A Transforming Growth Factor β2 (TGF-β2)-like Immunosuppressive Factor in Amniotic Fluid and Localization of TGF-β2 mRNA in the Pregnant Uterus

By David J. Altman, Sara L. Schneider, Diane A. Thompson, Hwei-Ling Cheng, and Thomas B. Tomasi

From the Department of Molecular Medicine and Immunology, Roswell Park Cancer Institute, Buffalo, New York 14263

Summary

This report describes a murine amniotic fluid (MAF) immunosuppressive factor that has properties similar to transforming growth factor β (TGF-β). The MAF factor exhibits TGF-β-like activity in stimulating soft agar colony formation by AKR-2B cells and inhibiting thymidine uptake by Mv1Lu cells. We demonstrate that both the immunosuppressive and TGF-β-like activities of the MAF factor are completely neutralized by anti-TGF-β2-specific antibodies and not by anti-TGF-β1-specific antisera. The immunosuppressive factor in MAF is novel in that it appears to be identical or very closely related to TGF-β2 and is active in its native state. This active and anti-TGF-β2-neutralizable factor chromatographs at ~70 kD on Sephadex at neutral pH and appears to be able to complex with α-fetoprotein in native amniotic fluid. Chromatography of native MAF under acidic conditions demonstrates a lower molecular mass protein that chromatographs on BioGel in the same position as the mature 25-kD TGF-β. This protein has the biological properties of TGF-β and is immunosuppressive. Both of these activities are neutralizable with anti-TGF-β2 but not with anti-TGF-β1 or other antisera. By Northern analysis, we find high levels of TGF-β2 mRNA (with little or no TGF-β1) in the pregnant uterus that peak around day 15 of gestation and then fall rapidly by day 19 as birth approaches. The TGF-β2-like factor could possibly play a role in maternal immunity, in the retention of the fetal allograft, as well as in regulating fetal and neonatal immunological competence.

Several factors have been implicated in the maternal tolerance to the semiallogeneic fetus and the immunological deficiencies described in pregnancy, as well as the fetus and neonate (reviewed in references 1 and 2). This includes evidence that deficiencies in MHC class I and class II antigen expression on fetal and maternal tissues may play a role in maternal tolerance. Different T cell subsets and especially suppressor cells have also been implicated in pregnancy-related immune suppression and in the immunotrophic promotion of fetal development (1, 2). Neonatal natural suppressor cells inhibit immune responses and may be involved in tolerance induction in the fetal and neonatal periods (3). In addition, soluble suppressive factors influencing immune development in the fetus and newborn, and immunity during pregnancy, have been described (1), and these may mediate and/or complement the cellular suppressors.

We have previously reported (reviewed in references 4 and 5) the immunosuppressive effects of murine amniotic fluid (MAF)[1] both in vitro and in vivo, and have suggested that the MAF suppressive factor could potentially be important in regulating immunity during pregnancy and in the newborn period. MAF has been found to inhibit splenic antibody responses to SRBC and the primary and secondary responses to other T-dependent antigens, as well as the MLR and T cell mitogen stimulation. The in vivo administration of MAF to newborn mice prolongs neonatal immunological immaturity (6), and we have demonstrated that MAF administered to mice intraperitoneally suppresses donor skin graft rejection across the H-Y histocompatibility antigen barrier (7). Although the suppressive activity in MAF has been found to co-purify with α-fetoprotein (AFP), the relationship of the immunosuppressive activity in MAF to AFP has not been clearly established (4, 5, 8). Variations in the suppressive

[1] *Abbreviations used in this paper:* AFP, α-fetoprotein; MAF, murine amniotic fluid; NMS, normal mouse serum.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/90/11/1391/11 $2.00
Volume 172 November 1990 1391-1401

potency of different AFP preparations have been variously attributed to the binding of small molecules, the presence of Cu^{2+} chelated at a critical histidine (4, 5), and/or a chemical isoform of AFP that is suppressive (9).

