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TRANSFORMING GROWTH FACTOR-β-INDUCED INHIBITION OF T CELL FUNCTION

Susceptibility Difference in T Cells of Various Phenotypes and Functions and Its Relevance to Immunosuppression in the Tumor-Bearing State[1]

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The present study investigates the nature of humoral component(s) generated in tumor-bearing hosts to induce immune dysfunction of T cells. Cell-free ascitic fluid and culture supernatant (SN) were obtained from the ascites and cultures allowing MH134 hepatoma cells to grow. These ascites and SN samples were tested for their abilities to influence the generation of CTL responses to TNP and alloantigens. The generation of the anti-TNP CTL responses that require self H-2-restricted CD4+ Th cells was markedly suppressed by addition of the ascites or SN under conditions in which these samples did not inhibit anti-allo CTL responses capable of using alternate pathways of allo-restricted CD4+ and CD8+ Th. The activation of CD8+ CTL precursors and CTL activity were also resistant to the ascites or SN. The ascites- or SN-induced suppressive effect to which CD4+ Th were most susceptible was found to be mediated by transforming growth factor-β (TGF-β) activity, because: 1) the TGF-β activity was detected in the MH134 ascites and culture SN; 2) the suppression of CD4+ Th function required for anti-TNP CTL responses was almost completely prevented by addition of anti-TGF-β antibody to cultures and; 3) rTGF-β also induced similar patterns of immunosuppression to those observed by ascites or SN. These results indicate that TGF-β produced by tumor cells induces deleterious effects on T cell, especially on the CD4+ Th subset, and provide an explanation for the molecular mechanism underlying the previously observed CD4+ Th-selective suppression in the tumor-bearing state.

Tumor-bearing hosts fail to reject malignant cells even in experimentally induced animal tumor models in which tumor cells express detectable levels of immunogenicity. Numerous reports showing that various suppressive mechanisms (1–10) are generated during the growth of immunogenic tumors have served to provide an explanation for the paradoxical growth of these tumors in immunocompetent syngeneic hosts.

Although it has been documented that T cell-mediated immunity is generally impaired in tumor-bearing hosts, we have recently found that CD4+ T cell subset is more susceptible to the immunosuppressive effect in the tumor-bearing state than CD8+ T cell subset including CD8+ CTL precursors and CD8+ Th cells (11). This was demonstrated by the findings that lymphoid cells from tumor-bearing hosts fail to generate anti-TNP-self CTL responses requiring the assistance of CD4+ Th, whereas portions of the same responding cells generate anti-allo-CTL responses capable of using CD8+ Th (11). The fact that anti-TNP CTL responses were inducible by lymphoid cells from tumor-bearing mice in the presence of exogenous IL-2 (11) also demonstrated that the activation/function of CTL precursors was not affected by the suppressive potential, and suggested that these mice had a defect in CD4+ Th function. Moreover, it has been shown that tumor-neutralizing activity was detectable in the CD8+ but not in the CD4+ T cell subset from tumor-bearing mice (12) although both CD4+ and CD8+ T cell subsets from tumor-immunized mice were capable of producing in vivo protection (13–15). These observations indicated the existence of immune dysfunction expressed preferentially on CD4+ T cell subset in the tumor-bearing state. Although suppressor cell activity capable of directly inhibiting the function of CD4+ T cell subset was not detected in lymphoid cells from tumor-bearing mice, the possibility existed that host's non-lymphoid cells and/or tumor cells generated suppressive potential through the production of humoral factor(s).

The present study investigates the nature of humoral component(s) generated during the tumor-bearing state to induce CD4+ T cell-selective dysfunction. The results show that similar CD4+ T cell dysfunction to that observed in lymphoid cells from tumor-bearing hosts is induced in cultures of normal lymphoid cells when added by cell-free ascitic fluid or tumor cell culture supernatants. Such dysfunction was found to be attributable to TGF-β produced by tumor cells, because CD4+ T cell dysfunction was: 1) prevented by addition of anti-TGF-β

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antibody; and 2) also induced by rTGF-β instead of tumor cell-derived components. The results are discussed in the context of the difference in the susceptibility to TGF-β between $CD4^+$ and $CD8^+$ T cells as well as the in vivo physiologic relevance of TGF-β-induced immunosuppression.

