Distinct Overexpression of Fas Ligand on T Lymphocytes in Aplastic Anemia

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Increased expression of Fas by hematopoietic progenitors in aplastic anemia (AA) suggests that Fas/Fas ligand (FasL) system plays a key role in the formation of severe pancytopenia. To further confirm the above hypothesis, T cells from 8 patients with AA were systematically studied for their FasL's distribution pattern, releasing manner and proapoptotic activity, compared with normal resting T cells and artificially activated T cell blasts. The results demonstrated that AA T cells abnormally expressed low levels of membrane-bound FasL and contained high levels of intracellular FasL which could be triggered to release by high-dose phytohemagglutinin (PHA) pulse-stimulation. The supernatants from the PHA-stimulated AA T cells had apparent cytotoxicity against FasL-sensitive Jurkat cells, which could be significantly inhibited by monoclonal antibody against FasL in a dose-dependent manner, or nearly completely abrogated by ultracentrifugation. The above phenomena also appeared on artificially activated T cell blasts, but this was not the case on normal resting T cells. These results indicate that AA T cell is a type of "preactivated" T lymphocyte, characterized by overexpression of FasL, especially intracellular FasL which can be stimulated to release in bioavtive exosomes-bound form. Taken together, our data provide further and direct evidence for the hypothesis that T cells might mediate the destruction of hematopietic progenitor in AA through Fas/FasL system. *Cellular & Molecular Immunology*. 2004;1(2):142-147.

Key Words: aplastic anemia, T cell activation, Fas, Fas ligand, exosome

Introduction

Aplastic anemia (AA) is dysfunction characterized by pancytopenia resulting from nonfunction of the bone marrow (BM) (1). Substantial clinical and experimental evidences suggest that T cell-mediated suppression of haemopoiesis is the most important pathogenic mechanism of BM failure in AA (2-10). It is not clear how T cells attack bone marrow cells. Increased level of Fas antigen was found on $CD34^+$ progenitors in patients with AA, indicating Fas-mediated cell death of hematopoietic progenitor and stem cells, maybe participate in severe pancytopenia (11, 12).

Fas ligand (FasL), a type Ⅱ transmembrane protein that belongs to the tumor necrosis factor (TNF) family, is predominantly expressed by activated T cells (13-15). It has recently been demonstrated in T cell lines that newly synthesized FasL is stored in secretory lysosomes and released after stimulated with T cell receptor (TCR)-mediated signals (16, 17).

It remains unknown, however, whether the phenomena viewed in T cell lines could also be seen in AA T cells. In addition, the significance of the Fas/FasL system in the pathogenesis of AA is ill-defined. In this study we were prompted to systematically investigate FasL's distribution pattern, releasing manner and cytotoxic activity of T cells in AA patients.

Materials and Methods

Patients and controls

Eight AA patients with a median age of 25 years (range 15~58 years) from Hematology Department of No.2 Affiliated Hospital of Suzhou University were included in the study. The diagnosis of AA was by bone marrow (BM) biopsy and peripheral blood (PB) cell counts according to the International Study of Aplastic Anemia. PB was collected from all patients at diagnosis. Normal control PB was obtained from 10 health donors at Suzhou Central Blood Bank.

Cells purification and culture

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll-Hypaque (Shanghai

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Abbreviations: AA, aplastic anemia; FasL, Fas ligand; FCM, flow cytometry; CLSM, confocal laser scanning microscopy; IFN-γ, interferon gamma; PHA, phytohemagglutinin.

second chemistry factory, China) density gradient centrifugation (d=1.077). T cells were purified by negative selection using mini-MACS system. Briefly, PBMCs were washed and incubated with a mixture of lineage-specific monoclonal antibodies (mAbs; anti-CD14, CD19, CD56) followed by incubation with magnetic beads conjugated with goat anti-mouse antibody (Ab). After magnetic separation, purity of T cells collected in negative fraction was >90%. FasL-sensitive Jurkat cells were cultured in the complete medium (RPMI1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomoycin). Seven-day T cell blasts were generated as follows. Purified T cells were stimulated with 10 μg/ml PHA for one day. Afterward, PHA was washed, and cells were resuspended in complete medium supplemented with 30 U/ml rhIL-2 and cultured for 6 days with medium changes every 48 hours.

