

mice on day 16 after the virus inoculation, if one copy of proviral DNA was postulated to reside in one CD4⁺ cell.

One of us (Y.K.) previously attempted to investigate the mechanism of CD4⁺ cell depletion seen in individuals with HIV-1 infection by employing a PBMC-transplanted NOD (NOD/Shi) *scid/scid* mouse system (24). Massive apoptosis was observed in HIV-1-uninfected CD4⁺ cells in the spleens of the HIV-1-infected NOD-*scid/scid* mice. A combination of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling and immunostaining for death-inducing tumor necrosis factor (TNF) family molecules showed that apoptotic cells were frequently found in conjugation with TNF-related apoptosis-inducing ligand (TRAIL)-expressing CD3⁺ CD4⁺ human T cells. Further observation that a neutralizing anti-TRAIL antibody inhibited the development of CD4⁺ cell apoptosis suggested that a large number of HIV-1-uninfected CD4⁺ cells undergo TRAIL-mediated apoptosis, contributing to the marked depletion of CD4⁺ cells (24). The observation by Miura and his colleagues that the number of TRAIL-positive cells was consistently higher in HIV-1-infected mice than in uninfected ones makes it apparent that TRAIL expression is induced upon HIV-1 infection (23, 24). In this regard, the present observation that AK602 and ddI potently blocked the decrease in CD4⁺ cells in spite of the rather increasing HIV-1 viremia in the face of AK602 or ddI (Fig. 7) suggests that the mere presence of viremia might not be sufficient for the HIV-induced apoptosis in CD4⁺ cells. Our observation that most surviving CD4⁺ cells in mice receiving AK602 or ddI were free of HIV-1 (see above) suggests that these anti-HIV-1 agents might block not only de novo HIV-1 infection, but also bystander killing of uninfected CD4⁺ cells. The present data also suggest that a certain factor(s) such as cytokines produced by the freshly HIV-1-infected cells might mediate the apoptosis of bystander CD4⁺ cells through the upregulation of TRAIL expression, death receptors (e.g., DR4 and DR5), and/or downregulation of decoy receptors (e.g., DcR1 and DcR2) (26, 27). However, experiments with a combination of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling and TNF family molecules have to be conducted for better understanding of the bystander killing in regard to AK602's effects.

It is of note that several CCR5 antagonists are currently in various stages of development. AK602 has recently been administered to healthy adult subjects in a phase I clinical trial and shown to bind to CCR5 for an extended period of time, suggesting that an oral formulation with fewer administrations and lower dosage is possible for AK602 as a therapeutic agent for HIV-1 infection (J. Demarest, K. Adkison, S. Sparks, A. Shachoy-Clark, K. Schell, S. Reddy, L. Fang, K. O'Mara, S. Shibayama, and S. Piscitelli, 11th Conf. Retrovir. Opp. Infect., abstr. 139, 2004). Taken together, our observations that plasma viral load reached ~10⁶ RNA copies/ml and that AK602 potently inhibited the replication of R5 HIV-1 strongly suggest that the present hu-PBMC-NOG mouse AIDS model could serve as a useful instrument for analyzing the pathogenesis of HIV-1 infection and testing the efficacy of antiviral agents.

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Alternatively spliced transcripts of Fas mRNAs in feline lymphoid cells

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Summary

Fas belongs to the tumour necrosis factor receptor family and transduces the death signal after binding to the Fas ligand. Five feline lymphoma cell lines were shown, by reverse transcription–polymerase chain reaction, to express six species of Fas transcripts. Based on sequence comparison of these Fas transcripts with the genomic Fas gene, five of the six transcripts were found to be generated through alternative splicing and to encode five different Fas proteins lacking the transmembrane domain. We also detected such alternatively spliced transcripts in primary tumour tissues from cats with naturally occurring lymphoma. These results suggest a possible association of the alternatively spliced Fas variants with the pathogenesis of feline lymphoma.

Introduction

Fas is a cell-surface receptor of type I membrane proteins belonging to the nerve growth factor (NGF) receptor/tumour necrosis factor (TNF) receptor superfamily (Itoh *et al.*, 1991). Fas induces apoptosis after binding to the Fas ligand or anti-Fas agonistic immunoglobulin. Fas consists of three domains: the extracellular domain; the transmembrane domain; and the cytoplasmic domain. The cytoplasmic domain contains a death domain essential for transduction of the death signal.

Human Fas is expressed in a wide variety of tissues and cells, such as the normal thymus, liver, heart and kidney, as well as malignant tumour cells (Nagata 1997). Authentic human Fas is a membrane-anchored protein, whereas its soluble form, lacking the transmembrane domain, blocks apoptosis induced by anti-Fas immunoglobulin (Cheng *et al.*, 1994). Cascino *et al.* (1995) reported two other Fas mRNA species in activated human peripheral blood mononuclear cells (PBMCs) and human tumour cell lines, in addition to the soluble form. These Fas mRNA variants were shown to have frameshifts that generate

premature termination codons and encode soluble proteins blocking the anti-Fas antibody-induced cell death.

It was recently reported that the amount of soluble Fas was increased in the sera of humans with various diseases, such as autoimmune diseases, including systemic lupus erythematosus (SLE) (Cheng *et al.*, 1994) and rheumatoid arthritis (RA) (Hasunuma *et al.*, 1997), and malignancies, including lymphoma (Yufu *et al.*, 1998; Niitsu *et al.*, 1999; Hara *et al.*, 2000), adult T-cell leukaemia (Sugahara *et al.*, 1997; Kamihira *et al.*, 1999), acute myeloid leukaemia (Inaba *et al.*, 1999), breast cancer (Kimura *et al.*, 1999; Ueno *et al.*, 1999), bladder cancer (Mizutani *et al.*, 1998) and gastric cancer (Lee *et al.*, 1998). In these disorders, the soluble Fas is considered to block the binding of Fas ligand to Fas, which may be one of the mechanisms used for the escape from cytotoxic T lymphocytes.

Previously, we cloned feline Fas cDNA (Mizuno *et al.*, 1998), but there has been no report on the variant transcript of the feline Fas gene. In the present study, we detected five species of Fas mRNAs, of different sizes, in feline lymphoid cells and characterized them in comparison to the genomic sequence of the feline Fas gene.

Materials and methods

Cell cultures

Five feline lymphoid tumour cell lines — FT-1 (Miura *et al.*, 1987; 1989), FL-74 (Theilen *et al.*, 1969), F422 (Rickard *et al.*, 1969), 3201 (Snyder *et al.*, 1978) and MCC (Cheney *et al.*, 1990) — were cultured (at 37 °C in a humidified atmosphere of 5% CO₂ in air) in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics. FT-1, FL74 and F422 cell lines were derived from lymphomas in cats infected with feline leukaemia virus (FeLV), whereas 3201 and MCC cell lines were negative for FeLV.

PBMCs were separated by the Ficoll–Hypaque density-gradient centrifugation of peripheral blood samples from two specific pathogen-free cats. The cells were resuspended in RPMI-1640 supplemented with 10% FCS, antibiotics, 100 U mL⁻¹ recombinant human interleukin-2 (IL-2) and 10 µM 2-mercaptoethanol. The resultant PBMCs were cultured in the presence of concanavalin A (Con A) (5 µg mL⁻¹) (Sigma Chemical Co., St Louis, MO) in a six-well plate at 37 °C for 3 d. The cells were then pelleted by centrifugation, frozen in liquid nitrogen and stored at –80 °C for subsequent RNA isolation.