MATERIALS AND METHODS

Mice and Tumors

Female C3H/He and BALB/c mice were obtained from Charles River Laboratory, Kanagawa, Japan and Shizuoka Experimental Animal Laboratory, Hamamatsu, Japan, respectively. These mice were used at 7 to 9 wk of age. MH134 hepatoma (16) and 4 additional tumors CSA1M fibrosarcoma (17), BCL1 B cell leukemia (18), LSTRA T cell leukemia (19), and MCH-11-AI fibrosarcoma (20) were used.

Cells and Reagents

Mv1Lu cells and NRK49F cells for TGF-β assays were obtained from American Type Culture Collection, Rockville, MD. Anti-TGF-β antibody was purchased from R & D Systems, Minneapolis, MN. Human rTGF-β was obtained from King Jozo Co. Ltd., Hyogo, Japan and rIL-2 (human) was kindly provided by Shionogi Pharmaceuntical Co. Ltd., Osaka, Japan.

Cell-Free Ascitic Fluid and Tumor Cell Culture SN[3]

C3H/He mice were inoculated i.p. with 10^6 viable MH134 hepatoma cells. Ten days later, ascites was collected and cell-free ascitic fluid was obtained by centrifugation. Tumor cell culture SN were obtained 1 day after culturing tumor cells in FCS-free RPMI 1640 medium.

Treatment of Culture SN with Acid

FCS-free culture supernatant was dialyzed against two changes of 1 M acetic acid, and then dialized against PBS, pH 7.4. This procedure was described as A/N treatment.

In Vitro Sensitization for Cytotoxic Effector Cells and Cytotoxicity Assay

In vitro sensitization and cytotoxicity assay were essentially the same as previously described (19). Briefly, 5×10^6 spleen cells were sensitized in vitro to irradiated (2000rad) allogeneic spleen cells (10^6) or syngeneic spleen cells (10^6) modified with 1 mM TNP-self in a 2-ml volume in 24-well culture plate. Effector cells generated after 5 days of culture were assayed on Con A-stimulated allogeneic or TNP-modified syngeneic blast target cells.

Detection of TGF-β Activity

Mv1Lu cell growth inhibition assay. The growth inhibition assay was performed with slight modifications according to the original method described by Cheifetz et al. (21). Briefly, Mv1Lu cells (1×10^4) were cultured with diluted samples or rTGF-β in a 0.2-ml volume of RPMI 1640 medium containing 5% FCS in 96-well microplates (Corning no. 25860; Corning Glass Works, Corning, NY) for 24 h in a CO_2 incubator. Cells were pulse-labelled with 20 KBq ^{3}H-TdR for the final 4 h, and the incorporated radioactivity was measured. Results are shown as the mean cpm ± SE of triplicate cultures.

Assay for growth in soft agar (soft agar growth assay). The procedure was essentially the same as previously described (22). Agar plates were prepared in 60-mm petri dishes (Corning no. 25010, Corning Glass Works) by first applying a 2-ml base layer of 0.5% agar (Difco, Agar Noble, Detroit, MI) in DMEM containing 10% FCS. Over this basal layer, an additional 2-ml layer of 0.3% agar in the DMEM/FCS containing appropriate concentrations of samples, 2×10^3 NRK-49F cells and 2 ng/ml of epidermal growth factor were applied. These plates were placed in CO_2 incubator (95% air/5% CO_2) for 7 days. Colonies were counted by using a microscope.

RESULTS

Selective suppression of $CD4^+$ Th-participating responses by tumor cell-derived humoral factor(s). We have confirmed the fact (11) that lymphoid cells from tumor-bearing hosts fail to generate anti-TNP CTL responses whereas portions of the same responding cells are capable of producing anti-allo-CTL responses (Fig. 1 *A* and *B*). Spleen cells from MH134 tumor-bearing C3H/He mice were stimulated with either TNP-conjugated syngeneic spleen cells (TNP-self) (Fig. 1*A*) or allogeneic BALB/c spleen cells (Fig. 1*B*). Effector cells generated 5 days after were assayed on TNP-self or BALB/c blast target cells. The results indicate selective inhibition of the generation of anti-TNP-self CTL responses, which require the participation of $CD4^+$ Th. In this report, studies were initiated to investigate whether some of humoral factors derived from tumor cells are responsible for such a $CD4^+$ Th-preferential suppression.

