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# Differential Expressions of Apoptosis Regulators and Protein Profiling by SELDI-TOF Mass Spectrometry in Human Testis with Obstructive and Non-obstructive Azoospermia

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## 폐쇄성과 비폐쇄성 무정자증 환자의 고환 내 세포자연사 관련 인자들의 발현 변화와 SELDI-TOF Mass Spectrometry를 이용한 단백질 발현 분석

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**김슬기[1] · 김호승[1] · 이호준[2] · 박용석[3] · 서주태[4*] · 윤용달[1*]**

**연구목적**: 본 연구에서는 비폐쇄성 무정자증 환자에서 나타나는 정자형성과정의 이상과 고환세포의 세포자연사와의 연관관계 여부를 확인하였다. 또한 SELDI-TOF MS 분석을 통하여 고환 내 단백질 발현 양상을 확인하고, 질환에 따른 효과적인 biomarker 개발 가능성 여부를 확인하였다.

**재료 및 방법**: RT-PCR 및 면역조직화학법을 사용하여 고환에서의 Fas, FasL, Bcl-2, Bax와 Caspase-3의 발현 양상을 확인하고, *in situ* DNA 3'-end-labelling 방법으로 고환세포의 세포자연사 양상을 확인하였다. SELDI-TOF MS 분석법에 의한 고환의 병리학적 소견에 따른 단백질 발현 변화는 소수성 칩 ($H_4$)을 사용하여 분자량 10~100 kDa 범위 내에서 분석하였다.

**결 과**: 정상적인 정자형성과정을 보이는 폐쇄성 무정자증 환자의 고환에 비해 지주세포 증후군 (Sertoli cell only syndrome)과 성숙정지 (maturation arrest)를 보이는 고환 내 생식세포와 지주세포에서 세포자연사가 현저하게 증가한 것을 확인할 수 있었다. 세포자연사 관련인자들의 발현 양상을 확인한 결과, 지주세포 증후군과 성숙정지 환자군에서 Fas와 FasL mRNA의 발현이 증가하였으나, bcl-2, bax와 caspase-3 mRNA 발현의 경우에는 두 질환 모두에서 유의한 차이를 확인할 수 없었다. FasL 단백질 발현의 경우, 세포자연사의 증가가 관찰되었던 지주세포 증후군과 성숙정지를 보이는 환자의 간질세포와 지주세포에서 증가하는 양상을 나타내었다. SELDI-TOF MS 분석 결과에서 폐쇄성 무정자증 환자군에 비해 전체적인 단백질 발현양이 지주세포 증후군과 성숙정지 환자의 고환에서 감소하는 양상을 보였으며, 특히, 16.730 kDa 단백질의 현저한 감소를 확인할 수 있었다.

**결 론**: 본 연구결과를 통해 비폐쇄성 무정자증 환자에서 나타나는 정자형성과정의 장애는 생식세포

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의 비정상적인 세포자연사와 연관되어 있으며, 고환 내 Fas와 FasL의 비정상적인 발현이 주된 원인인 것을 확인할 수 있었다. 또한, SELDI-TOF MS 분석법을 통한 단백질 발현 양상의 연구는 무정자증 환자에서의 다양한 병리학적 소견을 쉽게 파악할 수 있는 biomarker 발굴뿐만 아니라 질환의 원인규명을 위한 연구에도 유용하게 이용될 수 있을 것으로 사료된다.

**Key Words:** Apoptosis, SELDI-TOF MS analysis, Human, Testis, Azoospermia

Despite remarkable advances in treatment of severe male factor infertility using testicular sperm extraction and intracytoplasmic sperm injection (ICSI), significant numbers of men are not treatable because they lack the ability to produce mature sperm.[1] Recently, round spermatid injection and *in vitro* culture of testicular germ cells have been proposed for the treatment of non-obstructive azoospermia or severe oligozoospermia accompanying abnormal spermatogenesis, however, these therapeutic options are not entirely effective yet.[2~4] Because of deficiencies in our understanding of testicular failure pathophysiology, the therapeutic options for the non-obstructive azoospermia, such as maturation arrest (MA) and Sertoli cell-only syndrome (SCO), are very limited. Therefore, mechanisms responsible for abnormal spermatogenesis in the non-obstructive azoospermia need to be defined better to develop and improve successful treatment.

Spermatogenesis is a dynamic and complex process in which spermatogonial stem cells undergo many rounds of mitotic and meiotic cell divisions and differentiation to generate mature haploid spermatozoa. During this process, a considerable number of germ cells die by apoptosis throughout their development and differentiation.[5,6] Recent studies have demonstrated the increased apoptosis in some conditions of abnormal spermatogenesis in men,[7~9] however, its cause(s) and the molecular mechanism (s) are poorly understood.

