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Tenascin Suppresses CD3-Mediated T Cell Activation

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Tenascin (TN) is an extracellular matrix protein which interferes with fibronectin (FN)-dependent cell attachment and activation as a natural antagonist to FN action. In this study, we examined the inhibitory effect of TN on T cell proliferation induced by immobilized anti-CD3 Ab combined with various costimulators in a serum-free condition. Consistent with previous studies, human T cell activation induced by anti-CD3 plus FN was completely inhibited by the addition of TN. Interestingly, TN could interfere with T cell proliferations costimulated by various very late activation antigen (VLA) integrin/ligand interactions such as VLA-4/FN, VLA-5/FN and VLA-6/laminin. Furthermore, lymphocyte function-associated antigen 1 (LFA-1)-, CD2- and CD28-costimulated T cell activation were also inhibited by TN, while TN could not affect the phorbol ester-stimulated T cell proliferation. Collectively, TN inhibits anti-CD3-induced T cell proliferation irrespectively of costimulatory molecules, suggesting that TN acts as a generally immunosuppressive extracellular matrix protein which potentially interferes with T cell receptor/CD3-mediated T cell activation.

Tenascin (TN) is a large oligomeric extracellular matrix (ECM) protein that consists of an amino- terminal cystein-rich region involved in oligomerization, followed by linear segments of EGF-like and fibronectin-type III repeats and a fibrinogen-like region at the carboxyl terminal (1, 2). TN is widely expressed during embryonic development, especially in neural tissues (3, 4), and TN has been implicated in cellular processes including adhesion, migration, and proliferation (5, 6, 7). TN is also expressed in tissues of the immune system, including the bone marrow, thymus, and the T cell areas of secondary lymphoid organs (8, 9), but functional role of TN in immune responses has not been clarified yet. A recent report demonstrated that the ability of TN to support lymphocyte rolling might reflect its ability to support cellular migration (10). In contrast, several experimental data from cell culture experiments have demonstrated that TN exhibits antiadhesive effect on fibronectin (FN)-mediated cell adhesion (11, 12, 13). Hemesath and colleagues reported that TN prevented the expression of the interleukin 2 (IL-2) and IL-2 receptor genes by blocking the appearance of functional nuclear factor-AT-1 transcription factor following T lymphocyte stimulation mediated by very late activation antigen 5 (VLA-5)/FN interaction (14). However, T cells have been shown to interact with a number of ECM proteins including not only FN but also laminin, collagen, vitronectin and fibrinogen via integrin receptors, resulting in enhanced adhesion, migration and activation. In addition, other immune accessory molecules such as lymphocyte function-associated antigen 1 (LFA-1), CD2 and CD28 are expressed on T cells, and these costimulatory molecules play critical roles in interactions between T cells and antigen presenting cells (APCs). In the present study, we revealed that TN inhibits T cell activation via CD3 ligation combined with a variety of costimulatory molecules, and discuss the potential role of TN as an immunosuppressive molecule in antigen-specific T cell activation.

MATERIALS AND METHODS

Tenascin and costimulators. Human tenascin purified from U-251MG glioma cell line was purchased from Chemicon International Inc. (Temecula, CA), and was disolved in PBS at a concentration of 1 mg/ml and stocked at −80°C. TN used for T cell proliferation assay was added at a final concentration of 10 μg/ml. Human plasma fibronectin and laminin were purchased from Gibco-BRL (Gaithersberg, MD) and Mallinckrodt Speciality Chemical Inc. (Chesterfield, MO), respectively. Fibronectin fragments C-274, H-CS1 and purified human CD54 were kindly donated by Dr. I. Saiki (Toyama Medical and Pharmaceutical University) (15) and Dr. T. Kinashi (University of

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Tokyo) (16), respectively. Monoclonal antibodies against human CD3 (OKT3), CD2 ($T11_3$) and CD28 (TN228) were purified from ascites on protein A column. Polyclonal rabbit antibody against human tenascin was purchased from Cosmo-Bio Co., Ltd (Tokyo, Japan).