Transforming growth factor β (TGF-β) is a family of structurally and functionally related molecules (reviewed in references 10 and 11) that have been shown to be present in the mouse embryo (12, 13) and are differentially regulated in various tissues during development (13, 14). Other more distantly related developmental regulators that are members of the TGF-β superfamily include: the *Xenopus* Vg-1 gene product and its mammalian homologue Vgr-1, the decapentaplegic (dpp) protein of *Drosophila*, the bone morphogenetic proteins (BMP-2a, 2b, 3) Mullerian, inhibiting substance and the inhibins/activins (see review in reference 10). TGF-β has been shown to inhibit a variety of immune responses (15–20). We have reported in preliminary studies (21, 22) that TGF-β shares some of the characteristics of the amniotic fluid immunosuppressive factor. We also noted that both the amniotic fluid immunosuppressive factor and TGF-β modulated MHC class II gene expression induced by certain lymphokines and cytokines (21, 23). In this report, we demonstrate that the MAF suppressive factor is closely related to or identical with TGF-β2, and that it can complex with AFP in native amniotic fluid. Extremely high levels of TGF-β2 mRNA are found in uterine tissue after day 9.5 of pregnancy reaching a maximum by day 15.5, after which there is a rapid fall by day 19 as birth approaches. The TGF-β2-like factor may play an important role in the maintenance of pregnancy and in the regulation of immunity in the fetus and neonate.

Materials and Methods

Mice. The Swiss, DBA/2Ha, and C_3Hf/He mice were obtained from the West Seneca Laboratories (West Seneca, NY) of the Roswell Park Cancer Institute (RPCI), and the CBA mice were obtained from Cumberland Farms (Clinton, TN). Mice were cared for in the RPCI Animal Care Facility. The day of detection of vaginal plugs was considered day 0.5 of gestation.

MAF Preparation. Fluid was removed from the fetal amniotic sacs of 15-d pregnant Swiss mice, stored at 4°C, pooled, and the absorbance at 280 nm (A_{280}) was adjusted to 2.0 U/ml with PBS. The MAF was filter sterilized (0.2 μm), aliquoted, and stored at −70°C. Heat-treated MAF was prepared by heating MAF in a boiling water bath at >95°C for 7 min followed by centrifugation and removal of the supernatant. PBS or pooled Swiss normal mouse serum (NMS), diluted to 2.0 A_{280} U/ml, were used as controls. All MAF preparations were tested negative for endotoxin by the Limulus Amebocyte Lysate gel clot method (ACCU-Gel; Whittaker MA Bioproducts, Walkersville, MD).

Antigen-induced T Cell Proliferation Assay. Female CBA mice at 6–8 wk of age were immunized at the base of the tail and the footpad with 100 and 25 μg, respectively, of OVA in H37/Ra complete adjuvant (Difco Laboratories, Detroit, MI). After 7 d, the inguinal, para-aortic, and popliteal lymph nodes were removed, and a single cell suspension was prepared and cultured in 96-well flat-bottomed tissue culture plates (Corning Glass Works, Corning, NY) at 4×10^5 cells/well in 200 μl of RPMI 1640 with L-glutamine (Gibco Laboratories, Grand Island, NY), penicillin, streptomycin, 5×10^{-5} M 2-ME, and 1% fresh NMS. Triplicates were set up with and without 0.25 mg/ml OVA, and MAF, control NMS, or PBS were added at varying concentrations (5–10% [vol/vol] being standard). Porcine platelet-purified TGF-β1 and β2 (R & D Systems, Minneapolis, MN) were titered for activity and used at 2 ng/ml or less. Rabbit polyclonal neutralizing anti-TGF-β and anti-TGF-β2 (R & D Systems) were titered and used at 75–150 and 40 μg/ml or less, respectively. Turkey anti-TGF-β1 and anti-TGF-β2 obtained from Drs. Linda Dart and Michael Sporn (24, 25) were used at 1:5,000 to 1:100 dilutions with media. Culture plates were incubated for 72 h at 37°C in 5% CO_2, then pulsed for 18 h with 1 μCi/well of [^{3}H]TdR (2 Ci/mmol; New England Nuclear, Boston, MA), harvested, and counted in a scintillation counter. All experiments were repeated at least three times.