Several apoptosis-related factors have been reported. Fas and Fas ligand (FasL) have been identified as type 1 and type 2 transmembrane proteins, respectively, and binding of FasL to the Fas induces apoptosis of the Fas-bearing cells, possibly through the activation of caspases.[10,11] Within the rodent testis, apoptosis induced by FasL has been suggested to be one of the mechanisms limiting the number of germ cells during normal spermatogenesis or after different testicular injury, such as radiation exposure or administration of Sertoli cells toxicants, mono-(2-ethylhexyl)phthalate and 2,5-hexanedione.[12,13] Recent studies have shown that in human testis also, apoptosis is a conspicuous event during spermatogenesis and Fas-FasL interaction is involved in the regulation of this event.[14,15] The members of the Bcl-2 family also have been known to be involved in the regulation of apoptosis in various cell types, and may either inhibit (Bcl-2, Bcl-xL, Mcl-1, Bcl-w) or promote (Bax, Bad, Bcl-xs, Bak) apoptosis. The expression of Bcl-2 family proteins has been demonstrated in the normal human testis and their preferential expressions in various germ cell compartments strongly suggested that these apoptotic proteins may be involved in differentiation and maturation through the various stages of human spermatogenesis.[16]

Surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) is a novel approach to disease biomarker. In addition, SELDI-TOF MS allows a rapid protein expression profile from a variety of biological and clinical samples with minimal sample preparation. It has been used for discovering proteins with potential application as diagnostic markers for detection of prostate, bladder, breast and ovarian cancers as well as Alzheimer's disease.[17]

Thus, present study was designed to provide evidence that a pathological process in apoptosis

may participate in abnormal spermatogenesis in non-obstructive azoospermia and to understand the underlying molecular mechanisms. By means of *in situ* TUNEL staining, RT-PCR and immunohistochemistry, we have investigated apoptosis in testicular cells and the differential expressions of Fas, FasL, Bcl-2, Bax, and Caspase-3 in human testis. Profiling of differential protein expressions were also performed using SELDI-TOF MS analysis.

## MATERIALS AND METHODS

### 1. Testicular specimens

Testicular specimens were obtained by the open testicular biopsy from men with obstructive or non-obstructive azoospermia or oligozoospermia who visited the male infertility clinic in Samsung Cheil Hospital. Informed consent was obtained from all subjects. A piece of each testis was immediately snap-frozen in liquid nitrogen and stored at -70℃. Adjacent parts of tissue for histological observation were fixed with Bouin's solution before processing for paraffin embedding.

On the basis of standard qualitative interpretations of hematoxylin- and eosin-stained section, biopsies were classified as described below: complete Sertoli cell-only syndrome (SCO; n=6), all seminiferous tubules showed only Sertoli cells; maturation arrest (MA; n=6), all tubules showed arrested spermatogenesis; and normal (NOR; n=10), almost all tubules showed the developing of more than 10 elongating spermatids in each tubule cross-section.

### 2. *In situ* TUNEL labeling

Labeling of DNA fragmentation in each testis section was performed using an in situ apoptosis detection kit (Apoptaq; Oncor, Gaithersburg, MD). Paraffin sections were deparaffinized, hydrated, and treated with 20 μg/ml proteinase K for 15 min. DNA 3' end-labeling with digoxigenin-ddUTP (dig-ddUTP) was performed by incubation at 37℃ in a humidified chamber for 1 h 30 min. The reaction mixture, containing a terminal transferase reaction buffer, dig-ddUTP and terminal deoxynucleotidyl transferase (TdT) was used according to the supplier's guidelines. DNA strand breaks were revealed after incubation with an antidigoxigenin antibody conjugated to peroxidase at room temperature for 30 min and the subsequent detection of the enzyme activity using diaminobenzidine (DAB) as a substrate. A negative control was obtained by omitting TdT. The slides were then counterstained with Mayer's hematoxylin (Sigma Chemical Co., St Louis, MO) and mounted with Canada balsam (Sigma Chemical Co., St Louis, MO).

### 3. RT-PCR analysis

Total RNA from the testis tissue was isolated using TRIzol (Invitrogen; Carlsbad, Canada) according to the manufacturer's protocol. First-strand cDNA synthesis was carried out using 2 μg of total RNA in a total volume of 20 μl containing 20 U AMV reverse transcriptase (Roche, Penzberg, Germany) and the reaction mixture of 10 mM Tris (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1mM dNTP mix, 2.5 μM oligo-p$(dT)_{15}$-primer, 50 U RNase inhibitor. The reaction was allowed to proceed at 42℃ for 75 min followed by an increase to 95℃ for 5 min. The cDNAs obtained were used as a template for subsequent PCR amplification using 1 U of Taq DNA polymerase (Roche, Penzberg, Germany). Human-specific primers sequences for the genes of interest and their PCR conditions used in this study are summarized in Table 1. In these conditions, preliminary experiments demonstrated that the plateaus for the amplification were not reached. Finally, PCR products were analyzed by electrophoresis on 2% agarose gel pre-stained with ethidium bromide. The analysis of the respective bands was performed using an image analyzer