T cell preparation. Human T cells were purified from peripheral blood mononuclear cells (PBMC) of normal healthy donors by density gradient centrifugation on Ficoll-Hypaque (Pharmacia). Monocytes and B cells in PBMC were depleted by adherence to plastic dish, followed by nylon-wool column for 1h at 37°C. Plastic and nylon-wool non-adherent T cells resulted in >95% pure $CD3^+$ T cells, as determined by flow cytometry. Purified T cells were washed with serum-free RPMI1640 medium 3 times and suspended in AIM-V serum-free medium (Gibco-BRL).

T cell proliferation. Anti-CD3 antibody (OKT3, 100 ng/ml in PBS) was distributed onto 96-well microplate overnight at 4°C, followed by coating with human plasma fibronectin (FN), laminin (LM), FN fragments (H-CS1 and C-274), purified CD54, anti-CD28, or anti-CD2 mAbs at a concentration of 10 μg/ml in PBS for 3h at 37°C. Purified T cells in AIM-V medium were added at 2×10^4 cells/well in a final volume of 200 μl with or without TN, and plates were incubated at 37°C, 5% CO_2. After 3 or 4 days, the proliferative response was measured by supplementing the cultures with 0.5 μCi/well ^{3}H-thymidine(TdR) (DuPont/NEN, Boston, MA) for 8h prior to harvesting the cells onto glass fiber filters. Filter-bound ^{3}H-TdR incorporated into newly synthesized DNA was measured on a scintillation counter (Beckman Instruments, Fullerton, CA).

T cell attachment assay. T cell attachment was assessed in 96-well U-bottomed plates pre-coated with human plasma FN at 10 μg/ml. After blocking with BSA-PBS, human T cells (5×10^4 in 100 μl) resuspended in RPMI 1640 were added to each well in the presence of TN at various concentrations, and plates were incubated for 1h at 37°C, 5% CO_2. After washing to remove the unattached cells, the attached cells were stained with 0.2% crystal violet solution in 20% methanol for 10 min. After washing and drying, 50 μl of 1% sodium dodecyl sulfate was used to dissolve the cells and the absorbance at 560 nm was measured by Model 3550 Microplate Reader (Bio-Rad Lab.,Hercules,CA).

RESULTS

Inhibitory Effect of TN on FN Costimulation of Anti-CD3-Stimulated T Cell Proliferation

We first examined the effect of TN on proliferative response of anti-CD3-stimulated T cells when costimulated by FN. To avoid the contribution of other costimulatory extracellular matrix proteins in fetal bovine serum, proliferation assays were performed in serum-free medium as described previously (17). Similar to the previous reports, OKT3 or FN alone could not induce T cell proliferation in serum-free condition as shown in Figure 1A, but coimmobilization of OKT3 and FN enabled T cells to proliferate vigorously (Figure 1A). As shown in Figure 1B, the proliferative response induced by OKT3 and FN was inhibited by the addition of TN in a dose-dependent manner, with complete inhibition at concentrations over 20 μg/ml. Similarly, plate-coated TN was also able to inhibit T cell proliferation induced by OKT3 and FN (not shown). We then observed that T cell proliferative response was restored by the addition of neutralizing anti-TN antibody (Figure 1C), indicating that the suppression was indeed mediated by TN. To determine wether TN could interfere with the adherence of T cell to ECM, we examined the inhibitory effect of TN on FN-mediated T cell adhesion. As expected, TN could inhibit the T cell adhesion to FN in a dose-dependent manner (Figure 2). However, high dose of TN (> 10 μg/ml) which provided complete inhibition for T cell proliferation could not completely suppress T cell attachment (50% inhibition).

