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主論文I



# Membrane Fas ligand kills human peripheral blood T lymphocytes, and soluble Fas ligand blocks the killing

Takashi Suda*‖, Hideo Hashimoto*†‖, Masato Tanaka§, Takahiro Ochi†, and Shigekazu Nagata*§

*Osaka Bioscience Institute, Department of Molecular Biology, Suita, Osaka 565, Japan

†Osaka University Medical School, Department of Orthopaedic Surgery, and §Department of Genetics, Suita, Osaka 565, Japan

‖The first two authors contributed equally to this manuscript.

Running Title;

Differential cytotoxic activity of membrane and soluble Fas ligand

Address Correspondence to Takashi Suda, Osaka Bioscience Institute, Department of Molecular Biology, 6-2-4 Furuedai, Suita, Osaka 565, Japan.

TEL, 81-6-872-4850; FAX, 81-6-871-6686; e-mail, sudat@obi.or.jp

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**Summary**

It has been believed that Fas expressed on human peripheral blood T cells (PBT) is non-functional, because these cells are insensitive to agonistic anti-Fas/Apo-1 mAbs that efficiently kill *in vitro* activated T cells and many Fas-expressing cell lines. Here, we demonstrated that membrane Fas ligand (FasL) kills both fresh and *in vitro* activated PBT, indicating that Fas expressed on fresh PBT is functional. In contrast, soluble FasL kills only the latter. Naive T cells in natal code blood do not express Fas, but are induced to express Fas by IFNγ or by a combination of IL-2 and anti-CD28 mAb, and acquire sensitivity to membranous but not soluble FasL. Soluble FasL inhibited the killing of fresh PBT by membrane FasL. These results indicate that the shedding of membrane FasL is a mechanism that down-regulates at least a part of its killing activity.

## Introduction

Fas (Apo-1/CD95) and Fas ligand (FasL) play crucial roles in homeostasis and self-tolerance of lymphocytes in both humans and mice (1). FasL also works as a cytotoxic effector molecule of CTL and NK cells. FasL is constitutively expressed in the testis and eyes, and it has been implicated in the immune privileged property of these organs (2, 3). At the same time, the Fas-FasL system is a double-edged sword (4) in that both the diminishment and enhancement of this system have pathogenic effects. Loss-of-function mutations of Fas and FasL result in lymphoproliferative and autoimmune diseases. FasL expressed on activated T cells seems partly responsible for the tissue damage in fulminant hepatitis (5) and graft-versus-host disease (6, 7). It has been reported that thyrocytes in Hashimoto disease patients express both Fas and FasL and commit suicide like activation-induced cell death of T cells (8). It has been suggested that the Fas-FasL system plays a role in the loss of $CD4^+$ T cells in AIDS patients (9, 10). Therefore, strategies to up-regulate or down-regulate the Fas-FasL system might have therapeutic value for these diseases. However, since Fas is expressed rather ubiquitously in a wide range of normal tissues, side-effects of such treatments on normal cells should be carefully evaluated.

FasL is a 40kD type II transmembrane protein homologous to TNF, and induces apoptosis through its membrane receptor, Fas (11). TNF$\alpha$, which is also synthesized as a type II transmembrane protein, is cleaved into a 17kD soluble form by proteolysis, and it causes cachexia. Recently, the novel disintegrin metalloprotease (TNF alpha-converting enzyme, TACE) that mediates this process was identified (12, 13). Similarly, the TNF homologous portion of

membrane FasL is cleaved into a 26kD soluble form by a metalloprotease (14, 15), although it is unknown whether TACE cleaves FasL. This soluble human FasL efficiently induces apoptosis in some Fas expressing cells. In contrast, mouse soluble FasL loses its killing activity (16).

The function of Fas expressed on various cells has been investigated using agonistic anti-Fas/Apo-1 mAbs, and the results indicated that the expression of Fas does not necessarily mean susceptibility to these mAbs (17, 18). A large fraction of peripheral blood T cells (PBT) freshly isolated from adult humans express Fas. These $Fas^+$ PBT correspond to $CD45RO^+$ PBT cells that have been postulated to be memory T cells. In spite of readily detectable levels of Fas expression, these cells are totally resistant to agonistic anti-Fas mAbs (19-21). Virtually all murine T cells from peripheral lymphoid organs constitutively express Fas, but they are also resistant to anti-Fas mAb treatments (22). Therefore, it has been concluded that Fas expressed on peripheral mature T cells is non-functional. However, we recently discovered that recombinant soluble mouse FasL which carries the complete extracellular region of FasL (termed WX1) efficiently kills mature T cells in freshly isolated mouse splenocytes or lymph node cells (16). It should be noticed that, unlike naturaly processed mouse soluble FasL, WX1 which contains almost the entire extracellular region of mouse FasL is biologically active.

In this report, we examined the susceptibility of human PBT to the membrane and soluble human FasL. They exhibited differential cytotoxic activity.

## Materials and Methods

*Preparation of adult peripheral and natal code blood lymphocytes.* Heparinized adult peripheral and natal code blood were obtained from a healthy adult volunteers and new born babies delivered by Caesarean section after getting informed consent, respectively. Samples were diluted 2 fold with PBS and the lymphoid fraction was separated by gradient centrifugation using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden). ConA blasts were prepared from adult PBL by cultivation with 5 μg/ml of ConA (Sigma Chemical Co., St. Louis, MO) and 10 ng/ml of recombinant human IL-2 (Ajinomoto Pharmaceutical Co., Tokyo, Japan) for 48 h, followed by incubation in the presence of IL-2 alone for 5 days. Dead cells were removed by gradient centrifugation using Histopaque 1083 (Sigma). Cells were washed twice and suspended in RPMI 1640 medium (Nikken BioMedical Laboratory, Kyoto, Japan) supplemented with 10% FCS, 100 U/ml benzylpenicillin potassium (Meiji Seika Co., Tokyo, Japan), and 100 μg/ml streptomycin sulfate (Meiji Seika). To induce Fas expression in natal code blood lymphocytes, cells were cultured for 20 h with recombinant human IFNγ (10 ng/ml, Pepro Tech Inc., Rocky Hill, NJ), IL-2 (10 ng/ml) or in a plate coated with anti-CD28 mAb (PharMingen, San Diego, CA) at 10 μg/ml in 10 mM Tris-HCl pH8.0 at 37°C for 1 h. Further analysis of the expression and function of Fas in these cells were performed as described below.

*Induction of apoptosis by transformants expressing FasL, soluble FasL and anti-Fas monoclonal antibody.* Transformant 1A12 expressing human FasL lacking a cytoplasmic region (amino acid 8-69) was established as previously described (23). Soluble human FasL was purified from the supernatant of the 1A12 cells

using an affinity column coupled with anti-human FasL mAb (4H9) (24), and the purified protein was designated as AL-1. Recombinant soluble mouse FasL (WX1) consisting of the signal sequence derived from G-CSF and almost the entire extracellular region of mouse FasL (amino acid 101-279) was prepared as described previously (16), and purified using an anti-mouse FasL mAb affinity column. Anti-human Fas IgM monoclonal antibody (CH11) was purchased from MBL (Nagoya, Japan). To investigate the susceptibility of the cells to soluble FasL or anti-Fas mAb, 2 x $10^5$ fresh PBL or 6 x $10^5$ ConA blasts were cultured with various concentrations of WX1, AL-1, and CH11 in 200 µl of culture medium in a flat bottomed 96-well culture plate at 37°C in a 5% $CO_2$, humidified atmosphere for 14 h. The same number of fresh PBL were cocultured with 1A12 cells at various effector/target ratios for 12 h. In some experiments, 1A12 cells were preincubated with 10 µM of BB2116 matrix metalloprotease inhibitor (kindly provided by Dr. A. H. Drummond, British Biotech Pharmaceuticals, Oxford, UK) for 4 h, and it was continuously included in the following assay to obstruct the cleavage of membrane-bound FasL.

