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Journal of Immunological Methods 278 (2003) 201–209

JIM
Journal of Immunological Methods

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Molecular cloning, functional characterization, and enzyme-linked immunosorbent assay of cynomolgus monkey Fas ligand

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Received 31 January 2002; received in revised form 17 March 2003; accepted 17 March 2003

Abstract

Fas ligand (FasL) cDNAs were cloned and sequenced from cynomolgus, rhesus, and pig-tailed monkeys. The 840-bp cDNAs were identical among these three species of monkeys except for one nucleotide. The deduced 280 amino acids were completely identical and displayed 97% homology with human FasL (hFasL). Recombinant soluble FasL obtained from COS cells transfected with cynomolgus monkey FasL (cm-FasL) cDNA induced apoptosis in cells displaying human or cynomolgus monkey Fas-expressing cells. Several anti-human FasL monoclonal antibodies (mAbs) were able to neutralize the cytotoxic activity of monkey FasL, and a combination of mAbs was selected to obtain the most sensitive detection of monkey soluble FasL (sFasL) under sandwich enzyme-linked immunosorbent assay (ELISA). Plasma from normal monkey did not contain detectable levels of sFasL, whereas plasma from monkeys acutely infected with simian immunodeficiency virus (SIV) displayed increased levels of sFasL.
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Keywords: Nonhuman primates; Fas; Fas ligand; Molecular cloning; Apoptosis; ELISA

1. Introduction

Fas ligand (FasL) belongs to the tumor necrosis factor (TNF) family that includes TNF-α, lymphotoxin, TNF-related apoptosis-inducing ligand (TRAIL), CD40 ligand, CD27 ligand, CD30 ligand, and OX40 ligand (Nagata, 1997). Most members of the TNF family are type II membrane proteins, with the exception of lymphotoxin-α. FasL induces apoptotic cell death by binding to its receptor, Fas (also called APO-1 or CD95), which is a member of the TNF receptor family (Nagata and Golstein, 1995).

Abbreviations: mAb, monoclonal antibody; FasL, Fas ligand; sFasL, soluble FasL; ELISA, enzyme-linked immunosorbent assay; cm-FasL, cynomolgus monkey FasL; hFasL, human FasL; MMP, matrix metalloproteinase; SIV, simian immunodeficiency virus; RACE, rapid amplification of cDNA ends; HSCF, CD4-positive T cell line immortalized by *Herpesvirus saimiri*; PHA, phytohemagglutinin.

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doi:10.1016/S0022-1759(03)00187-X

FasL is predominantly expressed in activated T and NK cells (Tanaka et al., 1996; Arase et al., 1995), while Fas is ubiquitously expressed in various cells (French et al., 1995; Watanabe-Fukunaga et al., 1992). FasL-mediated cell death is involved in T or NK cell-mediated cytotoxicity and regulation of lymphocyte homeostasis (Nagata and Golstein, 1995). FasL-induced apoptosis has also been implicated in diseases such as hepatitis (Kondo et al., 1997), insulin-dependent diabetes (Chervonsky et al., 1997), and human immunodeficiency virus (HIV)-induced acquired immunodeficiency syndrome (AIDS) (Hosaka et al., 1998). FasL is also expressed in the testis (Bellgrau et al., 1995), eye (Griffith et al., 1995), and some malignant tumor cells (Hahne et al., 1996; Strand et al., 1996), and has been proposed as a mediator of immune privilege in such tissues to kill invading Fas-expressing effector cells.

A soluble form of FasL (sFasL) can be released from human FasL (hFasL) transfectants and induces apoptosis in Fas-expressing cells, indicating that hFasL can be processed from the membrane, like TNF-α (Tanaka et al., 1995; Kayagaki et al., 1995). Since matrix metalloproteinase (MMP) inhibitors block the processing of both FasL and TNF-α, MMP-like enzymes are considered responsible for processing membrane-bound FasL (Tanaka et al., 1996; Kayagaki et al., 1995) and TNF-α (Gearing et al., 1994; McGeehan et al., 1994). Recently, ADAM17 (TNF-α converting enzyme) and ADAM10, belonging to the ADAM (a disintegrin and metalloproteinase) family, have been shown to specifically process TNF-α (Black et al., 1997; Moss et al., 1997; Rosendahl et al., 1997). The processing enzyme for FasL has remained unidentified. Enzyme-linked immunosorbent assay (ELISA) systems for measuring human sFasL have already been established using anti-human FasL monoclonal antibodies (mAbs) (Tanaka et al., 1996; Kayagaki et al., 1995), and have been applied to detect sFasL levels in clinical samples. Elevated levels of sFasL have been reported in sera from patients with large granular lymphocytic leukemias and NK cell lymphomas (Tanaka et al., 1996), asymptomatic stage HIV infection (Bahr et al., 1997), and aggressive nasal lymphoma (Sato et al., 1996). These results have provided useful information in elucidating the role of sFasL and FasL/Fas-mediated apoptosis in human immune diseases.

No sensitive ELISA system has been available for detecting sFasL in nonhuman primates, although nonhuman primates serve as valuable models for studying (1) the efficacy of various vaccines and drugs against infectious diseases, including AIDS; (2) the pathogenic mechanisms of numerous infectious diseases; and (3) the biology of allogeneic and xenogeneic organ and tissue transplantation (King et al., 1988; Wolf et al., 1985; Letvin et al., 1985; Meisenberg et al., 1992; van Beusechem et al., 1992; Nakajima et al., 1995).