**Table 1.** List of primers used to analyze mRNA expressions by RT-PCR

| Target mRNA | Primer sequence | Annealing Tm (℃) | Product size (bp) |
|---|---|---|---|
| GAPDH | 5'-CCA CCC ATG GCA AAT TCC AT-3'<br>5'-AGT GGG GAC ACG GAA GGC CA-3' | 64 | 560 |
| Fas | 5'-CCA AGT GAC TGA CAT CAA CTC-3'<br>5'-CTC TTT GCA CTT GGT GTT GCT GG-3' | 58 | 426 |
| FasL | 5'-CTG GAA TGG GAA GAC ACC-3'<br>5'-AGA TTC CTC AAA ATT GAC CAG AGA GAG-3' | 58 | 338 |
| Bcl-2 | 5'-CAG CTG CAC CTG ACG CCC TT-3'<br>5'-GCC TCC GTT ATC CTG GAT CC-3' | 58 | 191 |
| Bax | 5'-CCA AGA AGC TGA GCG AGT GT-3'<br>5'-CAG CCC ATG ATG GTT CTG AT-3' | 56 | 250 |
| Bad | 5'-CGA GTG AGC AGG AAG ACT CC-3'<br>5'-CTG TGC TGC CCA GAG GTT-3' | 58 | 299 |
| Caspase-3 | 5'-ATA CTC CTT CCA TCA AAT AG-3'<br>5'-AAC ATC ACA AAA CCA TAA TC-3' | 55 | 410 |

(Viber Louvmat, Marne-la-Vallée, France). The intensity of each mRNA bands was normalized to the intensity of the corresponding GAPDH band. Statistical significance was determined using one-way analysis of variance (ANOVA) and paired student's *t* test was performed when the ANOVA indicated a significant defference. A $p<0.05$ was considered statistically significant.

### 4. Immunohistochemistry

Immunostaining was performed using commercial immunohistochemistry kit (Innogenex, San Ramon, CA) according to the manufacturer's protocol. Sections (4 μm thick) were placed on the Poly-lysine coated slides (Thermo Electron Corp., Cheshire, UK) and deparaffinized, then treated with 3% $H_2O_2$ in methanol for 10 min to suppress endogenous peroxidase activity. Antigen retrieval was performed by heating the sections in 10 mM citric acid solution (pH 6.0) using autoclave. The streptavidin-horseradish peroxidase method was used according to the manufacturer's instruction. Primary antibodies (Santa Cruz Biotechnology, Inc) for Fas (sc-714) and FasL (sc-834) were used. The optimal working dilution of each antibody was determined by incubating sections with various concentrations. Final coloring reaction was performed using liquid DAB. The slides were then counterstained with Mayer's hematoxylin and mounted with Canada balsam.

### 5. SELDI-TOF MS analysis

Tissue specimens (in numerical codes) weighing at least 0.1 mg were minced in Hanks' balanced salt solution (HBSS, Gibco BRL, Gaithersburg, MD). The tissue pieces were placed in RPMI1640 medium supplemented with 5% fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD) was added. The cell suspension was diluted with an equal volume of HBSS and centrifuged. The cell-free supernatant was stored frozen at -20°C. Supernatant (1 μl of 1/10 diluted in 5 mM HEPES, pH 7.4, and 0.01% Triton X100) was applied to reversed phase $H_4$ (hydrophobic) ProteinChip Arrays,

