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Molecules and Cells

Costimulatory Effect of Fas in Mouse T Lymphocytes

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To induce proper immune responses, T lymphocytes require two types of stimuli, antigen-specific and costimulatory signals. Among costimulatory molecules, CD28-engagement promotes the survival and proliferation of both naive and memory T cells. In addition, it is now believed that Fas may play a role in T cell activation in the human system. It is, however, controversial whether Fas can act as a costimulatory signal in the murine system. Thus, we investigated fundamental differences in the capacity to induce proliferation of T cells between Fas and CD28 in mice. Fas-mediated T cell proliferation was observed only with a full mitogenic dose of anti-CD3 antibodies, whereas CD28 engagement was able to enhance T cell proliferation in the presence of a suboptimal level of anti-CD3 antibody. Furthermore, Fas-engaged T cells showed faster response in the upregulation of CD25 and CD69 expression than CD28-engaged ones. Here, we report that Fas might play a role in mature T cell activation in the mouse system through a different mechanism from that in CD28 costimulation.

Keywords: CD28; Costimulation; Fas; Proliferation.

Introduction

Fas is a member of the tumor necrosis factor receptor superfamily, the cytoplasmic death domain of which can transduce signals leading to apoptosis (Chinnaiyan *et al*., 1995; Muzio *et al*., 1996; Sacffidi *et al*., 1998). During the antigen-specific T cell response, Fas is upregulated and the Fas ligand (FasL) is induced on activated T cells, which then may undergo Fas/FasL-mediated cell death, a phenomenon termed activation-induced cell death (AICD) (Ettinger *et al*., 1995; Renno *et al*., 1996). It is likely that the Fas-mediated death of T lymphocytes is most important to eliminate cells that repeatedly encounter persistent antigens, such as self-antigens (Parijs *et al*., 1998).

Treatment of monoclonal antibodies specific against Fas or co-culture with FasL expressing fibroblasts induce apoptosis in many types of cells including thymocytes (Dao *et al*., 1997; Ogasawara *et al*., 1993); however, ligation of Fas in mature T lymphocytes does not induce apoptosis as efficiently as that of thymocytes and some T cell lines. In fact, it has been reported that Fas-ligation even stimulates T cell proliferation in the human system, suggesting that the intracellular signal transduction pathways used by this receptor are regulated by different pathways (Alderson *et al*., 1993). The cytosolic domain of Fas is able to interact with several intracellular proteins, such as FADD (MORT-1), DAXX, RIP, FAF-1, FAP, and sentrin (Chinnaiyan *et al*., 1995). When Fas molecules are oligomerized by ligand or anti-Fas antibodies, intracellular aggregation of the Fas death domain recruits FADD, triggering the death response through a downstream component such as FLICE (caspase-8) (Muzio *et al*., 1996). In contrast, this signaling through FADD to FLICE is inhibited by FLIP, which is known to be differentially regulated in T cells during activation (Irmler *et al*., 1997; Rafaeli *et al*., 1998). Therefore, alternative expression of these death-prone or death-inhibitory molecules might determine the outcome of Fas signals in T cells so that Fas may transduce yet undefined activation signals.

For the immune response to occur, T lymphocytes must be exposed to two types of stimuli (Kim *et al*., 2000; Lenschow *et al*., 1996). The first signal is

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antigen-specific and the second signal includes costimulators and cytokines that promote clonal expansion of specific T cells and their differentiation into effector or memory cells. Furthermore, it has been demonstrated that $CD4^+$ T cells fail to proliferate if they are activated by one signal in the absence of the other signal (Parijs *et al.*, 1998). The best-defined costimulators for T cells are two members of the B7 family, B7-1 (CD80) and B7-2 (CD86), which are induced on antigen-presenting cells (Lenschow *et al.*, 1996). The CD28 receptors on T cells recognize B7 molecules and deliver activation signals that induce proliferation of both naive and memory T cells.

Desbarats *et al.* (1999) recently reported a similar dichotomy between naive and memory T cell responses to Fas engagement in the mouse system. As it was the first observation that showed the costimulatory effects of Fas in the murine system, it might be meaningful to investigate any fundamental differences between Fas and CD28 in their capacity to induce proliferation of T cells. Therefore, we compared the pathways involved in these two activation molecules in this study. These include the required dosage of anti-CD3 monoclonal antibody (mAb) to act as a costimulatory molecule as well as the surface expression profiles of T cell activation molecules such as CD69 and CD25 when Fas delivers an activation signal in the mouse system. Interestingly, we found that Fas-mediated costimulatory effects are not the same as those appearing during CD28 engagement in the mouse T cells.