In this context, we cloned monkey FasL cDNA, generated monkey FasL transfectants, purified recombinant monkey sFasL, and finally established a sensitive ELISA system for monkey sFasL. Recombinant monkey sFasL was functional in inducing apoptosis in human and monkey Fas-expressing cells and the newly established ELISA system was sensitive to picogram concentrations of sFasL. This system allows increasing levels of sFasL in plasma to be monitored in cynomolgus monkeys infected with simian immunodeficiency virus (SIV).

2. Materials and methods

2.1. Chemical

Synthesis of the MMP inhibitor KB-R8301 ([4-(*N*-hydroxyamino)-2R-isobutyl-3*S*-methylsuccinyl]-L-3-(5,6,7,8-tetrahydro-1-naphtyl) alanine-*N*-methylamide) was performed as previously described (Yamamoto et al., 1998).

2.2. Cloning and expression of monkey FasL cDNA

Peripheral blood was obtained from healthy cynomolgus, rhesus, and pig-tailed monkeys. Peripheral blood lymphocytes (PBL) were isolated by centrifugation on Ficoll–Pauque solution (Amersham Pharmacia Biotech, Buckinghamshire, UK) and cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS) containing 5 μg/ml of concanavalin A (ConA) (Sigma, St. Louis, MO) and 60 U/ml of human IL-2 (Invitrogen, Carlsbad, CA) for 7 days. ConA-activated T cell blasts were finally stimulated with 10 ng/ml phorbol myristic acetate (Sigma) and 500 ng/ml ionomycin (Sigma) for 4 h. Poly(A)$^+$

RNA was prepared using an mRNA isolation kit (Amersham Pharmacia Biotech). Single-strand cDNA was synthesized using either random hexamer oligonucleotides or oligo (dT)12-18 primer as primers in a cDNA synthesis kit (Invitrogen). Monkey FasL cDNA was amplified by PCR using an oligonucleotide corresponding to the first six codons as the sense primer and an oligonucleotide corresponding to the last six codons as the antisense primer, according to the published human FasL (hFasL) sequence (Takahashi et al., 1994). Sense (GGCCTCGAGAGATGCAGCAGCCCTTCAATTAC) and antisense (CGAGCGGCCGCTTAGAGCTTAGAGCTTATATAAGCCGAA) primers were tagged with a *Xho*I or a *Not*I site, respectively. The reaction mixture for PCR contained 10 pmol each of the sense and antisense primers and the reaction was initiated with the addition of 2.5 units of *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Conditions for PCR were 1 min at 94 °C, 1 min at 52 °C, and 2 min at 72 °C for 40 cycles. After *Xho*I and *Not*I digestion, PCR product was subcloned into pBluscriptII SK+ (Stratagene). Three independent clones from each species of monkey were sequenced using an ALFII DNA sequencer (Amersham Pharmacia Biotech). To determine the monkey FasL sequence in the region corresponding to the sense and antisense primers for hFasL, cDNA was synthesized from monkey poly(A)$^+$ RNA using a rapid amplification of cDNA ends (RACE) kit (Clontech, Palo Alto, CA) according to the manufacturer's instruction. Amplified 5′ and 3′ RACE cDNAs were subcloned into PCR-Script SK+ (Stratagene) and three independent clones were sequenced. Sequenced cynomolgus monkey FasL (cm-FasL) cDNA was then transferred into *Xho*I and *Not*I sites of the CDM8 (Invitrogen) expression vector. Transient expression of monkey FasL cDNA (cm-FasL/CDM8) in COS cells was performed using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Culture supernatant containing soluble FasL (sFasL) was collected after 5 days. Transient expression of hFasL cDNA was also performed using hFasL/CDM8 (Kayagaki et al., 1995) in the same manner.

2.3. Cytotoxic assay

Cytotoxic activity of sFasL in culture supernatants was tested against human Fas transfected WR19L (hFas/WR19L) using alamar Blue (Alamar Biosciences, Sacramento, CA) as described previously (Kayagaki et al., 1995). Briefly, 4×10^4 hFas/WR19L or WR19L cells were cultured with serially diluted culture supernatant of cm-FasL/COS or hFasL/COS cells in a total volume of 200 μl. After 16 h, 10 μl of alamar Blue was added and further incubated for 4 h. Fluorescence of the reduced alamar Blue was measured on a fluoroscan (MTP-32; Corona Electric, Tokyo, Japan) at 590 nm by excitation at 544 nm. To compare sensitivity to FasL-induced apoptosis, cynomolgus monkey T cell blasts activated by phytohemagglutinin (PHA), the CD4-positive T cell line immotalized by *Herpesvirus saimiri* (HSCF) CD4-positive T cell line immortalized by *H. saimiri* (Akari et al., 1996), and hFas/WR19L cells were treated with serially diluted cynomolgus monkey soluble FasL (cm-sFasL). Cell viability was determined using the alamar Blue method. For selecting anti-human FasL mAbs that cross-react with monkey sFasL, two mAbs, NOK-2 (mouse IgG2a, κ) and NOK-3 (mouse IgM, κ), generated previously (Kayagaki et al., 1995) and two mAbs (4A5 and 4H9, hamster IgG) purchased from MBL (Nagoya, Japan) were screened. In a neutralization test, serially diluted mAbs were added to the mixture of hFas/WR19L cells (4×10^4 cells) and cm-FasL/COS supernatant and cell viability was determined using the alamar Blue method.

