FADD-deficient T Cells Exhibit a Disaccord in Regulation of the Cell Cycle Machinery*

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FADD is an adapter protein that was originally isolated as a transducer of apoptotic signals for death domain-containing receptors. However, FADD-deficient mice are embryonic lethal and FADD−/− T cells developed from FADD−/− embryonic stem cells in the RAG-1−/− hosts lack the full potential to proliferate when stimulated through their T-cell receptor complex, suggesting that FADD protein might play a dualistic role in mediating not only cell death signaling but other non-apoptotic cellular pathways as well. Here we show that a substantial number of freshly isolated FADD−/− peripheral T cells are cycling but are defective in their co-stimulatory response when stimulated. Analysis of several cell cycle proteins shows normal down-regulation of p27 inhibitor but increased levels of p21, decreased levels of cyclin D2, and constitutive activation of several cyclin-dependent kinases in activated T cells. These data suggest that FADD is involved in the regulation of cell cycle machinery in T lymphocytes.

Members of the tumor necrosis factor (TNF) receptor super-family participate in a variety of biological processes, including proliferation, differentiation, and apoptosis (1, 2). A subset of this family, Fas, TNFR-1, DR3, DR4, and DR5, contains an intracellular death domain and can induce apoptotic cell death in a variety of cell types. Transduction of apoptotic signals by these receptors involves the adapter protein FADD (3–5), which recruits caspase-8 (6, 7) to initiate apoptosis. FADD-deficient cells are completely resistant to cell death mediated by Fas, TNFR, DR3, DR4, and DR5 (8–13). Mice with mutations in these receptors exhibit severe autoimmunity with symptoms include splenomegaly, lymphadenopathy, increased serum immunoglobulins, and kidney failure (14, 15). Additional mutation at the TNFRI locus exacerbates these symptoms further (16). Surprisingly, FADD-deficient mice are embryonic lethal with heart developmental problem and die around day 10 of gestation. Chimeric mice generated by injecting FADD−/− embryonic stem cells into the RAG-1−/− blastocysts do not develop any lymphoproliferative diseases. Initial analysis of these mice showed T-cell development abnormalities and mature T lymphocytes lack the full potential to proliferate when stimulated with cross-linking TCR antibodies. Transgenic mice expressing the human FADD dominant-negative mutant also exhibit defective TCR-mediated proliferation and abnormal T-cell development (17–20). More recently, analysis of T-cell-specific FADD-deficient mice showed an inhibition of T-cell development and the proliferative step between DN and DP transition (21). These observations suggest that FADD protein plays a role in mediating not only cell death signaling but T-cell proliferation as well. To understand the molecular defects of FADD−/− T proliferation, we have characterized FADD−/−→RAG-1−/− chimeras further. Here we show that a substantial number of resting FADD−/− peripheral T cells are cycling. However, they are defective in their co-stimulatory responses when stimulated with a combination of anti-CD3 and anti-CD28 antibodies. Western blot analyses of resting and stimulated T cells show normal regulation of the p27 inhibitor in the absence of FADD. However, all other cell cycle proteins examined are highly abnormal when FADD is not present. These data suggest that FADD is involved in the regulation of downstream cell cycle machinery in T lymphocytes.

MATERIALS AND METHODS

Antibodies and Western Blots—Anti-CD3 (500A2) and anti-CD28 (37.51) ascites were produced in house and titrated. The anti-CD3 ascites were used at the indicated dilutions. Anti-CD28 was used at a concentration of 5 μg/ml. The antibodies against CD4 (CT-CD4), CD8 (CT-CD8a), or CD62L (MEL-14) were purchased from Caltag (South San Francisco, CA). The anti-CD69 antibody (H1.2F3) was purchased from BD Pharmingen (San Diego, CA). Antibodies against cell cycle proteins as well as p35 were purchased from Santa Cruz Biotechnology. Cells were lysed by a 30-min incubation in a buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Nonidet P-40 (CalBiochem), 1 mM dithiothreitol, 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM sodium vanadate, 0.1 mM NaF, and a mixture of proteinase inhibitors (Sigma). Lysates (10 μg of total protein) were fractionated by 12% SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose. The filters were incubated with various antibodies as described previously (9) and developed with Renaissance (DuPont) per manufacturer’s instructions.