Flow cytometry (FCM) analysis

For analysis of membrane-bound FasL, T cells (5×10^5) were incubated for 30 minutes with 20 μg/ml mouse anti-human FasL mAb (Immunotech, Marseille, France), washed twice in phosphate-buffered saline (PBS), followed by 30 minutes with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Immunotech). For analysis of intracellular FasL, T cells were fixed in 2% paraformaldehyde, permeabilized for 20 minutes in PBS containing 0.5% bovine serum albumin (BSA) and 0.1% saponin, and then incubated for 30 minutes with 20 μg/ml mouse anti-human FasL mAb followed by FITC-labeled goat anti-mouse IgG. For negative control staining, mouse IgG1 (Immunotech) followed by FITC-labeled goat anti-mouse IgG was used. Samples were analyzed on EPICS-ALTRA flow cytometry (Beckman Coulter, USA) by EXPO2 software. Expression level of FasL was quantified as the mean fluorescence intensity (MFI).

Confocal laser scanning microscopy (CLSM) analysis

For CLSM analysis, all procedures were performed at room temperature. T cells were rinsed in PBS, fixed with 2% paraformaldehyde for 15 minutes and settled onto poly L-lysine-coated slides. Permeabilized cells were obtained by incubating the cells with 0.2% Triton X-100 (Sigma) for 30 minutes, followed by blocking with 1% BSA and 3% goat serum (Sigma) in PBS, for 60 minutes. Cells were incubated for 2 hours with mouse anti-human CD3 mAb (Immunotech) and polyclonal rabbit anti-human FasL Abs (Santa Cruz Biotechnology, Santa Cruz, CA), and stained for 1 hour in the dark with goat anti-mouse Cy3-labelled IgG and goat anti-rabbit FITC-labeled IgG (Immunotech). Images were obtained with a laser-scanning confocal microscope (Bio-Rad, Hercules, CA). Dual detection was performed with separate photomultiplier tubes, and resultant images were merged.

Cells stimulation and supernatants preparation

Purified T cells were prestimulated with 50 μg/ml PHA for 5 minutes; after PHA removed by centrifugation and washing, they were resuspended in complete medium and cultured at 37°C for 1 hour. Supernatants from 1-hour stimulated T cells were collected by low speed centrifugation (1000g, 10 minutes). The supernatants (6 ml) from

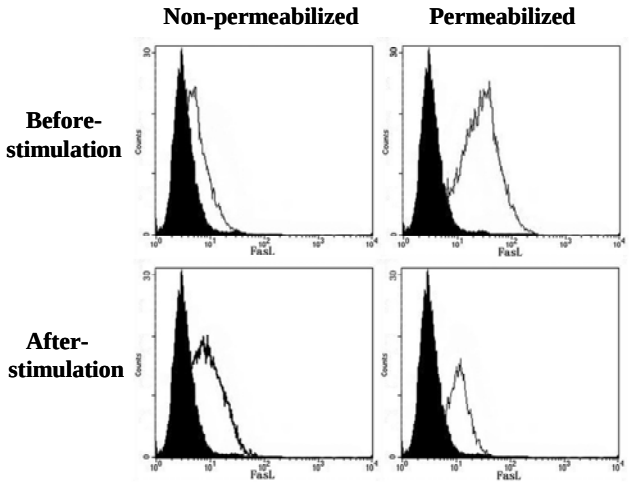

Figure 1. The expression of membrane-bound and intracellular FasL on T cells from AA patients before and after PHA-stimulation. Dark areas show isotype-control; clear areas show FasL. Histograms indicated with 'non-permeabilized' show membrane-bound FasL, Histograms indicated with 'permeabilized' show total FasL on T cells.