2.4. Western blotting

Supernatant of COS cells transfected with cm-FasL/COS or hFasL/COS was heated in 1 × SDS buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100 mM dithiothreitol). Samples were electrophoresed on 10–15% gradient polyacrylamide gel. Proteins were then transferred to PVDF membranes (Millipore, Bedford, MA). After blocking in PBS containing skimmed milk solution (Block Ace; Snow Brand Milk, Sapporo, Japan), the membrane was incubated for 1 h at room temperature with 200-fold diluted anti-human FasL polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS supplemented with 25% Block Ace. After washing three times with PBS containing 0.05% Tween 20, the membrane was incubated for 1 h at room temperature with 1000-fold diluted peroxidase-conjugated anti-rabbit IgG (Jack-

son ImmunoResearch Laboratories). The membrane was washed three times with PBS containing 0.05% Tween 20 and the proteins recognized by the antibody were visualized using the ECL system (Amersham Pharmacia Biotech).

2.5. Establishment of ELISA for monkey sFasL

To establish the sandwich ELISA for monkey sFasL, various combinations of capture antibody and detection antibody were set using mAbs cross-reacting with cm-sFasL. Immulon 600 (Greiner Japan, Tokyo) plates were incubated overnight with 100 μl/well of capture mAb (10 μg/ml). After washing with PBS containing 0.05% Tween-20 (PBS–Tween), wells were blocked by 200 μl/well of PBS with 10% Block Ace for 1 h at 37 °C. Following the removal of 10% Block Ace, 50 μl of the sample was added and incubated for 1 h at room temperature. After washing with PBS–Tween, 100 μl/well of biotinylated detection mAb (5 μg/ml) was added and the plate was incubated for 1 h at room temperature. Plates were washed with PBS–Tween, and 50 μl of 1:1000 diluted streptavidin-horseradish peroxidase (Amersham Pharmacia Biotech) was then added and incubated for 30 min. After washing with PBS–Tween, plates were developed with 100 μl of 1 mg/ml orthophenylene diamine in 50 mM citrate-phosphate buffer (pH 5.0) containing 0.03% H_2O_2, and stopped using 100 μl of 2N H_2SO_4. Optical density (OD) at 490 nm was measured using an automatic ELISA reader (Thermo Max; Molecular Devices, Sunnyvale, CA). The cm-sFasL was subsequently purified from the supernatant of cm-FasL/COS cells using an anti-hFasL (NOK-2) affinity column, and serial dilutions of purified cm-sFasL were used as the standard.

To evaluate the blocking ability of KB-R8301 for cm-FasL processing from COS cells transfectant, cm-FasL/COS or hFasL/COS cells (5×10^5) were cultured with or without KB-R8301 (from 0.625 to 10 μM) for 24 h and sFasL in the supernatant was determined by ELISA as described above.