MH134 tumor cell-free ascitic fluid and -culture SN were prepared and these samples were included into cultures of normal responding spleen cells that are stimulated in vitro with TNP-self or allogeneic cells. The results of Figure 1 *C*, *D*, *E*, and *F* demonstrate that addition of defined concentrations of ascitic fluid (1%) or culture SN (50%) to the cultures results in selective inhibition of anti-TNP-self but not of anti-allo-CTL responses. To test the effects of the ascitic fluid and culture SN on the activation of CTL precursors themselves, anti-TNP CTL cultures containing these suppressive materials were supplemented with rIL-2. The results of Figure 2 show that rIL-2 prevent the suppression of anti-TNP CTL responses that is induced by either ascites or concentrated culture SN, demonstrating that the activation of CTL precursors is not affected by the tumor-derived factor(s). It was also found that neither ascitic fluid nor culture SN did not affect CTL activity once generated (data not shown). These results indicate that some humoral factor(s) contained in ascitic fluid or culture SN induces similar patterns of immunosuppression to those

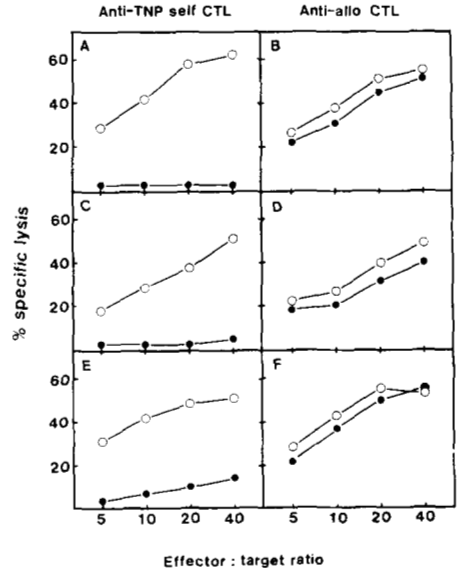

Figure 1. Anti-TNP self and anti-allo CTL responses of responding cells from tumor-bearing mice or normal responding cells in the presence of tumor-derived factor(s). Responding spleen cells (5×10^6) from C3H/He normal (○) or tumor-bearing mice (●) were sensitized to 1×10^6 TNP-self or allogeneic BALB/c cells (*A* and *B*). Normal responding spleen cells were sensitized to TNP-self or allogeneic cells in the absence (○) or presence (●) of 1% MH134 ascitic fluid (*C* and *D*) or 50% MH134 culture SN (*E* and *F*). Effectors generated 5 days after were assayed on TNP-self or BALB/c blast target cells.

[3] Abbreviations used in this paper: SN, supernatant; TNP-self, TNP-modified syngeneic cells; TGF-β, transforming growth factor-β; α2M, α2-macroglobulin.

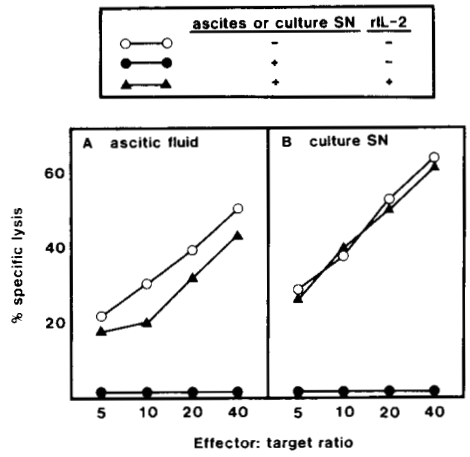

Figure 2. Reversal of ascites- or SN-induced suppression of anti-TNP self CTL responses by rIL-2. Normal responding spleen cells were sensitized to TNP-self in cultures containing 1% MH134 ascitic fluid (*A*) or concentrated culture SN (*B*) in the absence or presence of 40 U/ml of rIL-2. The concentrated culture SN was prepared by precipitating with saturated ammonium sulfate, centrifuging at 10,000 × *g* for 20 min, and then by dissolving the pellet with RPMI 1640 medium in 1/50 volume of the original sample.