TGF-β Activity Assays. TGF-β-like activity in MAF was assayed as follows. Soft agar colony formation of AKR-2B cells cultured in 0.4% agarose in 6-well plates (Corning Glass Works) at 7,500 cells/well in McCoy 5A (Gibco Laboratories) with 5% FCS (HyClone Laboratories, Logan, UT) was assayed as described (12, 26, 27) in response to the indicated concentrations of TGF-β or MAF (or PBS or NMS controls). Dishes were incubated at 37°C in 5% CO_2 for 10 d, and colonies were then counted (Gaertner Scientific Co., Chicago, IL). Assay of the growth inhibition of Mv1Lu mink lung epithelial cells (CCL-64; American Type Culture Collection, Rockville, MD) by TGF-β (27, 28) was modified as follows. Subconfluent cultures were trypsinized, washed, and seeded at 3×10^4 cells/well in 24-well culture plates (Corning Glass Works) in 1 ml of DMEM (Gibco Laboratories) with 10% supplemented calf serum (Hyclone Laboratories) and incubated at 37°C in 5% CO_2 for 72 h. Media was then changed to DMEM with 5% FCS, and 24 h later, TGF-β, MAF, heat-treated MAF, PBS, or NMS, and/or the various anti-TGF-β antibodies described above were added, and the plates were incubated for an additional 22 h. Media was then aspirated, and 1 μCi/well of [^{3}H]TdR (2 Ci/mmol; New England Nuclear) was added in 0.5 ml of MEM with 5% calf serum and incubated for 2 h. Cells were fixed with 10% ice-cold TCA, dissolved in 0.25 N NaOH, and counted in a scintillation counter.

IL-1 Thymocyte Proliferation Assay. The IL-1-dependent thymocyte proliferation assay was carried out essentially as described (16). Thymocytes were prepared from C_3Hf/He mice and cultured in 96-well tissue culture plates (Corning Glass Works) at 1.5×10^6 cells/well in RPMI 1640 with 1% fresh NMS. PHA was used at 1 μg/ml, and IL-1 (Genzyme Corp., Boston, MA) was added at 10 U/ml. TGF-β was added at 2 ng/ml and MAF at 10% (vol/vol). Plates were incubated for 72 h and then pulsed for 18 h with 1 μCi/well of [^{3}H]TdR, harvested, and counted as described above.

Bone Marrow Soft Agar Colony Assay. Bone marrow was flushed from the femurs and tibias of 8–10-wk-old female DBA/2Ha mice. The cells were washed and cultured at 10^5 cells/ml/well in 24-well culture plates in α-MEM (Gibco Laboratories), 10% FCS, penicillin, streptomycin, and 0.3% bactoagar (Difco Laboratories) in the presence or absence of rhuCSF-1 (Genzyme Corp.) at 100 CFU/ml (1 CFU is the amount required to produce one colony of >50 cells from 7.5×10^4 murine bone marrow cells in soft agar after 7 d), or with amniotic fluid, heat-treated amniotic fluid, or NMS at 10% (vol/vol). The plates were allowed to gel at room temperature and were then incubated at 37°C in 5% CO_2. Colonies of >50 cells were counted at 7 and 14 d.

Size Exclusion Gel Chromatography. MAF was concentrated on an ultrafiltration cell (model 52; Amicon Corp., Danvers, MA) with a Diaflo YM5, 5000 MWCO membrane, and dialyzed exhaustively against PBS. The concentrated MAF was applied to a column of Sephadex G-150 (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with PBS at pH 7.1, and fractions were collected,

