Immunoregulatory Role of Transforming Growth Factor β (TGF-β) in Development of Killer Cells: Comparison of Active and Latent TGF-β_1

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Summary

Using recombinant DNA technology, we have generated Chinese hamster ovary (CHO) cell lines that synthesize latent transforming growth factor β_1 (TGF-β_1) to study immune regulation by TGF-β_1. In vitro, latent TGF-β_1 synthesized by transfectants or added exogenously as a purified complex after activation inhibited CTL generation to a similar extent as seen with acid-activated recombinant human (rHu) TGF-β_1. In vivo, serum from *nu/nu* mice bearing CHO/TGF-β_1 tumors contained significant levels of latent TGF-β_1 in addition to depressed natural killer (NK) activity in spleens which paralleled that seen in C3H/HeJ mice treated with acid-activated rHuTGF-β_1. rHuTGF-β_1 treatment of mice receiving heart allografts resulted in significant enhancement of organ graft survival. Because of possible regulated tissue-specific activation, administration of latent rather than active TGF-β may provide a better route to deliver this powerful immunosuppressive agent in vivo.

Transforming growth factors encompass a family of polypeptides that regulate the growth and differentiation of both normal and transformed cells (1, 2). One of these, TGF-β_1, has been isolated and purified from various normal and neoplastic cells. Molecular cloning and sequencing of the cDNA for TGF-β_1 (3) in conjunction with protein structural data (4–6) showed that TGF-β_1 is synthesized as a precursor protein of 391 amino acid residues and subsequently processed after signal peptidase and proteolytic cleavage to a 112 residue (M_r 12,500) form. The active or "mature" TGF-β_1 homodimer (25 kD) is noncovalently associated with the remainder of its precursor form (75-kD dimer), and in platelets covalently linked with one molecule of TGF-β_1 binding protein (135 kD) (5, 7, 8) or in serum associated with α2-macroglobulin (9) to form a high molecular weight inactive "latent" complex unable to bind TGF-β cellular receptors (10). TGF-β_1 can be activated by exposure to extremes of pH, heat, or by treatment with chaotropic agents, proteases, or glycosidases (11–14). However, the physiological processes by which latent TGF-β_1 is activated have not yet been fully elucidated.

Numerous studies have described the pleiotropic nature of the biologically active form of TGF-β_1. In addition to its potent effects on cell proliferation and differentiation, TGF-β_1 plays an active role in wound healing, tissue repair, and regulation of immune responses (1, 15, 16). The immunoregulatory properties of TGF-β_1 include inhibition of thymocyte proliferation (17–19), T and B cell proliferation, production of IgM or IgG (20–22), but not IgA synthesis which TGF-β enhances (23, 24), cytokine production (25), NK cell activity (26), cytotoxic T cell development (27–29), and lymphokine-activated killer (LAK)[1] cell activity (28, 30). With regard to monocytes/macrophages, TGF-β displays both inhibitory and stimulatory effects. TGF-β can induce monocyte chemotaxis, and enhance mRNA expression for TNF-α and IL-1 (31, 32) while deactivating macrophages by reducing their capacity to release H_2O_2, cytotoxicity activity, class II and $Fc_\epsilon R2$ expression, as well as TNF-α and IL-1 production, thus perhaps imposing negative feedback and limiting the detrimental effects of toxic monokines (33–36).

Many of the studies that establish TGF-β as a regulator of immune function have examined the immunoregulatory effects of acid-activated TGF-β in vitro. However, the potential clinical usefulness of TGF-β as an immunosuppressant relies also on in vivo studies, few of which have been reported. Second, physiological TGF-β is first presented in vivo as a

[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary cells; B6, C57BL/6; dhfr, dihydrofolate reductase; Hu, human; LAK, lymphocyte activated killer cell; LAP, latency-associated peptide.

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latent complex incapable of binding TGF-β receptors (10) and regulating immune functions. It is not clear from previous studies using activated TGF-β that latent or endogenous TGF-β can be activated both in vitro and in vivo to regulate immune responses. To address these questions, we investigate the immunoregulatory properties of TGF-β using latent recombinant human TGF-β_1 (rHuTGF-β_1) produced in Chinese hamster ovary (CHO) cells and compare the immune activities of latent and acid-activated rHuTGF-β_1. We conclude that mechanisms exist in vitro and in vivo to activate latent TGF-β which in an autocrine or paracrine manner can suppress immune function. The possible role of active TGF-β in immune surveillance is discussed.

Materials and Methods

Cell Culture and cDNA Transfection. Chinese hamster ovary (CHO) cells deficient in dihydrofolate reductase (dhfr) activity (37) were grown in DME/F12 (50:50) medium (Gibco Laboratories, Grand Island, NY) supplemented with 1 g/liter glucose, 10% FCS (HyClone, Logan, UT), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Gibco) and 0.133 mM glycine, 0.11 mM hypoxanthine, 0.02 mM thymidine (GHT). CHO dhfr$^-$ cells (2×10^6) were cotransfected with 1 μg of the expression vector p(SBβ) containing the TGF-β_1 cDNA (3) driven from the cytomegalovirus immediate early promoter in pRK5 (kindly provided by R. Klein and D.V. Goeddel, Genentech, Inc.) and 100 ng of pFD11 which encodes the *dhfr* gene. Cells stably expressing the *dhfr* gene were selected in DME/F12 lacking GHT and 10% dialyzed FCS. Individual transfectant clones were selected, expanded in culture, and screened for TGF-β_1 production by either RIA (6) or bioassay (described below). Confirmation of rHuTGF-β_1 production was also determined by SDS-PAGE (6). The integrated plasmid copy number was amplified by culturing cells in the presence of up to 1-2 μM methotrexate in selective medium. For some experiments we used active rHuTGF-β_1 produced by CHO transfectant cells, purified to 0.8 mg/ml (1 pg endotoxin/μg protein) and acid-activated by dialysis against 20 mM sodium acetate, pH 4.0. Latent rHuTGF-β_1 in CHO transfectant–conditioned medium was purified by sequential cation exchange and hydrophobic chromatography (12). The latency of purified rHuTGF-β_1 was confirmed both before and after its isolation by inactivity in bioassay or TGF-β receptor binding studies. The structure of purified latent rHuTGF-β_1 is representative of the small latent TGF-β complex composed of the active 25-kD TGF-β dimer noncovalently associated with the 75-kD precursor remnant, but lacking the 135-kD binding protein found in the platelet large latent complex (6). For ease in reading the text the following abbreviations are used; CHO for parental CHO dhfr$^-$ cells, CHO dhfr$^+$ and CHO/TGF-β_1 refer to CHO dhfr$^-$ mutant cell line that has been transfected and expresses dhfr, dhfr and rHuTGF-β_1 activities, respectively.