Blastocyst Complementation—FADD-deficient ES cells were previously described (9). These FADD−/− ES cells were injected into blastocysts isolated from pregnant RAG-1−/− mice to generate FADD−/−→RAG-1−/− chimeras.

Flow Cytometry and Cell Cycle Analysis—Cell suspensions were prepared from lymphoid organs of mice (6–10 weeks of age). Briefly, lymph node cells and splenocytes were depleted of red blood cells by treatment for 5 min at room temperature with red blood cell lysis buffer (Sigma). For cell staining and flow cytometric analysis, the lymphoid cells were cultured in anti-CD3 ascites (1:3000)-coated 24-well plate with 10⁵ cells per well. Anti-CD28 was added to the medium as a soluble component. Cells were filtered and washed in phosphate-buffered saline with 4% fetal calf serum and 1 mM NaCl. Cells were stained at the times indicated in the aforementioned buffer for 20 min. Flow cytometry analysis was performed on the Beckman-Coulter (Fullerton, CA) EPICS XL-MCL.

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Cell Cycle Abnormality in the Absence of FADD

Fig. 1. FADD−/− T cells have a limited proliferative capacity, possibly because of a lack of co-stimulation. A, purified lymph node and splenic T cells (>85% purity) from FADD−/− chimeras and C57BL/6 (B6) were cultured with anti-CD3 antibodies and various amounts of anti-CD28 antibodies as indicated. [3H]thymidine was added 48 h poststimulation. B, purified T cells from FADD−/− chimeras and C57BL/6 (B6) were cultured with anti-CD3 or a combination of anti-CD3 and anti-CD28 antibodies. [3H]thymidine (1 μCi) was added per well 10 h prior to the time point indicated.

For cell cycle analysis, CD4+ and CD8+ peripheral T cells were sorted on a Beckman-Coulter EPICS Elite ESP Sorter. Cells were taken at 0, 24, and 48 h postactivation with plate-bound anti-CD3 and anti-CD28. At each time point, cells were fixed in 100% ethanol and kept at 4 °C for at least 4 h. Cells were then pelleted and washed with phosphate-buffered saline twice. Finally, the cells were resuspended in propidium iodide (PI, Sigma), RNase (Roche Molecular Biochemicals, Indianapolis, IN) (10 μg/ml PI and 100 μg/ml RNase) buffer and incubated for 30 min at 37 °C. Cells were then analyzed immediately by flow cytometry.

RESULTS

In our previous analyses of FADD−/−→RAG-1−/− chimeric mice, we showed that the total splenocytes or lymph node cells responded minimally to stimulation by anti-CD3 antibodies, Con A, or a combination of phorbol 12-myristate 13-acetate and ionomycin (9). To further investigate the role of FADD in mature T-cell activation, we generated more chimeric mice and examined the proliferation responses with purified T cells. Peripheral CD4+ or CD8+ T cells were isolated by magnetic beads and treated with various concentrations of anti-CD3 antibodies in the presence of anti-CD28 co-stimulation. Similar to the results obtained with unpurified populations, proliferation of FADD−/− T cells was dramatically reduced when compared with that of wild type cells over a range of antibody concentrations (Fig. 1a). To see if co-stimulation response is affected, we treated T cells with anti-CD3 antibody in the presence and absence of anti-CD28 antibody. Whereas CD28 cross-linking greatly enhanced the proliferative responses of wild-type T cells, it failed to provide co-stimulatory effects to FADD−/− T cells (Fig. 1b). The level of CD28 on the cell surface of FADD−/− T cells is comparable with that on the wild-type T cells (data not shown). These data indicate that the reduced proliferation of FADD-deficient T cells may result from their inability to respond to co-stimulation.