TN Inhibits T Cell Proliferation Costimulated by Sub-domains of FN and LM

A previous report demonstrated that soluble TN interfered with T cell activation via RGD-dependent VLA-5 integrin receptor (14). It has been shown that resting T cells constitutively express VLA-4, VLA-5 and VLA-6 which act as costimulatory molecules in T cell activation (18, 19). To examine whether TN also suppress T cell activation mediated by VLA-4 and VLA-6, we investigated proliferative responses of T cells cultured on plates coated with anti-CD3 plus fragments of FN (H-CS1 and C-274) or LM. As shown in Figure 3, T cell proliferation induced by H-CS1 (bind to VLA-4), C-274 (bind to VLA-5) or LM (bind to VLA-6) were significantly inhibited by the addition of TN at 10 μg/ml, suggesting that TN could suppress T cell activation costimulated not only via RGD-dependent VLA-5 but also via RGD-independent VLA-4 and VLA-6 integrins.

Inhibition of T Cell Proliferation Induced by Other Costimulatory Signals

It has been shown that several costimulatory molecules expressed on APCs greatly augment the proliferative response of anti-CD3-stimulated T cells (19, 20). We next examined whether TN could suppress T cell activation induced by another costimulatory molecules such as LFA-1, CD28 and CD2. As shown in Figure 4, costimulatory effects were observed with purified CD54 protein, anti-CD28 and anti-CD2 mAbs that induced vigorous T cell proliferation. Unexpectedly, we found that TN could also suppress the T cell proliferation induced by these costimulators. Our preliminary data indicated that TN did not alter the binding ability of T cells to CD54, anti-CD28 or anti-CD2 mAb (data not shown). These results suggest that TN might suppress some intracellular signal transduction pathway mediated by CD3 or costimulatory molecules.

TN Is Unable to Affect T Cell Proliferation Induced by PMA

Treatment with phorbol myristate acetate (PMA), a pharmacological activator of protein kinase C, in com-

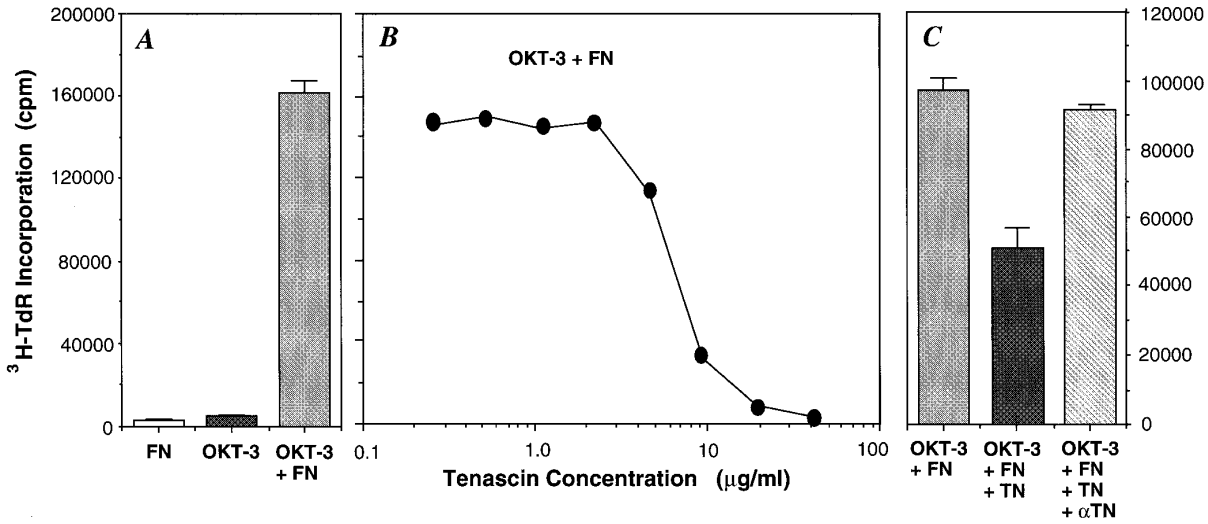

FIG. 1. T cell proliferation induced by coimmobilization of anti-CD3 Ab and FN. (*A*). Purified human T cells (2×10^4) were cultured for 4 days in a 96 well microtiter plate coated with OKT3 and/or FN, as indicated. Cultures were supplemented with 0.5 μCi of ^{3}H-thymidine(TdR) for the final 8 hours. Data are represented as mean cpm + SD of triplicate wells. (*B*). Inhibition of T cell proliferation by TN. Human T cells were stimulated with OKT3 plus FN for 4 days in the presence of TN at the indicated concentrations (abscissa). Data represent mean cpm of incorporated ^{3}H-TdR of triplicate wells. (*C*). Inhibitory effect of anti-TN antibody on TN-mediated suppression of T cell proliferation. T cells stimulated with OKT-3 and FN were cultured with TN with or without antibody to TN. After culturing for 3 days, proliferative response of T cells was measured by ^{3}H-thymidine incorporation assay.