TGF-β Bioassay. A subclone of the Mv1Lu mink lung cell line (American Type Culture Collection, Rockville, MD) isolated in our laboratory was used to measure TGF-β activity (38). Conditioned medium or serum were assayed either untreated or after treatment with 0.12 N HCl for 15 min at room temperature followed by neutralization with 0.1M HEPES buffer containing 0.144 M NaOH (acid-activated). Samples diluted in MEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonesential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin (complete MEM [CMEM]) (all reagents from Gibco Laboratories) and 0.1% FCS (HyClone) were added to cultures in 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA). The plates were incubated at 37°C with 5% CO_2 for 24 h and pulsed during the last 4 h with 1 μCi of [^{3}H]thymidine in CMEM (6.7 Ci/mmol; Amersham Corp., Arlington Heights, IL). Cells were harvested using a PHD Cell Harvester (Cambridge Technology, Inc., Watertown, MA) and [^{3}H]TdR incorporation was measured with a liquid scintillation counter. Results (picograms/milliliter of TGF-β) were calculated based on percent decrease in [^{3}H]TdR incorporation compared with a rHuTGF-β_1 standard. The lower detection limit of the bioassay was 10 pg/ml TGF-β. Samples containing rHuTGF-β that show negligible bioactivity without acid-activation are considered "latent," i.e., biologically inactive.

Animals. 6–8-wk-old BALB/c *nu/nu* female mice and C57BL/6 (B6) female mice were purchased from Charles River Breeding Laboratories, Wilmington, MA, C3H/HeJ female mice were obtained from Jackson Laboratories, Bar Harbor, ME. For the transplantation studies adult (8–10 wk) male C3H/km mice and neonatal (24–48 h) unsexed BALB/c mice were obtained from the Department of Radiobiology, Stanford University Medical Center, Stanford, CA.

Animals Studies. BALB/c *nu/nu* mice were injected subcutaneously with 10^7 CHO or CHO/TGF-β transfected cells (>95% viable as judged by Trypan blue exclusion) washed with PBS (0.15 M NaCl, pH 7.0) and tumor size determined based on the average of the two cross-sectional diameters of each tumor. Serum samples were withdrawn for quantitation of TGF-β_1 by bioassay and spleens were assayed for NK activity. C3H/HeJ mice were injected by various routes with various doses of acid-activated rHuTGF-β_1 and spleen cells were assayed for NK activity.

For NK assays, spleen effector cells harvested from *nu/nu* or C3H/HeJ mice were washed three times in CMEM and seeded at various concentrations into 96-well round-bottomed tissue culture plates (CoStar) in CMEM supplemented with 10% FCS. YAC-1 target cells were labeled with 150 μCi of $Na^{51}CrO_4$ (5 mCi/ml; Amersham Corp.) for 45 min at 37°C and after three washes with CMEM, 10^4 target cells were added to effector cells at various concentrations in 96-well round-bottomed microtiter plates (Costar). After 4 h of incubation at 37°C, culture supernatants were harvested (Skatron, Sterling, VA) and radioactivity was quantitated using an automatic gamma counter (Micromedic Systems, Hersham, PA). Percent specific lysis was calculated as $100 \times \{$[cpm of combined effector and target cells supernatants (experimental release) − cpm of supernatants of target alone (spontaneous release)]/[cpm after lysis of targets with 2% NP-40 (maximum release) − spontaneous release]$\}$. Results are expressed as mean of triplicate determinations ± SEM. Spontaneous release of target cells alone was <10% of maximum for all experiments.

Cytotoxic T Lymphocyte Generation. CTLs were generated in 5-d cultures by incubation in T25 culture flasks of 2.5×10^7 B6 responding spleen cells and 2.5×10^6 stimulator CHO or CHO/TGF-β_1 transfectant cells (irradiated 5,000 rad) in 10 ml of CMEM + 10% FCS. It should be noted that CHO/TGF-β_1 cells used in the one-way MLC still accumulate TGF-β_1 into culture medium after 5,000-rad irradiation. For some experiments B6-anti-CHO CTLs were generated in the presence of various concentrations of acid-activated rHuTGF-β_1, purified latent rHuTGF-β_1, or acid-activated purified latent rHuTGF-β_1. After 5 d of culture effector cells were assayed for cytolytic activity against various ^{51}Cr-labeled, CHO cell lines that were labeled with ^{51}Cr as indicated for YAC-1 cells, and cytotoxicity assays were performed as described (27).

Mouse Heterotopic Cardiac Transplantation Model. A mouse pinna (ear)-heart transplant model was used in which a newborn BALB/c

donor heart is transplanted into a surgically constructed subdermal pocket on the left dorsal pinna of an adult C3H/km recipient. Recipient mice were anesthetized via intraperitoneal injection of chloral hydrate dissolved in saline (25 mg/kg) and a 2-mm epidermal/dermal incision was made parallel to the body axis and 2–3 mm distal to the ear-skull junction. An oval subcutaneous pouch measuring 3–4 mm in diameter was formed on the ear by inserting one arm of a small curved forceps into the incision separating the epidermal/dermal layer from the underlying cartilage. The donor heart was extracted en bloc via thoracotomy and both atria were removed. Excess air and fluid were expressed from the pouch with a cotton swab to facilitate maximal contact between donor and recipient tissues.