*Evaluation of Fas expression and cell death by flow cytometry.* After incubations to induce apoptosis, aliquots of the cells were stained with optimal doses of FITC-conjugated anti-Fas mAb (UB-2, MBL), PE-conjugated mAbs against either CD3, CD4 or CD8 (Becton Dickinson, San Jose, CA), and 2 µg/ml of propidium iodide (PI) in 50 µl of staining solution for 30 min on ice, and then washed 3 times with staining solution. These cells were immediately analyzed in a FACScan® (Becton Dickinson) equipped with a 488-nm argon laser, and the data were analyzed using CELLQuest software. % Specific Cell Killing was calculated as follows.

% Specific Cell Killing = (% $PI^+$ cells - % spontaneous $PI^+$ cells in medium only) / (100 - % spontaneous $PI^+$ cells in medium only)

**Results**

*WX1, but not soluble human FasL or anti-Fas mAb kills freshly isolated PBL.* About 50% of $CD4^+$ and $CD8^+$ T cells in freshly isolated PBL from a healthy adult volunteer and virtually all Concanavalin A (ConA) blasts generated from the PBL expressed readily detectable levels of Fas (data not shown). Consistent with previous reports, agonistic anti-Fas mAb (CH11) efficiently killed ConA blasts, whereas fresh PBT were resistant to the mAb (Figure 1a). We previously established a transfectant expressing human FasL which lacks most of its cytoplasmic region. This cell line (1A12) secretes soluble FasL by a proteolytic mechanism (23). The soluble FasL was purified using an affinity column with anti-human FasL mAb. This purified soluble human FasL (named AL-1) showed similar target specificity to CH11 (figure 1b). In contrast, biologically active recombinant soluble mouse FasL, WX1 killed both ConA blasts and fresh PBT (Figure 1c-g). Since WX1 specifically killed $Fas^+$ cells in PBT, which consist of about 50% of the total PBT (Figure 1d,e), the dose-response curves of WX1-induced death in ConA blasts and fresh $Fas^+$ PBT are comparable. There was no difference between CD4 and CD8 T cells in terms of susceptibility to various Fas agonists.

*Membrane FasL kills freshly isolated PBL, and soluble FasL inhibits this activity.* The cytotoxicity of membrane-bound FasL to fresh PBT was investigated using a transfectant (1A12) expressing human FasL and its parental cell line (WR19L). The metalloprotease inhibitor BB2116 prevents proteolytic secretion of FasL from 1A12 cells (23). As shown in Figure 2a, 1A12 killed fresh PBT in a dose dependent manner, and BB2116 dramatically enhanced the

cytotoxicity of 1A12. WR19L showed no cytotoxicity even in the presence of BB2116. BB2116 did not enhance the cytotoxicity of WX1 (data not shown), excluding the possibility that the weak toxicity of BB2116 rather than its protease inhibitor activity caused the enhancement of 1A12-mediated killing. Fas$^+$ PBT were specifically killed by 1A12 (Figure 2b-d). Since 1A12 cells produce soluble FasL in this condition, it was then tested whether soluble FasL inhibits the cytotoxicity of 1A12 against PBT. As shown in Figure 2e, exogenously added soluble FasL inhibited the cytotoxicity of 1A12 in the presence of BB2116 in a dose dependent manner. Similarly, anti-Fas mAb inhibited killing of PBT by 1A12 (Figure 2f). These results indicate that membrane FasL is capable of killing fresh Fas$^+$ PBT, whereas soluble FasL and anti-Fas mAb behaves as an Fas-antagonist against these cells in these conditions.

*IFNγ and a combination of IL-2 and immobilized anti-CD28 mAb induces Fas expression and susceptibility to 1A12 cells and WX1 but not to AL-1 or CH11 in PBL.* Essentially all code blood T cells (CBT) are CD45RO$^-$ naive T cells and do not express Fas at detectable levels. Accordingly, they are not killed by any forms of FasL or anti-Fas mAb (data not shown). Previously, it was reported that CBT cultured with phytohemagglutinin for 5 days express Fas (20). Here, we found that IFNγ induces Fas expression on both CD4$^+$ and CD8$^+$ CBT within 20 h (Figure 3a,f). IL-2 alone induced FasL weakly, while other stimuli including IL-1, IL-4, IL-6, TNFα and immobilized anti-CD28 mAb did not, when added separately (Figure 3b-h, and data not shown). However, a combination of IL-2 and anti-CD28 mAb showed Fas-inducing activity comparable to IFNγ (Figure 3d, i). The levels of Fas expression induced by IFNγ plus

anti-CD28 mAb were not much different from those induced by IFNγ alone (data not shown).

Then we tested the susceptibility of Fas-induced CBT to various forms of FasL and anti-FasL mAb. As shown in Figure 4k, CBT treated with IFNγ or a combination of IL-2 and anti-CD28 mAb were killed by 1A12 cells in the presence of BB2116 and WX1, whereas the same cells were resistant to AL-1 and CH11.

**Discussion**

We have shown that the membrane form of FasL is capable of killing $Fas^+$ PBT. The $Fas^+$ fraction of PBT overlaps with $CD45RO^+$ memory T cells (20). In contrast, naive T cells in adult or natal code blood do not express Fas, hence they are not killed by any types of FasL or anti-Fas mAb. However, IFNγ and a combination of IL-2 and anti-CD28 mAb rapidly induce Fas expression in naive T cells from cord blood, and as a result, these cells become sensitive to membrane FasL. We have previously reported that freshly isolated mouse T cells from peripheral lymphoid organs, which are resistant to anti-mouse Fas mAb, are susceptible to WX1 (16). The cytotoxicity of membrane FasL against peripheral T cells is not specific to transfectant expressing high levels of FasL, because activated T cells from B10-$Thy1.1^a$ (Thy1.1) mice induced cell death in naive T cells from B10 (Thy1.2) mice in a FasL dependent manner (data not shown). Collectively, these results demonstrated that Fas expressed on peripheral T cells of humans and mice is functional. Why fresh PBT, which is potentially useful cells, should be killed by membrane FasL? T cells are activated through TCR in an Ag-specific manner. However, once T cells are activated, they produce a variety of cytokines and cell surface co-stimulative molecules that can activate bystander T cells in an Ag-nonspecific manner. Therefore, when some naive or memory T cells with inappropriate Ag-specificity accidentally encounter activated T cells at an inflammatory site, they should be killed probably through FasL expressed by activated T cells; otherwise they would be activated Ag-nonspecifically. We previously demonstrated that TCR engagement induces FasL-resistance in mouse splenic T cells. Therefore, naive or memory T cells with appropriate TCR, which are

thereby acquiring antigenic signals, would not be killed by activated T cells (16).

Soluble human FasL is biologically active in that it has potent cytotoxicity against some Fas expressing cell lines (14). In contrast, its mouse counterpart is inactive. Since Fas is constitutively expressed in various tissues, if soluble FasL is fully functional, it may be detrimental to humans. Here, we found that soluble human FasL possesses a selective killing activity. Potentially useful PBT are not killed by AL-1. In contrast ConA blasts are killed by AL-1. The latter cells may represent useless or even dangerous senescent activated T cells. Human recombinant soluble FasL produced in yeast, which has similar target selectivity to naturally processed soluble FasL (data not shown), induces lethal hepatic failure, but only when a high dose (500 μg/mouse) of the FasL is intravenously injected in mice (24). In contrast, a small number of a CTL clone can induce lethal hepatitis in mice (25), and the lethal activity is mediated by FasL (5). These results suggest that cytotoxicity of soluble FasL against hepatocytes is also limited compared to that of membrane FasL. This notion has been confirmed by an *in vitro* cytotoxicity assay against primary culture hepatocytes (MT and SN, manuscript in preparation). It has been shown that membrane TNFα is far more efficient than soluble TNFα in activating type II TNF receptor (26). These results indicate that the membrane-bound form of FasL and TNFα is the primary activator of their receptors. We discovered that soluble human FasL competitively inhibits the killing of PBT by membrane FasL. Collectively, these results indicate that the cleaving of membrane ligands is a mechanism that partly down-regulates their activities.