pooled, and tested for suppressive activity in the antigen-induced T cell proliferation and mink cell inhibition assays (described above). Acid (pH 2.3) chromatography was performed on BioGel P100 (100–200 mesh; Bio-Rad Laboratories, Richmond, CA) equilibrated with 1 M acetic acid at pH 2.3. MAF was first dialyzed against 1 M acetic acid, then applied to the column, and the collected fractions were neutralized and tested for suppressive activity. The immunosuppressive activity (U/μg) of the chromatographic fractions were expressed as units of the biologic response causing 50% inhibition of maximal stimulation in the T cell assay per microgram of protein in the fraction. Human platelet-purified TGF-β1 was prepared as previously described (29). Controlled pore glass beads (CPG Inc., Fairfield, NJ) were of 343-Å mean pore diameter and 120/200 particle size. Rabbit anti-mouse AFP was prepared against AFP from ascites fluid (ICN Immunobiologicals, Lisle, IL) and was used without further purification. Goat anti-NMS (Accurate Chemical & Scientific Corp., Westbury, NY) and anti-mouse IgG (ICN Immunobiologicals) were also used (as controls) without further purification.

RNA Isolation. Maternal and fetal tissues were removed from Swiss mice at days 9.5, 12.5, 15.5, and 19 of gestation with careful dissection (30, 31), and total RNA was isolated by the guanidine thiocyanate–CsCl method (32). Briefly, tissues were rapidly disrupted in 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.1 M 2-ME, and 0.5% sodium *N*-laurylsarcosine using a Tissumizer homogenizer (Tekman Industries, Cincinnati, OH). The homogenates were carefully layered over 5.7 M cesium chloride and centrifuged in a SW50.1 rotor (Beckman Instruments, Inc., Fullerton, CA) at 36,000 rpm for 16–18 h at 20°C. The RNA pellet was dissolved in 7.5 M guanidine hydrochloride, 25 mM sodium citrate, pH 7.0, and 10 mM 2-ME. The RNA was precipitated by adding 0.025 vol of 1 M acetic acid and 0.5 vol of absolute ethanol, pelleted by centrifugation, and resuspended in water. RNA was reprecipitated by adding 2 M potassium acetate, pH 5, (0.1 vol) and ethanol (2.5 vol). The RNA pellet was dissolved in water and quantified spectrophotometrically. The PC-3 adenocarcinoma cell line (American Type Culture Collection) was used as a control and grown in DMEM with 10% FCS. PC-3 RNA was prepared identically to the tissue RNAs.

Northern Analysis. Total RNA (30 μg/lane) was denatured by incubation at 50°C for 1 h in glyoxal-DMSO and subjected to 1% agarose gel electrophoresis. After electrophoresis, the gel was dried in a gel slab dryer (model 224; Bio-Rad Laboratories) with heat on for 1.5 h, and hybridization was performed directly on the dried gel (33). Both prehybridization and hybridizations were performed in 5× SSC, 25 mM sodium phosphate (pH 7), 0.1% SDS, 5× Denhardt's solution (0.1% Ficoll, 0.1% polyvinyl pyrrolidone, and 0.1% BSA), and 100 μg/ml sonicated, denatured salmon sperm DNA, at 65°C overnight. DNA fragments were ^{32}P labeled by the random primer technique (34) to a sp act of 10^9 cpm/μg DNA. Heat-denatured ^{32}P-labeled probes (1–2 × 10^6 cpm/ml hybridization solution) were added. After hybridization, gels were washed for 30 min at room temperature with 2× SSC, 0.05% Sarkosyl, and 0.02% sodium pyrophosphate; for 2 h at 50°C with 0.2× SSC, 0.05% Sarkosyl, and 0.01% sodium pyrophosphate; and for 30 min at 55°C with 0.2× SSC, 0.05% Sarkosyl, and 0.01% sodium pyrophosphate. The resulting gels were blot dried and autoradiographed at −70°C on Kodak XAR-5 film, utilizing Dupont Cronex intensifying screens. RNA ladders (Bethesda Research Laboratories, Gaithersburg, MD) processed identically to the tissue RNAs were used as molecular weight standards.