Treatment groups received intraperitoneal injection of either activated rHuTGF-β_1 at various doses or normal saline scheduled at various intervals for 13 d after surgery. On the sixth postoperative day and on every other day thereafter until graft failure, the grafts were visually examined for contractions by using a stereomicroscope (Stereozoom No. 4; Bausch and Lomb Co., Rochester, NY) at 10–2-fold magnification. Graft viability was evaluated in a binary fashion (+ or −) based upon the presence or absence of graft contractions. All animals were weighed periodically to monitor drug toxicity.

Results

Production of Latent TGF-β_1 by CHO Transfectants. Our initial studies used CHO cells lines 12/20/6 and 61-1/7C3C12A engineered to produce recombinant TGF-β_1 (CHO/TGF-β_1). To demonstrate that the TGF-β_1 secreted from the CHO/TGF-β_1 transfectants was latent, conditioned medium from CHO/TGF-β_1 cells was analyzed for bioactivity with or without acid-treatment. The data indicate that clones 12/20/6 and 61-1/7C3C12A produced significant quantities of recombinant latent TGF-β_1 after 2 d of in vitro culture of which >90–98% was detectable by bioassay only after acid-activation (Table 1); the rHuTGF-β_1 secreted by the higher producing clone 12/20/6 was also incapable of binding TGF-β cellular receptors as determined by radioreceptor assay (Dr. Lalage Wakefield, personal communication). Media obtained from parental CHO cells showed background levels of TGF-β. SDS-PAGE analysis showed that the rHuTGF-β_1 synthesized by CHO transfectants formed a high molecular weight complex with molecular species of M_r 100,000 and 75,000, consistent with the presence of processed precursor and precursor remnant, respectively, similar in structure to platelet latent complex but lacking the 135-kD binding protein (data not shown, reference 6).

rHuTGF-β_1 Inhibits CTL Generation In Vitro. We have shown previously that activated rHuTGF-β_1 can inhibit the generation of CTL in vitro but not their cytolytic activity (27). The studies presented now further examined whether (*a*) latent TGF-β–secreting CHO cell lines can inhibit CTL generation in vitro and (*b*) such CHO cells can serve as targets for B6 anti-CHO–specific CTL. The data in Fig. 1 indicate that B6 spleen cells generated potent CTL against the CHO parental and CHO dhfr$^+$ cells but not CHO/TGF-β_1 stimulator cells. In contrast, B6 anti CHO–specific CTL exhibited similar cytolytic activity against the CHO parental cells, CHO dhfr$^+$ cells and CHO/TGF-β_1 cells in 4-h CTL cytolytic assays (Fig. 1). These results indicate that transfection per se did not affect the ability of CHO cells to serve as stimulators or targets for CTLs and that CHO cells that secrete TGF-β are poor stimulators for CTL generation. Since latent TGF-β itself cannot bind cellular receptors, mechanisms

Table 1. *rHuTGF-β_1 Synthesized by CHO Transfectant Is Biologically Latent*

	TGF-β_1 concentration*	
	Untreated CM	Acid-treated CM
	pg/ml	
CMEM	<161	206 ± 6
CHO	230 ± 50	340 ± 77
CHO/TGF-β_1 clone 12/20/6	394 ± 18	17,783 ± 1,269
CHO/TGF-β_1 clone 61-1/7C3C12A	<161	1,813 ± 359

* Test samples of conditioned media (CM) were assayed either untreated or acid-activated as described in Materials and Methods. Results were obtained with 10^7 cells incubated for 48 h in CMEM and 10% FCS. Data are from one representative experiment.

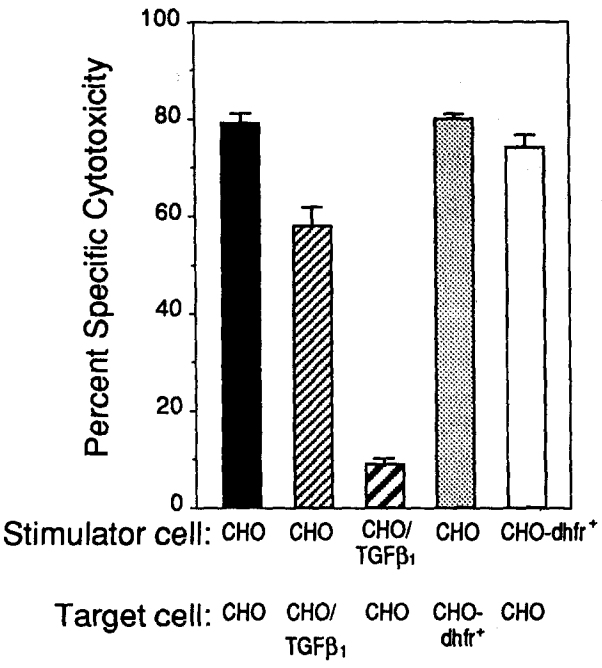

Figure 1. Effect of TGF-β_1 on in vitro CTL generation. B6-anti-CHO (or CHO transfectant)-CTLs were generated in 5-d cultures as described in Materials and Methods and tested in a 4-h ^{51}Cr-release assay against indicated target cells. Data presented are the mean of triplicate determinations ± SEM performed at an E/T ratio of 100:1. Similar results were obtained at all E/T ratios tested (data not shown).