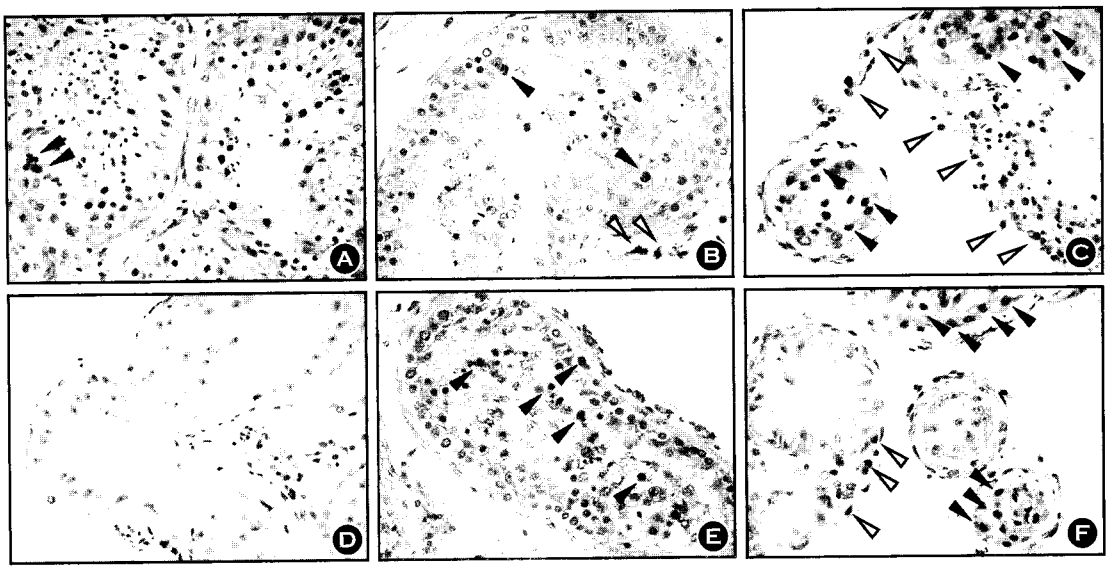

**Figure 1.** In situ detection of apoptotic cells in normal human testis with normal and abnormal spermatogenesis. **A,** Testis with normal spermatogenesis; **B** and **E,** Testis with maturation arrest; **C** and **F,** Testis with Sertoli cell-only syndrome; **D,** Negative control generated by substituting $dH_2O$ for the TdT during the labeling step. All arrowheads indicate apoptotic testicular cells. Open arrowheads in **B, C** and **F** indicate apoptotic interstitial cells. Magnification: ×200.

pre-wetted with acetonitrile (ACN). Samples were washed in a gradient of ACN in $dH_2O$ (5% or 50%, v/v) prior to adding the energy absorbing molecule, sinapinic acid (SPA). SPA was reconstituted in 500 μl with 50% ACN and 0.5% trifluoroacetic acid, and 0.5 μl was added in 2 applications. The arrays were then inserted into the ProteinChip Reader (Ciphergen Biosystems, Fremont, CA), a TOF mass spectrometer. Analyzed data were collected by an automated protocol and interpreted by ProteinChip Software, version 3.1b (Ciphergen Biosystems, Fremont, CA). We chose to focus on peptide species in the mass range of approximately 10,000 to 100,000 Da.

## RESULTS

### 1. Identification of apoptotic cells in testis

In case of normal spermatogenesis, apoptotic primary spermatocytes, round spermatids and Sertoli cells were occasionally observed in seminiferous tubules (Figure 1A). No apoptotic Leydig cells were observed in the normal testis. In case of MA, increased apoptosis was observed in primary spermatocytes and interstitial Leydig cells compared with the normal testis (Figure 1, B and E). Massive apoptotic Sertoli cells were observed in 4 out of 6 SCO cases (Figure 1, C and F) while rare apoptotic Sertoli cells were observed in the rest of 2 cases. In addition, apoptotic signals were also observed in the interstitial Leydig cells in a case of SCO which showed hypertrophy of Leydig cells (Figure 1C).

### 2. Transcriptional expressions of apoptosis regulators

The expression of FasL mRNA in the testis from patients with SCO was significantly increased compared to that of patients with normal spermatogenesis (Figure 2). In case of the expression of Fas mRNA, there was a huge variation at the different patients. Although no statistically significant changes in the average of Fas mRNA expression in the testes with SCO and MA were obser-

ved, some of the patients showed significantly high expressions of Fas mRNA. The expressions of Bcl-2, Bax, and Caspase-3 mRNA in the testes with SCO and MA were not significantly changed compared to those with normal spermatogenesis.

### 3. Localization of Fas and FasL protein expressions

Intense FasL staining was found in the basal compartment of the seminiferous tubules and in interstitial Leydig cells of testes with normal spermatogenesis (Figure 3A). The positive staining in the seminiferous tubules was confined to Sertoli cells. A diffuse immunostaining for FasL was detected in the Sertoli cells and Leydig cells of testes with SCO and MA and the staining intensities levels were shown to be increased compared to the testes with normal spermatogenesis (Figure 3,