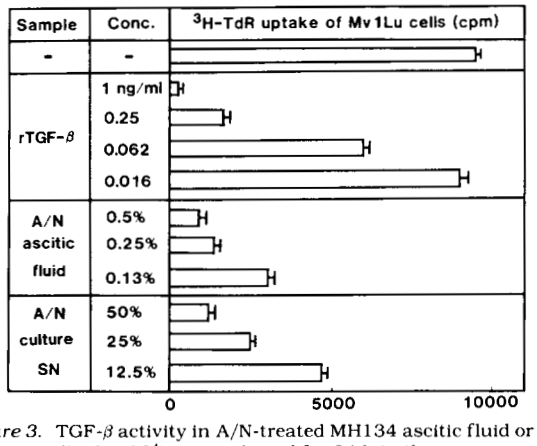

Figure 3. TGF-β activity in A/N-treated MH134 ascitic fluid or culture SN. Mv1Lu cells (1 × 10^4) were cultured for 24 h in the presence of rTGF-β or A/N-treated MH134 ascitic fluid or culture SN at indicated concentrations. Cells were pulse-labeled with 20KBq ^{3}H-TdR and incorporated ^{3}H-TdR was determined.

observed by lymphoid cells from tumor-bearing mice (Fig. 1 *A* and *B*).

Presence of TGF-β activity in MH134-ascitic fluid and culture SN. We have investigated whether MH134 ascitic fluid and culture SN contain TGF-β activity. TGF-β activity was assessed by using two assay systems (growth inhibition assay and anchorage-independent growth assay in soft agar). The results of Figure 3 demonstrate that growth inhibition assay using Mv1Lu mink lung epithelial cells detects potent TGF-β activity in A/N-treated MH134 ascitic fluid and culture SN. The production of an active form of TGF-β by various tumor cells including MH134 hepatoma was also examined by using untreated crude culture SNs (Fig. 4). The results illustrate that some of the five tumor cell lines tested produced detectable levels of TGF-β activity in an active form as detected in crude (A/N-untreated) culture SN, although most of tumor cell types except for MCH-11-Al exhibited more potent TGF-β activity in A/N-treated SN (total TGF-β activity). Because this assay system was unable to detect TGF-β activity in untreated ascitic fluid probably due to inclusion of some interferring factors in the ascites, inclusion of an active form of TGF-β activity in untreated ascitic fluid was determined by another assay, the soft agar assay. The results of Table I illustrate that untreated MH134-ascitic fluid stimulates the growth of NRK-49F fibroblasts in soft agar containing epidermal growth factor, indicating the presence of TGF-β activity in untreated MH134-ascitic fluid as well.

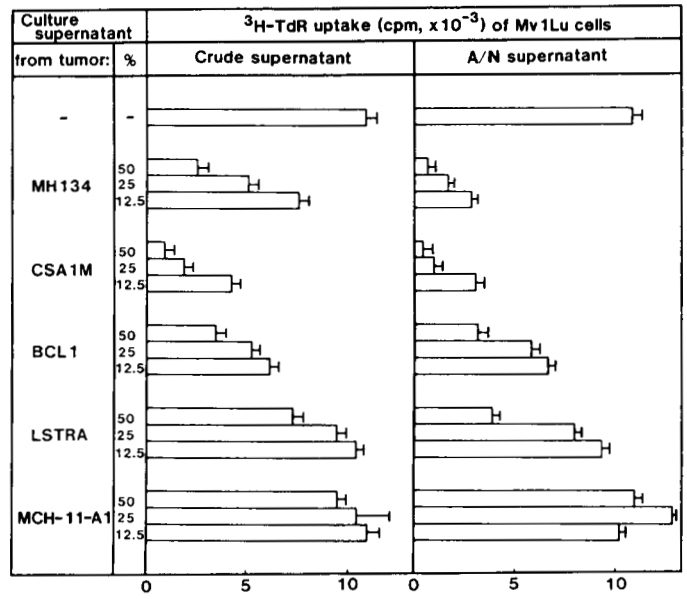

Figure 4. Production of an active form of TGF-β by various tumor cell lines. TGF-β activity in an active form or in total (active and latent forms) was detected in crude (untreated) or A/N-treated culture SN of various tumor cell lines.

TABLE I

TGF-β activity in crude MH134 ascitic fluid

Sample[a]	Dose	EGF[b]	No. of Colony	
			Expt. 1	Expt. 2
		−	0	0
		+	38	0
rTGF-β	1.0 ng/ml	+	ND	638
	0.3	+	398	244
	0.03	+	152	104
Ascitic fluid	1.0%	−	0	0
	1.0%	+	494	420
	0.5%	−	0	0
	0.5%	+	344	396
	0.25%	−	ND	0
	0.25%	+	ND	240

[a] rTGF-β or untreated (crude) MH134 ascitic fluid was added at indicated concentrations to soft agar in anchorage-independent growth assay using NRK-49F cells.