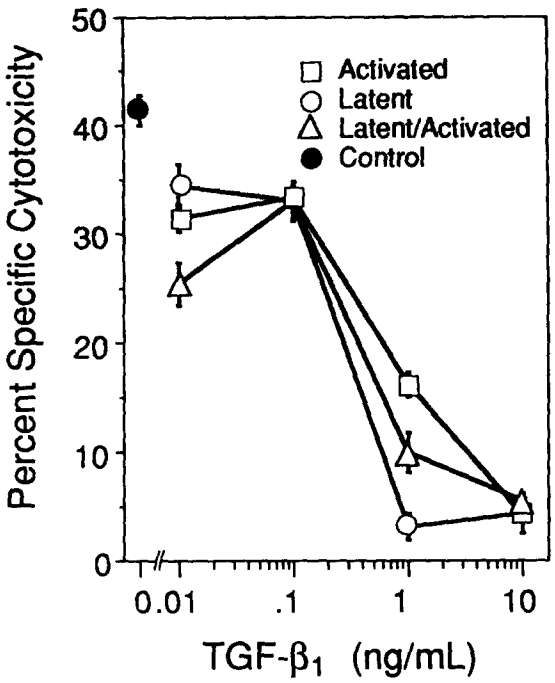

Figure 2. Effect of latent TGF-β_1 on CTL generation. B6-anti-CHO CTLs were generated in the presence of 10 ng/ml acid-activated (□), latent (○) and acid-activated (△) rHuTGF-β_1, or non-TGF-β_1-containing buffer (●), and were tested in a 4-h cytotoxicity assay against ^{51}Cr-labeled CHO target cells as described in Materials and Methods. Data shown are the mean of triplicate values ± SEM performed at an E/T ratio of 25:1. Similar results were obtained at all other E/T ratios tested (data not shown).

exist in vitro to convert latent TGF-β into a biologically active form that can regulate antitumor responses.

We next investigated whether latent/activatable rHuTGF-β_1 was as effective in suppressing CTL generation in vitro as acid-activated rHuTGF-β_1. Both untreated and acid-activated latent rHuTGF-β_1 inhibited CTL generation that was dose-dependent and in a manner similar to that of activated rHuTGF-β_1 (Fig. 2). The concentration necessary to inhibit to 50% (ID_{50}) the maximal CTL response was 0.63, 0.73 and 0.83 ng/ml acid-activated, latent/acid-activated, and latent rHuTGF-β_1, respectively, for the experiment shown (Fig. 2). These data suggest that latent TGF-β is effectively converted to its active form in a manner quantitatively similar to activation by acid pH. Although the latent preparation contained ~5% active fraction (as determined by radioreceptor assay), the [ID_{50}] for latent, latent/activated, and activated rHuTGF-β_1 suggest that the inhibitory activity is not the result of pre-existing activated TGF-β but rather the conversion of latent rHuTGF-β_1 to active polypeptide.

Tumor Growth and TGF-β Production In Vivo. Tumor growth was observed in *nu/nu* mice injected subcutaneously with 1×10^7 CHO/TGF-β_1 cells (Table 2). Over the 30-d examination period, the tumors grew progressively but were nonlethal. Tumor formation was observed in some animals as early as the first day after cell inoculation, with all animals demonstrating measurable tumors by day 5 after the study start. Elevated TGF-β_1 levels were observed in the CHO/TGF-β_1 bearing *nu/nu* mice compared with baseline levels of untreated controls (Table 2). Serum levels of activatable TGF-β increased as the study progressed, and tumors increased in size. In the two animals, which regressed the CHO tumor (one on day 14 and the other on day 30) the serum TGF-β levels were significantly lower in comparison with those obtained for the tumor bearing mice (data not shown). These results suggest that the CHO/TGF-β_1 cells lines continue to synthesize rHuTGF-β_1 in vivo which circulates in a latent but potentially activatable form.

Effect of rHuTGF-β_1 on Murine Lymphoid Functions. Acid-activated human platelet-derived TGF-β_1 has been shown to inhibit NK activity in vitro (26). However, the effects of both latent and acid-activated TGF-β_1 on NK activity in vivo have not been reported. As shown in Fig. 3 *A*, the i.p. injection of 1 μg acid-activated rHuTGF-β_1 suppressed NK activity even after a single administration. Maximum inhibition was observed when rHuTGF-β_1 was administered daily. Less sup-

Table 2. *Serum TGF-β Levels and Growth of CHO/TGF-β_1 Cells in nu/nu Mice*

	Day of study	Average serum levels TGF-β (pg/ml)*	Tumor incidence	Average tumor size (mm)‡
CHO/TGF-β_1 clone	5	23,256 ± 11,574	3/3	3.25 ± 0.48
61-1/7C3C12A	10	76,063 ± 50,216	4/4	3.63 ± 0.31
	14	105,775 ± 62,404	3/4	5.67 ± 1.42
	30	186,368 ± 59,785	3/4	9.33 ± 1.92
Untreated control mice	30	25,374 ± 2,710	0/4	0

BALB/c *nu/nu* mice were used; injections, tumor measurements, and determinations of serum TGF-β levels were performed as indicated in Materials and Methods

* Represents serum levels of activated TGF-β ± SEM obtained after acid-activation for tumor-bearing animals. All nonacid-activated serum contains less than detectable levels of TGF-β (detection limit 20 pg/ml).

‡ Average size ± SEM includes only tumor-bearing animals.

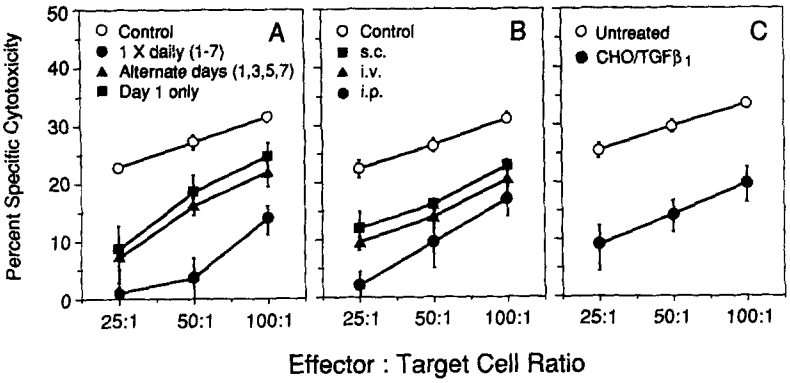

Figure 3. TGF-β_1-mediated inhibition of murine NK activity. NK activities are shown for: (*A*) C3H/HeJ mice injected intraperitoneally with 1 μg dosage of acid-activated rHuTGF-β_1 given daily (days 1–7) (●), on alternative days (days 1,3,5,7) (▲), day 1 only (■), or control (○); (*B*) C3H/HeJ mice given 2 μg once daily (days 1–7) dosage of rHuTGF-β_1 as a function of route of administration, or (*C*) untreated control (○), or experimental *nu/nu* mice injected subcutaneously 30 d before with CHO/TGF-β_1 cells (●). Each data point represents the mean NK activity ± SEM determined from five animals.