We noticed that a fraction of freshly isolated FADD−/− T cells from all mutant chimeric mice exhibit characteristics of activated T-cell blasts as visualized by microscopical examination and are also indicated by increased forward scatter profiles by flow cytometry (data not shown). In addition, a majority of freshly isolated peripheral mature FADD−/− T cells express higher levels of the activation marker CD69 (Fig. 2). These cells also dramatically down-regulated Mel-14 (CD62L) expression. Freshly isolated FADD−/− T cells are apparently cycling as they incorporate [3H]thymidine even without stimulation (data not shown). These results imply that some of the peripheral, circulating FADD−/− T cells are in an activated state and attempt to undergo cell cycle. To confirm this possibility, we analyzed the cell cycle profile of freshly isolated T cells by staining for DNA content and flow cytometry. As indicated in Fig. 3, almost all (98%) freshly isolated peripheral, wild-type T cells are in G0 phase. In contrast, a fraction of FADD−/− cells are in the S phase and the G2/M phase (~10%), which is a 10-fold increase over the wild-type level. This result agrees with the thymidine incorporation experiment and further indicates that FADD−/− cells are cycling constitutively at low levels and fail to establish and maintain the resting state in vivo.

Stimulation with anti-CD3/CD28 antibodies did not further increase the percentage of S phase FADD−/− cells, unlike the wild-type T cells that rapidly undergo G0 to S transition. By 48 h of stimulation, more than 60% of the wild-type cells are cycling. Thus, whereas a small fraction of FADD−/− T cells have spontaneously progressed into the S phase, the majority failed to do so with or without stimulation through their T cell antigen receptors.

Progression through the G1 phase is dictated by the presence of D-type cyclins in complex with the G1 phase cyclin-dependent kinases CDK4 and CDK6 (22, 23). In addition, cyclin E associates with CDK2 to enforce the G1 to S phase transition and to facilitate entry into S phase. Cyclin A in conjunction with CDK2 pushes cells through the S phase (24). Activities of these cyclin/CDK holoenzymes are tightly regulated by a number of inhibitors such as p16, p21, and p27. To determine whether FADD deficiency affects regulation of the cell cycle in T cells, we examined expression of a panel of cell cycle proteins by Western blot analyses. Total proteins were extracted from purified T cells, and the levels of cyclin D2/D3, CDK6, CDK2, CDK2, cyclin A, cyclin E, p21, and p27 inhibitors were assessed. Because of a small number of T cells that can be obtained from a limited number of FADD−/−
stimulation with anti-CD3 and anti-CD28 antibodies. Consti-
pression of these CDK and cyclins did not increase further upon

We have previously shown that FADD−/− mature T cells

generated in RAG-1−/− chimeras are defective in their ability
to proliferate in response to anti-CD3 or phorbol 12-myristate
13-acetate/ionomycin stimulation (9). Recently, we generated
T-cell-specific FADD−/− mice and found that deletion of FADD
at the DN stage results in an inhibition of DN to DP transition
at the proliferative stage (21). These results argue strongly
that in addition to its function in apoptosis, FADD also plays a
role in proliferation. We show here that FADD−/− T cells
are defective in co-stimulation and exhibit abnormal cell cycle
regulation. Surprisingly, some of the unstimulated
FADD−/− T cells are in the G0 stage of the cell cycle with basal
levels of cyclin D2 and cyclin D3 and dramatic induction of p21 may
account for the lack of G1 to S cell cycle progression in FADD−/−
T cells.

**DISCUSSION**

CDK and cyclins expressed in normal T cells are those at time 0 where the percent of cells in Go/G1 and G2/M and S (S) are listed above each histogram. The %S for 24 and 48 h are also indicated to the right of each histogram.

FIG. 3. FADD−/− T cells are cycling in the absence of TCR stimulation and are unable to progress through a complete cycle. Purified lymph node and splenic T cells from FADD−/− chimeras and C57BL/6 were cultured with anti-CD3 and anti-CD28. Cell cycle analysis was performed at 0, 24, and 48 h poststimulation and 10000 events were collected for each histogram. The histograms displayed are

FIG. 4. Cell cycle protein analysis. Cellular proteins were ex-
tracted from sorted CD4+ or CD8+ peripheral T cells, which were
untreated or treated with anti-CD3 and anti-CD28 antibodies for 24 h. Cell cycle protein levels were detected by Western blots with various antibodies as described under “Material and Methods.” wt denotes T
cells from C57BL/6.