Materials and Methods

Mice and the isolation of mouse T cells Female C57BL/6 (B6) mice were obtained from Seoul National University Breeding Laboratories. Mouse T cells were first isolated by mechanical disruption of fresh mouse spleen and lymph nodes in a ground-glass tissue grinder and used *ex vivo*. The T cell population was further isolated from single cell suspensions as described previously (Rosok *et al.*, 1997). Briefly, mononuclear lymphocytes were incubated with immunomagnetic beads coated with anti-mouse Ig Dynabeads (Dynal, Oslo) for 30 min at 4°C followed by isolation of magnet-attached B lymphocytes. Unattached cells were used to induce activation and the T cell purity was confirmed by flow cytometric analysis.

Cell culture and antibodies Purified T cells were suspended at 2×10^6 cells in a final volume of 1 ml of RPMI culture medium containing 10% FCS. The monoclonal antibodies used were 2C11 (anti-mouse CD3 mAb), 37.51 (anti-mouse CD28 mAb), and Jo2 (anti-mouse Fas mAb) (all from Pharmingen, San Diego, CA) and the concentration applied was 5 μg/ml unless otherwise indicated. Cell phenotypes were determined by staining cells with anti-CD4, anti-CD8, anti-B220, anti-CD25, and anti-CD69 antibodies (Becton-Dickinson, Mountain View, CA).

Proliferation assays Cells were cultured for 24, 48, or 72 h on antibody-coated plates at densities of 2×10^5 cells per 96-well plate. At indicated times, the cells were pulsed with [^{3}H]-thymidine (1 μCi/well) and cultured for another 18 h. The wells were harvested onto filters, and the incorporated radiation was quantified using a Packard scintillation counter.

Flow cytometric analysis Purified T cells were incubated in antibody-coated 24-well plates and the cells were harvested at indicated times. In order to determine T cell phenotypes, triple color staining was performed. The antibodies used were FITC-conjugated anti-CD8 mAb (Pharmingen), cychrome-conjugated anti-CD4 (Becton-Dickinson), PE conjugated anti-CD25 (Becton-Dickinson), and PE conjugated anti-CD69 (Becton-Dickinson) mAbs. The data were acquired on a FACScan (Becton-Dickinson) and were analyzed using CellQuest software.

Results and Discussion

Costimulatory effect of Fas mAb on mouse T lymphocytes It has been demonstrated that Fas ligation is able to transduce the activation signal as well as to induce apoptosis in the human system (Alderson *et al.*, 1993). Until recently, however, its activation effect was not found in the murine system. In order to investigate the effect of Fas on T cell activation in the mouse system, mouse T cells were isolated from spleen and lymph nodes.

The T cells were isolated as described in Materials and Methods and the purities were more than 95% as confirmed by flow cytometric analysis with CD4 and B220 mAbs (data not shown). Purified T cells were cultured on antibody-coated 96-well plates and the incorporated [^{3}H]-thymidine was counted at the time point indicated. As shown in Fig. 1, anti-mouse Fas antibody, Jo2, was able to induce T cell activation in combination with anti-CD3 mAb; however, Jo2 mAb alone could not induce T cell proliferation and the maximum costimulatory effect was less strong than that of CD28 (Fig. 1, day 3). In addition, Fas costimulation was not observed when used along with Con A (data not shown). Therefore, Fas seemed to work with CD3 as a costimulatory molecule in mouse T cells and the effect was comparable with that in the human system (Alderson *et al.*, 1993). This implies that Fas is able to deliver activation signals only when antigen-specific activation is preceded. In addition, the costimulatory effect of Fas is comparable to that of CD28 on day 1, suggesting that Fas may be involved in the early events of T cell activation.