2.6. Measuring sFasL levels in monkey plasma

To measure concentrations of sFasL in monkey plasma, plasma samples were obtained from normal monkeys and SIVmac239-infected monkeys. These monkeys were bred in the Tsukuba Primate Center of the National Institute of Infectious Diseases, and were serologically negative for B virus, simian T cell leukemia virus-type I, and simian-type D retrovirus. The plasma was obtained from two male monkeys aged 5 years, immediately before and 10 and 17 days after intravenous inoculation of 100 tissue culture infectious doses ($TCID_{50}$) of a pathogenic clone, SIVmac239 (Akari et al., 1998). Plasma was separated by centrifuge and stored at −80 °C until use. The sFasL levels were determined by the ELISA method using 4H9 for capture and biotinylated 4A5 for detection.

```
Monkey  MQQPFNYPYP QIYWVDSSAS SPWAPPGTVL PCPTSVPRRP GQRR-PPPPP   49
Human   .......... .......... .......... .......... ....P.....   50

Monkey  PPPPLPPPPP SPLPPLPLPP LKKRGNHSTG LCLLVMFFMV LVALVGLGLG   99
Human   ...LP..... P......... .......... .......... ..........  100

Monkey  MFQLFHLQKE LAELRESTSQ KHTASSLEKQ IGHPSPPPEK KEQRKVAHLT  149
Human   .......... .......... M......... .......... ..L.......  150
                                    **

Monkey  GKPNSRSMPL EWEDTYGIVL LSGVKYKKGG LVINETGLYF VYSKVYFRGQ  199
Human   ..S....... .......... .......... .......... ..........  200

Monkey  SCTNLPLSHK VYMRNSKYPQ DLVMMEGKMM SYCTTGQMWA HSSYLGAVFN  249
Human   ..N....... .......... .......... .......... R.........  200

Monkey  LTSADHLYVN VSELSLVNFE ESQTFFGLYK L  280
Human   .......... .......... .......... .  281
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Fig. 1. Comparison of amino acid sequences for monkey and human Fas ligands. Identities are indicated by dot plot. The putative transmembrane domain is underlined. The amino acids at the position for hFasL processing are indicated by asterisks.

3. Results and discussion

3.1. Cloning of monkey FasL cDNA

The nucleotide sequences of cynomolgus, rhesus, and pig-tailed monkeys FasL have been deposited to DDBJ under accession numbers AB035138, AB035139, and AB035140, respectively. Monkey FasL cDNA nucleotide sequences among these three species of macaque monkeys were almost identical except for one nucleotide change at nucleotide position 522 (cynomolgus: G; rhesus: A; pig-tailed: A), a silent change which does not change the encoding amino acid. The monkey FasL cDNA sequence contained a long open reading frame (ORF) of 840 bp and displayed 97% homology to human FasL. The ORF codes a polypeptide comprising 280 amino acids, and is completely identical for all three species. The predicted amino acid sequence of monkey FasL was one amino acid shorter than human FasL, with a calculated Mr of 31 kDa. As with human FasL (Takahashi et al., 1994), monkey FasL is a type II membrane protein, with high homology to human FasL. An alignment of the amino acid sequences of monkey and human FasL is shown in Fig. 1. Eight amino acid changes (five in extracellular domains and three in intracellular domains) and one amino acid deletion (position 45) are present in monkey FasL compared to human FasL. Compared to the previously reported rhesus FasL cDNA (Wang et al., 1998), six nucleotide changes (our G^{150}, A^{192}, G^{218}, C^{459}, T^{825}, C^{829} vs. their A^{150}, G^{192}, A^{218}, T^{459}, C^{825}, T^{829}) and one amino acid change (our R^{43} vs. their K^{43}) were identified. This may be attributable to polymorphism. Conservation of these residues among the other two species of monkeys and human suggests a more likely sequence of G^{150}, A^{192}, G^{218}, C^{459}, T^{825}, C^{829} in the rhesus monkey, as determined in the present study.

3.2. Functional characterization of monkey FasL

Since FasL cDNA sequences of three species of macaques were identical, cynomolgus monkey FasL (cm-FasL) cDNA was inserted into a mammalian expression vector CDM8 and transiently transfected into COS cells as a representative. Recent studies have reported the presence of a functional soluble form of human FasL (Tanaka et al., 1995; Kayagaki et al., 1995). We therefore collected supernatant from the cm-FasL/COS cell culture at 5 days after transient transfection, and examined cytotoxicity against the human Fas-expressing cell line, hFas/WR19L. Both cm-FasL/COS cell supernatant and hFasL/COS cell supernatant exhibited specific cytotoxicity against hFas/WR19L cells in a dose-dependant manner (Fig. 2A). This result indicated that cm-FasL/COS cells could produce functional monkey recombinant FasL that might undergo processing

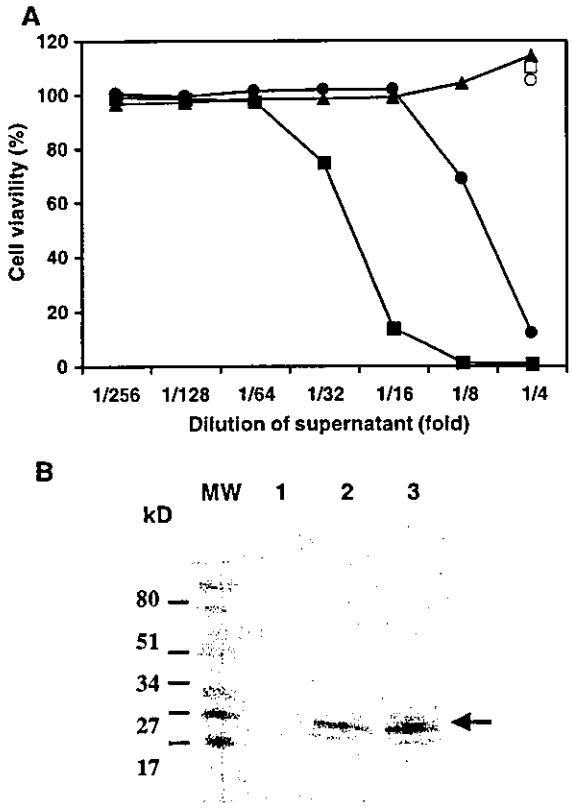