pression was obtained when 0.1 μg rHuTGF-β was administered (data not shown). Administration of 2 μg of activated rHuTGF-β_1 daily by either the intraperitoneal, intravenous, or subcutaneous route reduced NK activity to a similar degree (Fig. 3 *B*). The i.p. administration of rHuTGF-β_1 proved consistently the most effective in suppressing NK activity. As our previous data demonstrated that predominantly latent rHuTGF-β_1 was produced in vivo by CHO/TGF-β_1 cells we questioned whether mice bearing these CHO-TGF-β_1 tumors exhibited changes in NK activity. The results demonstrated a significant reduction in NK activity in the tumor-bearing mice (Fig. 3 *C*). NK activity for *nu/nu* mice bearing CHO dhfr$^+$ cell tumors was similar to that of untreated control animals (data not shown). The spleen cells from mice administered 1 μg rHuTGF-β_1 intraperitoneally which showed reduced NK activity also demonstrated a suppressed responsiveness to Con A in comparison with untreated control animals (data not shown). However, it is interesting that in certain studies, mice treated with rHuTGF-β_1 demonstrated reduced NK levels without the concomitant suppression of Con A responses (data not shown). The findings support that latent TGF-β activated in vivo can suppress NK activity and the ability to respond to mitogenic stimuli.

Treatment with rHuTGF-β_1 Prolongs Organ Graft Survival in Heart Transplant Recipient Mice. Additional studies were performed to determine the efficacy of activated rHuTGF-β_1 as an immunosuppressant in organ transplantation. C3H/km recipients of BALB/c heart allografts were treated intraperitoneally with 1 μg or 5 μg activated rHuTGF-β_1 on days 1–13 or 4–13 after transplant. The drug delivery schedule included the days before and after allograft vascularization, day 4, and the period of allograft rejection, days 10–14. Allograft survival time was defined as the first day on which graft contraction, i.e., rejection, ceased. The data in Table 3 indicate that percent heart graft survival in rHuTGF-β_1–treated groups was significantly prolonged using both rHuTGF-β_1 doses compared with the saline treated control group. No significant difference in mean graft survival time was observed between treatment groups administered activated rHuTGF-β_1 on either day 1 or day 4 after transplantation (Table 3). rHuTGF-β_1 was an effective immunosuppressant, with graft survival time of 16.0 d compared with 10.6 d for control using the 5 μg/2.5 μg combination dose. Cyclosporin A required higher doses of 3–6 mg/kg in this model to achieve similar graft survival (data not shown). In addition, activated rHuTGF-β_1 also suppressed the T cell–mediated increased lymph node enlargement occurring in host-versus-graft popliteal lymph node assay (data not shown).

Discussion

We have examined the immunoregulatory effects of TGF-β using CHO lines that synthesize and secrete latent rHuTGF-β_1. Latent TGF-β was chosen as substrate to define the conditions under which immune cells process latent TGF-β to a functional polypeptide. The latency of rHuTGF-β_1 was determined by inactivity in biological growth inhibition and radioreceptor assays. While it is generally true that most cells synthesize latent TGF-β (3), some cell lines such as the human breast cancer cell lines MCF-7 and T-47D, and human glioblastoma cells have been reported to secrete TGF-β that is biologically active without activation by acid, pH, or urea (39–41). The different mechanisms regulating the decision whether to release TGF-β as active or latent product is currently unclear. Since only active TGF-β can bind to cellular receptors (10), elucidation of those cells that can either acti-

Table 3. *Effect of rHuTGF-β_1 on an Heterotopic Murine Heart Graft Survival*

rHuTGF-β_1 treatment	*n*	Schedule	Mean graft survival time*	*p* Value vs. control
		d	*d*	
None	105	1–13	10.6 ± 0.2	–
1 μg	10	1–13	12.9 ± 1.5	<0.00006
1 μg	4	4–13	12.5 ± 1.6	0.002
5 μg/ 2.5 μg	3	1–6/ 7, 9, 11, 13	16.0 ± 4.3	<0.0003

BALB/c neonatal hearts were transplanted into C3H/km mice as described in Materials and Methods. 1 or 4 d after surgery, mice were injected intraperitoneally with indicated amounts of rHuTGF-β_1 or saline control.
* Results are reported as mean ± the 95% confidence limit.

vate TGF-β directly, or are in proximity to those cells that can, is important for the understanding of autocrine or paracrine regulation by TGF-β. However, at least for some cells, the masking of active TGF-β by association with "latency-associated peptide" (LAP) appears an important means to protect themselves from the potent regulatory effects of TGF-β they synthesize (1, 8, 20–22, 42).

In keeping with the protective role of LAP, we found that CHO/TGF-β_1 cells injected into *nu/nu* mice secrete rHuTGF-β_1 into the animal's circulation as latent protein. Since activated TGF-β_1 was not found systemically at detectable levels in CHO/TGF-β_1 recipient *nu/nu* mice, but is required to decrease NK activity, the results suggest that TGF-β_1 activation is a localized event in vivo, safeguarding the release of large amounts of this growth regulator. The localized activation of latent TGF-β may provide a means for cells to regulate TGF-β activity only in specific tissues.