PHA-stimulated T cells (Initial-supernatants, Init-SS) were centrifugated again at 10,000g for 20 minutes at 4°C to eliminate cell debris, and then the obtained supernatants were further ultracentrifugated at 100,000g for 8 hours at 4°C. The ultercentrifugated supernatants (Ultra-SS) were recovered, and the pellets, containing exosomes, were resuspended in 1.5 ml fresh complete medium, then concentrated supernatants (Con-SS) were obtained.

Cytotoxicity assays

The cytotoxicity of different supernatants (Init-SS, Ultra-SS, and Con-SS) was tested as Marinez-Lorenzo et al. (18) described. Briefly, FasL-sensitive Jurkat cells (5×10^4) were suspended in 100 μl aliquots of supernatants described above. After incubation for 16 hours, cytotoxicity was determined by the MTT assay. Jurkat cells suspended in complete medium were used as control. Cell viability was determined by the MTT reduction method and was expressed as percentage of the corresponding values for control cells.

Statistical analysis

FasL expression by AA and normal T cells, and cytotoxicity of different supernatants on Jurkat cells, were analyzed with Student's unpaired *t*-test using SPSS software. The values were presented as mean ± SD. Values of $p<0.05$ were considered statistically significant.

Results

FasL's distribution pattern on AA T cells

The expression of FasL was measured on AA T cells by FCM analysis, compared with normal resting T cells and T cell blasts. It was found that membrane-bound FasL was expressed very low levels on the surface of AA T cells,

Table 1. *The mean fluorescence intensity (MFI) of FasL on T cells before and after PHA–stimulation.*

	normal T (n=10)		T blasts (n=10)		AA T (n=8)	
	mFasL	iFasL	mFasL	iFasL	mFasL	iFasL
Before-stimulation	ND	6.5±0.8	8.8±0.9	87.5±9.4*	4.3±0.5	28.1±6.1*
After-stimulation	ND	6.8±1.5	19.9±1.6	27.7±4.2*	7.7±0.6	10.4±1.3#

Note: ND, no detetcable; mFasL, membrane-bound FasL; iFasL, intracellular FasL. Compared with "Normal T", # $p<0.05$, * $p<0.01$.

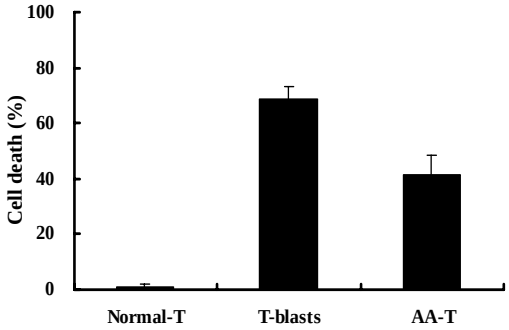

Figure 3. The cytotoxicities against Jurkat cells of supernatants from PHA-stimulated T cells.

however, it was not detectable on the surface of normal resting T cells (Figure 1, Table 1). As it has recently been confirmed that activated T cells prefer to retain FasL in their cytoplasm and FasL's surface expression is tightly regulated, we analyzed intracellular FasL in T lymphocytes permeabilized with saponin and subjected to flow cytometry. Levels of intracellular FasL in AA T cells were significantly higher than those of their surface-bound FasL, and, of course, also higher than those in normal resting T cells (Figure 1, Table 1). Interestingly, artificially activated T blasts also expressed higher levels of intracellular FasL and moderate levels of surface-bound FasL (Table 1). In addition to FCM analysis, indirect immunofluorescence CLSM analysis was also used to directly visualize the subcellular distribution of FasL on AA T cells. FasL-specific signals in the cytoplasm of AA T cells were apparently stronger than those on their surface (Figure 2 d-f). While in normal resting T cells, tiny FasL-specific signals only distributed in their cytoplasm (Figure 2 a-c). These results demonstrated that AA T cell was a typical kind of abnormally activated cells marked with elevated expression of FasL, especially high levels of intracellular FasL.