Fig. 2. Release of functional sFasL from cm-FasL-transfected COS cells. (A) Cytotoxic activity of sFasL in supernatant of vector (▲), cm-FasL (■, □), or hFasL (●, ○)-transfected COS cells was tested against hFas/WR19L cells (■, ●) or WR19L cells (□, ○) according to the alamar Blue method. (B) Western blotting. Supernatant (10 μl) from vector-transfected (Lane1), cm-FasL (Lane2), or hFasL (Lane3)-transfected COS cells was analyzed using anti-hFasL antibody as described in the Materials and methods, heated in SDS sample buffer and resolved by electrophoresis on a 10–15% gradient polyacrylamide gel. After blotting to a PVDF membrane, sFasL was detected using ECL system. The arrow indicates the band corresponding to monkey sFasL protein.

from membrane-bound FasL. Recently, Schneider et al. (1998) reported the cleavage site of hFasL, located between Ser 126 and Leu 127 in hFasL protein. As shown in Fig. 1, monkey FasL protein displayed the same amino acids surrounding the cleavage site of hFasL protein. We observed a 27-kDa protein in the supernatant of cm-FasL/COS cells as well as of hFasL/COS cells by Western blotting using anti-hFasL polyclonal Ab (Fig. 2B). These results suggest that monkey FasL can be processed to create a functional soluble form (sFasL) like human FasL. As shown in Fig. 3, monkey sFasL also exhibited potent cytotoxicity against Fas-expressing cynomolgus monkey T cell blasts and the HSCF cell line (Akari et al., 1996).

3.3. Characterization of anti-human FasL mAbs against monkey FasL

Cross-reactivity of four different anti-hFasL mAbs (NOK-2, NOK-3, 4A5, and 4H9) was characterized with cm-sFasL on the basis of neutralization of cm-sFasL cytotoxicity against hFas/WR19L cells. In these mAbs, four mAbs, NOK-3 (mouse IgM, κ), 4A5 (hamster IgG), and 4H9 (hamster IgG), neutralized the cytotoxic activity of cm-sFasL in a dose-dependent manner (Fig. 4). As presented in Fig. 4, among these four mAbs cross-reacting with cm-sFasL, 4A5 mAb neutralized cm-sFasL from 50- to 100-fold more efficiently than 4H9, NOK-3, and NOK-2.

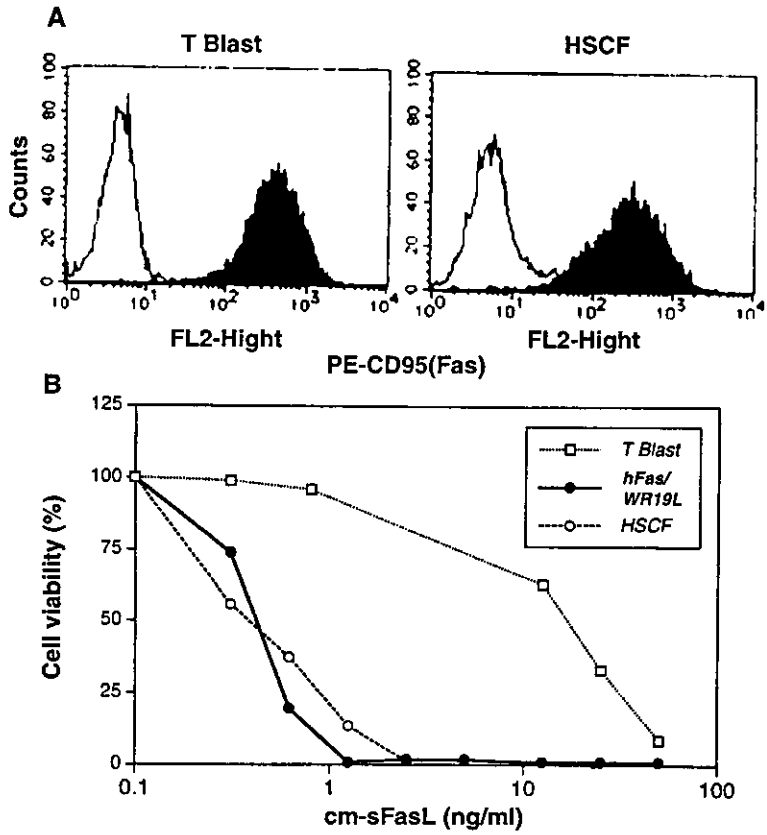

Fig. 3. Recombinant monkey sFasL induced apoptosis in Fas-positive cynomolgus monkey cells. (A) Cynomolgus monkey PHA-activated T cell blasts and T cell line (HSCF) were stained with PE-conjugated anti-human CD95 mAb (filled diagrams) and analyzed by flow cytometry. Open histograms represent staining with isotype-matched control mAb. (B) Cytotoxic activity of purified recombinant cynomolgus monkey soluble FasL (cm-sFasL) was tested against hFas/WR19L (●), HSCF (○), and PHA-activated T cell blasts (□) according to the alamar Blue method.

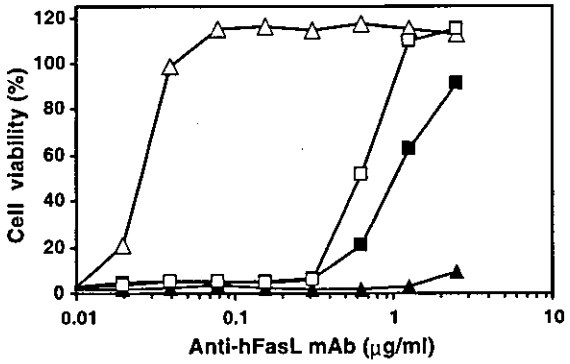

Fig. 4. Comparison of neutralizing activity among anti-human FasL mAbs cross-reacting with monkey sFasL. Cytotoxic activity of sFasL in the supernatant of cm-FasL-transfected COS cells was tested against hFas/WR19L cells in the presence of serially diluted NOK-2 (▲), NOK-3 (■), 4A5 (△), and 4H9 (□). Cytotoxicity was determined according to the alamar Blue method.

3.4. Establishment of an ELISA system for monkey sFasL measurement

Next, establishment of a sandwich ELISA system for monkey sFasL was attempted using all combinations of the four mAbs for capture and detection. As a result, 4A5 was most efficient for detection, in combination with 4H9 for capture. A sandwich ELISA system was therefore established using 4H9 for capture and biotinylated 4A5 for detection. The correlation between optical density and cm-sFasL contents ranging from 5 to 400 pg/ml was significant ($r>0.9$, $P<0.001$), indicating that this assay system is very sensitive for measuring both monkey sFasL (Fig. 5A) and human sFasL (Fig. 5B). Using this ELISA system, we examined the effect of MMP inhibitor (KB-R8301) on monkey sFasL release from cm-FasL/COS cells. When cm-FasL/COS cells were cultured for 24 h in the presence of KB-R8301, KB-R8301 inhibited sFasL release in a dose-dependent manner (60% inhibition at 10 μM and 36% inhibition at 2.5 μM) (data not shown), in addition to inhibiting human sFasL release from hFasL/COS (Kayagaki et al., 1995). This result indicated that release of monkey FasL is likewise processed by an MMP-like enzyme. Finally, this assay system was utilized to evaluate levels of sFasL in plasma from SIV-infected cynomolgus monkeys. The sFasL was undetectable in normal monkey plasma (data not shown). Conversely, levels of sFasL in SIV-infected cynomolgus monkeys increased and became detectable at 10–17 days after infection with pathogenic SIV (Table 1). These results demonstrated that the presently established sandwich ELISA system is applicable to monitor changes to sFasL concentrations in monkey disease

Table 1
Soluble FasL levels in SIV-infected monkey plasma

Monkey #	Dpi	CD4+ T cells (%)	Plasma p27 (ng/ml)	sFasL (pg/ml)
#028	0	47.9	0.00	0.0
	10	47.3	3.15	4.1
	17	30.5	2.91	14.3
#323	0	43.9	0.00	0.0
	10	48.9	1.63	0.0
	17	47.3	0.71	24.8

Dpi: days post infection of SIVmac239 into cynomolgus monkeys.