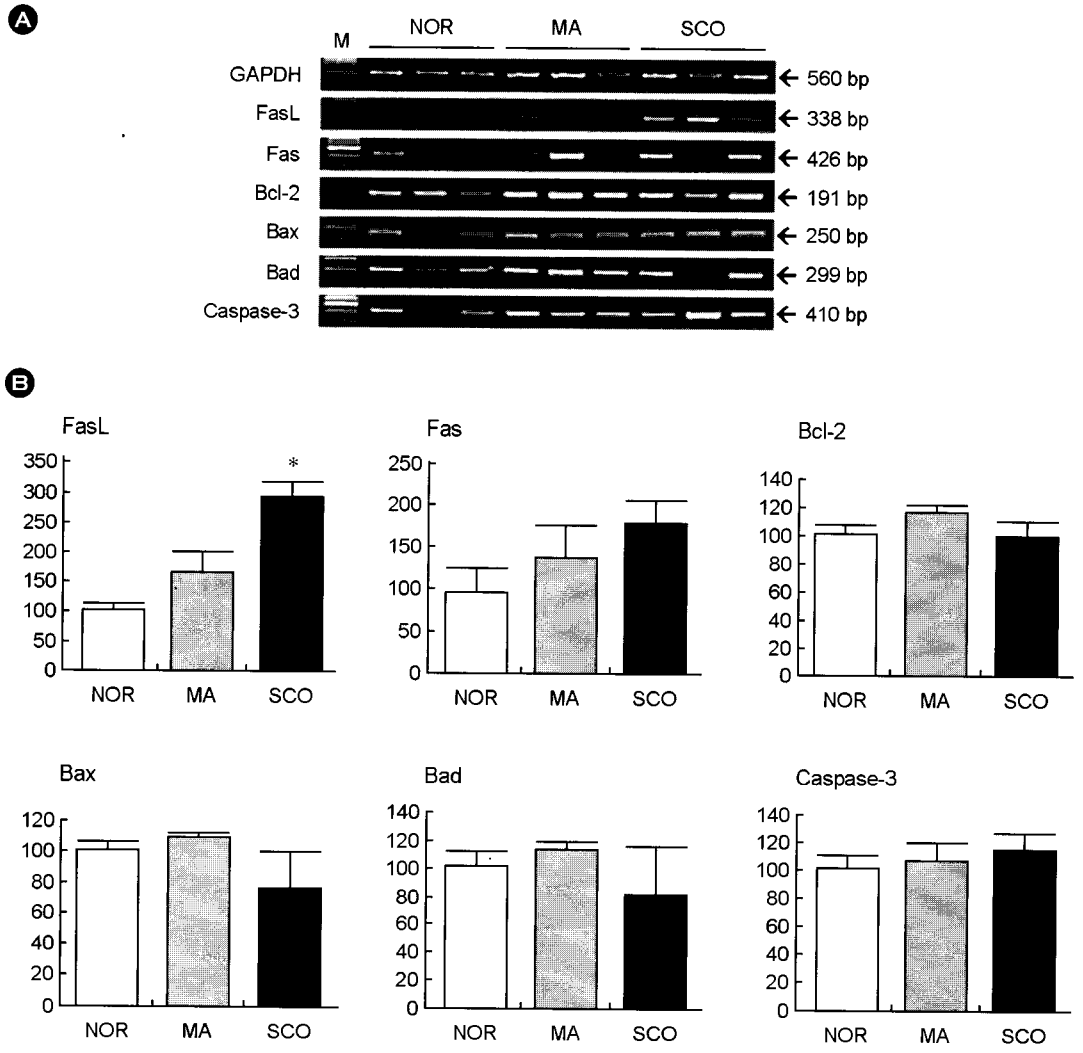


**Figure 2.** Transcriptional expressions of apoptosis related genes in human testis with normal and abnormal spermatogenesis. The mRNA expressions of FasL, Fas, Bcl-2, Bax, Bad, and Caspase-3 were evaluated by RT-PCR. **A,** Representative PCR products stained with ethidium bromide. **B,** Relative ratios of expressions of target genes to GAPDH, respectively. Abbreviation: M, 100 bp DNA Marker; NOR, normal spermatogenesis; MA, maturation arrest; SCO, Sertoli cell-only syndrome; bp, base pair. Significantly differences from the normal values are denoted as *, $p<0.05$

