landsmeer, the Netherlands). The virion concentration was considered to be half the virus RNA concentration.

Detection of HIV-1 RNA and DNA

The samples of sperm suspension, culture medium or plasma were centrifuged at 35 500 × *g* for 1 h at 4°C. RNA and DNA were extracted from the precipitate using QIAamp UltraSens Virus Kit (Qiagen, Tokyo, Japan). One fourth of the eluate was tested in quadruplicate by RT-nested PCR as follows. The RT reaction was performed by incubation at 42°C for 10 min in a

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