Fas signals versus CD28 signals in mouse T cell activation Anti-CD28 monoclonal antibody has been well demonstrated to act as a costimulatory molecule even in the absence of APC when coimmobilized with a submitogenic dose of anti-CD3 mAb (Yashiro *et al.*,

1998). Consequently, we questioned whether Fas has the same effect as CD28 on T cells. Figure 2 shows T cell stimulation by immobilized anti-Fas and anti-CD3 mAbs at indicated doses. When stimulated with anti-mouse CD28 mAb, a submitogenic dose (0.2 μg/ml) was capable of stimulating T cell proliferation in the absence of APC and 1 μg/ml of anti-CD3 mAb with anti-CD28 mAb had an almost maximal effect on T cell activation. However, in case of Fas, more anti-CD3 mAb (more than 1 μg/ml) was needed for activation than in the CD28 case, as a submitogenic dose of anti-CD3 mAb is not able to induce T cell proliferation. In addition, to obtain the maximal costimulatory effect of Fas, 5 μg/ml of anti-CD3 mAb was required. In order to verify whether CD3 ligation simply upregulates Fas expression on the surface, its expression was checked by flow cytometric analysis upon CD3 engagement. No obvious augmentation of Fas expression was detected after the treatment with anti-CD3 mAb (data not shown); therefore, this implies that, although the expression level of Fas is similar in naive and activated T cells, the Fas signal is turned on only after certain amounts of TCR-mediated signal transduction occurred.

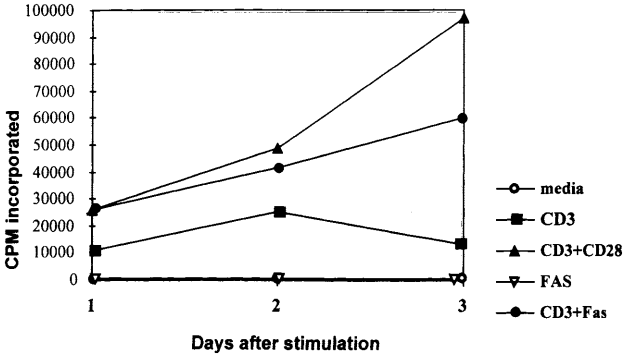

Fig. 1. Activation kinetics through CD28 and Fas molecules in mouse T cells. The T cell proliferation assay was performed by counting the incorporated [^{3}H]-thymidine in each treatment at indicated times as described in Materials and Methods. 2×10^5 cells in each 96-well plate were used and incubated for various times with the antibody indicated. The antibodies used were anti-mouse CD3 mAb, 2C11, anti-mouse CD28 mAb, 37.51, and anti-mouse Fas mAb, Jo2, and 5 μg/ml of each antibody was applied to coat the plates. The costimulatory effect of Fas ligation is evident in mouse T cells in the absence of APC.

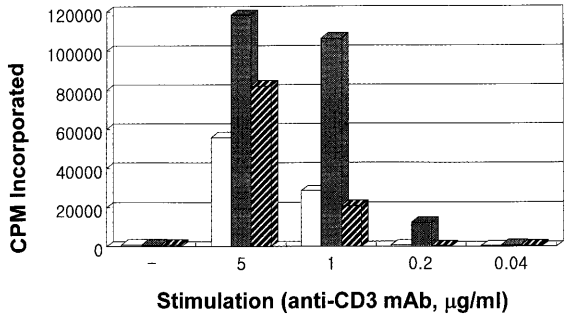

Fig. 2. Costimulatory effects of anti-CD28 and anti-Fas mAbs in submitogenic stimulation of anti-CD3 mAb. Various concentration of anti-mouse CD3 antibody was used along with a fixed concentration of anti-CD28 or anti-Fas antibody to induce T cell proliferation. In the case of CD28 signaling, detectable proliferation was observed with submitogenic concentration of anti-CD3 mAb, 0.2 μg/ml; however, the Fas signal needed more anti-CD3 mAb to activate mouse T cells. Various concentrations of anti-mouse CD3 mAb, 2C11, alone (open bars), with 5 μg/ml of anti-mouse CD28 mAb, 37.51, (closed bars) or 5 μg/ml of anti-mouse Fas mAb, Jo2, (shaded bars) were used to activate mouse T cells for 2 d and this was followed by [^{3}H]-thymidine uptake analysis. The amount of anti-CD3 mAb used is indicated on the *x*-axis.

Expression kinetics of activation molecules by Fas costimulation As shown in Fig. 1, the costimulatory effect of Fas seemed to be involved in the early event of activation. Therefore, we investigated the expression kinetics of activation molecules in both Fas and CD28 cases by performing a flow cytometric analysis of T cell activation molecules such as IL2 receptor (IL2R, CD25) and CD69 after the costimulation of T cells through mouse Fas mAb treatment. In fact, it was reported that Fas stimulation augmented the expression of activation molecules, CD25 and CD69, in the human system (Alderson *et al.*, 1993).

The early activation antigen, CD69, and the low-affinity chain of IL2R, p55, were analyzed after stimulation with anti-CD3 mAb along with anti-mouse Fas or anti-CD28 mAb. Table 1 shows the upregulation

Table 1. Intensity of activation molecules expressed on activated T cells by Fas or CD28 constimulation[a].