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Primer name	Sequence	Nucleotide position	PCR product
6S	5'-GGC GGG GCG CTC CGC AGC C-3'	-19-1	360 bp
6R	5'-TTC TAA GCC ATG CTT TCA T-3'	322-341	
7S	5'-GAA GAA GCG AAG GAC TAC ACA GAC-3'	255-278	442 bp
7R	5'-GTT CCG CAA TGC TAC TGA TG-3'	677-696	
8S	5'-GAA TCT ACA GTC TCA GTT AC-3'	619-631	354 bp
8R	5'-GCA GTT TCC ATT CTC AAG-3'	945-972	

Table 1. Primer pairs used for reverse transcription-polymerase chain reaction (RT-PCR) of feline Fas

Primary tumour tissues

Specimens of tumour tissues were obtained from 11 cats with lymphoma referred to the Veterinary Medical Center of the University of Tokyo for diagnosis and treatment. The tumour tissues obtained at biopsy or necropsy were rapidly frozen in liquid nitrogen and stored at -80°C for subsequent RNA extraction.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells and tissues by acid guanidium-phenol-chloroform with RNazol (Bio-tecx, Houston, TX). After removal of contaminating DNA by treatment with DNase I (Life Technologies, Rockville, MD), cDNA was generated from 0.5 μg of total RNA using an RNA PCR core kit (Perkin Elmer Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Oligo dT primer was used to prime the first-strand cDNA synthesis for each of the reactions. Three primer pairs (6S/6R, 7S/7R and 8S/8R) were synthesized, based on the sequence of feline Fas cDNA (Mizuno *et al.*, 1998), to amplify the full-length coding region of feline Fas (Table 1). The PCR reaction consisted

of 40 cycles of denaturation at 94°C for 1 min and annealing/polymerization at 60°C for 1 min, followed by a final extension at 72°C for 7 min. These amplified products were electrophoretically separated through a 12.5% acrylamide gel and visualized by silver staining using a GenePhor electrophoresis unit (Amersham Pharmacia Biotech, Bucks., UK).

Nucleotide sequence determination of the PCR products

The PCR products were gel purified and subcloned into the pGEM-T Easy vector (Promega, Madison, WI). At least four clones from each of the differently sized PCR bands were sequenced by the dideoxy chain-termination reaction using a 377 Genetic Analyser (Perkin Elmer Applied Biosystems).

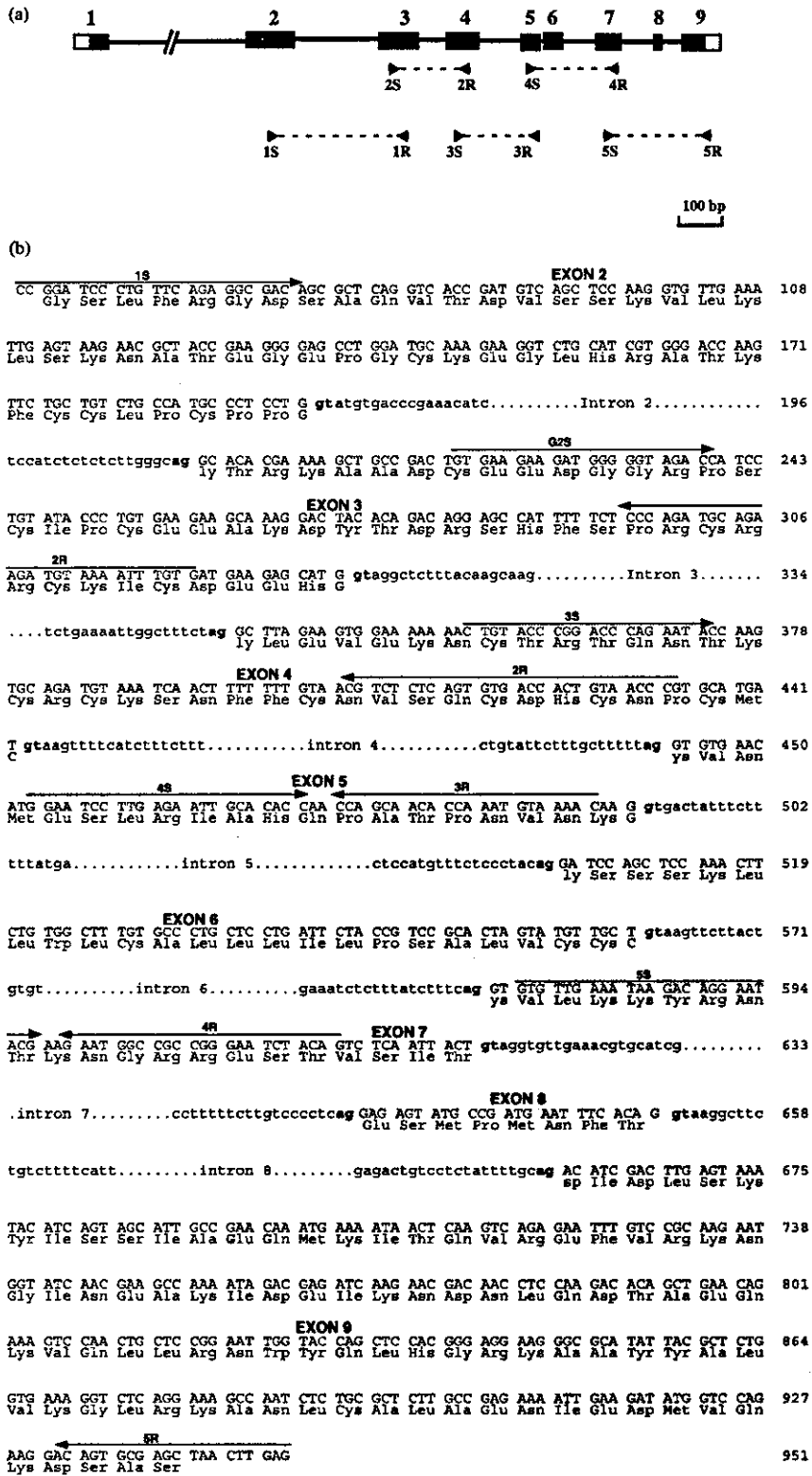
Cloning and sequencing of genomic DNA

Genomic DNA (500 ng), extracted from normal cat spleen, was amplified using an LA-PCR kit (Takara, Kyoto, Japan). Primer pairs used for PCR cloning of the feline genomic Fas gene were 1S/1R, 2S/2R, 3S/3R, 4S/4R and 5S/5R (Table 2, Fig. 1a & b). Sequences of the primers were based on the previously reported sequence of feline Fas

Table 2. Primer pairs used for genomic polymerase chain reaction (PCR) of feline Fas

Primer name	Sequence	Nucleotide position	Corresponding exon
1S	5'-CCG GAT CCC TGT TCA GAG GCG ACA-3'	47-70	2
1R	5'-CAC AAA TTT TAC ATC TTC TGC ATC TGG G-3'	295-322	3
2S	5'-GTG AAG AAG ATG GGG GTA GAC C-3'	218-239	3
2R	5'-CGG GTT ACA GTG GTC ACA CTG AGA GAC G-3'	408-435	4
3S	5'-CTG TAC CCG GAC CCA GAA TAC-3'	354-374	4
3R	5'-GTT TAC ATT TGG TGT TGC TGG-3'	478-499	5
4S	5'-GGA ATC CTT GAG AAT TGC ACA CC-3'	454-476	5
4R	5'-CTG TAG ATT CCC GGC GGC CAT TC-3'	600-622	7
5S	5'-GTG TTG AAA AAG TAC GGG AAT ACG-3'	572-592	7
5R	5'-CTC AAG TTA GCT CGC ACT GT-3'	967-986	9

Figure 1. Structure of the feline genomic Fas gene. (a) Exon/intron organization of the feline genomic Fas gene. Exons are represented by boxes and putatively numbered from the sequence alignment of the feline Fas gene and with the homologues of other species. Introns are represented by horizontal lines. Coding sequences in the exons are shown as filled boxes. Locations of the primers for cloning of the feline genomic Fas gene are indicated by arrowheads. (b) Nucleotide and predicted amino acid sequences of the feline genomic Fas gene. Nucleotides at the intron/exon boundaries are shown in bold type. Nucleotide numbering was based on the sequence of feline Fas cDNA previously reported (Mizuno *et al.*, 1998). Amino acids are numbered from the first methionine. Primers used for genomic polymerase chain reaction (PCR) are indicated by arrows.



cDNA (Mizuno *et al.*, 1998). The PCR products were cloned into the pGEM-T Easy vector and sequenced using a 377 Genetic Analyser (Perkin Elmer Applied Biosystems).