[b] The concentration of EGF was predetermined to provide conditions in which EGF helped TGF-β to induce optimal anchorage-independent growth of NRK-49F cells but produced marginal numbers of colonies by itself. Thus, 2 ng/ml EGF was included in soft agar.

Although the above results strongly suggested the presence of TGF-β activity in ascitic fluid and culture SN, this was confirmed by examining the blocking of such TGF-β activity by anti-TGF-β antibody. The results of Figure 5 demonstrate that TGF-β activity as detected by the growth inhibition assay can be suppressed almost completely by addition of anti-TGF-β antibody. Thus, taken collectively, these results indicate that TGF-β is contained in MH134 ascitic fluid and culture SN that induced CD4+ Th-selective suppression.

Prevention of suppression of CD4+ Th function by anti-TGF-β antibody. The preceding results prompted us to test whether anti-TGF-β antibody can prevent the

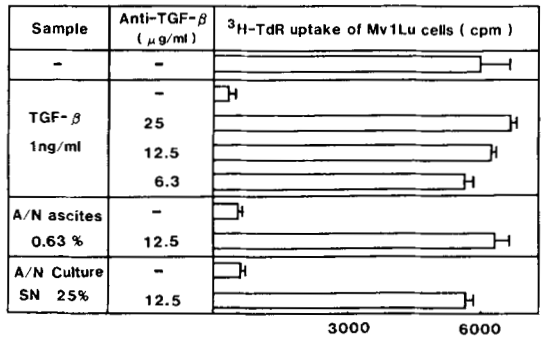

Figure 5. Blocking of TGF-β activity by anti-TGF-β antibody. Mv1Lu cells were cultured with various samples (shown) in the absence or presence of anti-TGF-β antibody. Normal rabbit IgG was used as control.

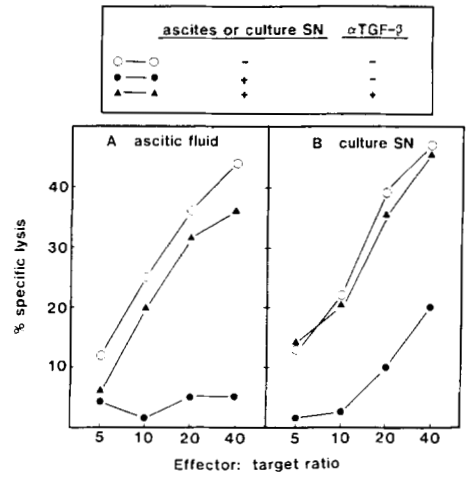

Figure 6. Prevention of ascites- or culture SN-induced suppression of anti-TNP self CTL responses by anti-TGF-β antibody. Normal responding spleen cells were sensitized to TNP-self in cultures containing 1% MH134 ascitic fluid or 25% MH134 culture SN in the absence (normal IgG) or presence of anti-TGF-β antibody (12.5 μg/ml).

inhibition of the generation of anti-TNP-self CTL responses induced by MH134 ascitic fluid or culture SN. As shown in Figure 6, the addition of anti-TGF-β antibody to cultures almost completely restored the generation of anti-TNP-self CTL responses that would be otherwise inhibited by ascitic fluid or culture SN. These results indicate that TGF-β contained in ascitic fluid or culture SN is responsible for inhibiting the activation of CD4+ T cell subset.

Suppression of CD4+ Th function by rTGF-β. We finally investigated whether rTGF-β induces the suppression of CD4+ Th function. Various doses of rTGF-β were included into cultures generating TNP-self or allo-CTL responses (Fig. 7). The results demonstrate that rTGF-β suppresses the generation of anti-TNP-self, but not of anti-allo CTL responses when added to cultures at the concentration of 1 to 2 ng/ml, although this cytokine inhibits the generation of both anti-TNP-self and anti-allo CTL responses at its higher (>6 ng/ml) concentrations.

Thus, taken collectively, these results indicate that selective suppression of self Ia-restricted CD4+ Th function that is observed in the tumor-bearing state is inducible in vitro by the addition to cultures of rTGF-β as well as tumor-derived humoral products containing TGF-β activity.