Effects of PHA-stimulation on AA T cells' FasL distribution

In order to perform its proapoptotic activity, intracellular FasL must be released from T cells' cytoplasm. Recently, studies on T cell lines indicated that signals triggering the intracellular FasL releasing were delivered by TCR. We

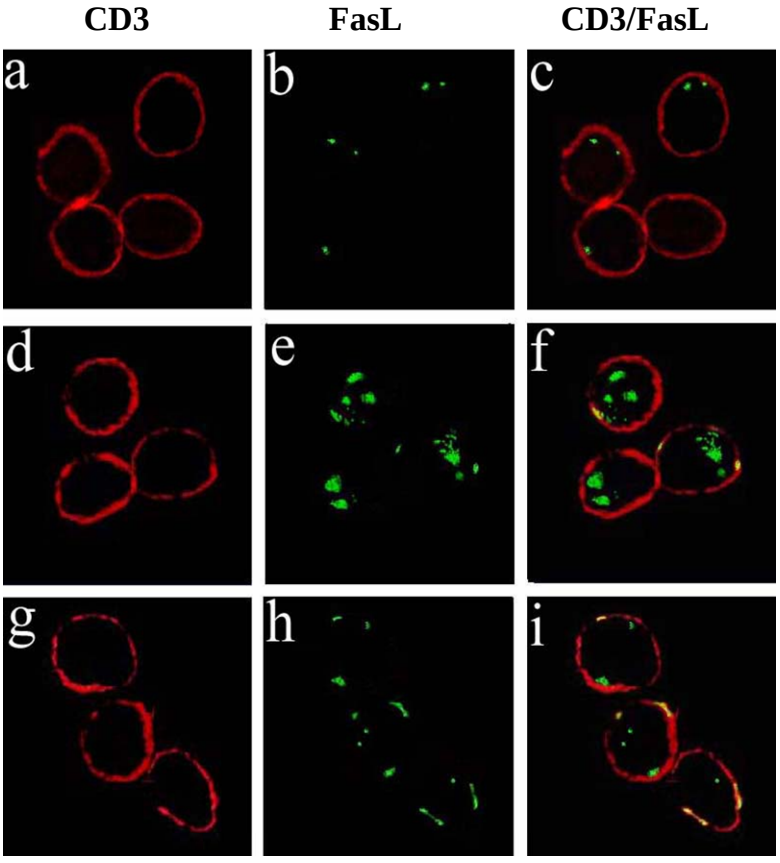

Figure 2. Confocal microscopy analysis of FasL distribution on AA T cells before and after PHA pulse-stimulation. *a-c*, normal T cells (normal control); *d-f*, AA T cells; *g-i*, AA T cells pulse-stimulated with PHA. CD3 is indirectly labeled by IgG-Cy3 and depicted in red. FasL is indirectly labeled by IgG-FITC and depicted in green. Overlap of CD3 with FasL is depicted in yellow.

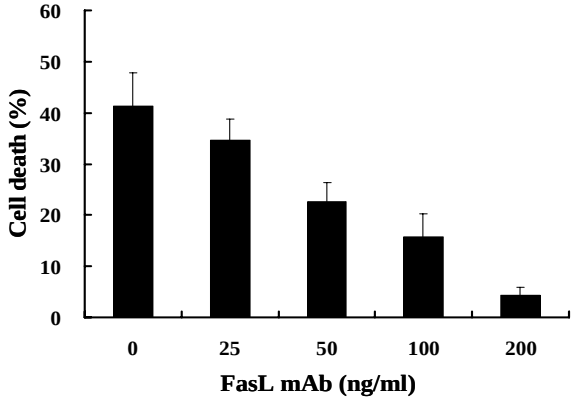

Figure 4. The inhibitory effects of FasL mAb on cytotoxicities of supernatants from PHA-stimulated AA T cells.