Results

Genomic organization of the feline Fas gene

Based on the sequence of feline Fas cDNA, to identify the genomic organization of the feline Fas gene a feline genomic DNA sample was subjected to PCR to amplify the introns and determine the exon/intron boundaries. A fragment amplified using primers 1S and 1R contained a region of the Fas gene corresponding to exons 2–3. Similarly, four more fragments corresponding to exons 3–4 (2S/2R), exons 4–5 (3S/3R), exons 5–7 (4S/4R), and exons 7–9 (5S/5R) were cloned. The exons of the feline Fas gene were putatively numbered from the exon numbers of Fas genes of other species previously reported (Behrmann *et al.*, 1994; Cheng *et al.*, 1995; Yoo *et al.*, 1996). Sequences of the amplified DNA fragments were determined and compared with that of feline Fas cDNA reported previously (Mizuno *et al.*, 1998). All of the exon/intron boundaries in the cloned genomic Fas gene were consistent with the established GT/AG rule for splicing (Breathnach *et al.*, 1978) (Fig. 1a & b). The genomic organization of the exons and introns of the feline Fas gene was apparently the same as those of Fas genes of other species, including humans (Behrmann *et al.*, 1994; Cheng *et al.*, 1995) and cattle (Yoo *et al.*, 1996), reported previously.

Identification and characterization of Fas mRNA variants in feline lymphoid tumour cell lines

To characterize feline Fas mRNA, RNA samples from five feline lymphoma cell lines were analysed by RT-PCR. We carried out a series of RT-PCR amplifications by using specific primers to amplify three overlapping fragments of feline Fas mRNA (Table 1 and Fig. 3). By using two sets of primers (6S/6R and 8S/8R), PCR products of 360 bp and 354 bp, respectively, were amplified. The sizes of these fragments were the same as those expected from the sequence of authentic feline Fas cDNA reported previously (Mizuno *et al.*, 1998). RT-PCR, using another primer pair (7S/7R) encompassing exons 3–7, generated four or five bands of different sizes in all of the five feline lymphoma cell lines used in this study (Fig. 2a). In addition to a fragment of 442 bp corresponding to the authentic Fas mRNA (band A), five other fragments, of smaller sizes, were observed (bands B, C, D, E and F) (Fig. 2a). Band A, derived from the authentic Fas transcript, was the most intense. Bands B and D were distinct in all five cell lines but were less intense than band A. Band C was very faint in all cell lines. Bands E and F were slightly less intense than bands B and D, and band F was not detected in one (MCC) of the five cell lines.

Nucleotide sequences of the five bands B, C, D, E and F were compared with that of a band derived from the

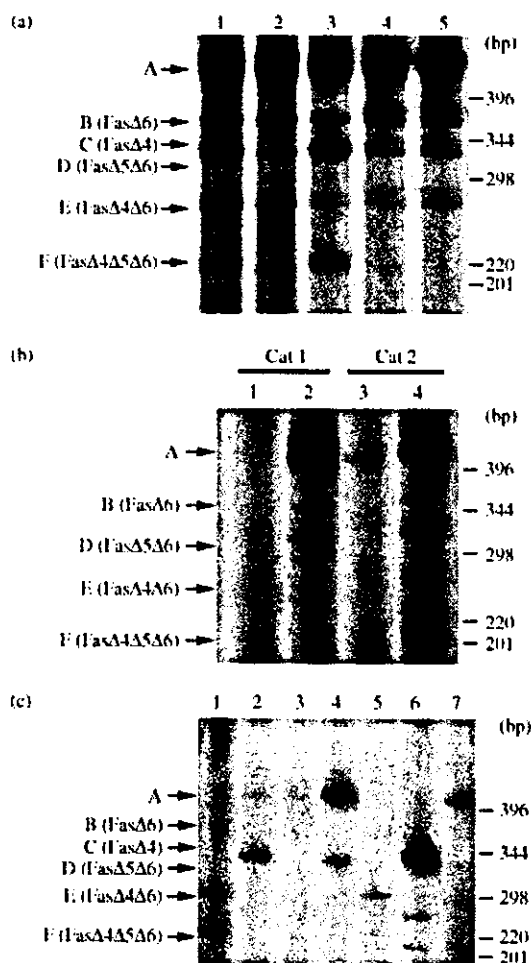


Figure 2. Reverse transcription–polymerase chain reaction (RT-PCR) analysis for Fas transcripts in feline lymphoid cell lines (a), concanavalin A (Con A)-activated feline peripheral blood mononuclear cells (PBMCs) (b), and primary tumour tissues from cats with lymphoma (c). Total RNA was extracted from the feline lymphoma cell lines, FT-1 (lane 1), FL-74 (lane 2), F422 (lane 3), 3201 (lane 4) and MCC (lane 5) (a), from unstimulated PBMCs (lanes 1 and 3) and Con A-stimulated PBMC (lanes 2 and 4) (b), and from primary tumour tissues (lane numbers indicate cat numbers) (c), and were subjected to RT-PCR analysis. The RNA samples were amplified by RT-PCR with primers 7S/7R. The PCR products were separated through a 12.5% polyacrylamide gel.

authentic feline Fas mRNA (band A) (Fig. 3a & b). Band B was shown to be derived from an mRNA species with a deletion of 69 bp corresponding to exon 6 (FasΔ6). The deletion found in the FasΔ6 mRNA did not generate a frameshift, and the FasΔ6 was shown to encode a smaller protein lacking its transmembrane domain. Nucleotide sequencing of band C revealed a deletion of 110 bp, corresponding to exon 4 (FasΔ4), a frameshift from the beginning of exon 5 and the generation of a premature termination codon at nucleotide position 544 in exon 6. Band D was shown to lack a fragment of 127 bp, which

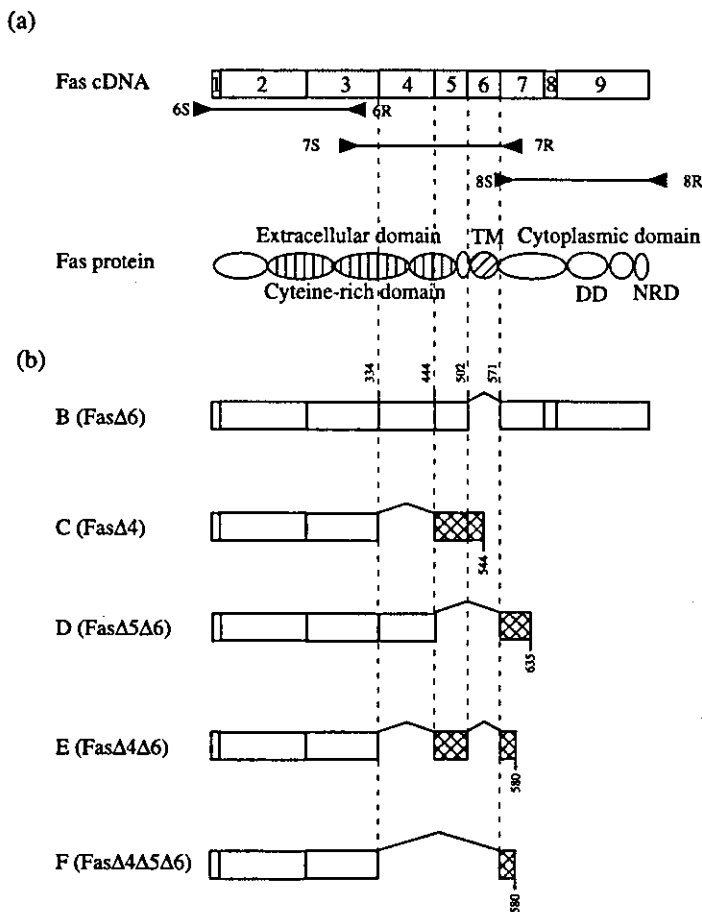


Figure 3. Schematic representation of normal and variant feline Fas mRNAs. (a) Nine exons encoding feline Fas are shown. TM, DD and NRD indicate the transmembrane domain, death domain and negative regulatory domain, respectively. (b) Structures of variant Fas mRNAs. Regions lacking in Fas mRNA variants are indicated by broken lines. The coding regions are shown in boxes. Cross-hatched boxes indicate exons with frameshifts.

encompassed exons 5 and 6 (FasΔ5Δ6) and to generate a frameshift and a premature stop codon at nucleotide position 577 in exon 7. Lack of exons 4 and 6 was found in the band E sequence (FasΔ4Δ6), generating a frameshift of exons 5 and 7 and a termination codon at nucleotide position 577 in exon 7. In the sequence of the smallest band F, exons 4, 5 and 6 were shown to be deleted (FasΔ4Δ5Δ6), generating a frameshift of exon 7 and a premature stop codon at nucleotide position 634. All of the sequences from the splicing variants were submitted to GenBank (GenBank accession nos: AB072009, AB072010, AB072011, AB072012, AB072013 and AB072014).