CD4+ T cells: percentages of CD4+ lymphocyte subsets.
Plasma p27: p27 SIV antigen level in plasma.
sFasL: sFasL level by ELISA using anti-hFasL antibodies (4H9 and biotinylated 4A5).

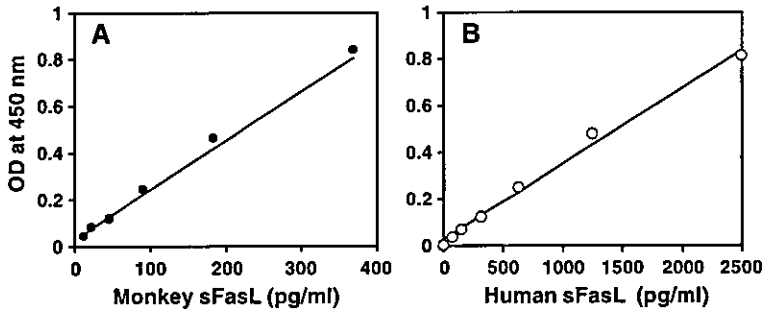

Fig. 5. Standard curves for monkey sFasL and human sFasL determined by ELISA. Purified monkey (A) and human (B) recombinant sFasL were serially diluted and the reactivity was determined by ELISA using 4H9 for capture and biotinylated 4A5 for detection.

models. Since the amino acids sequence is completely identical among three species of macaque monkeys, this ELISA system is also potentially useful for rhesus and pig-tailed monkeys. Although the role of sFasL in the progression of disease remains controversial, shedding of FasL from the membrane plays an important role in controlling Fas/FasL-induced apoptosis. Both the in vitro assay system for apoptosis induced by recombinant monkey sFasL and the highly sensitive monkey sFasL measuring system established in the present study will be useful for exploring the significance of Fas/FasL-induced apoptosis in monkey disease models, including AIDS (Letvin et al., 1985), malaria (Matsumoto et al., 2000), and organ transplantation (van Beusechem et al., 1992).

Acknowledgements

This work was supported by the Japan Health Sciences Foundation. We thank Drs. Hirofumi Akari of National Institute of Health Japan and Hiroshi Ishiguro of Nippon Organon for helpful discussions.

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Primates (2003) 44:291–294
DOI 10.1007/s10329-002-0016-6

SHORT COMMUNICATION

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Familial early onset macular degeneration in cynomolgus monkeys (*Macaca fascicularis*)

Received: 12 August 2002 / Accepted: 30 November 2002 / Published online: 14 March 2003
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Abstract The mode of inheritance of macular degeneration was determined with 45 cynomolgus monkeys (18 females and 27 males) who were the offspring of one breeding male with typical macular degeneration. In the first generation, 27 offspring (10 females and 17 males) were born from mating between the macular degeneration-affected founder male and 5 normal female breeders. Among them, 18 monkeys (9 females and 9 males) were judged as having macular degeneration (affected). Next, the distribution of affected offspring was examined with 18 offspring who were born from 3 different mating pairs, normal vs normal, affected vs normal and affected vs affected, when they became 2 years old. All of the 9 monkeys (4 females and 5 males) obtained from the 2 pairs of normal vs normal were normal. On the other hand, 6 affected monkeys (3 females and 3 males) were detected in 8 offspring from the mating pair of affected vs normal, and the single offspring produced by the mating pair of affected vs affected was affected. These results showed that this degeneration must be early onset familial macular degeneration controlled by autosomal dominant gene(s).

Keywords Cynomolgus monkey · Macular degeneration · Family study

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Introduction

The study of age-related eye diseases is an important research field because the number of aged is rapidly increasing. So far there is no suitable animal model for tardive eye diseases such as glaucoma, cataracts, and retinal degeneration. The establishment of an eye disease model bearing a close resemblance to human diseases is important to elucidate causes, diagnosis, treatments, and/or prophylaxis for age-related human ophthalmologic diseases. Since the primate-specific eye diseases such as macular degeneration of the retina do not occur in rodents (Rubin 1974a), the use of monkeys which are the most closely related animals to humans is essential for the elucidation of these problems. Research into such primate-specific eye diseases and their development is much needed.

Although several hereditary familial eye diseases including familial macular degeneration have been reported in humans (Ohba 1988), there is no reported evidence of familial eye disease in nonhuman primates. We have already reported that a high distribution rate of macular degeneration was determined among offspring from one male cynomolgus monkey who was judged to have typical macular degeneration (Suzuki 1998). In this paper, the mating experiment was performed to determine whether this degeneration is an hereditary eye disease.

Methods

Subject animals

The ocular fundi was observed from 6 wild-originated (1 male and 5 females) and 60 laboratory-born cynomolgus monkeys (26 males, 34 females) all of which were reared in Tsukuba Primate Center for Medical Science (Honjo 1985). They were kept under clean indoor environmental conditions where temperature and relative humidity were set at 25 ± 2°C and 60 ± 5%, respectively. Fresh air was provided by a ventilation cycle of 10 times/hour, and artificial lighting was put on for 14 continuous hours/day. The animals were fed with