bination with ionomycin or some costimulatory molecules results in proliferation of T and B lymphocytes (21). We finally examined whether TN inhibits PMA-induced T cell proliferation. As shown in Figure 5, suboptimal doses of PMA alone could not induce obvious T cell proliferation, while the combination with ionomycin, anti-CD28 or FN induced vigorous T cell proliferation. In contrast to the valid inhibitory effect of TN against anti-CD3-stimulated T cell proliferation, the addition of TN exhibited only a weak (15%) inhibitory effect on T cell proliferation induced by PMA plus FN but did not affect T cell proliferation induced by PMA plus ionomycin or anti-CD28 Ab (Figure 5). Moreover, TN was unable to inhibit the IL-2-induced T cell proliferation (data not shown). These results indicate that TN does not inhibit T cell proliferation generally but inhibits the TcR/CD3-mediated proliferative response irrespectively of costimulatory signals.

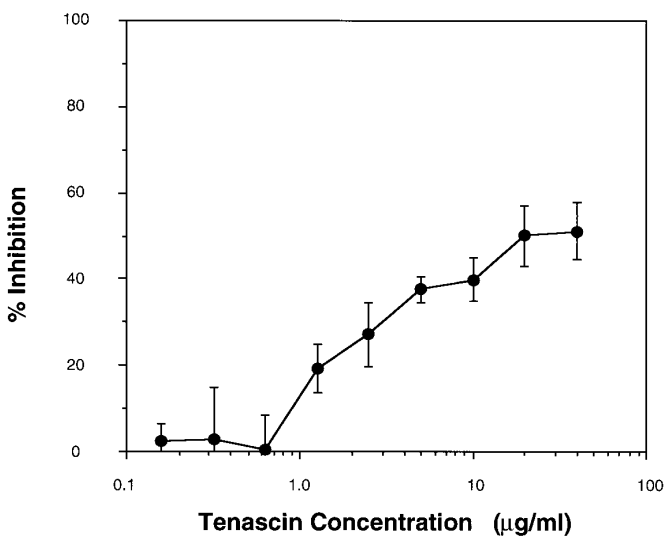

FIG. 2. Inhibitory effect of TN on T cell adhesion to FN. Human T cells were incubated on FN-coated plate for 1h in the presence of TN at the indicated concentrations (abscissa) and the number of the attached cells were assessed by crystal violet staining. Data represent mean ± SD of triplicate wells.

DISCUSSION

In this study we investigated the regulatory effect of TN on T cell activation induced by a variety of costimulators, including ECMs (FN, FN fragments and LM), ICAM-1 (CD54), and agonistic antibodies (anti-CD2 and CD28 Abs). A previous report demonstrated that TN inhibited T cell proliferation induced by soluble antigen, alloantigen, or mitogen (22). However, the inhibitory mechanism of TN has not been characterized in detail. Generally, two signals through their TcR/CD3 complex and costimulatory molecules are required for inducing T cell proliferation. Recently, Hemesath et al reported that interference of T cell adhesion to FN by