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cells (Fig. 2). This can be caused by the defective co-stimulatory activity in FADD-deficient cells or T-cell hyporesponsiveness as a consequence of proliferation problems.

Analysis of the cell cycle proteins in FADD-deficient T cells indicate that molecules involved with all phases of cell cycle, except for p27, are expressed aberrantly. Many of the molecules associated with G1 to S and G2/M are expressed constitutively at high levels, consistent with our observations that substantial fractions of FADD-deficient T cells have spontaneously overcome the G0 block in vivo. However, these cells have failed to cycle back to the G0 state, indicating a possible block at the G2/M phase. Paradoxically, FADD−/− T cells fail to transit through G1 to S and to G2/M phases following TCR stimulation, perhaps because of the exaggerated expression of p21. We thus conclude that FADD is intimately involved in the downstream events of cell cycle regulation in T cells, and FADD deficiency results in problems at multiple cell cycle checkpoints. It remains unclear how FADD modulates induction and degradation of cell cycle proteins but it is unlikely to involve death domain-containing cell surface receptors. Such proteins that are known to associate with FADD include Fas, TNFR-I, and the TRAIL receptors (DR4 and DR5 in human, mDR4/5 in mouse). However, mice with mutations in Fas, TNFR-I, or double mutation of Fas/TNFR-I do not exhibit an impaired T-cell proliferation. Inhibition of DR4/5 signals by injection of TRAIL-blocking antibody actually leads to an increase of T-cell proliferation (31). In addition, deficiency of FADD in T cells arrests T cell development at the DN to DP transition (21), whereas Fas and TNFR-I double deficiency allows normal T-cell development. Analysis of the nearly complete human genome database did not reveal any new member of the TNF receptor superfamily (1, 2). It is thus possible that FADD regulates proliferation through its interaction with a distinct set of cytoplasmic proteins during cell cycle. In support of this hypothesis, FADD is phosphorylated at the G2/M phase of the cell cycle and associates with a cell cycle-regulated kinase (32).

Recently, the status of cell cycle proteins was examined in mouse T cells using a dominant-negative mutant of human FADD (33). Expression of this human dominant-negative protein has been shown to inhibit T cell proliferation. Human FADD dominant negative (FADD-DN) inhibits G0 to S entry of T cells when stimulated with anti-CD3 plus CD28 antibodies, consistent with our findings with the FADD-deficient T cells. However, unlike our findings, these FADD-DN transgenic T cells were shown to be impaired in their ability to flux calcium, suggesting that early event of TCR/CD28 signaling is compromised. Regulation of cell cycle proteins also appears normal in FADD-DN transgenic T cells, and these cells maintain characteristics of resting cells in the absence of mitogenic stimulation, in sharp contrast to FADD−/− T cells that appear to enter the cell cycle spontaneously. The discrepancy between our results and that of dominant-negative mutant transgenic mice is not completely clear, but might reflect the differences in the nature of the two systems where the functions of FADD have been blocked. Although it is efficient at blocking Fas-mediated apoptosis, FADD-DN may only partially block its proliferative function. Alternatively, FADD-DN may exert nonspecific effects. For example, FADD-DN has been shown to inhibit cycloheximide-induced cell death (34), a process not known to involve any receptors. In addition, the phenotypes of FADD-DN transgenic mice vary significantly among reports (17–19, 35, 36). We have found that expression of a mouse FADD-DN protein at a high level did not perturb T-cell development nor inhibit T-cell proliferation (35), suggesting that human and murine FADD may interact with slightly different sets of target when overexpressed. Future biochemical and genetic experiments are necessary to further illuminate the molecular mechanisms by which FADD targets the cell cycle machinery.

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