Why membrane-bound and soluble FasL have different target

spectrums? In relation to this phenomenon, various Fas agonists including soluble and membrane FasL as well as anti-Fas mAbs are separable into two groups based on their killing activity against fresh PBT and ConA blasts. Membrane FasL and WX1 kill both fresh PBT and ConA blasts. Whereas "agonistic" anti-Fas mAbs and AL-1 kill ConA-activated T cells but not fresh PBT. Furthermore, the latter group inhibits the killing activity of the first group, indicating that they can bind to Fas on fresh PBT. Since both AL-1 and WX1 are soluble proteins, solublility does not separate the two groups. The fact that purified WX1 can kill fresh PBT makes unlikely the possibility that an unidentified molecule coexpressed with membrane FasL on 1A12 cells is required as a co-stimulative molecule to facilitate the FasL-mediated death of fresh PBT. Analytical gel filtration indicated that AL-1 exists as a trimer (M.T., S.N., unpublished observation) whereas a majority of WX1 is oligomer larger than a decamer (16). Therefore, it is possible that the trimerization of Fas is sufficient in killing ConA blasts, whereas the formation of larger complexes of Fas is required to kill fresh PBT. However, this possibility seemes unlikely because the anti-human Fas mAb, CH11 that belongs to the second group is a decavalent IgM Ab. Furthermore, super-cross-linking of Fas on PBT by sequential treatments with CH11 and anti-mouse IgM antibodies induced only a minimum death in $CD4^+$ cells, and it had absolutely no effect on $CD8^+$ cells (data not shown). Affinities or avidities of various agonists may determine their target spectrum. Recently, Fadeel et al. (27) reported that various anti-Fas mAbs that recognize the same epitope exhibit different effects on Fas mediated apoptosis, and that those mAbs with a moderate affinity killed Fas expressing cells, while high affinity Abs were antagonistic

and low affinity Abs showed no biological effect. However, the reason why AL-1 inhibits the cytotoxicity of membrane FasL is probably not because AL-1 has a very high affinity, since rather large doses of AL-1 were required to inhibit cytotoxicity of membrane FasL. Alternatively, membrane FasL and WX1 but not anti-Fas mAbs or AL-1 may interact with, in addition to Fas, another molecule expressed in fresh PBT, and this interaction may be necessary to kill fresh PBT. Since WX1, which is a soluble protein, somehow mimics membrane FasL rather than soluble FasL, careful comparison of WX1 and AL-1 may elucidate this question. WX1 is also a useful tool to investigate Fas sensitivity of a group of cells that are only killed by membrane FasL or WX1.

Since agonistic anti-Fas/Apo-1 mAbs show strong cytotoxicity against a variety of tumors (28, 29) or abnormally proliferated synovial cells in rheumatic arthritis (30), a possible therapeutic value of these mAbs for these diseases has been discussed. The main concern has been the toxicity of these molecules against normal cells. Our finding that some soluble FasL and an anti-Fas mAb show a selective killing activity opens up the possibility that some tumor cells or abnormally activated cells are more sensitive than normal cells to these molecules. If this is the case, these molecules at a sublethal dose may be therapeutic for tumor or rheumatoid arthritis patients.

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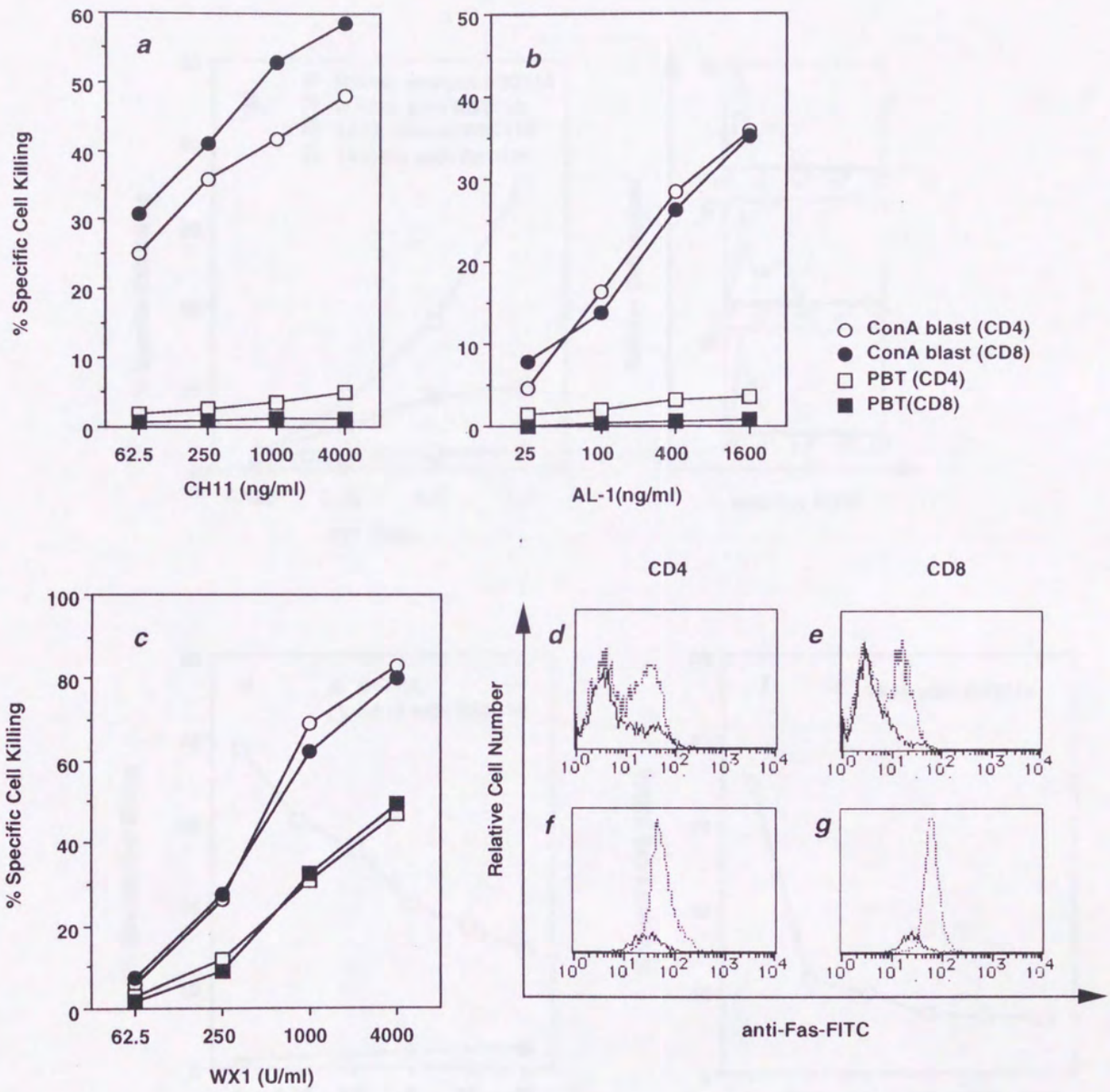
## Figure Legends

**Figure 1.** Susceptibility of fresh PBT and ConA blasts to anti-Fas mAb (CH11), soluble human FasL (AL-1) and recombinant soluble mouse FasL (WX1). a-c, Fresh PBL or ConA blasts were cultured in the presence of the indicated concentrations of CH11 (a), AL-1 (b) and WX1 (c) for 14 h. Cells were then stained with FITC-anti-Fas mAb, PE-conjugated mAb against either CD4 or CD8, and PI. % Specific Cell Killing of $CD4^+$ and $CD8^+$ cells was determined as described in the Experimental Procedure. d-g. Fresh PBL (d,e) and ConA blasts (f,g) cultured with (solid line) or without 4000 units/ml of WX1 (dotted line) for 14 h were stained as described above, and $5 \times 10^4$ total cells (including both viable and dead cells) were analyzed in a FACScan®. Staining profiles for FITC-anti-Fas mAb of viable ($PI^-$) $CD4^+$ (d,f) or $CD8^+$ cells (e,g) are shown.