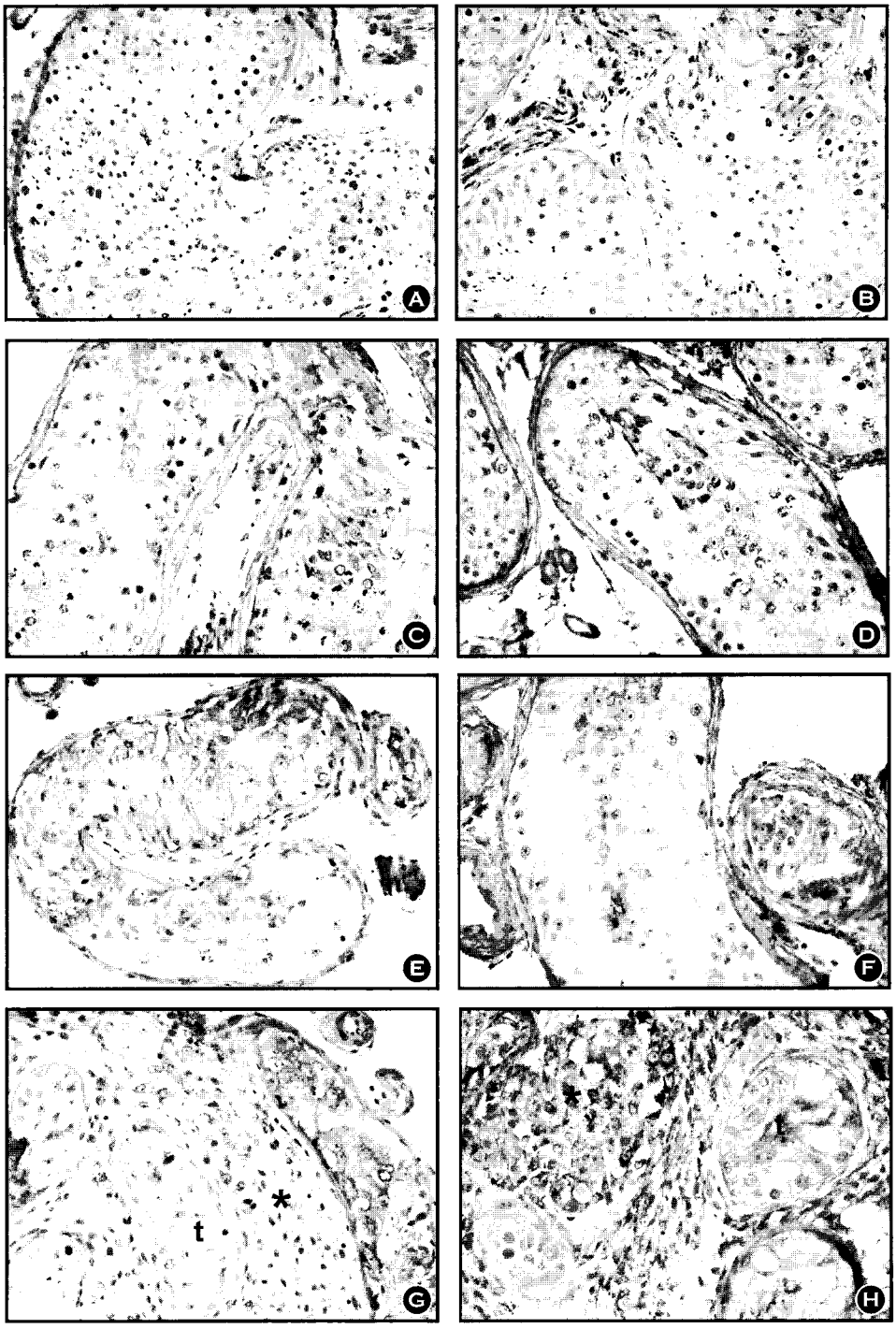


**Figure 3.** Immunohistochemical analyses of FasL and Fas expressions in human testis with normal and abnormal spermatogenesis. **A** and **B** show testis with normal spermatogenesis stained with FasL and Fas, respectively; **C** and **D** show testis with maturation arrest stained with FasL and Fas, respectively; **E** and **F** show testis with Sertoli cell-only syndrome of no hyperplastic Leydig cells; **G** and **H** show testis with Sertoli cell-only syndrome of hyperplastic Leydig cells (*) stained with FasL and Fas, respectively. t, seminiferous tubule. Magnification: ×200.

**Table 2.** The proteomic pattern analysis in profiling of differential protein expression of SCO, MA and normal cases at 16.7, 22.2, 23.1, 24.5, 25.4 and 27.9 kDa

| M.W./type | 16.7 kDa | 22.1 kDa | 23.6 kDa | 24.5 kDa | 25.4 kDa | 27.8 kDa |
|---|---|---|---|---|---|---|
| Normal | +++ | ++ | - | ++ | + | - |
| SCO | ++ | ++ | ++ | - | ++ | ++ |
| MA | + | - | ++ | + | +++ | - |

C, E and G).

In case of normal spermatogenesis, Fas expression was detected in occasional seminiferous tubules, mainly in cytoplasm of primary spermatocytes. Intense staining of Fas was also detected in interstitial Leydig cells (Figure 3B). In case of MA, intense immunostaining of Fas was observed in degenerating primary spermatocytes (Figure 3D). Especially, Fas expression was detected in Sertoli cell and hyperplastic interstitial cells in the testes of SCO (Figure 3, E-H).

### 4. Protein expression profiling by SELDI-TOF MS

The profiling of differential protein expression in SCO, MA and normal cases was examined by SELDI-TOF MS using $H_4$ ProteinChip array. At least 12 protein peaks were observed between 16.0 kDa and 36.0 kDa. A major peak was observed at 16.7 kDa. Other significant peaks were found at 22.2, 23.6, 24.5, 25.4 and 27.9 kDa. The intensity of 16.7 kDa peaks in SCO and MA was lower than normal. In normal and SCO, the peaks at 22.2 and 23.6 kDa were observed except in MA. However, the peak at 24.5 kDa was showed in SCO and MA but not in normal. The peaks at 25.4 kDa were observed in all the groups of normal, SCO and MA while the peak at 27.9 was only observed in SCO (Table 2).