well-washed apples and oranges (100 g each) in the morning and 70 g monkey chow (AS type; Oriental Yeast, Tokyo) in the afternoon. Tap water was supplied ad libitum. New born animals were nursed by their mothers for about 15 weeks after birth until weaning. After that, a group rearing system by age was adopted until they became 3–4 years old, where plural animals of the same age were reared in one cage. Thereafter, they were caged individually.

Observation of ocular fundi

About 20 min before examining the ocular fundi, one drop of tropicamide-phenylephrine hydrochloride (Mydrin-P; Santen Pharmaceutical) was instilled into both eyes of each animal for dilation of the pupils and then the monkeys were anesthetized by intramuscular injection of 10 mg/kg ketamine-HCl (Ketalar-50; Sankyo, Tokyo). Photographs were taken with a Kowa RC-2 ophthalmoscope camera with daylight-type color film (Fujichrome ASA 100; Fuji Photo Film, Tokyo).

Mating experiments

One wild-originated founder male monkey (Fig. 1 fM) affected by typical macular degeneration was mated with 5 normal wild-originated female monkeys (Fig. 1 family A–E), and produced 27 F_1 monkeys (Fig. 1 family A–E, 0–10 years of age; 10 females and 17 males). Four different mating pairs were set to determine whether this macular degeneration is a hereditary eye disease. Two pairs consisted of two normal F_1 males and ten unrelated normal females (Fig. 1 H, I). One pair consisted of the affected F_1 male and five normal unrelated females (Fig. 1 F) and one of the affected male (fM) and an affected F_1 female (Fig. 1 G). In total 18 F_2 monkeys (8 females and 10 males) were obtained from 15 different mating pairs (Fig. 1). The ocular fundi was observed with F_2 offspring at 2 years old. The protocol of the experimental procedures was approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases. All monkeys were cared for and treated humanely in accordance with the rules for care and management of the Tsukuba Primate Center (Honjo 1985), and with the Guiding Principles for Animal Experiments using Non-Human Primates formulated by the Primate Society of Japan (Primate Society of Japan 1986).

Results

Figure 1 shows the distribution of macular degeneration-affected monkeys among offspring of one wild-originated affected male breeder (fM). The ocular fundi could be examined from 27 F_1 monkeys born from the matings between the affected male and five wild-originated normal females (A–E). Among them, 18 monkeys (9 males and 9 females) were judged as affected (66.7%). The incidence of affected monkey is 90% (9/10) in female and 65.3% (9/17) in male offspring. In F_2 monkeys, a normal ocular fundi was observed with all nine F_2 monkeys who were born from two mating pairs of normal males and ten normal females (H and I). An affected ocular fundi was observed in six F_2 monkeys (three males and three females; 75%) among eight offspring obtained from the mating pair of one affected male and five normal females (F). Only one affected F_2 male monkey was determined from the mating pair composed of the original affected founder male and affected daughter (G). All affected monkeys showed normal behavior in individual cages.

Fine dots colored in grayish white or yellowish white indicated the lesions and were observed in the maculae. The locations of the lesions mostly fell within the region centered on the fovea centralis with the same diameter as one optic disk. No other abnormalities were observed in the optic disk, the blood vessels of retina or peripheral regions of the retina (Fig. 2).

Table 1 summarizes the distribution of affected F_1 and F_2 monkeys obtained from three different mating pairs, normal vs normal, affected vs normal and affected vs affected. There is no difference ($X^2 = 3.182$, $df = 1$) in incidence of affected monkeys between female and male offspring obtained from the mating pair of affected and normal monkeys.

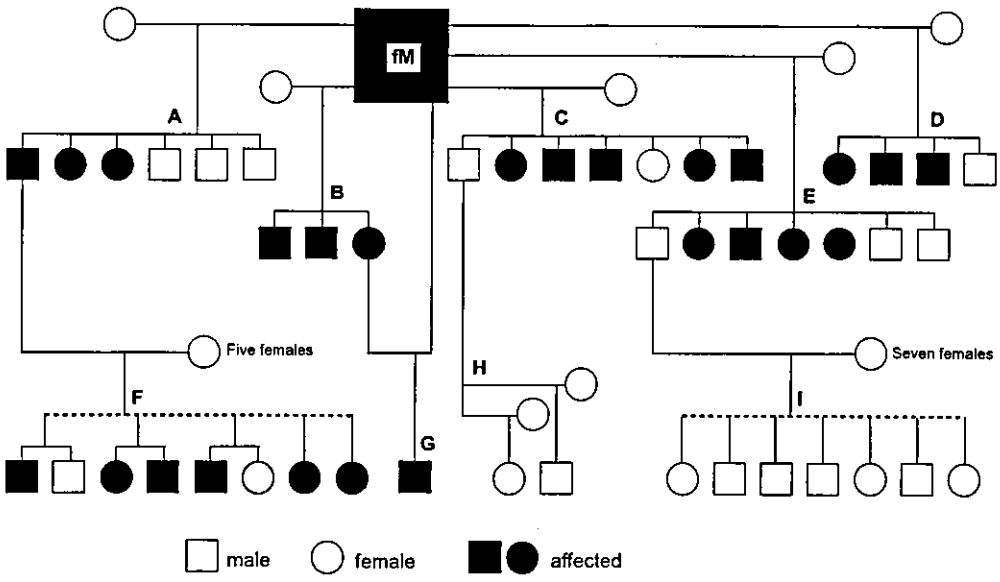

Fig. 1 Pedigree of cynomolgus monkeys with macular degeneration. *fM* Original founder breeding male monkey with typical macular degeneration. A *solid line* shows full sibs and *dashed line* shows half sibs

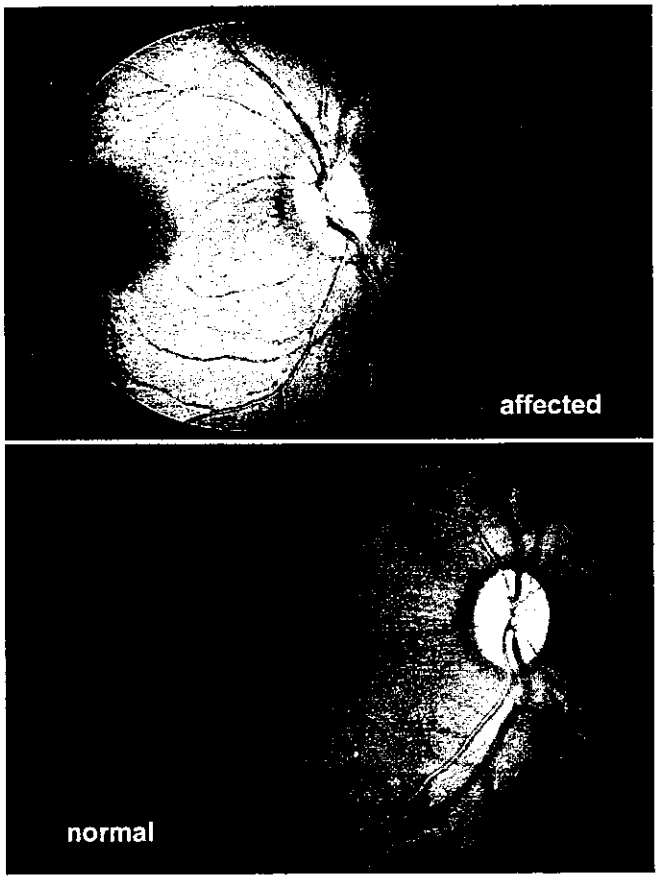

Fig. 2 Fundus photographs of a cynomolgus monkey with macular degeneration. *Upper* affected 5-year-old male cynomolgus monkey. Fine dots colored in grayish white or yellowish white can be seen in the macula. *Lower* normal 8-year-old female cynomolgus monkey

Table 1 Distribution of macular degeneration among offspring

Mating pair		Offspring			
Male	Female	Male		Female	