In addition to being sensitive to the inhibitory effects of TGF-β_1, most lymphoid cells produce latent TGF-β. The presumed conversion of latent or endogenous rHuTGF-β_1 to an active form that can inhibit immune functions was shown to occur both in vitro and in vivo. Kehrl et al. have recently reported that polyclonal antibodies to active TGF-β can enhance B-cell proliferation and Ig synthesis (22). In addition, Lucas and co-workers have shown that mAbs to active TGF-β can enhance the proliferation of PBMC to IL-2 and PHA/PMA as well as the generation of lymphokine-activated killer cells. These findings suggest that endogenously produced latent TGF-β can be activated by normal physiological mechanisms which can then downregulate in an autocrine fashion these immune functions (43). The studies presented here further demonstrate that endogenous latent TGF-β can be activated and suppress both CTL generation in vitro and NK activity in vivo.

A key towards understanding the target specificity of TGF-β action is elucidation of the processes by which latent TGF-β becomes biologically active. Recent studies have suggested that activated macrophages have the potential to both secrete and activate latent TGF-β, although the mechanisms involved in this conversion are not known. Acidic conditions can convert latent TGF-β to active (11), suggesting that acidic cellular environments may provide a physiologic milieu in which activation may occur (44, 45). From microelectrode studies it has been shown that macrophages, as well as osteoclasts, contain such acidic microenvironments (46) that may be sufficiently low to activate TGF-β. Moreover, the observation that activated macrophages express sialidase activity (47), coupled with the finding that removal of sialic-acid–containing carbohydrate structures in the TGF-β precursor remnant can activate TGF-β (14), further substantiates the possible role of the macrophage as an activator of latent TGF-β. We have found that LPS-stimulated human peripheral blood–derived adherent macrophages did not significantly activate exogenously added purified latent TGF-β_1 (data not shown). This result does not preclude the possibility that antigen-presenting macrophages can activate latent TGF-β, but may indicate the need for cognate help by other cell types during activation. In support of this hypothesis, Antonelli-Orlidge et al. report that the conditioned media of endothelial cells and pericytes each contained latent TGF-β, but only during their co-culture can activated TGF-β be produced (48). At the current time we have no information to support or exclude the possibility that CHO or CHO-TGF-β_1 cells themselves can activate latent TGF-β. Our results showing that activation of latent TGF-β occurs during the generation of immune cytolytic cells may indicate that de novo induction of lymphoid maturation proteins or cell surface differentiation markers are pre-requisites for TGF-β activation.

Once activated, TGF-β_1 displays potent immunosuppression in vivo of NK cytolytic activity, the ability of splenocytes to respond to mitogenic stimuli, and CTL generation in vitro, which are shown in this report and previously (26–28). As suggested by Rook et al., TGF-β may affect the continuous recruitment and activation of "pre-NK" cells by blunting their ability to respond to IFN-α, an important growth factor for NK activation and function (26). The blunting of IFN-α responsiveness may reflect downregulation of IFN-α receptor numbers expressed on NK cells by TGF-β. In addition, TGF-β may affect the differentiation of lymphoid cells as shown for Ig-secreting B cells (21, 22), CTLs (27), and LAK cells (30), possibly by lowering lymphocyte cell receptors for IL-2 (20). Alternatively, TGF-β may inhibit the generation of CTLs, and also LAK cells, by dampening TNF-α production (25), an important modulator of both CTL and LAK cell development (27, 30). Interestingly, TGF-β does not effect the ability of either lymphoid cells (27) or tumor cells (data presented here) to serve as targets for lysis by mature CTLs. This finding suggests that TGF-β_1 might not affect MHC class I expression on CHO cells, in contrast to its ability to modulate class II HLA-DR surface antigen expression on human cells (34, 35).

Within the framework of the complex immune cell network, TGF-β is also shown to downregulate both NK activity and primary CTL responses in vivo (49). Tumor cells that produce TGF-β may therefore potentially reduce the numbers or generation of circulating cytolytic CTL and NK cells and thus promote escape of the tumor from immune surveillance. Clinically, patients with glioblastomas, which secrete TGF-β2 (50), demonstrate a generalized immune suppression; manifested especially by an inability of normal T cells to respond to mitogens (51). Moreover, TGF-β–producing tumors can grow progressively in transiently immunosuppressed mice while retaining expression for class I MHC (49). Thus, TGF-β by inhibition of host immune responses may provide a mechanism to suppress allograft rejection and may possibly accelerate processes of carcinogenesis.