Examples of SELDI-TOF spectra from five patients in the preliminary set [five normal (n=5) and three of SCO (n=3) and two of MA (n=2)] are shown in Figure 4. The optimum discriminatory pattern for testicular pathologies was defined by the amplitudes at the key *M/Z* values, 22.1, 23.6, 24.5, 25.4 and 27.9 kDa. Those peaks were clearly distinguishable from the background noise.

## DISCUSSION

In this study, we examined the incidence of apoptosis and the expressions of the apoptosis regulators in human testis with normal and abnormal spermatogenesis. Using an in situ end-labeling technique, this study demonstrated significantly increased apoptosis in histopathologic states of MA and SCO. In rodent model, increased apoptosis is directly associated with abnormal sperm production.[18,19] Recently, in human testis, apoptosis is also considered to play an important role in spermatogenesis,[20] and increased apoptosis occurred in MA and hypospermatogenesis in infertile men.[7~9] These previous studies and our study suggest that increased apoptosis contributes significantly to the impaired spermatogenesis in MA. Noteworthy, these previous studies could not observe any apoptotic signal in SCO, however, prominent apoptosis of Sertoli cells was observed in the present study. Recently, Oldereid et al.[16] reported occasional apoptotic Sertoli cells in normal human testis and the possibility that Sertoli cells themselves may undergo apoptosis. However, further studies are needed to evaluate the relationship between apoptosis of Sertolic cells and germ cell depletion in SCO.