		Normal	Affected	Normal	Affected
Normal	Normal	5	0	4	0
Affected	Normal	9	12	2	12
Affected	Affected	0	1	0	0

Discussion

A variety of reports have been published concerning hereditary retinopathy of humans (Ohba 1988). Even if the types of retinopathy are limited to those which occur in the macular, many conditions, including Stargardt disease, cone dystrophies, vitelliruptive macular degeneration, inverse retinitis pigmentosa and others, have been mentioned (Ohba 1988). For these eye diseases, a lot of work has been done to trace their origins while conducting treatments, and analysis taking advantage of genetic engineering is currently being undertaken (Uehara 1988).

In terms of spontaneous macular degeneration (central retinal degeneration) in mammals, reports which describe the disease in cats, monkeys and others have been published (Bellhorn 1981; Dawson et al. 1989; Fukui et al. 1985; Nicolas et al. 1996; Rubin 1974a, 1974b; Stafford et al. 1984; Vainisi et al. 1974). For monkeys, rhesus monkey (*Macaca mulatta*; Bellhorn 1981; Dawson et al.1989; Rubin 1974b; Stafford et al. 1984), Guinea baboon (*Papio papio*; Vainisi et al.1974), and cynomolgus monkey (Nicolas et al. 1996) have been mentioned as developing the disease.

The outcome of the present study is that the lesions in the macular can be observed as dots colored grayish white or yellowish white with regular fundus photography used as a part of clinical tests for morphological analysis. A few or up to 20 dots can be observed per macular. These findings were first obtained in the 1 wild-originated male monkey and his 18 offspring.

At present the disease is considered as hereditary based on the results of mating experiments using F_1 male monkeys as breeders. The F_2 monkeys born from the mating between the affected F_1 male and normal females did develop the disease, while the other F_2 monkeys born from the mating between normal F_1 male and normal females did not. In addition, there is no difference in distribution of affected monkeys between female and male offspring obtained from the mating between affected males and normal females. It is appropriate to consider that the disease is controlled by autosomal dominant gene(s). If we assume the wild-originated founder male has the heterozygous dominant character as its genotype, mating with unaffected female monkeys of bottom recessive character must produce both affected F_1 monkeys of heterozygous dominant character and unaffected normal F_1 monkeys. In fact, a total of 14 F_1 monkeys obtained from the experiments actually developed the disease and were thought to be of that character. Moreover, while some of the F_2 monkeys derived from the affected F_1 generation (heterozygous dominant character) developed the disease, not even a single F_2 derived from the unaffected F_1s (bottom recessive) did. The likelihood that the wild-originated male monkey is of simple homozygous dominant or recessive character can be considered negligible due to the fact that some of the F_1s developed the disease. Another possibility that the naturally-derived male monkey is of homozygous dominant character with a certain penetrance can also be denied; if he was, all of the F_1s would be of heterozygous dominant character with the disease developed. However, there could still be an alternative possibility which considers the existence of a certain penetrance so that while all the F_1s carry the gene for the disease, some F_1s might have a phenotype which does not develop any lesions in the macular. This theory might explain why some F_1s do not seem to develop the disease. However, in accordance with this theory, because the F_1s would carry the gene for the disease as heterozygotes even if they didn't develop the disease, some of the F_2 monkeys derived from these F_1s should

develop the disease. The clear result of the mating experiments where not even a single F_2 derived from the unaffected F_1s developed the disease make the latter possibility untenable.

Diabetes mellitus observed in black Celebes apes (*Macaca nigra*) has been the only report of hereditary disease in monkeys (Howard 1977). The report on black Celebes apes, however, described a case study only tracing up to the F_1 generation. Here, we report on the outcome of hereditary disease models with macular degeneration based on a case study embracing the F_2 generation. Moreover, maintenance of this disease model is a relatively easy procedure, because the present disease is considered to be a dominant hereditary disease. The establishment of this model monkey with early onset macular degeneration is expected to contribute to the elucidation of the causes, diagnosis, treatments, and/or prophylaxis of the same disease in humans in the immediate future.

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