Probes for Hybridization. The probe for TGF-β2 used in this study is a TGF-β2-specific 929-bp EcoRI-HindIII fragment generated by restriction digestion of plasmid pPC-21 (2.3 kb), a cDNA clone of human TGF-β2 (35). The 929-bp EcoRI-HindIII fragment contains 179-bp of 5′ noncoding as well as 750-bp of precursor coding region. This eliminates the 3′ terminal mature region of the cDNA, which is the region of highest sequence conservation in the TGF-β family. The probe specific for TGF-β1 is a 1,600-bp EcoRI fragment obtained from restriction digestion of plasmid pTGF-β (1)-2, a cDNA clone of simian TGF-β1 (36), which contains the entire TGF-β1 precursor coding region as well as the 3′ terminal mature region. Both pPC-21 and pTGF-β (1)-2 were kindly provided by Dr. A. F. Purchio. Plasmid pL-7, a cDNA clone of mouse ribosomal protein L7 (37), was a gift from Dr. R. P. Perry. pL-7 was cleaved with EcoRI and HindIII to generate a 540-bp fragment and was used as an internal hybridization intensity control.

Results

Immunosuppressive Activity of MAF and TGF-β. MAF suppresses the in vitro antigen-induced proliferation of ovalbumin sensitized lymph node T cells in a dose-dependent manner (Fig. 1). Thymidine incorporation is significantly inhibited by 1% MAF, while 5% MAF inhibits by >90%, and 10% MAF by 97% with no effect on cell viability. Syngeneic adult normal mouse serum (at the same protein concentration) has no effect. Both TGF-β1 and TGF-β2 at 2 ng/ml suppress T cell proliferation to approximately the same extent as 5% MAF. Concentrations of TGF-β2 as low as 0.5 ng/ml significantly suppress this response (TGF-β1 exhibited about half the suppressive potency of TGF-β2; data not shown). Suboptimal concentrations of MAF and TGF-β2 exhibit additive inhibition to the maximum seen with optimal concentrations of either. Similar suppression by MAF, and TGF-β1 and TGF-β2, is observed in the MLR, T cell mitogen stimulation (data not shown), and the IL-1-dependent stimulation of murine thymocyte proliferation (Fig. 2).

Characterization of the TGF-β-like Activities of MAF. Biological activities classically attributed to TGF-β are present in MAF. Since TGF-β stimulates AKR-2B mouse embryonic

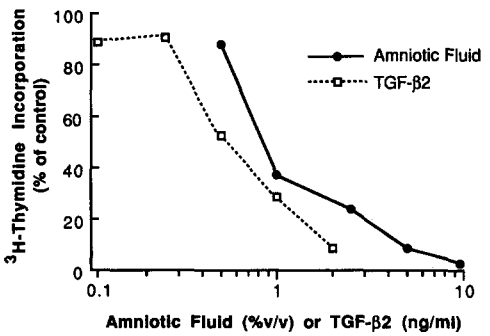

Figure 1. Dose response of amniotic fluid and TGF-β-induced suppression of antigen-specific T cell proliferation. OVA-primed mouse LN cells were cultured, in the presence or absence of OVA, with murine amniotic fluid, TGF-β2, or PBS and normal mouse serum controls, for 72 h. Results are the mean of triplicate cultures (variability <15%) of one representative experiment of more than three independent experiments.

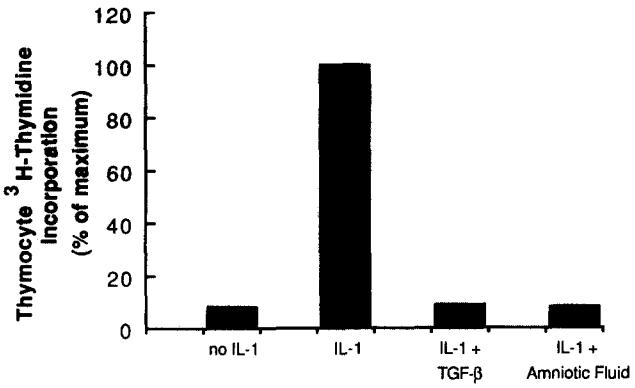

Figure 2. Amniotic fluid and TGF-β suppression of IL-1-induced thymocyte stimulation. Murine thymocytes were cultured, in the presence or absence of sub-optimal levels of PHA and 10 U/ml of rIL-1, with murine amniotic fluid at 10% (vol/vol), TGF-β2 at 2 ng/ml, or PBS and normal mouse serum controls at 10% (vol/vol).