Detection of Fas mRNA variants in Con A-activated normal PBMC

RT-PCR analysis indicated that expression of Fas mRNA was increased in normal feline PBMCs after stimulation with Con A. To establish the presence of the alternatively spliced transcripts of Fas mRNA in the activated PBMCs, the Con A-activated PBMCs from two normal cats were subjected to RT-PCR analysis for Fas mRNA. RT-PCR analysis of Con A-activated feline PBMCs with two

primers pairs (6S/6R and 8S/8R) gave bands of 360 bp and 354 bp, respectively, which coincided with the size of bands derived from normal feline Fas mRNA. Nevertheless, RT-PCR with a different primer pair, 7S/7R, generated three to four bands in addition to the most intense band corresponding to the authentic Fas transcript (Fig. 2b).

Nucleotide sequencing of these DNA fragments showed that the four fragments detected in the PBMCs from cat 1 corresponded to FasΔ6, FasΔ5Δ6, FasΔ4Δ6 and FasΔ4Δ5Δ6 transcripts observed in the lymphoid tumour cell lines. Three bands detected in the PBMC sample from cat 2 were shown to correspond to FasΔ6, FasΔ5Δ6 and FasΔ4Δ6 transcripts. These results indicate that some splicing variants of feline Fas mRNA are also present in Con A-activated normal feline PBMCs.

Detection of Fas mRNA variants in primary tumour tissues from cats with spontaneous lymphoma

To investigate the significance of the alternatively spliced variants of Fas mRNA in feline lymphoma, we examined the presence of the splicing variants in primary tumour tissues from cats with naturally occurring lymphoma. We

used primary tumour tissues from 11 cats with lymphoma of different forms, such as thymic, multicentric and alimentary forms. Fas mRNA was detected in seven of the 11 lymphoma samples by RT-PCR, whereas β -actin, as an internal control, was amplified to a similar level in all the samples. In the seven samples containing Fas mRNA, RT-PCR with two primer pairs (6S/6R and 8S/8R) generated bands derived from the authentic Fas gene transcript, but RT-PCR with 7S/7R primers gave various splicing variants (Fig. 2c).

All of the bands detected by RT-PCR were sequenced and their sequences were compared with those of authentic and alternatively spliced feline Fas mRNA-derived cDNAs. In cases 4 and 7, a band derived from the authentic Fas mRNA (band A) was the most intense as compared with other bands, but in other cases, it was faint (cases 2 and 3) or absent (cases 1, 5 and 6). In case 7, in addition to the band of authentic transcript (band A), RT-PCR generated all of the five spliced variants of Fas Δ 6 (band B), Fas Δ 4 (band C), Fas Δ 5 Δ 6 (band D), Fas Δ 4 Δ 6 (band E) and Fas Δ 4 Δ 5 Δ 6 (band F) transcripts.

Discussion

The presence of several transcript variants of the human Fas gene has been reported previously (Cascino *et al.*, 1995; Liu *et al.*, 1995; Schumann *et al.*, 1997). In the cell lines from lymphoma, leukaemia and other tumours in humans, five Fas mRNA variants, which lacked exon 6, exon 4, exons 4 and 6, exons 3 and 4, and exons 3, 4 and 6, were reported (Cascino *et al.*, 1995; Papoff *et al.*, 1996). In addition to these variants, another isoform (lacking exons 4 and 7) was detected in human ventricular myocardium (Schumann *et al.*, 1997). These Fas mRNA variants were shown to be generated by alternative splicing and to lack the transmembrane domain because of the absence of exon 6 or its frameshift. In another report, a Fas mRNA variant, lacking exon 8, which contains the death domain, was found in human lymphoma cell lines resistant to apoptosis (Cascino *et al.*, 1996). In this study, we detected five splicing variants of feline Fas mRNA lacking exon 4, exon 6, exons 5 and 6, exons 4 and 6, and exons 4, 5, and 6 in feline lymphoid tumour cell lines. Of these alternatively spliced variants of Fas mRNA, those lacking exons 5 and 6, and exons 4, 5, and 6, shown in this study, have not been detected previously. As all of these splicing variants of Fas mRNA identified in this study were shown to encode Fas proteins lacking a transmembrane domain, they might be translated to soluble proteins, as reported in human and murine Fas conceivably associated with the pathogenesis of various diseases such as tumours and autoimmune diseases. Unfortunately, the presence of the soluble Fas protein could not be identified in the cat system because monoclonal antibodies directed to human and murine Fas did not cross-react with feline Fas and anti-feline Fas immunoglobulin was not available.

A mechanism for the alternative splicing, mentioned above, is called exon skipping. Meanwhile, in mice,

abnormal splicing of the Fas gene, owing to insertions of some unrelated exons, has also been reported (Adachi *et al.*, 1993; Kobayashi *et al.*, 1993; Chu *et al.*, 1993; Wu *et al.*, 1993). It has been shown that *lpr* mice cannot express normal functional membrane-bound Fas, and this results in generalized lymphadenopathy and autoimmune disease (Kobayashi *et al.*, 1993). *lpr* mice were shown to have an insertion of the *Etn* transposon between exon 2 and exon 3 of the Fas gene, producing a truncated, deficient form of Fas. Of the mRNA variants of the feline Fas gene detected in this study, there was no variant mRNA as a result of such insertion of unrelated exons.

In human PBMCs, the expression of Fas and its splicing variants was detected, even in the absence of mitogen stimulation (Cheng *et al.*, 1994). Stimulation of human PBMCs with phytohaemagglutinin was shown to increase the amount of authentic Fas mRNA and decrease that of its splicing variants (Liu *et al.*, 1995). In contrast, we were unable to detect Fas mRNA in unstimulated feline PBMCs, but observed the distinct expression of Fas mRNAs (including its authentic transcript and splicing variants in PBMCs) after Con A stimulation. A band derived from the authentic transcript was more intense than other bands derived from splicing variants in Con A-activated feline PBMCs. Therefore, in feline PBMCs, unlike human PBMCs, Con A stimulation was considered to enhance the expression of authentic and variant Fas mRNAs.

In this study, we detected alternatively spliced variants of Fas mRNA in primary tumour cells from naturally occurring feline lymphomas, as well as in feline lymphoma cell lines. A splicing variant of Fas mRNA lacking exon 6 has been often detected in various malignancies, such as leukaemias (Sugahara *et al.*, 1997; Kamihira *et al.*, 1999; Inaba *et al.*, 1999) and carcinomas (Mizutani *et al.*, 1998; Kimura *et al.*, 1999; Ueno *et al.*, 1999; Nonomura *et al.*, 2000), in humans, but no other splicing variant of the Fas gene has been identified in primary neoplastic tissues and cells in humans. In the present study, various splicing variants of Fas mRNA lacking exon 4, exon 6, exons 5 and 6, exons 4 and 6, and exons 4, 5 and 6 were observed, even in the absence of authentic Fas transcript in feline primary lymphoma cells. Several reports have indicated that the amount of soluble Fas in serum is correlated with the clinical parameters and the degree of malignancy in adult T-cell leukaemia patients (Sugahara *et al.*, 1997; Kamihira *et al.*, 1999); however, we were unable to establish a relationship between the Fas mRNA pattern of the splicing variants and the clinical prognosis of lymphoma. An *in situ* RT-PCR study revealed the presence of normal Fas and soluble Fas in samples from gastric adenocarcinoma and its metastatic lymph node (Lee *et al.*, 1998). In these reports, on human malignancies, the amounts of variant and normal Fas transcripts were shown to be proportional to those of membrane-bound and soluble Fas proteins, respectively. Although, in this study, we were unable to measure the amount of soluble Fas protein in cats with lymphoma, Fas proteins lacking the transmembrane domain derived from

the alternatively spliced mRNAs may exist as soluble forms in the sera of these cats.

A variety of alternatively spliced variants of Fas mRNA were detected in feline lymphoid cells in this study. The alternatively spliced variants identified in this study, and the putative resultant soluble forms of Fas, may be associated with the pathogenesis of tumours, autoimmune diseases, and several other diseases in cats. Further studies are required to establish an assay system to measure the level of soluble Fas protein in cats and to investigate the role of soluble Fas in pathogenesis.