Days[b]	Activation[c]	CD25	CD69
1	Media	110.4[d]	ND[e]
	CD3	133.4	142.1
	CD3 + CD28	201.1	132.8
	CD3 + Fas	730.4	337.5
3	Media	124.1	135.6
	CD3	423.9	476.5
	CD3 + CD28	1159.5	635.7
	CD3 + Fas	564.5	487.1

[a] Surface expression of early activation molefules, CD25 and CD69, were checked by FACS analysis.
[b] Purified T cells were incubated for the time indicated and then the flow cytometric analysis was performed.
[c] To deliver activation signals, antibodies were immobilized on 24 well plates at a concentration of 5 μg/ml of each antibody.
[d] The mean fluorescence intensity (MFI) was calculated in each case of activation.
[e] ND, not determined.

of both molecules in the case of Fas costimulation. Interestingly, the expression of CD25 and CD69 molecules was enhanced on day 1 with Fas costimulation, while no significant increase of expression was detected with CD28 costimulation as well as TCR (CD3) stimulation (Table 1). This may explain the early stimulation of T cell proliferation observed by [^{3}H]-thymidine uptake assay in Fas costimulation as shown in Fig. 1. Thus, these results indicate that Fas costimulation induces T cell proliferation only in the presence of a full mitogenic dose of anti-CD3 mAb, which induces the surface expression of CD25 and CD69 molecules in an earlier stage than the costimulation with anti-CD28 mAb.

Costimulatory effects by Fas could be different from those by CD28 in several aspects. In fact, it is now clear that CD28 is not the only molecule that plays a role in costimulation of T cells. For instance, ICOS, the third member of the CD28/CTLA-4 family, is known to be crucial for effective T cell dependent immune responses in a CD28-independent manner (Coyle *et al.*, 2000; Hutloff *et al.*, 1999; Yoshinaga *et al.*, 1999). The natural ligands of CD28, B7-1 and B7-2 molecules, are usually expressed on antigen-presenting cells, while FasL can be expressed on T cells themselves rather than an antigen-presenting cells (Lenschow *et al.*, 1996). In addition, CD28-induced costimulation is able to affect both memory and naive T cells in contrast to Fas costimulation, in which only memory cells are the targets of activation (Desbarats *et al.*, 1999). It is also reported that inhibiting CD28-B7 interaction results in impaired TCR-induced tyrosine phosphorylation of the signal-transducing zeta chain and ZAP-70 (Tuosto and Acuto, 1998), while the stress-activated protein kinase is involved in Fas signaling (Deak *et al.*, 1998). The engagement of CD28 by B7 results in the expression of Bcl-x_L in T cells and the production of cytokines, such as IL-2, and promotes survival of primary stimulated T cells, while Fas-mediated costimulation is not an absolute requirement for a primary immune response (Parijs *et al.*, 1996). In addition, ligation of Fas on memory T cells by mAb or recombinant FasL in the presence of TCR stimulation leads to enhanced proliferation (Alderson *et al.*, 1995). However, this model could not fully explain why proliferation of $CD4^+$ memory T cells is enhanced by Fas engagement.

Our data support another possibility suggested by Desbarats *et al.* (1999); Fas-mediated costimulation might contribute to the increased efficiency and accelerated kinetics displayed during memory response. As Fas and FasL are expressed on activated T cells, Fas–FasL interaction induces AICD in primary activated T cells, while this interaction might augment the T cell proliferation in memory responses. In addition, the fact that the enhanced proliferation by Fas ligation is observed in T cells activated with a full mitogenic dose of anti-CD3 mAb suggests that high-affinity T cells may have advantages in T cell proliferation costimulated by an Fas signal than low-affinity T cells; therefore, our data suggest the possibility that Fas-induced proliferation of memory T cells might contribute to the selective evolution of high-affinity T cells during the immune response (McHeyzer-Williams *et al.*, 1999). To investigate the physiological roles of Fas-induced T cell proliferation, it is worthwhile examining whether Fas−/− mice have defective memory responses as well as lpr-cg mice, which express impaired Fas molecules on their surfaces. Furthermore, it will be interesting to study if Fas engagement is able to deliver costimulatory signals to CD4 and/or CD8 T cells and which molecules are involved in this signaling processes. This may help us to understand what is the downstream of an Fas signal as a costimulatory molecule.

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