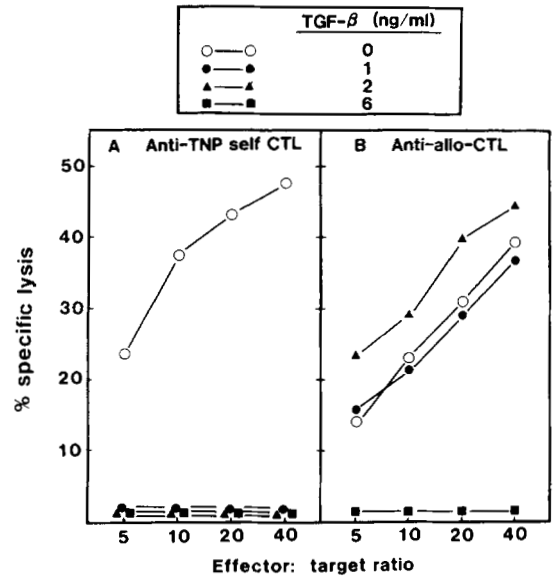

Figure 7. Effect of rTGF-β on the generation of anti-TNP-self (*A*) or anti-allo CTL responses (*B*). Normal responding spleen cells were sensitized to TNP-self in the presence of various concentrations of rTGF-β.

DISCUSSION

TGF-β is a hormonally active polypeptide that is produced by many types of cells, irrespective of whether they are nontransformed or transformed (23, 24). This unique growth factor was originally discovered as one of molecules with transforming activities able to support the anchorage-independent growth of normal cells (22, 25, 26), and its complementary DNA sequence has been recently determined (27). In addition to such mitogenic effects on some fibroblast systems, it has been reported that the TGF-β exerts multiple actions on both normal and transformed cells (23), including a negative growth modulatory effect (28). Among a variety of TGF-β actions, recent studies have defined various immunoregulatory properties of TGF-β for immune system, including inhibition of T and B cell proliferation and lymphokine production (29–33). However, it has not been determined how host's immune reactivity is influenced by tumor cell-derived TGF-β in the tumor-bearing state.

An earlier study from our laboratory demonstrated that CD4+ T cell-mediated immune responses are more severely suppressed in the tumor-bearing state than CD8+ T cell-mediated immunities (11). Preferential suppression of CD4+ T cell immunity was demonstrated for CD4+ T cells responsible for mediating in vivo tumor-neutralizing activity (14), as well as providing help for CTL responses against tumor antigens (34). It was also shown in a previous report (11) that such CD4+-selective immune dysfunction is: 1) not due to the loss or decrease in the number of CD4+ T cells that has been described to explain immune dysfunction in symptomatic AIDS or graft-vs-host disease (35, 36); 2) reversible after the removal of tumor mass; and 3) not ascribed to suppressor cell activity capable of directly inhibiting the function of CD4+ T cell subset. Thus, these observations raised the possibility that host's non-lymphoid cells or tumor cells generate suppressive potential through the production of humoral factor(s).

In the present study, the nature of a humoral component(s) generated by tumor cells to induce such an im-

mune dysfunction was investigated by monitoring its suppressive effect on CD4+ Th function. The results showed that the addition of MH134 tumor cell culture SN and ascitic fluids at a given concentration to cultures of normal responding cells resulted in selective suppression of CD4+ Th but not of CD8+ Th or CTL precursors that was similar to that observed in responding cells from tumor-bearing mice. Our results also demonstrated that 1) the above suppression was prevented by anti-TGF-β antibody and 2) rTGF-β induced CD4+ Th-selective suppression when included in CTL cultures of normal responding cells. This illustrates the inclusion of TGF-β in tumor cell culture SN and ascitic fluids and mediation of the CD4+ Th-selective suppression by TGF-β included. Because several types of TGF-β may be contained in these tumor cell-derived preparations, the exact type(s) of TGF-β will have to be determined. In addition, an aspect of the TGF-β effect should be noted from our studies. In considering that the function of spleen cells from tumor-bearing mice are still suppressed even after washing these cells prior to in vitro CTL cultures (Fig. 1) (11), it is suggested that the effect of TGF-β persists in the absence of TGF-β once susceptible cells are exposed to TGF-β. In fact, our recent study has revealed that the induction of anti-TNP CTL was also inhibited by preincubating normal responding spleen cells in the presence of rTGF-β (unpublished observations). Thus, our results indicate that TGF-β is one of several potential humoral factors that are produced to induce immune dysfunction in the tumor-bearing state.