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Expression of apoptosis-related gene mRNAs in feline T-cells infected with feline immunodeficiency virus (FIV)

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Abstract

In the present study, full length of feline *bax*, *bcl-2*, *bcl-xL* and *caspase 3* genes were sequenced and the expression of these mRNAs were also investigated in FIV-infected lymphocytes.

The full length cDNA sequence of *bax* (646 bp), *bcl-2* (1423 bp), *bcl-xL* (1163 bp) and *caspase 3* genes (1208 bp) contained a single open reading frame of 579 bp coding 193 amino acids, 708 bp coding 236 amino acids, 702 bp coding 234 amino acids and 834 bp coding 278 amino acids, respectively.

Number of apoptotic Kumi-1 cells gradually increased after FIV infection and approximately 70% were apoptotic and 30% were viable in the cells infected with FIV after 8-day incubation, though approximately 80% were non-apoptotic and 20% were dead in non-infected cells.

The expression of *bcl-2* mRNA in lymphocytes of established cell line was increased by FIV. The amounts of mRNAs of *bax*, *caspase 3* and *bcl-xL* in FIV-infected cells were not different from those in uninfected control cells.

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Keywords: *bcl-2*; *bax*; *bcl-xL*; *Caspase 3*; Feline immunodeficiency virus

1. Introduction

Feline immunodeficiency virus (FIV) is a lentivirus and associated with slow progressive disease in domestic cats. FIV replicates in T-lymphocytes (CD4⁺ cells and CD8⁺ cells), B-lymphocytes, macrophages, and astrocytes (Sellon, 1998). The hallmark of FIV pathogenesis is the progressive disruption of normal

immune function, the mechanisms of which are under intense investigation. Within months to years after infection, an immune deficiency stage similar to acquired immunodeficiency syndrome (AIDS) in humans develops. Therefore, the FIV infection has been studied as a model for study of HIV infection.

Early immunologic abnormalities after spontaneous and experimental infection are decreases of CD4⁺ cells in total number and relative proportion. CD4⁺/CD8⁺ ratios were not different extremely in FIV-infected cats between with and without clinical signs, though loss of CD4⁺ cells leads to inversion

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of the CD4⁺/CD8⁺ ratio in humans with HIV infection. The decrease of CD4⁺ cells could include the decreased production secondary to bone marrow or thymic damage due to the infection, lysis of infected cells induced by FIV itself (cytopathic effects), destruction of infected cells by the immune system, and induction of cell death by apoptosis. Apoptosis induced by Fas ligand (L) and TNF- α was reported in FIV-infected lymphocytes (Ohno et al., 1994; Mizuno et al., 1997; Mizuno et al., 1998). Tompkins et al. (2002) reported that flow cytometry revealed high percentages of CD8⁺ and CD4⁺ cells expressing B7.1 + B7.2 CTLA4 + T cells and that apoptosis occurred in these cells in lymph node. The B7.1 and B7.2 costimulatory molecules on antigen-presenting cells provide second signals for regulating T-cell immune responses via CD28 and cytotoxic T-lymphocyte antigen 4 (CTLA4) on T-cells.

On the other hand, cells obtained from HIV-infected patients and cells infected with HIV in vitro were deviated in the regulation of Fas and FasL expression. Acute HIV infection of the promonocytic cell line U937 is associated with viral replication-dependent apoptosis that is characterized by the increased membrane expression of Fas and FasL, by the down-regulation of antiapoptotic proteins, *Bcl-2* and *Bcl-xL*, and by concomitant increase in proapoptotic proteins, *Bcl-xS* and *Bax* (Badley et al., 2000). Interestingly, though the expression of *Bax*, *Bcl-xL*, and *Bcl-xS* in cells infected with HIV does not differ from that of uninfected controls, the expression of *Bcl-2* was increased in T-cells from HIV-infected patients. (Badley et al., 2000).

However, the precise molecular mechanism of apoptosis in FIV-infected lymphocytes is unknown at present. In the present study, full length sequences of feline *bax*, *bcl-2*, *bcl-xL* and *caspase 3* genes were sequenced and their expression of mRNAs were also investigated in FIV-infected lymphocytes.

2. Materials and methods

2.1. Preparation of cDNA for feline *bax*, *bcl-2*, *bcl-xL* and *caspase 3* genes

The feline T-lymphocyte cell line of FT-1 (Miura et al., 1987, 1989) was cultured in Dulbecco's mod-

ified eagle medium supplemented with 5% fetal calf serum. Total RNA was extracted from 5×10^6 cells with RNeasy total RNA kit (QIAGEN, CA, USA). Reverse transcription of the poly(A)⁺ RNA was performed with a OmniscriptTM Reverse Transcriptase kit (QIAGEN).

A series of 5'- and 3'-RACE-PCR experiments were carried out to determine the full length cDNA sequence of the feline *bax*, *bcl-2*, *bcl-xL* and *caspase 3* genes. The sequences of gene-specific primers were designed from the sequence of the conserved region in *bax*, *bcl-2*, *bcl-xL* and *caspase 3* genes of human and mouse, respectively (Table 1).

The 5'- and 3'-sides of each gene were determined, respectively, according to the user manuals of 5'-RACE system for rapid amplification of cDNA ends (GIBCOBRL, MD, USA) and 3'-RACE system for rapid amplification of cDNA ends (GIBCOBRL). The PCR products of 5'- and 3'-sides of each gene were sequenced by dideoxy chain termination method using an ABI PRISM 310 Genetic Analyzer (ABI Prism, Foster City, USA).

2.2. Preparation of cDNA from FIV-infected feline T-lymphocyte cell line and determination of the viability and apoptosis rate

FIV inoculation was performed by a previous report (Mizuno et al., 2003). The feline T-lymphocyte cell line of Kumi-1 (Hohdatsu et al., 1996) was cultured in RPMI1640 medium supplemented with 5% fetal calf serum, 100 U/ml of human recombinant IL-2 and 50 μ mol of 2-mercaptoethanol. Kumi-1 cells were inoculated with FIV of Sendai-1 strain (Hohdatsu et al., 1996) (500 cpm/ml per 1×10^6). After adsorption for 2 h at 37 °C, the cells were washed in RPMI1640 medium three times and resuspended to a final concentration of 5×10^5 cells/ml.

Three, six, and eight days after infection, the cells were harvested to measure the viable cell counts with trypan blue stain and to confirm apoptosis with TUNEL assay. The TUNEL assay was performed using DeadEnd colorimetric apoptosis detection system, following the manufacturer's instructions (Promega Corporation, Madison, USA) (Oguma et al., 2000). Total RNA was extracted from cells at each day with RNeasy total RNA kit (QIAGEN, CA, USA). Reverse transcription of the poly(A)⁺ RNA was per-

Table 1
PCR primers, annealing temperature and amplified fragment size for real-time PCR

Gene	Sequence	Annealing temperature (amplified fragment size)
<i>bax</i>		
Sense	5'-CCAGCTCTGAGCAGATCAT-3'	65 °C (212 bp)
Reverse	5'-CACTCCC GCCACAAAGATGG-3'	
<i>bcl-2</i>		
Sense	5'-GGAGGATTGTGGCCTTCT-3'	65 °C (223 bp)
Reverse	5'-GTTATCCTGGATCCAGGTGT-3'	
<i>bcl-xL</i>		
Sense	5'-CTTGATGGCCACTTACCTGA-3'	58 °C (278 bp)
Reverse	5'-TCTTCTGGTCATTTCCGACTGAAG-3'	
<i>caspase 3</i>		
Sense	5'-AGCCATGGTGAAGAAGGAA-3'	65 °C (208 bp)
Reverse	5'-TGTTGCCACCTTTCGGTT-3'	
GAPDH		
Sense	5'-TGGTGAAGCAGGCATCAGAG-3'	60 °C (157 bp)
Reverse	5'-CAGGAAATGAGCTTGACAAAGTGG-3'	

formed with a OmniscriptTM Reverse Transcriptase kit (QIAGEN).

2.3. Real-time PCR

The quantification of *bax*, *bcl-2*, *bcl-xL* and *caspase 3* transcripts in FIV-infected lymphocytes was carried out with the LightCycler PCR system (Roche Diagnostics, Meylan, France) using the DNA binding SYBR Green I dye for the detection of PCR products. The feline glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Harley et al., 1999) gene was used as reference for mRNA expression. Quantities of each gene and GAPDH transcripts were determined by comparison to a DNA external standard. The forward and reverse-specific primer sequences used, the size of the amplified fragment and the annealing temperature were listed in Table 1.