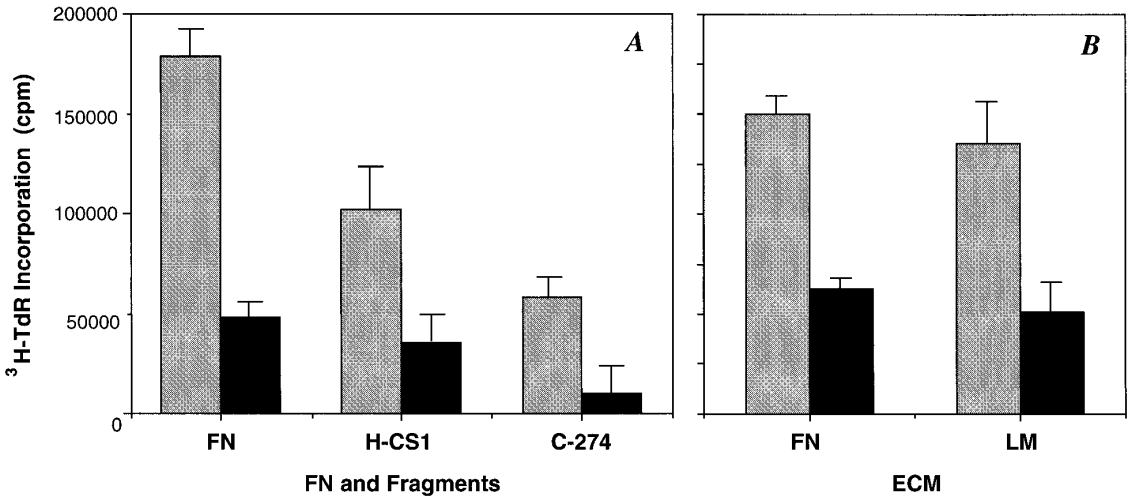

FIG. 3. Inhibitory effect of TN on T cell proliferation induced by sub-domains of FN and LM. (*A*). Inhibition of T cell proliferation costimulated with FN or FN fragments (H-CS1 or C-274) by TN. Human T cells were cultured in plates coated with OKT3 plus FN, H-CS1 or C-274 in the presence (solid bars) or absence (shaded bars) of 10 μg/ml TN. Data represent mean + SD of triplicate wells. (*B*). Proliferative response of human T cells cultured on LM. Human T cells were cultured in plates coated with OKT3 plus FN or LM in the presence (solid bars) or absence (shaded bars) of TN. Data represent mean + SD of triplicate wells.

soluble TN led to the inhibition of human T cell proliferation (14). However, we found that TN could completely inhibit the T cell proliferation despite of partial inhibition of adherence to FN. To further clarify the immunosuppressive activity of TN, we examined the inhibitory effect of TN on T cell proliferation induced by a variety of costimulators. T cell proliferation induced by immobilized anti-CD3 Ab and FN was completely inhibited by both soluble and immobilized TN, and TN also inhibited T cell proliferation costimulated via VLA-4/H-CS1, VLA-5/C-274 and VLA-6/LM interactions. Consistent with the previous observation that TN suppressed IL-2 production (14), the addition of exogeneous IL-2 to the cultures reversed the inhibition by TN (data not shown). Furthermore, we found that TN also inhibits the LFA-1-, CD28- or CD2-costimulated T cell proliferation, while TN could not block the IL-2- and PMA-induced T cell proliferation. In addition, it was reported that TN prevented the appearance of NF-AT1 in nuclei

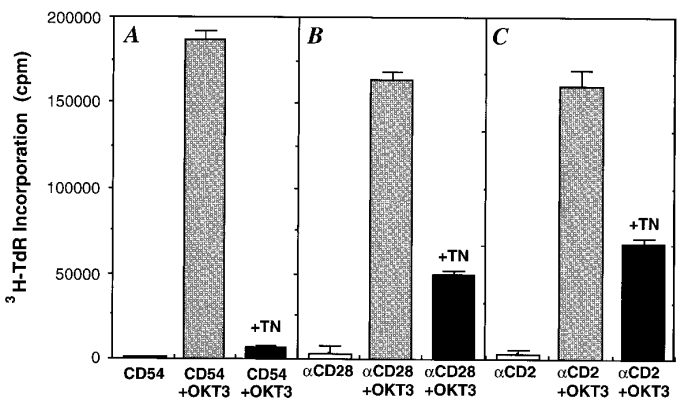

FIG. 4. Effect of TN on T cell proliferation induced by other costimulatory signals. Anti-CD3 Ab (OKT3) plus purified human CD54 protein (*A*), anti-CD28 Ab (*B*) or anti-CD2 Ab (*C*) were coated onto 96 well microtiter plates overnight, followed by culturing human T cells in the presence (solid bars) or absence (shaded bars) of 10 μg/ml TN. Data represent mean + SD of triplicate wells.