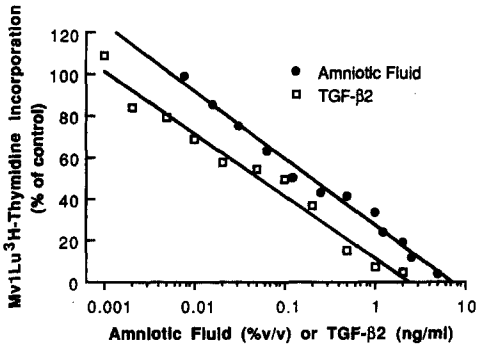

Figure 4. Dose response of amniotic fluid and TGF-β suppression of Mv1Lu growth. Mv1Lu mink lung epithelial cells (CCL-64) were cultured, as described in Materials and Methods, with the indicated concentrations of murine amniotic fluid, TGF-β2, or PBS and normal mouse serum controls. Results are the mean of triplicate cultures (variability <10%) of three independent experiments.

fibroblast cells to form colonies in soft agar (26), we tested MAF in this assay. As shown in Fig. 3, TGF-β supports AKR-2B colony formation at doses ranging between 0.2 and 30 ng/ml, while MAF stimulates increasing numbers of AKR-2B colonies at concentrations ranging from 1 through 20%. TGF-β inhibition of the growth of the Mv1Lu (CCL-64) mink lung epithelial cell line is a sensitive assay for TGF-β (27, 28). Fig. 4 shows that 0.07 ng/ml of TGF-β2 inhibits Mv1Lu thymidine incorporation by 50%, while 2 ng/ml inhibits by >95%. This inhibition is duplicated by MAF, which demonstrates a dose-dependent suppression of Mv1Lu thymidine uptake with 50% inhibition at 0.15% MAF, and >95% inhibition at 5% MAF (Fig. 4).

To further define the nature of the suppressive activity in MAF and its relation to TGF-β, we evaluated the effect of four different neutralizing anti-TGF-β antibodies in mink lung epithelial cell growth inhibition and in antigen-induced T cell proliferation assays in the presence of MAF, TGF-β2, or TGF-β1. Two of the anti-TGF-β preparations are rabbit polyclonal antibodies. One rabbit antibody neutralizes mink lung cell proliferative inhibition by both TGF-β1 and TGF-β2. This antisera, at 75–150 μg/ml, completely neutralizes the suppression of antigen-induced T cell proliferation caused by 10% MAF, as well as by 2 ng/ml of either TGF-β1 or TGF-β2. This antibody also completely neutralizes the inhibition of IL-1-dependent thymocyte proliferation caused by either TGF-β or MAF when added at the initiation of the proliferation assay (data not shown). The second rabbit antibody neutralizes only TGF-β2. As shown in Fig. 5, this antibody abolishes mink lung epithelial cell proliferative inhibition caused by MAF as well as TGF-β2, but not by TGF-β1. Complete neutralization of 1 ng/ml of TGF-β2 is seen with 20 μg/ml of this antibody, and the same amount totally abrogates the inhibition caused by 5% MAF, while having no effect on TGF-β1. The TGF-β2-specific rabbit antibody also completely neutralizes, at 10–20 μg/ml, the immunosuppressive effects caused by 10% MAF or 2 ng/ml of TGF-β2 in the T cell proliferation assay, but does not significantly affect the activity of TGF-β1 (Fig. 6 *A*).

Two additional neutralizing anti-TGF-β antibodies raised

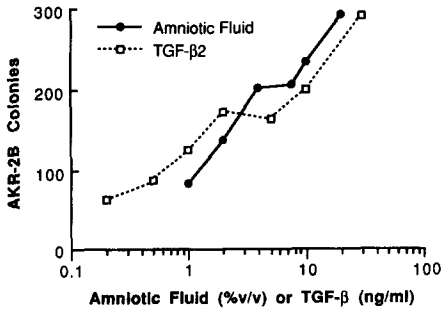

Figure 3. Amniotic fluid and TGF-β stimulation of AKR-2B cell colony formation in soft agar. AKR-2B mouse embryonic fibroblast cells were cultured in soft agar in the presence or absence of murine amniotic fluid and TGF-β2 (or PBS and normal mouse serum controls), as described in Materials and Methods.