DNA standards were prepared from PCR using cDNA of cells. The amount of extracted DNA was quantified by spectrophotometry and expressed as copy number. A serial dilution was used to generate each standard curve. After 2 min of denaturation at 95 °C, the reaction were cycled 40 times for 5 s at 95 °C, 10 s at the annealing temperature, and 15 s at 72 °C. Product specificity was determined by melting curve analysis as described in the LightCycler handbook. We used feline GAPDH gene as a RT-PCR

control (Harley et al., 1999). The amounts of transcripts in each sample is given as copy number. The results are expressed as ratios of each transcript to GAPDH transcripts.

3. Results

3.1. Full length sequences of feline *bax*, *bcl-2*, *bcl-xL* and *caspase 3* cDNAs

Using cDNA of FT-1 cells as a template, full length sequences of feline *bax*, *bcl-2*, *bcl-xL* and *caspase 3* genes were cloned using 5'- and 3'-RACE methods.

The full length cDNA sequence of the *bax* gene (646 bp) contained a single open reading frame of 579 bp coding 193 amino acids (Fig. 1). The sequence was deposited in the DDBJ database (accession no. AB080724, feline *bax* gene, complete cds).

The amino acid sequence of feline *bax* shared 98–92% sequence identity with the human (GenBank accession no. L22473) and the mouse (L22472) *bax* genes (Fig. 1).

The full length cDNA sequence of the *bcl-2* gene (1423 bp) contained a single open reading frame of 708 bp coding 236 amino acids (Fig. 2). The sequence was deposited in the DDBJ database (accession no. AB096611, feline *bcl-2* gene, complete cds).

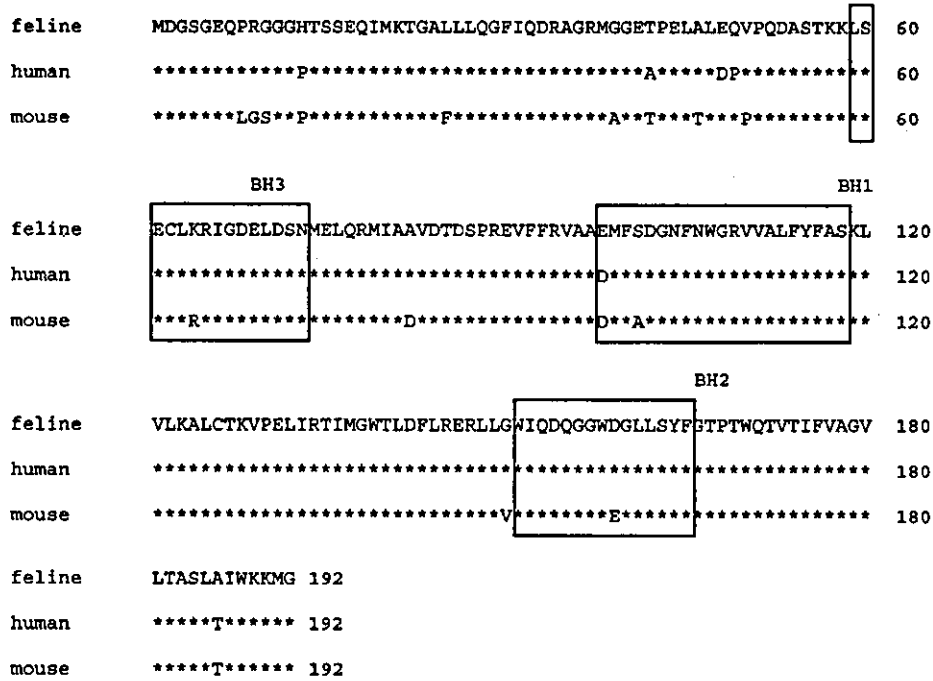


Fig. 1. Comparison of homologous regions of the predicted protein sequences of *bax* among feline, human (L22473) and mouse (L22472). Asterisk indicates identity with the amino acids of the feline *bax*. BH1–3 indicate *Bcl-2* homology domain 1–3, respectively.

The amino acid sequence of feline *bcl-2* shared 90–86% sequence identity with the human (GenBank accession no. M14745) and the mouse (M16506) *bcl-2* genes (Fig. 2).

The full length cDNA sequence of the *bcl-xL* gene (1163 bp) contained a single open reading frame of 702 bp coding 234 amino acids (Fig. 3). The sequence was deposited in the DDBJ database (ac-

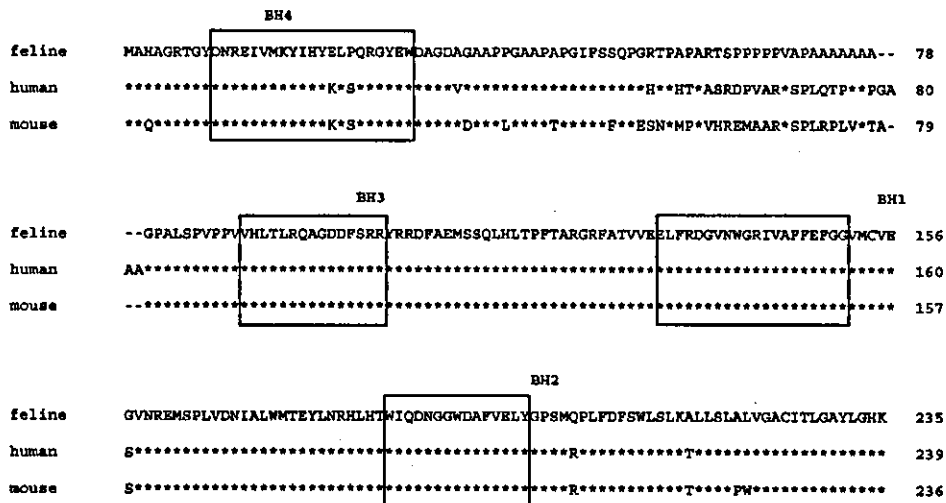


Fig. 2. Comparison of homologous regions of the predicted protein sequences of *bcl-2* among feline, human (M14745) and mouse (M16506). Asterisk indicates identity with the amino acids of the feline *bcl-2*. BH1–4 indicate *Bcl-2* homology domain 1–4, respectively.



Fig. 3. Comparison of homologous regions of the predicted protein sequences of *bcl-xL* among feline, human (Z23115) and mouse (U51278). Asterisk indicates identity with the amino acids of the feline *bcl-xL*. BH1–4 indicate *Bcl-2* homology domain 1–4, respectively.

cession no. AB080951, feline *bcl-xL* gene, complete cds).

The amino acid sequence of feline *bcl-xL* shared 99–96% sequence identity with the human (GenBank accession no. Z23115) and the mouse (U51278) *bcl-xL* genes (Fig. 3).

The full length cDNA sequence of the *caspase 3* gene (1208 bp) contained a single open reading frame

of 834 bp coding 278 amino acids (Fig. 4). The sequence was deposited in the DDBJ database (accession no. AB090246, feline *caspase 3* gene, complete cds).

The amino acid sequence of feline *caspase 3* shared 87–87% sequence identity with the human (GenBank accession no. AY219866) and the mouse (Y13086) *caspase 3* genes (Fig. 4).



Fig. 4. Comparison of homologous regions of the predicted protein sequences of *caspase 3* among feline, human (AY219866) and mouse (Y13086). Asterisk indicates identity with the amino acids of the feline *caspase 3*.