Although control and mechanisms of male germ

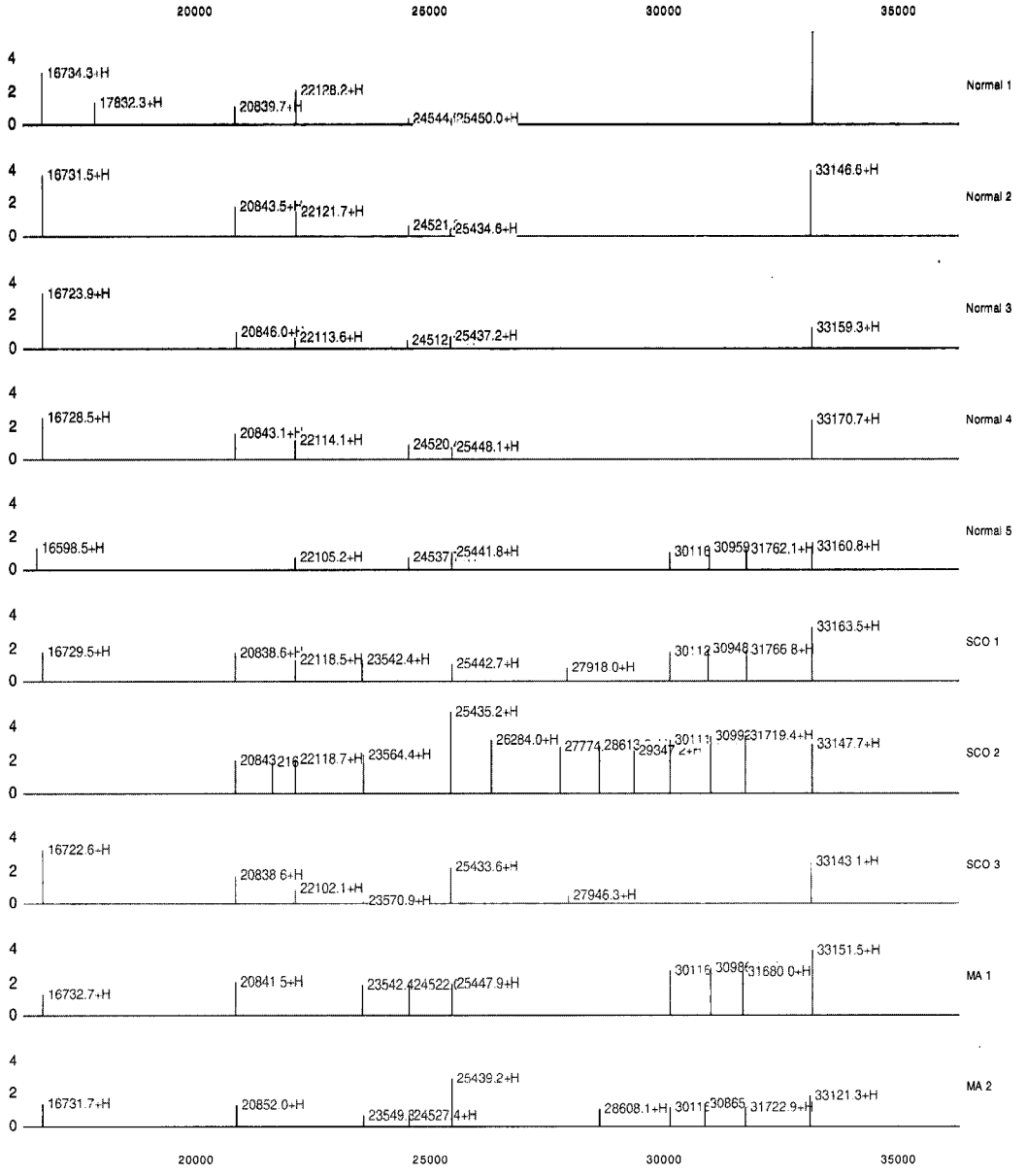


**Figure 4.** SELDI profiling performed by using hydrophobic chips ($H_4$) to compare and identify differences in the protein expression patterns between human testis with normal and abnormal spermatogenesis at the molecular weight from 1.6 to 3.6 kDa (i.e., using SPA as a matrix).

cell apoptosis has been well characterized in various animal models, few studies regarding the expressions of different apoptotic regulators in the human testis has been reported. Possible involvement of Bcl-2 family members in regulating spontaneous germ cell apoptosis in normal human testis have been suggested by preferential expression of Bax, Bcl-x, Bcl-2, Bad and Bak proteins in the various germ cell compartment.[16] Moreover, increased germ cell apoptosis and decreased anti-

apoptotic factor, bcl-xl in the human testis following anti-androgen treatment were reported.[21] In our study, we could not observe any changes on the transcriptional expressions of Bcl-2 family members in testis with MA and SCO compared to the normal testis, however, to clarify their possible involvement in abnormal apoptotic process in MA and SCO, their expressions at the protein level has to be evaluated in the future studies.

By means of RT-PCR and immunohistochemistry, expressions of Fas-FasL mRNA and protein were evaluated in normal testis and testicular pathologies. In normal testes, Fas immunoreactivity was observed in interstitial Leydig cells and in the cytoplasm of isolated germ cells while FasL immunoreactivity was observed in Sertoli cells and Leydig cells.[14] Significant increased mRNA expression of FasL was observed in SCO and increased immunoactivities of FasL were prominent in both MA and SCO. The tight correlation observed between the increased number of apoptotic cells and the intensities of FasL expressions in testis with MA and SCO suggests that up-regulation of FasL expression might be associated with abnormal apoptosis in the testicular failure. Possible involvement of Fas-FasL system in the spermatogenic cell apoptosis in human testis with MA has been reported by others.[14,22,23] To our knowledge, no report has been published with regard to increased apoptosis and coexpressions of Fas and FasL in Sertoli cells of SCO. Further studies with larger samples may help to define a role for abnormal apoptosis in SCO.

Although coexpression of Fas and FasL in Leydig cells was observed in all the testicular specimens, no apoptotic Leydig cells were obsèrved in normal testis, confirming the observation of Francavilla et al.[14] However, degenerating Leydig cells were occasionally observed in MA and especially, massive apoptotic Leydig cells were observed in a case of SCO which showed hypertrophy of Leydig cells. In endometrial glandular cells, the coexpression of both molecules does not trigger apoptosis that is indeed inhibited by the antiapoptotic factor Bcl-2.[24] It is possible that under normal conditions apoptosis of Leydig cells is inhibited by an antiapoptotic factor downstream from the Fas-FasL system. Therefore, it may be worthy to further evaluate whether those antiapoptotic factors were normally regulated in testis with MA and SCO or not.

In addition, SELDI analysis was performed to compare and identify differences in the protein expression patterns between testis with normal and abnormal spermatogenesis at the molecular weight from 10 to 100 kDa. As the intensity of a major peak observed at 16.7 kDa was lower in case of SCO and MA, it could be used as a biomarker. Several other peaks at 22.1, 23.6, 24.5, 25.4 and 27.9 kDa could be also used as a biomarker subset. When the SELDI profiling of the human testis with normal and abnormal spermatogenesis is performed, if the peaks at 22.1, 23.1 and 25.4 kDa exist, it might be a normal. If sample has the peaks at 22.1 23.6 25.4 and 27.9 kDa, it might be SCO. And if sample has the peaks at 23.6, 24.5 and 25.4 kDa, it might be MA cases. If peaks at 16.7, 22.1, 23.6, 24.5, 25.4 and 27.9 kDa as total biomarker sets are used, the screening will be clearer.

In conclusion, present study showed that the frequency of degenerating cells was correlated with that of FasL-expressing Sertoli cells and Fas-expressing germ cells in human testis with normal spermatogenesis, MA and SCO. Especially, upregulation of FasL gene and protein expression in Sertoli cells triggering apoptotic elimination of Fas-expressing germ cells might be associated with altered germ cell maturation in MA and possibly in germ cell depletion in SCO. Present study also showed that application of the SELDI-TOF MS technique to evaluate the differential protein expressions would be useful for disease identification

and lead us to a better understanding of abnormal spermatogenesis states in human male infertility.

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