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References

1. Sporn, M.B., A.B. Roberts, L.M. Wakefield, and R.K. Assoian. 1986. Transforming growth factor-β: Biological function and chemical structure. *Science (Wash. DC).* 233:532.
2. Roberts, A.B., M.A. Anzano, L.M. Wakefield, N.S. Roche, D.R. Stern, and M.B. Sporn. 1985. Type β transforming growth factor: A bifunctional regulator of cellular growth. *Proc. Natl. Acad. Sci. USA.* 82:119.
3. Derynck, R., J.A. Jarrett, E.Y. Chen, D.H. Eaton, J.R. Bell, R.K. Assoian, A.B. Roberts, M.B. Sporn, and D.V. Goeddel. 1985. Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells. *Nature (Lond.).* 316:701.
4. Gentry, L.E., M.N. Lioubin, A.F. Purchio, and H. Marquardt. 1988. Molecular events in the processing of recombinant type 1 pre-pro-transforming growth factor beta to the mature polypeptide. *Mol. Cell. Biol.* 8:4162.
5. Miyazono, K., U. Hellman, C. Wernstedt, and C.-H. Heldin. 1988. Latent high molecular weight complex of transforming growth factor β_1: Purification from human platelets and structural characterization. *J. Biol. Chem.* 263:6407.
6. Wakefield, L.M., D.M. Smith, S. Broz, M. Jackson, A.D. Levinson, and M.B. Sporn. 1989. Recombinant TGF-β_1 is synthesized as a two-component latent complex that shares some structural features with the native platelet latent TGF-β_1 complex. *Growth Factors.* 1:203.
7. Pircher, R., P. Jullien, and D.A. Lawrence. 1986. β-Transforming growth factor is stored in human blood platelets as a latent high molecular weight complex. *Biochem. Biophys. Res. Commun.* 136:30.
8. Wakefield, L.M., D.M. Smith, K.C. Flanders, and M.B. Sporn. 1988. Latent transforming growth factor-β from human platelets. A high molecular weight complex containing precursor sequences. *J. Biol. Chem.* 263:7646.
9. O'Connor-McCourt, M.D., and L.M. Wakefield. 1987. Latent transforming growth factor β in serum: A specific complex with α2-macroglobulin. *J. Biol. Chem.* 262:14090.
10. Wakefield, L.M., D.M. Smith, T. Masui, C.C. Harris, and M.B. Sporn. 1987. Distribution and modulation of the cellular receptor for transforming growth factor-beta. *J. Cell Biol.* 105:965.
11. Lawrence, D.A., R. Pircher, and P. Jullien. 1985. Conversion of a high molecular weight latent β-TGF from chicken embryo fibroblasts into a low molecular weight active β-TGF under acidic conditions. *Biochem. Biophys. Res. Commun.* 133:1026.
12. Brown, P.D., L.M. Wakefield, A.D. Levinson, and M.B. Sporn. 1990. Physicochemical activation of recombinant latent TGF-beta's, 1,2,3. *Growth Factors.* 3:35.
13. Lyons, R.M., J. Keski-Oja, and H.L. Moses. 1988. Proteolytic activation of latent transforming growth factor-β from fibroblast-conditioned medium. *J. Cell Biol.* 106:1659.
14. Miyazono, K., and C.-H. Heldin. 1989. Role for carbohydrate structures in TGF-β_1 latency. *Nature (Lond.).* 338:158.
15. Palladino, M.A., R.E. Morris, H.F. Starnes, and A.D. Levinson. 1990. The transforming growth factor-betas: A new family of immunoregulatory molecules. *Ann. NY Acad. Sci.* 593:181.
16. Roberts, A.B., and M.B. Sporn. 1988. Transforming growth factor β. *Adv. Cancer Res.* 51:107.
17. Ellingsworth, L.R., D. Nakayama, P. Segarini, J. Dasch, P. Carrillo, and W. Waegell. 1988. Transforming growth factor-βs are equipotent growth inhibitors of interleukin-1-induced thymocyte proliferation. *Cell. Immunol.* 114:41.
18. Kim, K.J., J. Abram, M. Alphonso, M. Pearce, G.J. Thorbecke, and M.A. Palladino. 1990. Role of endogenously produced interleukin-6 as a second signal in murine thymocyte proliferation induced by multiple cytokines: regulatory effects of transforming growth factor-beta. *Cell. Immunol.* In press.
19. Chantry, D., M. Turner, and M. Feldmann. 1989. Interleukin 7 (murine pre-B cell growth factor/lymphopoietin 1) stimulates thymocyte growth: regulation by transforming growth factor beta. *Eur. J. Immunol.* 19:783.
20. Kehrl, J.H., L.M. Wakefield, A.B. Roberts, S. Jakowlew, M. Alvarez-Mon, R. Derynck, M.B. Sporn, and A.S. Fauci. 1986. Production of transforming growth factor-β by human lymphocytes and its possible role in the regulation of T cell growth. *J. Exp. Med.* 163:1037.
21. Kehrl, J.H., A.B. Roberts. L.M. Wakefield, S. Jakowlew, M.B. Sporn, and A.S. Fauci. 1986. Transforming growth factor β is an important immunomodulatory protein for human B lymphocytes. *J. Immunol.* 137:3855.
22. Kehrl, J.H., A.S. Taylor, G.A. Delsing, A.B. Roberts, M.B. Sporn, and A.S. Fauci. 1989. Further studies of the role of transforming growth factor-β in human B cell function. *J. Immunol.* 143:1868.
23. Sonoda, E., R. Matsumoto, Y. Hitoshi, T. Ishii, M. Sugimoto, S. Araki, A. Tominaga, N. Yamaguchi, and K. Takatsu. 1989. Transforming growth factor β induces IgA production and acts additively with interleukin 5 for IgA production. *J. Exp. Med.* 170:1415.
24. Coffman, R.L., D.A. Lebman, and B. Shrader. 1989. Transforming growth factor β specifically enhances IgA production by lipopolysaccharide-stimulated murine B lymphocytes. *J. Exp. Med.* 170:1039.
25. Espevik, T., I.S. Figari, M.R. Shalaby, G.A. Lackides, G.D. Lewis, H.M. Shepard, and M.A. Palladino. 1987. Inhibition of cytokine production by cyclosporin A and transforming growth factor β. *J. Exp. Med.* 166:571.
26. Rook, A.H., J.H. Kehrl, L.M. Wakefield, A.B. Roberts, M.B. Sporn, D.B. Burlington, H.C. Lane, and A.S. Fauci. 1986. Effects of transforming growth factor β on the functions of natural killer cells: depressed cytolytic activity and blunting