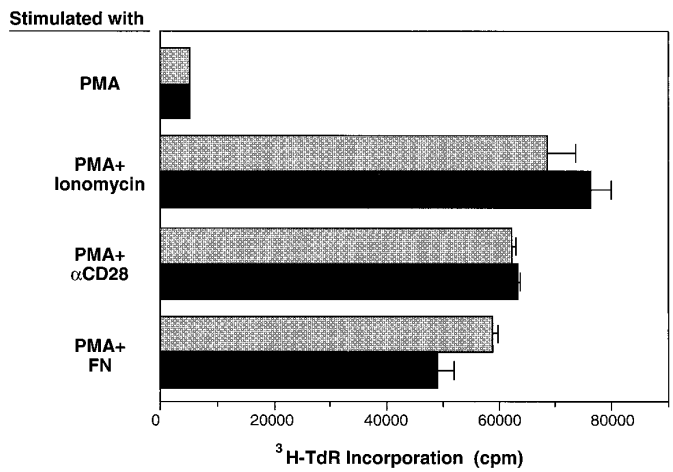

FIG. 5. Effect of TN on T cell proliferation induced by PMA plus costimulators. Human T cells were stimulated with PMA plus ionomycin, anti-CD28 Ab or FN in the presence (solid bars) or absence (shaded bars) of 10 μg/ml TN. ^{3}H-TdR incorporation was measured for the last 8 hours of a 3 day culture. Data represent mean + SD of triplicate wells.

of activated T cells (14). Collectively, TN inhibit T cell proliferation costimulated not only by ECM receptor integrins but also by LFA-1 and Ig superfamily molecules such as CD2 and CD28, when stimulated by anti-CD3 mAb but not by PMA. Therefore, it seems more likely that TN may interfere with the TcR/CD3-mediated signal transduction in T cell activation. The effect of TN on intracellular events that are induced by TcR/CD3 ligation, such as CD3-ζ chain tyrosine phosphorylation and ZAP-70 activation, remains to be determined by further studies.

A number of molecules have been reported to be a TN receptor. Several ECMs such as proteoglycan, collagen, and FN were found to interact with TN (23, 24, 25). Chung et al. reported that TN binds preferentially to soluble FN and matirx fibrils and acts as an antagonist to FN (25). Several cell surface integrins such as $\alpha_8\beta_1$, $\alpha_9\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_6$ bind to TN via the TNfn3 domain containing FN type III repeats with RGD sequence (26, 27, 28). Furthermore, non-integrin proteins including annaxin II, neurocan, phosphacan, perlecan were also identified as TN receptors (29, 30). However, all these molecules have not been found on resting T cells. Previous results demonstrated that TN interacted with human monocytes, T cells and Epstein-Barr virus-transformed B cells and alters their activation properties (22). In addition, Clark and colleagues suggested that binding to TN is not dependent on any molecules previously identified as TN receptors and is likely to involve a novel TN receptor on lymphocytes (10). Identification of TN receptor on lymphocytes will be essential to calify the molecular mechanisms for the T cell unresponsiveness induced by TN.

In a recent study using the TN-deficient mice, no anatomical or histological abnormalities were observed in any tissues, raising a doubt on proposed roles of TN in normal development (31). Besides the physiological function of TN in development, TN is also found in the matrices of a number of tumors including glioma, neuroblastoma, astrocytoma, small cell lung carcinoma (32, 33, 34). Previously, Ruegg and co-workers reported that TN inhibited T cell activation induced by soluble antigen and alloantigen (22). Our preliminary data showed that allogeneic T cell response against small cell lung carcinoma expressing TN was partially enhanced by the addition of anti-TN antibody (not shown). In view of the immunosuppressive function of TN observed in this study, we hypothesize that immunosuppression by tumor-derived TN is a potent mechanism of tumor evasion from immune surveillance.

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