**Figure 2.** Membrane FasL kills fresh PBT and soluble FasL inhibits the killing. a. Fresh PBL were cultured with the transfectant expressing human FasL (1A12) or its parental cell line (WR19L) at the indicated effector/target (E/T) ratios for 12 h in the presence or absence of 10 μM of BB2116. Cells were then stained with FITC anti-Fas, PE-anti-CD3 mAb and PI, and analyzed in a FACScan®. b-d. Staining profiles for FITC anti-Fas mAb of $PI^-CD3^+$ cells after culture with WR19L in the absence of BB2116 (b), or with 1A12 in the absence (c) or presence of BB2116 (d) at the E/T ratio of 1.0 are shown. Dotted profiles indicate negative controls stained with FITC-labeled mouse IgG1. e,f. Fresh PBL were cultured with 1A12 plus 10 μM BB2116 or with WR19L cells at the E/T ratio of 1.0 in the presence of the indicated concentrations of purified AL-1 (e) or

CH11 (f) for 12 h.

**Figure 3.** Function of Fas on CBT induced by IFNγ or a combination of IL-2 and immobilized anti-CD28 mAb. a-h. Natal code blood lymphocytes were cultured for 20 h with medium only (dotted line in each panel), IFNγ (10 ng/ml, a,e), IL-2 (10 ng/ml, b,f), immobilized anti-CD28 mAb (coated on plate at 10 μg/ml, c,g), or IL-2 plus immobilized anti-CD28 mAb (d,h), respectively. Cells were then stained with FITC-anti-Fas mAb, PE-conjugated mAb against either CD4 or CD8, and PI. Staining profiles for FITC-anti-Fas mAb of viable ($PI^-$) $CD4^+$ (a-d) or $CD8^+$ cells (e-h) are shown. i,j. CBT treated with IFNγ or with IL-2 plus anti-CD28 mAb as described above were cultured with CH11, WX1, AL-1 or 1A12.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CD8
Relative Cell Number
anti-Fas-FITC

Fig.1

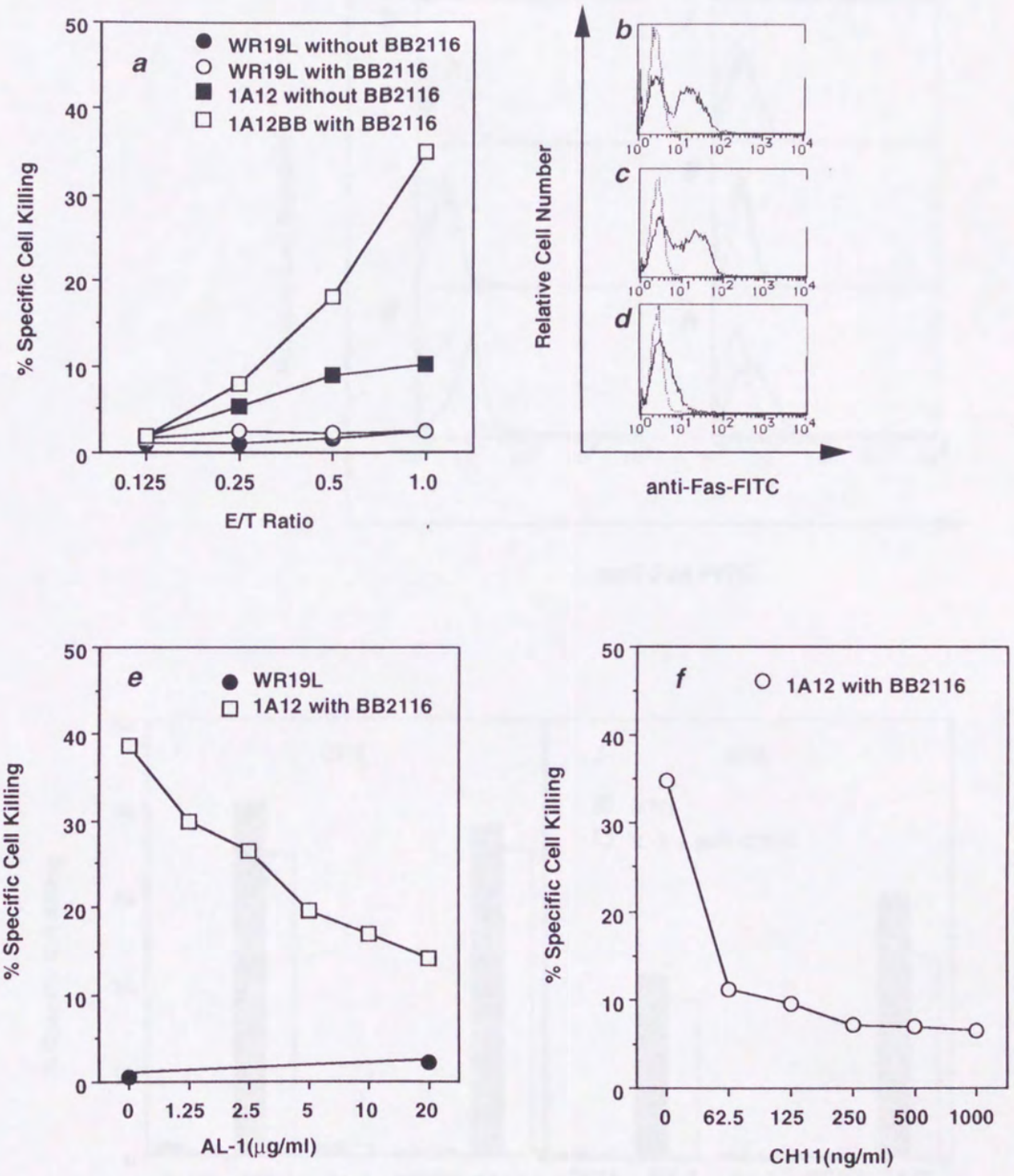
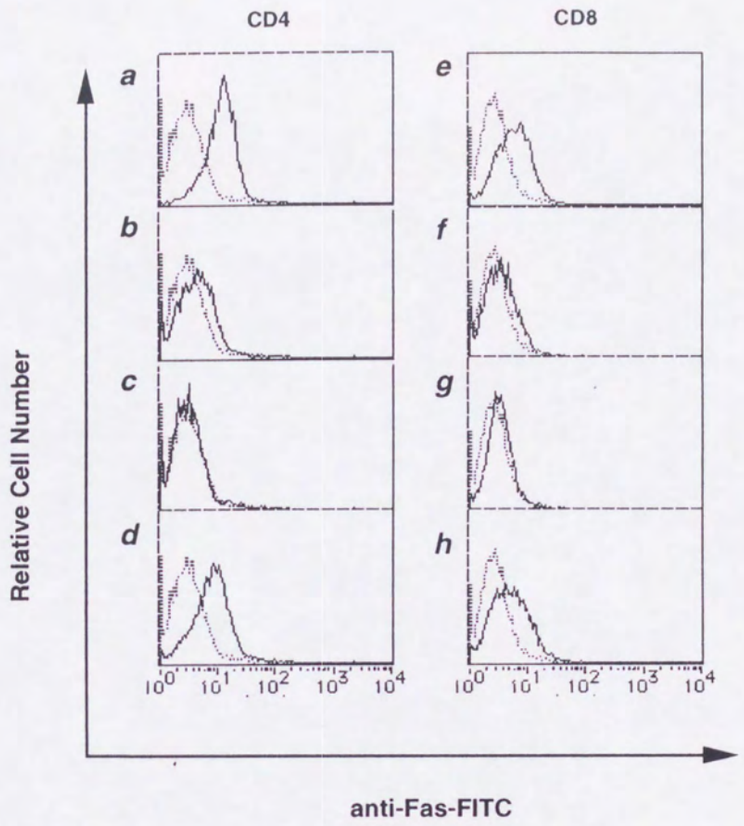