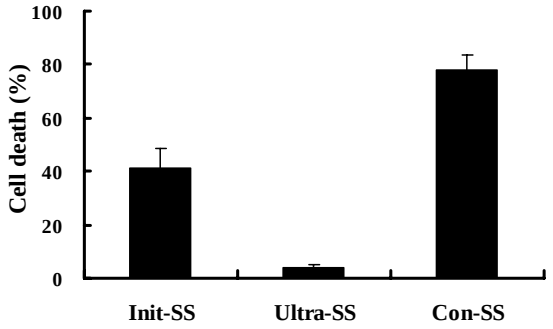

Figure 5. The influence of ultracentrifugation on cytotoxicities of supernatants from PHA-stimulated AA T cells. SS: supernatants; Init-: Initial; Ultra-: Ultracentrifugation; Con-: Concentrated.

hypothesized that the phenomena viewed on T cell lines might occur on AA T cells. Therefore, we used 50 μg/ml PHA to stimulate AA T cells, and then detected PHA-stimulation's effects on their FasL distribution by FCM or CLSM analysis. After PHA stimulation, membrane-bound FasL on the surface of AA T cells were slightly elevated, while the intracellular FasL drastically decreased (Figure 1, Table 1, Figure 2 g-i). These results suggested that high-dose PHA pulse-stimulation triggered the great majority of the stored FasL in AA T cells to release into the cells culture medium.

Released intracellular FasL maintains its proapoptotic activity

We further investigated whether the supernatants from PHA-stimulated AA T cells had proapoptotic activity by using cytotoxicity tests targeted to FasL-sensitive Jurkat cells. In consequence, we found that after PHA stimulation the supernatants of AA T cells had apparent cytotoxicity on Jurkat cells (the percent of cell death was 41.3 ± 6.9), which was similar to that of T blasts (the percent of cell death was 68.8 ± 4.6), but this was not the case with normal resting T cells (Figure 3). To further confirm that the Jurkat cells' death was specifically caused by FasL, we performed blocking experiments by using FasL mAb. Significant dose-dependent inhibition on Jurkat cells' death was observed when the target cells were coincubated with increased concentrations of FasL mAb (Figure 4). The percent of cell death were 34.7 ± 4.2, 22.7 ± 3.8, 15.7 ± 4.5 and 4.3 ± 1.5 respectively, after adding 25 ng/ml, 50 ng/ml, 100 ng/ml and 200 ng/ml FasL mAb respectively. The above results indicated that Jurkat cells' death was indeed FasL-mediated and the released FasL in the supernatants of stimulated AA T cells maintained its proapoptotic activity.

Released intracellular FasL ws associated with exosomes

Two isoforms of FasL can exist in a suspended form after common-speed centrifugation. One is soluble FasL, another exosomes-bound FasL. The latter is a specific membrane-bound FasL and has more efficient proapoptotic activity. To verify this possibility that FasL in the supernatants from PHA-stimulated AA T cells might be exosomes-bound FasL, the common-speed centrifugation supernatants were subjected to ultracentrifugation, under the conditions of which exosomes could be separated from supernatants and deposited as pellet. The Ultra-SS showed little cytotoxicity on Jurkat cells (the percent of cell death was 3.8 ± 1.5), whereas the Con-SS caused more Jurkat cells to death (the percent of cell death was 78.2 ± 5.7). Moreover, the cytotoxicity in the Con-SS was higher than that observed in the Init-SS before ultracentrifugation because of the concentration of exosomes in the pellet (78.2 ± 5.7 versus 41.3 ± 6.9) (Figure 5). Furthermore, gravity-filtration experiments demonstrated that Init-SS filtrated through the 0.1 μm pore size filter lost most of the cytotoxicity, but this was not the case after filtration through the 0.22μm pore size filter (data not shown), indicating that the size of the particles should be between 100~220nm. These results suggested that bioactive FasL released from PHA-stimulated AA T cells was associated with exosomes.