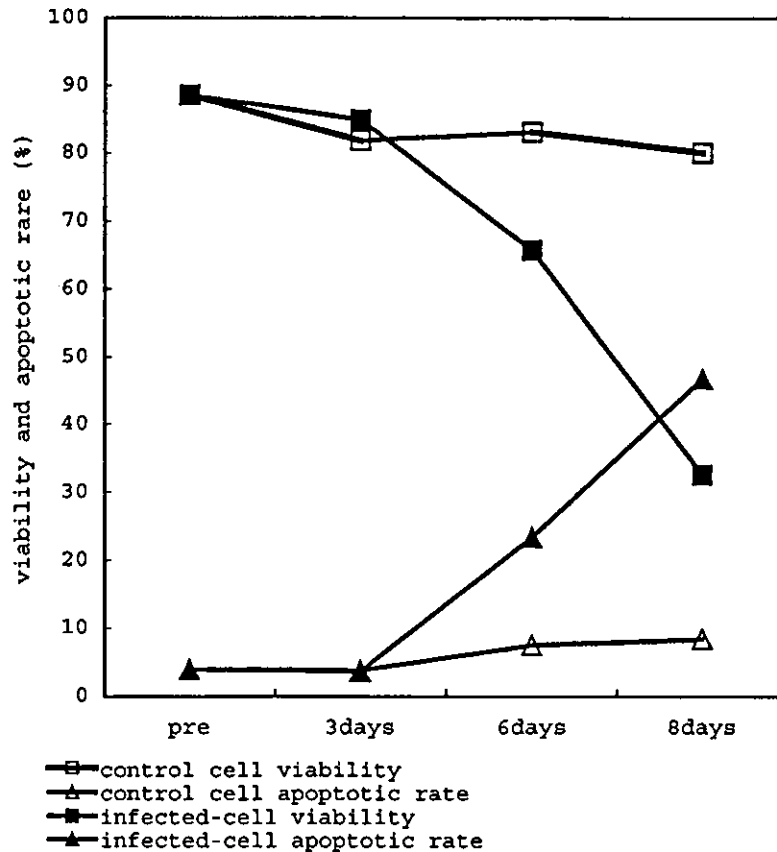


Fig. 5. The viability and apoptotic rate of infected cells and control cells. Apoptotic Kumi-1 cells gradually increased after FIV infection. Approximately 70% had been apoptotic and 30% were viable in the cells infected with FIV but approximately 80% were non-apoptotic and 20% had been dead in non-infected cells after 8-day incubation.

3.2. The viability and apoptosis rate in FIV-infected cells

Apoptotic Kumi-1 cells gradually increased after FIV infection (Fig. 5). Approximately 70% cells had been apoptotic and 30% were viable in the cells infected with FIV while incubation after 8 days approximately and 20% had been dead 80% were non-apoptotic in non-infected control cells.

3.3. Analysis of *bax*, *bcl-2*, *bcl-xL* and *caspase 3* gene mRNAs by real-time PCR

By real-time PCR analysis, *bax*, *bcl-2*, *bcl-xL* and *caspase 3* gene mRNAs were detectable in lympho-

cytes pre- and post-FIV infection (Fig. 6a–d). Although the expression of *bcl-2* mRNA was increased in FIV-infected cells, mRNAs of *bax*, *caspase 3* and *bcl-xL* were expressed in the infected cells as in the non-infected control cells (Fig. 6a–d).

4. Discussion

In the present study, full length sequences of feline *bax*, *bcl-2*, *bcl-xL* and *caspase 3* genes were sequenced and their expression of mRNAs were also investigated in FIV-infected lymphocytes.

The amino acid sequences of feline *bax*, *bcl-2*, *bcl-xL* and *caspase 3* genes were highly conserved in comparison with those of human and mouse, respec-

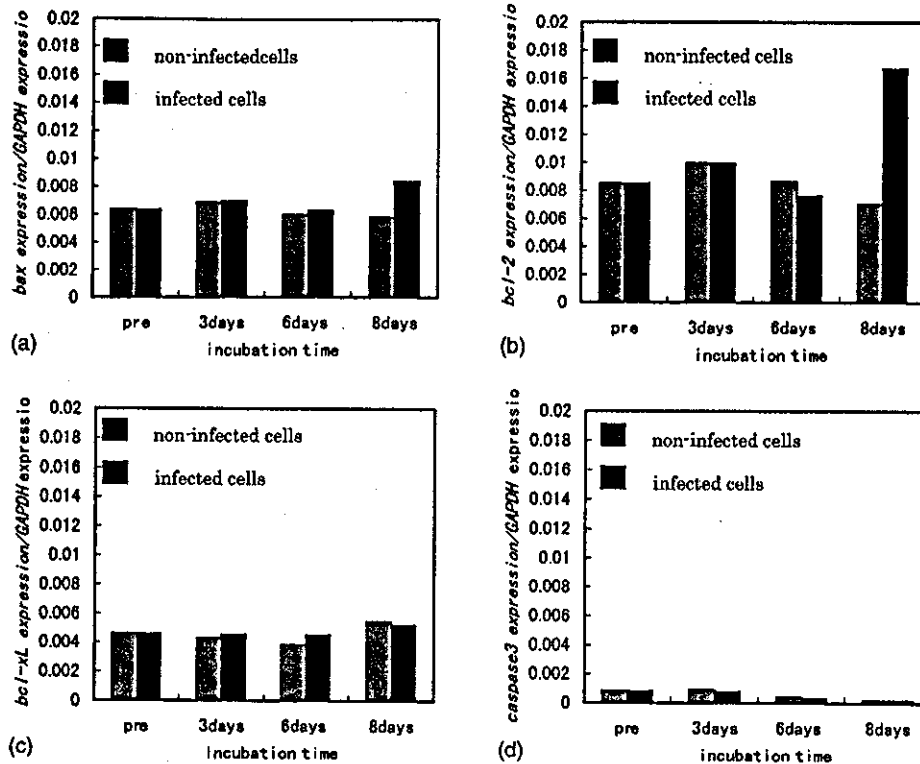


Fig. 6. Quantification of (a) *bax*, (b) *bcl-2*, (c) *bcl-xL* and (d) *caspase 3* mRNAs by LightCycler after infection by FIV for 0, 3, 6, and 8 days. The results are expressed as ratios (Y-axis) of each transcripts to GAPDH transcripts. The black and gray columns indicate the expression in infected cells and non-infected cells, respectively.

tively, suggesting that these feline genes might play similar roles to human and mouse genes.

Approximately 70% were apoptotic and the other 30% were viable in the cells infected with FIV, though the approximately 80% were non-apoptotic and 20% were dead in non-infected cells after 8-day incubation (Fig. 5). The expression of *bcl-2* mRNA was increased in FIV-infected cells after 8-day incubation (Fig. 6b), indicating the correlation between expression of *bcl-2* and viral replication in lymphocyte culture. The increased expression of antiapoptotic gene of *bcl-2* in FIV-infected cells, as in T-cells from HIV-infected patients (Sandstrom et al., 1996) might play some antiapoptotic role in FIV-infected cells. The expression of *bcl-2* was reported to associate with enhanced HIV replication in infected cells (Sandstrom et al., 1996). Tat protein of HIV was proved to stimulate the *bcl-2* mRNA and *Bcl-2* protein expression in HIV-infected T-cell line (Zauli et al., 1995), suggesting that tat might

be a promotor for *bcl-2* mRNA expression to inhibit host cell apoptosis (Zauli et al., 1995). Tat gene was also confirmed to be in FIV genome and related with virus replication in host cells (de Parseval and Elder, 1999). Therefore, FIV tat might also stimulate the expression of *bcl-2* mRNA in infected feline T-cells.

Apoptotic cells in FIV-infected Kumi-1 cells increased progressively, indicating that FIV infection-induced cell death in these cells. Therefore, amount of apoptosis induction genes such as *bax* and *caspase 3* mRNAs was expected to increase in FIV-infected cells. However, the expression of *bax* and *caspase 3* genes in the infected cells did not differ from that in non-infected control cells. The results were consistent to the expression of apoptosis induction genes in T-cells from HIV-infected patients (Badley et al., 2000). Therefore, another apoptosis inducible mechanisms such as ceramide might be concerned in FIV- and HIV-infected cells.

The results in this study suggested that molecular mechanisms of FIV-associated lymphocyte apoptosis and antiapoptosis might be similar as in the HIV infections.

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Prevalence of Canine Distemper Virus, Feline Immunodeficiency Virus and Feline Leukemia Virus in Captive African Lions (*Panthera leo*) in Japan

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ABSTRACT. Sero-prevalences of canine distemper virus (CDV), feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) were evaluated in 20 captive lions in two Japanese zoos. Anti-CDV antibody was detected in 13 of 20 lions. We could pursue antibody responses against CDV in three lions back to 1996. Sera collected in 1996 were negative for anti-CDV antibody, therefore, all of them showed sero-conversion in 2000. This result suggested that the epidemic of CDV infection in this zoo might have happened between 1996 and 2000. The lions were also examined for FIV and FeLV infections. We had no evidence for FeLV infection but eight lions were sero-positive for anti-FIV antibody.