a
WR19L without BB2116
WR19L with BB2116
1A12 without BB2116
1A12BB with BB2116
% Specific Cell Killing
E/T Ratio
0.125
0.25
0.5
1.0
b
c
d
Relative Cell Number
anti-Fas-FITC
e
WR19L
1A12 with BB2116
% Specific Cell Killing
AL-1(μg/ml)
0
1.25
2.5
5
10
20
f
1A12 with BB2116
% Specific Cell Killing
CH11(ng/ml)
0
62.5
125
250
500
1000

Fig.2


CD4
CD8
a
b
c
d
e
f
g
h
Relative Cell Number
anti-Fas-FITC

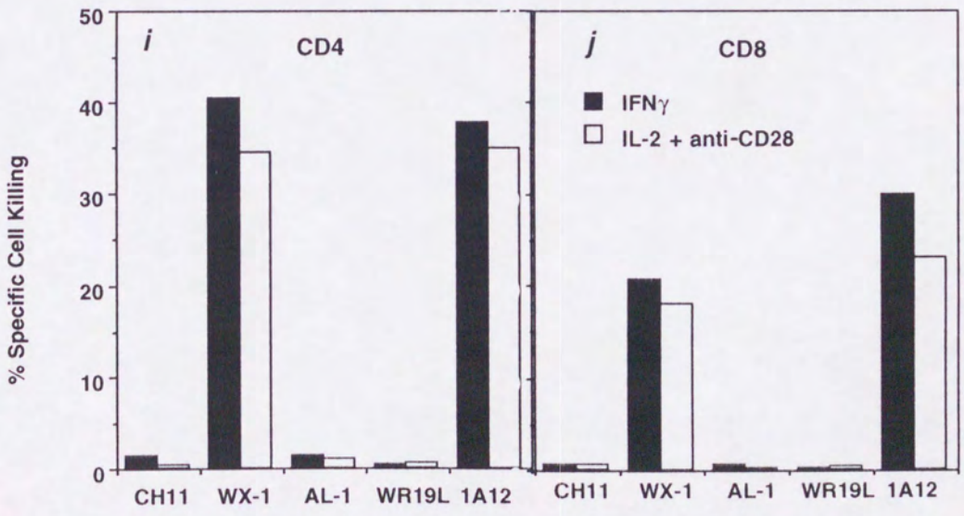

i
CD4
j
CD8
IFNγ
IL-2 + anti-CD28
% Specific Cell Killing
50
40
30
20
10
0
CH11
WX-1
AL-1
WR19L
1A12

Fig.3

主論文Ⅱ

# Soluble Fas Ligand in the Joints of Patients with Rheumatoid Arthritis and Osteoarthritis.

Hideo Hashimoto *§,MD, Masato Tanaka †,MD, PhD, Takashi Suda §,PhD, Tetsuya Tomita *,MD, PhD, Kenji Hayashida *,MD, PhD, Eiji Takeuchi*,MD, Motoharu Kaneko*,MD , Hiroshi Takano*,DDS, Shigekazu Nagata †§,PhD, and Takahiro Ochi*,MD, PhD.

*Osaka University Medical School, Department of Orthopaedic Surgery, and †Department of Genetics, Suita, Osaka 565, Japan

§Osaka Bioscience Institute, Department of Molecular Biology, Suita, Osaka 565, Japan

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Address reprint requests to Hideo Hashimoto, MD, Osaka University Medical School, Depertment of Orthopaedic Surgery, 2-2 Yamada-oka, Suita, Osaka 565, Japan.
TEL: 81-6-879-3552 FAX: 81-6-879-3559
e-mail: hideo@ort.med.osaka-u.ac.jp.

The disorder of Fas-FasL system causes autoimmune diseases. In this study, we demonstrated that synovial fluid lymphocytes (SFL) from RA patients expressed FasL, and soluble FasL (sFasL) was accumulated in the inflamed joints. Membrane FasL could induce apoptosis to RA and OA synovial cells, but naturally processed sFasL could not. Depending on the different killing activity, the cleavage of membrane FasL may regulate the Fas-mediated apoptosis of the synovial cells.

***Objective.*** To investigate the expression and function of Fas ligand (FasL) ,which can be in a membrane bound or soluble form (sFasL), in the joints of patients with rheumatoid arthritis (RA) and osteoarthritis (OA).

***Methods.*** The concentration of sFasL in the serum and the synovial fluid (SF) from 24 OA and 38 RA patients was measured using an enzyme-linked immunosorbent assay (ELISA). The expression of FasL on synovial fluid lymphocytes (SFL) and peripheral blood lymphocytes (PBL) was assessed by RT-PCR analysis. The cytotoxic killing asssay of membrane-bound FasL and purified sFasL against cultured synovial cells was performed.

***Results.*** sFasL was detected in the SF of patients with RA and OA, but not in the serum of the patients. The concentration of SF-sFasL was remarkably higher in severe RA patients than in mild RA or OA patients. RT-PCR analysis showed that SFL, but not PBL from RA patients expressed mRNA for FasL. Membrane-bound FasL induced apoptosis in the cultured synovial cells from the RA and OA patients, but naturally processed human sFasL did not.

***Conclusion.*** SFL from RA patients expressed FasL and cleaved sFasL accumulated in the synovial fluid of the inflamatory joints. The different killing activity of membrane FasL and sFasL against the synovial cells may regulate Fas-mediated apoptosis in synovial cells.

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation and progressive joint destruction (1). Synovial hyperplasia is typical in the inflamed joints of RA patients. RA synovial cells have transformed phenotypes, which express proto-oncogenes, ras, myc, fos, or jun (2). These proto-oncogenes upregulated the proliferation of synovial cells and induced the production of cysteine proteinase, collagenase, and stromelysine, which degraded matrix proteins (3). RA synovial cells also secrete inflammatory cytokines, IL-1, IL-6, TNF-α, and GM-CSF, which perpetuate arthritis (4). Considering these facts, the synovial cells have been thought to play a pivotal role in the pathogenesis of RA. Recently Fas-mediated apoptosis in RA synovial cells was suggested to lead the regression of the hyperplastic synovial cells (5, 6). Therefore Fas-mediated apoptosis is thought to be a useful tool for the treatment of rheumatic arthritis (7, 8).

FasL is a 40kD type Ⅱ transmembrane protein that belongs to the TNF family (9), and it induces apoptosis through its membrane receptor, Fas. Human FasL was found to be converted to the 26kD sFasL by the action of metalloproteinase(MMP)-like enzyme (10) as found with TNF (11, 12). sFasL was detected in the serum of patients with LGL leukemia or NK lymphoma (13), but the pathological role of sFasL remains unknown. Quite recently we found that membrane FasL, sFasL, and agonistic anti-Fas mAb had the selective

killing activities against Fas-expressing cells (14). For instance, membrane FasL and recombinant soluble mouse FasL, which carries the complete extracellular region of FasL (termed WX1), killed fresh human pripheral blood T cells (PBT) that were resisitant to sFasL and agonistic anti-Fas mAb (CH11), and the killing was inhibited by sFasL and CH11.