of interferon responsiveness. *J. Immunol.* 136:3916.
27. Ranges, G.E., I.S. Figari, T. Espevik, and M.A. Palladino. 1987. Inhibition of cytotoxic T cell development by transforming growth factor β and reversal by recombinant tumor necrosis factor α. *J. Exp. Med.* 166:991.
28. Mulé, J.J., S.L. Schwarz, A.B. Roberts, M.B. Sporn, and S.A. Rosenberg. 1988. Transforming growth factor-beta inhibits the in vitro generation of lymphokine-activated killer cells and cytotoxic T cells. *Cancer Immunol. Immunother.* 26:95.
29. Fontana, A., K. Frei, S. Bodmer, E. Hofer, M.H. Schreier, M.A. Palladino, and R.M. Zinkernagel. 1989. Transforming growth factor-β inhibits the generation of cytotoxic T cells in virus-infected mice. *J. Immunol.* 143:3230.
30. Espevik, T., I.S. Figari, G.E. Ranges, and M.A. Palladino. 1988. Transforming growth factor-β_1 (TGF-β_1) and recombinant human tumor necrosis factor-α reciprocally regulate the generation of lymphokine-activated killer cell activity: Comparison between natural porcine platelet-derived TGF-β_1 and TGF-β2, and recombinant human TGF-β_1. *J. Immunol.* 140:2312.
31. Wahl, S.M., D.A. Hunt, L.M. Wakefield, N. Francis-McCartney, L.M. Wahl, A.B. Roberts, and M.S. Sporn. 1987. Transforming growth factor type β induces monocyte chemotaxis and growth factor production. *Proc. Natl. Acad. Sci. USA.* 84:5788.
32. Wiseman, D.M., P.J. Polverini, D.W. Kamp, and S.J. Leibovich. 1988. Transforming growth factor-beta (TGFβ) is chemotactic for human monocytes and induces their expression of angiogenic activity. *Biochem. Biophys. Res. Commun.* 157:793.
33. Tsunawaki, S., M. Sporn, A. Ding, and C. Nathan. 1988. Deactivation of macrophages by transforming growth factor-β. *Nature (Lond.).* 334:260.
34. Czarniecki, C.W., H.H. Chiu, G.H.W. Wong, S.M. McCabe, and M.A. Palladino. 1988. Transforming growth factor-β_1 modulates the expression of class II histocompatibility antigens on human cells. *J. Immunol.* 140:4217.
35. Zuber, P., M.C. Kuppner, and N. deTribolet. 1988. Transforming growth factor-β2 down-regulates HLA-DR antigen expression on human malignant glioma cells. *Eur. J. Immunol.* 18:1623.
36. Tanaka, M., K. Lee, J. Yodoi, H. Saito, Y. Iwai, K.-M. Kim, M. Morita, M. Mayumi, and H. Mikawa. 1989. Regulation of Fc_ϵ receptor 2 (CD23) expression on a human eosinophilic cell line EoL3 and a human monocytic cell line U937 by transforming growth factor β. *Cell. Immunol.* 122:96.
37. Urlaub, G., and L.A. Chasin. 1980. Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. *Proc. Natl. Acad. Sci. USA.* 77:4216.
38. Danielpour, D.L., L.L. Dart, K.C. Flanders, A.B. Roberts, and M.B. Sporn. 1989. Immunodetection and quantitation of the two forms of transforming growth factor-beta (TGF-β_1 and TGF-β2) secreted by cells in culture. *J. Cell. Phys.* 138:79.
39. Knabbe, C., M.E. Lippman, L.M. Wakefield, K.D. Flanders, A. Kasid, R. Derynck, and R.B. Dickson. 1987. Evidence that transforming growth factor-β is a hormonally regulated negative growth factor in human breast cancer cells. *Cell.* 48:417.
40. Arrick, B.A., M. Korc, and R. Derynck. 1990. Differential regulation of expression of three transforming growth factor β species in human breast cancer cell lines by estradiol. *Cancer Res.* 50:299.
41. Bodmer, S., K. Strommer, K. Frei, C. Siepl, N. de Tribolet, I. Heid, and A. Fontana. 1989. Immunosuppression and transforming growth factor-β in glioblastoma: Preferential production of transforming growth factor-β2. *J. Immunol.* 143:3222.
42. Assoian, R.K., B.E. Fleurdelys, H.C. Stevenson, P.J. Miller, D.K. Madtes, E.W. Raines, R. Ross, and M.B. Sporn. 1987. Expression and secretion of type β transforming growth factor by activated human macrophages. *Proc. Natl. Acad. Sci. USA.* 84:6020.
43. Lucas, C., L.N. Bald, B.M. Fendly, M. Mora-Worms, I.S. Figari, and M.A. Palladino. The autocrine production of transforming growth factor-β1 during lymphocyte activation: A study with a monoclonal antibody-based ELISA. *J. Immunol.* 145:1415.
44. Jullien, P., T.M. Berg, and D.A. Lawrence. 1989. Acidic cellular environments: Activation of latent TGF-β and sensitization of cellular responses to TGF-β and EGF. *Int. J. Cancer.* 43:886.
45. Oreffo, R.O.C., G.R. Mundy, S.M. Seyedin, and L.F. Bonewald. 1989. Activation of the bone-derived latent TGF beta complex by isolated osteoclasts. *Biochem. Biophys. Res. Commun.* 158:817.
46. Silver, I.A., R.J. Murrills, and D.J. Etherington. 1988. Microelectrode studies on the acid microenvironment beneath adherent macrophages and osteoclasts. *Exp. Cell. Res.* 175:266.
47. Pilatte, Y., J. Bignon, and C.R. Lambre. 1987. Lysosomal and cytosolic sialidases in rabbit alveolar macrophages: Demonstration of increased lysosomal activity after in vivo activation with bacillus Calmette Guerin. *Biochim. Biophys. Acta.* 923:150.
48. Antonelli-Orlidge, A., K.B. Saunders, S.R. Smith, and P.A. D'Amore. 1989. An activated form of transforming growth factor β is produced by cocultures of endothelial cells and pericytes. *Proc. Natl. Acad. Sci. USA.* 86:4544.
49. Torre-Amione, G., R.D. Beauchamp, H. Koeppen, B.H. Park, H. Schreiber, H.L. Moses, and D.A. Rowley. 1990. A highly immunogenic tumor transfected with a murine transforming growth factor type β_1 cDNA escapes immune surveillance. *Proc. Natl. Acad. Sci. USA.* 87:1486.
50. Wrann, M., S. Bodmer, R. de Martin, C. Siepl, R. Hofer-Warbinek, K. Frei, E. Hofer and A. Fontana. 1987. T cell suppressor factor from human glioblastoma cells is a 12.5-kd protein closely related to transforming growth factor-β. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1633.
51. Miescher, S., T.L. Whiteside, S. Carrel, and V. von Fliedner. 1986. Functional properties of tumor infiltrating and blood lymphocytes in patients with solid tumors: effects of tumor cells and their supernatants on proliferative responses of lymphocytes. *J. Immunol.* 136:1899.