TGF-β has been shown to inhibit the generation of CTL responses (37). Because TGF-β does not inhibit the cytotoxic activity of CTL once activated (37) (our unpublished observations), it is conceivable that TGF-β functions to inhibit the activation of Th and/or interfere with the development of CTL precursors by influencing their reactivity to lymphokines provided by Th. Our study investigating the effect of TGF-β on the generation of CTL responses to modified self and alloantigens in parallel has more clearly identified the T cell functions affected by TGF-β. It has been well established that CTL responses against modified self Ag such as TNP-self and tumor Ag requires the participation of self H-2-restricted CD4+ Th. In contrast, anti-allo CTL responses can be generated by using three different pathways: self-restricted CD4+ Th; allo-restricted CD4+ Th; and allo-restricted CD8+ Th (38, 39). A given concentration of rTGF-β as well as tumor cell culture SN or ascitic fluid containing TGF-β activity selectively suppressed anti-modified self (TNP-self) CTL responses, under conditions in which the magnitudes of anti-TNP-self CTL responses generated by normal responding cells were comparable to those of anti-allo CTL responses. It was also shown that rIL-2 exogenously provided was capable of correcting the failure to generate anti-TNP CTL responses. These observations suggest that self H-2-restricted CD4+ Th are more susceptible to the inhibitory action of TGF-β than allo-reactive Th (either or both of CD4+ and CD8+ Th). Further studies will be required to determine molecular mechanisms underlying the susceptible difference to TGF-β action between self H-2-restricted CD4+ Th vs allo-reactive CD8+ or CD4+ Th or CD8+ CTL precursors, including differences in the number and affinity of TGF-β receptors and intracellular signal transduction. Thus, our present data not only add to a growing list of immunoregulatory properties of TGF-β, but also determine more accurately subsets of lymphocytes on which TGF-β imposes its immunoregulatory function.

Another important aspect of the present study is concerned with the detection of active form of TGF-β produced in various tumor cell culture SN and ascitic fluid. Whereas many of cell types have potential to produce TGF-β, they have been reported to secrete TGF-β in an inactive (latent) form (23, 24, 26, 40, 41). There are only few reports of the production of activated TGF-β by tumor cells in vitro (42). Therefore, the physiologic relevance of TGF-β as an immunomodulator appears to rest on the production of active form of TGF-β and/or the regulation of the activation of TGF-β produced in a latent form. In this context, it should be noted that various tumor cells examined here produced some or appreciable levels of active TGF-β as detected by highly sensitive growth inhibition assay and that the production in vivo of high levels of active TGF-β in ascitic fluid by one tumor cell type was also detected in anchorage-independent growth assay (the original assay system for the detection of active TGF-β). Moreover, recent studies have shown that an activated form of TGF-β is also produced by endothelial cells through interaction with pericytes, providing the observation that non-transformed cells are capable of producing active TGF-β (43). Thus, it is increasingly evident that transformed as well as non-transformed cells produce an active form of TGF-β to a various extent depending on the conditions.

Even though tumor cells produce an active form of TGF-β, such an active TGF-β would be rendered inactive through binding to α2M in serum (44). However, it has recently been reported that heparin or heparin-like molecules such as heparin sulfate residing at cell surfaces function to dissociate TGF-β-α2M complex, resulting in free (active) TGF-β molecules (45). Conversion of both inactive (TGF-β-α2M complex and latent TGF-β) to active forms in vivo would be a central issue concerned with the physiology of TGF-β.

Our results illustrate that tumor cells produce TGF-β in vitro and in vivo, and this cytokine inhibits selectively at a given concentration, the function of CD4+ T cell subset that is responsible for mediating in vivo tumor neutralization and assisting CTL responses to modified self Ag. In considering that the participation of CD4+ T cells is an absolute requirement for generating anti-tumor immune responses, TGF-β-mediated immunosuppression may represent a major aspect of immune dysfunction in the tumor-bearing mice. By demonstrating CD4+ T cell-preferential suppression by TGF-β, the present study could provide important insights into the effects of tumor growth on immunity to the tumor itself.

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