In this study, we examined the expression of sFasL in the synovial fluid (SF) from RA and OA patients. To further elucidate the role of FasL in arthritis, the function of membrane FasL and sFasL against RA and OA synovial cells was investigated.

## Patients and Methods

**Patients.** 38 patients with RA (5 male and 33 female) and 24 patients with OA (6 male and 18 female) participated in this study. All the patients were treated at Osaka University Hospital or related facilities. The average age of the RA patients and OA patients was 52.1 and 68.1 years, respectively. The average disease duration of RA was 14.8 years. The diagnosis of RA was based on the 1987 revised criteria of the American Collage of Rheumatology (15) and that of OA was based on its clinical and radiological features. We previously classified patients with RA into three disease subsets (16), least erosive disease (LES), more erosive disease (MES), and mutilating disease (MUD), by assessing the

joint destruction. In this study, the 38 RA patients consisted of 16 LES and 22 MES. The samples of synovial fluid (SF) and peripheral blood (PB) were obtained simultaneously at the time of surgical treatment or therapeutic arthrocentesis after obtaining informed consent. For the study of synovial cells, synovial tissue from several RA or OA patients was obtained during surgical operation.

**ELISA for sFasL.** The SF and PB from the patients were centrifuged and the supernatants were stored at -80℃ until the examinations. The concentration of sFasL was measured by ELISA system as previously described (13). In brief, 96-well plates (Maxisorb, Nunc Co.) were coated with 1 μg/ml of anti-human FasL mAb (4H9) in 50mM bicarbonate buffer (pH 9.6), washed with PBS and incubated with 5% skim milk. The SF or PB samples from patients were diluted 5 times with PBS, then 100 μl aliquots were added to the wells. After incubation at room temperature for 1 hour, the wells were washed with PBS containing 0.1 % Tween 20 (T-PBS). The wells were incubated at room temperature for 1 hour with 5 μg/ml of biotinylated anti-human FasL monoclonal antibody (4A5), washed 5 times with T-PBS and incubated with 1:500 diluted peroxidase-conjugated streptavidin (Boehringer, Ingelheim, Germany) for 30 min. The peroxidase activity was detected using o-phenylenediamine as the substrate and a peroxidase- detection kit (Sumitomo Co., Tokyo). The absorbance was measured at 492 nm using a Micro-ELISA reader. Purified human recombinant

FasL produced in Pichia pastoris was used as the standard. In some sera which contain high level of rheumatoid factor, a control hamster mAb was included during the reaction with biotinylated 4A5 mAb at a final concentration of 50 μg/ml.

**RT-PCR for FasL.** SFL and PBL were isolated by a Ficoll-Paque Plus (Pharmacia LKB Biotechnology, Piscataway, New Jersey) gradient centifugation from heparinized SF and PB. The total RNA was prepared using Isogen (Nippon Gene Co., Tokyo, Japan). One microgram of the total RNA was reverse-transcribed with 200 units of Super Script Ⅱ (Gibco BRL, Grand Island, New york) in 20 μl of reaction buffer containing 0.5 mM dNTP, 10 mM DTT, and 100 ng of the antisense primer (5'-GCCGAAAAACGTCTGAGATT) for human FasL. After incubation at 42°C for 60 min, one microliter of the reaction mixture was used for PCR. The PCR was carried out with 2.5 units of Taq DNA polymerase (Pharmacia) in 50 μl of reaction buffer containing 0.25 mM dNTP, 600 ng each sense primer (5'-TGTTTCAGCTCTTCCACCTA) and antisense primer (5'-GTTCCTCATGTAGACCTTGT). The conditions for PCR are at 94℃ for 1 min, at 55℃ for 2 min, and at 72℃ for 3 mim, for 25 cycles with a thermal cycler (Perkin Elmer, Norwalk, Connecticut). To amplify the human G3PDH mRNA, 100 ng of random hexamers (Gibco) was used in the cDNA synthesis, and a 30 PCR cycles

(at 94℃ for 1 min, at 55℃ for 2 min, and at 72℃ for 3 min) was performed using a commercial primer set (Clontech, Palo Alto, California). The amplified products were subjected to electrophoresis on 1.2% agarose gels and visualized by ethidium bromide staining. The predicted size of the fragment for FasL was 344 base pairs, and that for G3PDH was 983 base pairs.

**Preparation of cultured synovial cells and flow cytometry.** The synovial tissue from the RA patients and OA patients was minced with scissors and digested with 1 mg/ml of collagenase (Sigma Chemical Co., St. Louis. MO) for 2 hour at 37℃. The single cells that passed through a nylon mesh were suspended in DMEM with high glucose and glutamine (Nikken BioMedical Laboratory, Kyoto, Japan) supplemented with 10% FCS, 100 U/ml benzylpenicillin potassium (Meiji Seika Co., Tokyo, Japan), and 100μg/ml streptomycin sulfate (Meiji Seika). The synovial cells were cultured and the cells from the forth through sixth passage were used for further experiments.

For flow cytometry analysis, the synovial cells were dislodged with 1 mM of EDTA, washed once in staining solution (PBS containing 2% FCS and 0.02% sodium azide), and incubated on ice for 30 min with 20 μg/ml of anti-Fas mAb (CH11, MBL, Nagona, Japan) or mouse IgM (Seikagaku-kougyou, Tokyo, Japan). Afer washing twice with the staining solution, the cells were incubated with 10 μg/ml of FITC-labeled goat anti-mouse IgM for 30 min, and washed three times

with the staining solution. The cells were then analyzed by FACScan (Becton Dickenson), and the data was analyzed using CELLQuest software.

**Assay for the killing activity of membrane FasL and sFasL against synovial cells.** Transformant (1A12) expressing human FasL that lacked a cytoplasmic region (amino acid 8-69) (13) was used as the membrane FasL. Naturally processed human sFasL (AL-1) was purified from the supernatant of the 1A12 cells using an affinity column coupled with anti-human FasL mAb (4H9) (17). Recombinant soluble mouse FasL (WX1) consisted of the signal sequence derived from G-CSF and almost the entire extracellular region of mouse FasL (amino acid 101-279) (18) was purified using an anti-mouse FasL mAb affinity column. The cultured synovial cells were seeded 50000 cells per well in 96-well microtiter plates and grown for 24 hr. The cells were incubated at 37°C for 12 hr with various concentrations of AL-1 or WX1. For the 1A12 cell-mediated cytotoxicity, synovial cells were cocultured with 1A12 cells at various effector/target ratios in the presence or absence of 10 μg/ml of 4H9 for 12 hr. The viable synovial cells were stained for 20 min at room temperature with 0.75% crystal violet in 50% ethanol, 0.25% NaCl, and 1.75% formaldehyde. After extensive washing with water to remove the non-adherent cells, dye uptake was mesured by absorbance at 540nm using a Micro-ELISA reader.

**Statistical analysis.** Statistical analysis was performed by the Mann-

Whitney U test and a p-value less than 0.05 was considered significant.