KEY WORDS: CDV, Lion, Retroviruses.

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Recently, many cases of interspecies transmission of viral diseases between humans and animals between animal species have been reported [8, 13, 25–27] and wildlife is no exception. An incident that threatened the lives of African lions (*Panthera leo*) occurred at Serengeti National Park of Tanzania in 1994. Roelke-Parker *et al.* and others finally clarified that canine distemper virus (CDV) had infected the lions by breaking through the species barrier, and induced the death of infected lions in this area [14, 23]. In addition, it was also reported that the captive large felids, including leopards (*Panthera pardus*), tigers (*Panthera tigris*), lions and jaguars (*Panthera onca*), were affected with CDV and died [1, 3, 11]. On the other hand, some feline retrovirus infections were also detected in large felids. The cross-reactive antibodies to feline immunodeficiency virus (FIV) have been found in wild felids including lion, cheetah (*Acinonyx jubatus*), puma (*Felis concolor*), bobcat (*Felis rufus*) and Florida panther (*Panthera concolor coryi*) [2, 7, 17, 21]. However, the lentiviruses isolated from these felids were highly adapted in each species and genetically distant from FIV in domestic cats. These viruses were designated as lion lentivirus (LLV) or puma lentivirus (PLV) [16, 17, 21]. The infection of feline leukemia virus (FeLV) was found in puma, clouded leopard (*Neofelis nebulosa*), bobcat, European wildcat (*Felis silvestris*) and cheetah [4, 9, 18, 19, 24]. The fundamental features of the retroviruses described above has poorly understood in wild felids and it will be required the further analyses considering the potential of retroviruses for mutation and difficulties of virus elimination. Research on viral infections of wild animals has advanced in the last few decades. Continuous efforts in this field are

indispensable for prevention of the spread of lethal infectious diseases and the preservation of wild animal species. Most of the large felids in Japan are housed in zoos. It is important to understand the status of virus infections in them from the epidemiological, virological and economical points of views. In the present study, we evaluated the status of CDV, FIV (or LLV) and FeLV infections of 20 lions in two Japanese zoos.

Details of the 20 lions examined in this study are shown in Table 1. These lions ranged from 3-months to 11-years-old in age, except an unknown case (lion B1). Eleven were male and the others were female. All lions housed in zoo A and six of these in zoo B were born at the respective zoo, but three (lions B1, B2 and B3) were transferred from other zoos (zoo C or D) to B zoo at 1 or 3 years before the sample collection. All twenty lions were healthy and showed no obvious clinical signs at the time of sampling. In addition, they have no history of vaccinations for CDV, FIV and FeLV infections. Blood samples were collected from these 20 lions of two zoos under anesthesia with xylazine and ketamine in a squeeze cage. Serum and peripheral blood mononuclear cells (PBMC) were prepared and subjected to the following serological and molecular analyses, respectively.

Anti-CDV humoral immune responses were examined by enzyme-linked immunosorbent assay (ELISA) as described previously [12]. This assay showed that 12 of the 20 lions possessed the anti-CDV antibodies (Table 1). Nine out of eleven (81.8%) and three out of nine (33.3%) lions were positive for CDV antibody in zoo A and zoo B, respectively. We also examined the existence of neutralizing antibody against CDV in the same samples as described previously [15]. Neutralizing antibody titers were well correlated with the results of ELISA (Table 1). One exceptional case (B5)

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Table 1. Clinical profile and Serological status for CDV, FIV and FeLV infections of lions examined in this study

Animal ID	Zoo	Sex ¹⁾	Age	Origin	Transfer date	CDV Ab (ELISA)		CDV neutralization Ab titer (2000)	FIV Ab	FeLV Ag
						2000	1996			
A1	A	M	9Y	A	-	+	ND ²⁾	905	-	-
A2	A	F	7Y	A	-	+	ND	320	-	-
A3	A	N	3M	A	-	-	ND	<10	-	-
A4	A	F	5Y	A	-	+	ND	1522	-	-
A5	A	F	3Y	A	-	+	ND	381	-	-
A6	A	M	6Y	A	-	+	ND	95	-	-
A7	A	F	6Y	A	-	+	-	1280	-	-
A8	A	F	4M	A	-	-	ND	<10	-	-
A9	A	M	5Y	A	-	+	ND	320	-	-
A10	A	F	6Y	A	-	+	-	113	-	-
A11	A	M	6Y	A	-	+	-	113	-	-
B1	B	F	Unknown	C	11/21/97	-	ND	<10	+	-
B2	B	M	2Y	D	3/15/99	-	ND	<10	+	-
B3	B	M	3Y	D	3/15/99	-	ND	<10	-	-
B4	B	M	11Y	B	-	+	ND	190	+	-
B5	B	F	8Y	B	-	-	ND	95	+	-
B6	B	F	8Y	B	-	+	ND	113	+	-
B7	B	M	8Y	B	-	+	ND	320	+	-
B8	B	M	5Y	B	-	-	ND	<10	+	-
B9	B	M	2Y	B	-	-	ND	<10	+	-

M, male; F, female; ND, not done.

was judged as negative for anti-CDV antibody in ELISA but possessed neutralizing antibody. Neutralizing antibody responses are generally stricter with each infectious agent than ELISA. Thus, thirteen lions in zoo A and B were diagnosed as having been infected with CDV from the findings in ELISA and the virus neutralizing assay. Fortunately, we could review the status of CDV infection in the sera from three lions (lions A7, A10 and A11) housed in zoo A back to 1996. In 1996, sera from all three lions were negative for anti-CDV antibody. However, all of them showed sero-conversion against CDV in 2000 (Table 1). These results suggested that an epidemic of CDV infection in zoo A might have occurred between 1996 and 2000. Additionally, the lack of infection in cubs indicates that the prevalence occurred before birth. The lions in zoo A were shown to be highly affected with CDV. This may be due to keeping style of lions. In zoo A, the lions are able to contact each other in the display field. Although how the first infection occurred is still unclear, the explosive expansion of the infection might have happened from the first affected lion through the direct contact. In zoo B, transferred lions (lions B1, B2 and B3) and young lions (B8 and B9 were less than 6 years old) were sero-negative for CDV. This finding also suggests that active infection occurred more than 5 years ago in B zoo.

Although exact infectious route cannot be determined, two possible routes are considered for CDV infection. One is that the domestic dogs or free ranging dogs around the zoo might have been a source of the virus as described previously [6, 10, 23]. In this case, however, it is difficult to suppose that there could be a direct contact of lions with dogs because of complete separation of lions from outside. Therefore, it is conceivable that some agents (including

human and foods) have played a role as a vector or carrier for CDV infection in lions. It is also conceivable that exchanges of lions between zoos to avoid inbreeding might have spread the virus from one zoo to other zoos. On the pathogenicity of the CDV in lions, there is a major difference between the previous epidemics in Africa and the United States, and current ones in Japanese zoos. It has been reported no cases of large felids which showed typical symptoms of CDV infection in Japan. Although we could not molecularly characterize these viruses, this inconsistency may be due to the differences in the CDV strains. Further molecular analyses of the viruses are required to clarify this point.

We next analyzed the status of FIV and FeLV infections by detecting the anti-FIV antibody against p24 gag protein or FeLV p27 antigen in serum using a commercial kit (Idex Laboratories, Portland, ME). All lions housed in zoo A were negative for anti-FIV antibody, whereas eight of nine lions in zoo B were positive for it. FeLV p27 antigen was not detectable in all lions examined in the present study. Since FIV or LLV infection was strongly suspected from the findings of serological survey, PCR was performed to detect viral RNA genome in sera or provirus in PBMC from two lions (B2 and B3). In the PCR analyses, three primer sets were used for FIV *env* or LLV *pol* region. Nested primers 5'-GAG TAG ATA C(A/T)T GGT T(G/A)C AAG-3' (VE-1S), 5'-CAT CCT AAT TCT TGC ATA GC-3' (VE-1R), 5'-CAA AAT GTG GAT GGT GGA A(T/C)-3' (VE-2S) and 5'-ACC ATT CC(A/T) ATA GCA GT(G/A) GC-3' (VE-2R) were designed based on the sequences conserved among a number of FIV isolates [20, 22]. For amplification of LLV genome, two primer sets, 5'-CCT ATA TTT TGC