Discussion

To date AA is regarded as a special bone marrow failure disease which is usually mediated by abnormally activated T cells, sharing pathophysiologic mechanisms with other organ-specific autoimmune diseases, like mutiple sclerosis, ulcerative colitis, uveitis, and type Ⅰ diabetes, in which Th1/Tc1 cells effect organ-specific destruction (12). The increased expression of Fas on $CD34^+$ progenitors from AA patients suggested that activated AA T cells probably induced Fas-mediated cell death of hematopoietic progenitor and stem cells, ultimately resulting in a remarked decrease in the number of these cells leading to severe pancytopenia. However, the direct evidence for the participation of T cells in Fas-mediated killing of AA progenitors is still lacking.

In the present study, by using FCM and CLSM analysis, we identified that AA T cells contained higher levels of intracellular FasL than normal resting T cells. Interestingly, artificially activated T cell blasts also contained large amounts of FasL in their cytoplasm, indicating that AA T cells shared some characteristics of activation with T blasts. Accordingly, AA T cells are at the "preactivated" status, a status that cells can express activation molecules without

the requirement for *in vitro* stimulation. The conclusion drawn from our results is consistent with a recent finding of examining the level of IFN-γ stored in AA T cells by intracellular cytokine staining. In that report, Soland and colleagues found that AA T cells showed increased IFN-γ content in their cytoplasm without stimulation (19). In addition to intracellular FasL, we also observed that, though significantly lower than intracellular FasL, membrane-bound FasL on the surface of AA T cells was slightly elevated compared with normal resting T cells. Since it had recently been found that FasL expressed on the surface of T cells could deliver reverse costimulatory signals during T cell activation (14), we hypothesized that the surface-bound FasL on AA T cells should preferably play a role in reverse signal transduction. This hypothesis seemed partially to be verified by our further experiments which showed that high level of intracellular FasL in AA T cells was able to be stimulated to release to perform its apoptotic-inducing activity.

Intracellular accumulation and release upon demand have emerged as a novel and efficient mechanism regulating the availability of immune regulation molecules. CD40 ligand, another type Ⅱ membrane protein of the TNF family, is found to be stored in cytoplasm of platelets and can be released within minutes upon activation (20). Flt3 ligand (FL) , a hematopoietic cytokine with a broad range of activities at early stage of hematopoiesis, is retained intracellularly in T cells and released in response to stem cell deficiency (21, 22). Studies on T cell lines show that intracellular FasL can be triggered to release by TCR delivered signals (16, 17). According to our data, FasL accumulated in preactivated AA T cells might share the releasing mechanism with that in T cell lines, being released from cytoplasm after high-dose PHA pulse-stimulation. The results of FCM, CLSM and cytotoxicity assays targeted to FasL-sensitive Jurkat cells demonstrate that the majority amounts of intracellular FasL in AA T cells were released into the cell culture medium in the form of whole, nonproteolyzed proteins. Furthermore, ultra-centrifugation and gravity-filtration experiments defined that the released FasL existed in the supernatants from stimulated AA T cells was associated with exosomes, particulates designated as ultracentrifugable microvesicles (23). The secretion of these death messengers on the surface of exosomes guarantees their cytotoxic potential, because they retain their multimerization ability, which is necessary and sufficient for death signal initiating (18, 24). Moreover, exosomes' tiny surface endows FasL assembled on their surface with a very high cross-linking efficiency, even greater than that of cell surface FasL. In this sense, exosome associated with FasL represents a more efficient death signaling device (25). It seems hence conceivable that AA T cells can send out death signals by releasing high amounts of intracellular FasL in the exosomes-bound form, and receive reverse costimulatory signals through their moderate expressed surface-bound FasL. However, this possibility needs to be further explored.

Taken together, our present data showed that FasL's expression was elevated on AA T cells. These results not only unveil a novel mechanism through which AA T cells induce Fas-mediated target cell death, but also provide a direct evidence for the hypothesis that T cells might mediate the destruction of hematopoietic progenitors in AA through Fas/FasL system, which may help to elucidate the mechanism of hematopoietic suppression in AA.

Acknowlegement

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