## Results

**Elevated levels of sFasL in SF from RA patients.** Using an ELISA, sFasL was detected in the SF from the RA patients and some of the OA patients (Figure 1-a). The concentration of sFasL in the SF from the RA patients (951± 162 pg/ml) was significanly higher than that from the OA patients (354±115 pg/ml, $p<0.001$). RA patients were divided into the severe subset (MES) and the mild subset (LES) based on our previous criteria (16). The level of sFasL in the SF from MES (1301±258 pg/ml) was significantly higher than that from LES (469±83 pg/ml, $p<0.01$) (Figure 1-b). The serum sFasL levels of all RA and OA patients were under the detectable level (50 pg/ml), except for some serum samples in which we could not determine the sFasL level because of high back ground aparently due to rheumatoid factor (anti-IgG Ab) in the serum.

**Expression of FasL mRNA on SFL and PBL from RA and OA patients.** To investigate the expression of FasL in the PBL and SFL, RT-PCR analysis was performed (Figure 2). The FasL mRNA was detected in the SFL from the RA patients. Because the SF from OA patients contained few lymphoid cells, we only had enough SFL for RT-PCR from two OA patients. These patients had a

relatively high amount of sFasL in the SF compared to the other OA patients. Consistently the FasL mRNA was detected in their SFL. Under the same conditions, the FasL mRNA was not detected in the PBL from the RA or OA patients (Figure 2) or the healthy volunteer (H.H. 32 y.o. male, data not shown) by RT-PCR.

**Differential Fas-dependant cytotoxic activity among membrane-bound FasL (1A12), sFasL (AL-1, WX1), and agonistic anti-Fas mAb (CH11) against synovial cells.** Cultured synovial cells from both OA and RA patients expressed Fas abundantly on their surface (Figure 3-a) and were killed by CH11 in a dose dependant manner (Figure 3-b). The OA synovial cells were slightly more resistant than RA synovial cells to a high dose of CH11. Next we used the transformant expressing FasL, 1A12, to examined the killing activity of membrane FasL against synovial cells. 1A12 cells were co-cultured with synovial cells in the presence of the 10 μM MMP inhibitor, BB2116, to prevent membrane FasL from proteolyzing into sFasL. The 1A12 cells markedly killed the OA and RA synovial cells (Figure 3-c,d). The 1A12 cells were less efficient at killing in the absence of BB2116 than in the presence of it (data not shown). The cytotoxicity of the 1A12 cells was inhibited by anti-human FasL neutralizing Ab (4H9), and the parental cells (WR19L) had no killing activity, indicating that the cytotoxicity depended on the Fas-FasL interaction. On the contrary, the OA and RA synovial

cells were resistant to the naturally processed sFasL (AL-1). But the recombinant mouse sFasL (WX-1) was markedly cytotoxic against synovial cells like membrane FasL. (Figure 3-e,f).

## Discussion

In this study, we demonstrated that SFL from RA patients expressed FasL mRNA whereas PBL did not. This result was possibly due to the difference of the SFL and PBL cell populations. The SFL from the RA patients contained more NK cells and CTL (19-21) than PBL. The Fas-FasL system is a major pathway for CTL-mediated cytotoxity along with perforin (22). We considered that activated NK cells and CTL in SFL expressed FasL, and a flow-cytometric analysis was performed. But we could not detect FasL on their cell-surface (data not shown). The reason for this result is likely that a membrane FasL is very easy to be converted to sFasL because the levels of MMPs in SF from RA patients are considerably high (23). In the present study, such as the OA1 patient in Figure 2, the expression of FasL mRNA on SFL did not indispensably correlated to the level of sFasL in SF. The activity of FasL-converting enzyme might partly regulate the concentration of sFasL in SF.

Recently Okamoto et al reported that the JNK/AP-1 signaling pathway contributed to the difference in the sensitivity to Fas mediated-apoptosis of RA

and OA synovial cells (24). In our study, the OA synovial cells were more resistant to CH11 than the RA synovial cells only at the high dose of CH11. The sensitivity of Fas-expressing synovial cell to CH11 did not seem so different between RA and OA. These results suggested that OA synovial cells were oligo-clonal and consisted of populations which were either sensitive or insensitive to CH11. But the RA and OA synovial cells were similarly sensitive to membrane FasL. We recently discovered that membrane FasL could kill the insensitive cells to CH11 such as fresh PBT (14). Collectively membrane FasL had wider target spectrum than CH11. In the present study, we nominated the transfected cell line, 1A12, as the effector cell against synovial cells. The level of FasL expressed in 1A12 was likely to be higher than in NK cells or CTL in SFL. We also showed that activated T cells (ATC) expressing FasL could induce apoptosis in naive T cells, and this killing was inhibited by soluble human Fas ligand (AL-1) (14). Considering these results, the level of FasL on ATC is likely to be enough to induce apoptosis in physiological Fas-bearing cells. Wheras, both RA and OA synovial cells were resistant to sFasL (AL-1). These data suggested that membrane FasL was deprived of its killing activity by convertion to its soluble form. Considering the high accumulation of sFasL in the SF of severe RA patients, sFasL cleaved from membrane FasL might contribute to the down-regulation of Fas-mediated apoptosis in RA synovial cells to perpetuate the

arthritis. An MMP-inhibitor was reported to alleviate the cartilage and bone destruction in adjuvant arthritis (25). These results may be partly due to the protection of membrane FasL on SFL from the cleavage to soluble form.

Recently it has been demonstrated that the administration of agonistic anti-Fas mAb improved the rheumatic arthritis in MRL/gld mice (8), which were defective in functional FasL, and HTLV-1 tax transgenic mouse (7) by inducing apoptosis in the synovial cells. In the present study, WX1 was significantly cytotoxic like membrane-bound FasL, and it was more cytotoxic than CH11. Therfore WX1 is likely an appropriate effector against synovial cells rather than CH11. If the possible toxicity of WX1 against normal tissues is overcome, WX1 can be used for the treatment.

The sFasL levels in SF from the severe RA patients was significantly higher than that from the mild RA patients or OA patients. Considering our results, sFasL is the probable exacerbating factor for RA. Then we examined the correlation between the levels of sFasL in SF and other clinical parameters, which reflected acute inflammation, C-reactive protein, erythrocyte sedimentation, and rheumatoid factor, but there was no correlation among them (data not shown). It is possible that the concentration of sFasL in SF reflects chronic disease activity leading to joint destruction instead of acute inflammation. We previously reported that the high concentration of C1q (16)

and BST-1 (bone marrow stromal cell antigen 1) (26) were frequently observed in the serum of the severe RA patients. The abnormal myeloid linage cells producing a high level of IL-1 were also found in the bone marrow from the RA patients who have severe joint destruction. These characteristic abnormalities have been found persistently during their clinical course, and are helpful for distinguishing the severe subset of RA. In this regard, it will be interesting to measure the concentration of sFasL in the SF for the prognosis of RA. A following observation and further analysis of the patients with high concentration of sFasL in the SF are necessary before a final conclusion can be made.

In conclusion, a high concentration of sFasL was accumulated in the SF of severe RA patients. The cleavage of membrane FasL to its soluble form plays an important role in the regulation of synovial apoptosis.

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**Figure Legend**

Figure. 1

***a,*** Concentration of sFasL in SF from OA and RA patients. The levels of sFasL in the SF from the RA patients were significantly high compared with the OA patients.

***b,*** RA patients in this study were divided into two groups based on our criteria, mild RA patients (LES) and severe RA patients (MES). The statistical analysis showed that the differences between two groups were significant .

Each bar represents the mean ± SEM of each group.

Figure. 2

Expression of FasL on PBL and SFL examined by RT-PCR as described in the patients and methods section. The FasL mRNA was expressed in SFL (Lane 3,5,7,9), but not in PBL (Lane 2,4,6,8). The patients had high sFasL levels in the SF (OA1=1750 pg/ml, LES1=1092 pg/ml, MES1=1467 pg/ml, and MES2=2734 pg/ml). As a positive control, the plasmid contained cDNA of whole human FasL was amplified by PCR (Lane 1). The predicted size of the fragment for FasL was 344 basepairs, and that for G3PDH was 983 basepairs.

Figure. 3

***a,*** Staining profiles for Fas expression on cultured synovial cells from OA and RA. The cells were incubated with anti-human Fas mAb (CH11, solid line) or purified mouse IgM (dotted line) and subsequently stained with FITC-anti-mouse IgM mAb.

***b,*** sensitivity of synovial cells from OA (○)or RA (●) patients to agonistic anti-Fas mAb (CH11).

***c,d,*** Cytotoxic activity of a transfectant that expressed FasL (1A12) or its parental cell (WR19L) against cultured synovial cells. Synovial cells from OA(***c***) or RA(***d***) were co-cultured with WR19L (▲) or 1A12 in the presence (○) or absence (●) of 10 μg/ml of anti-FasL neutralizing Ab (4H9) for 12 hr.

***e,f,*** Differential cytotoxic activity of two kinds of sFasL against synovial cells. Cells from OA (***e***) or RA (***f***)●; with WX1, ▲; with AL-1) were incubated with naturally processed human sFasL (AL-1; ○) or mouse recombinant sFasL (WX1; ●) at the indicated concentrations for 12 hr. 1 unit was defined as the amount of sFasL that produced the half-maximal cytotoxicity against $7.5\times10^4$ W4 cells,which were transformant expressing mouse Fas, in 100μl. 1 ng of AL-1 was equivalent to 10 units.

***b-f*** represent the average of three independent experiments. Vertical bars indicate the SD.

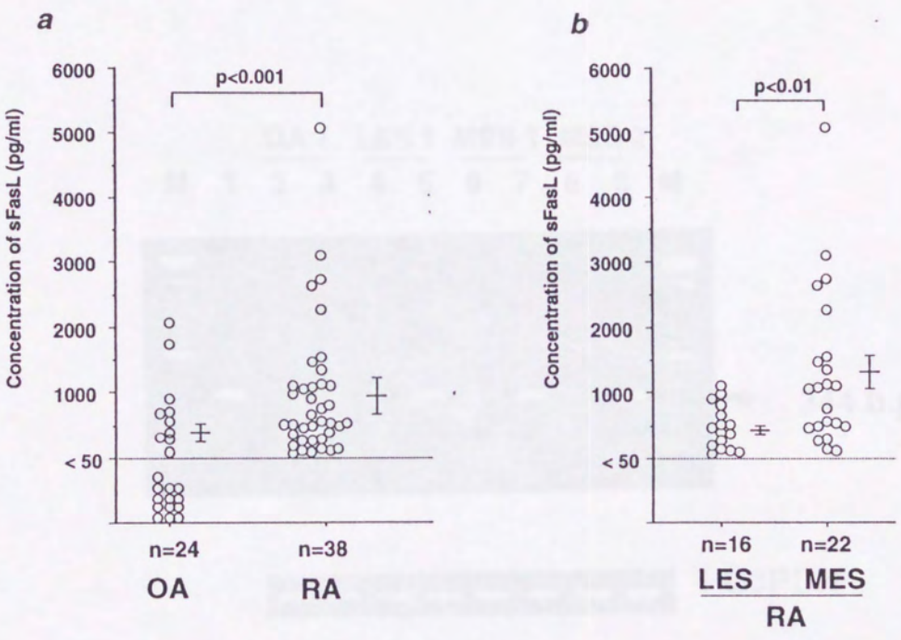


Figure. 1, H.Hashimoto et al. "sFasL in the joints of RA and OA"

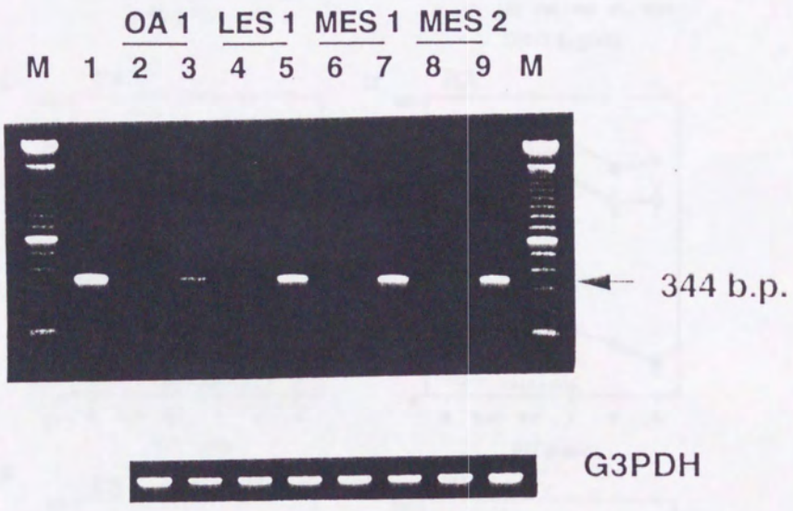


Figure. 2, H.Hashimoto et al. "sFasL in the joints of RA and OA"

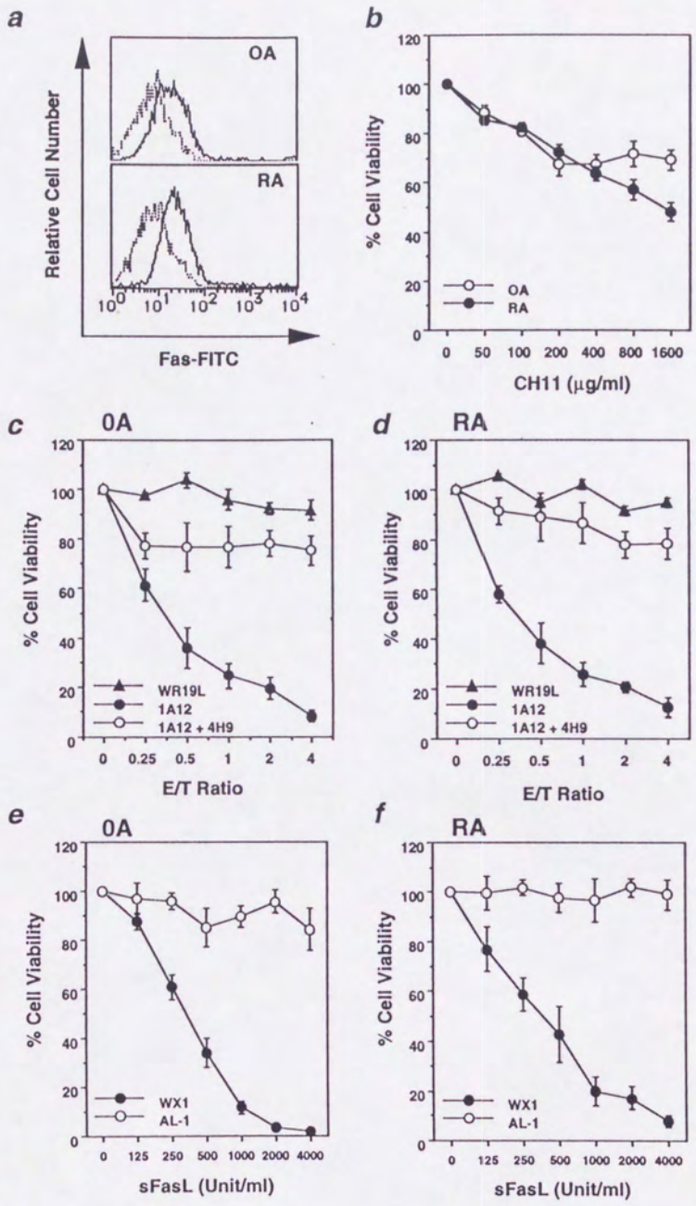


Figure. 3, H.Hashimoto et al. "sFasL in the joints of RA and OA"