Figure 5. Anti-TGF-β2-specific antibodies, but not anti-TGF-β1, neutralize amniotic fluid suppression of Mv1Lu growth. Mv1Lu mink lung epithelial cells were cultured with murine amniotic fluid at 5% (vol/vol), or TGF-β1 or TGF-β2 at 1 ng/ml, in the presence or absence of 20 μg/ml of rabbit anti-TGF-β2-specific antibody, or 1:100 dilutions of turkey anti-TGF-β2-specific or anti-TGF-β1-specific antibodies.

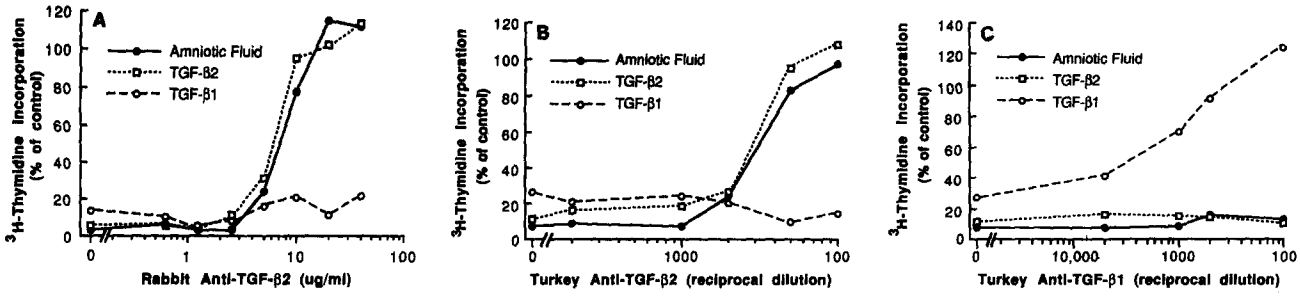

Figure 6. Anti-TGF-β2-specific antibodies, but not anti-TGF-β1, neutralize amniotic fluid immunosuppression. Dose response of anti-TGF-β2 and anti-TGF-β1 on the suppression of antigen-induced T cell proliferation by murine amniotic fluid at 10% (vol/vol), TGF-β2 and TGF-β1 at 2 ng/ml. (*A*) Rabbit anti-TGF-β2-specific antibody; (*B*) turkey anti-TGF-β2-specific antibody; (*C*) turkey anti-TGF-β1-specific antibody.

in turkeys (24, 25) were also used to examine the TGF-β-like activity in MAF. The first is specific for TGF-β2 alone and abolishes the inhibition of mink lung epithelial cell growth caused by MAF as well as by TGF-β2, but not by TGF-β1 (Fig. 5). These anti-TGF-β2-specific antibodies completely neutralize the suppression of antigen-induced T cell proliferation by 2 ng/ml of TGF-β2 or 10% MAF at dilutions from 1:1,000 to 1:100, without affecting TGF-β1 suppression (Fig. 6 *B*). The second turkey anti-TGF-β is specific for TGF-β1 and blocks mink lung epithelial cell growth inhibition by TGF-β1, but not by TGF-β2 or by MAF (Fig. 5). At dilutions of 1:1,000 to 1:100, this antiserum also abolishes TGF-β1 suppression in the T cell proliferation assay but does not affect TGF-β2 or MAF immunosuppression (Fig. 6 *C*).

CSF-1 Activity in Amniotic Fluid. MAF contains high levels of macrophage (CSF-1) activity in bone marrow colony assays in soft agar (Table 1). We have found that the CSF-1 activity in MAF, equivalent to ~1,000 CFU/ml of rCSF-1 (Genzyme Corp.), can be separated from the immunosuppressive activity (measured by antigen-induced T cell proliferation) by size exclusion chromatography. Heat treatment of MAF (>95°C for 7 min) also eliminates CSF-1 activity, leaving the immunosuppressive activity unchanged (see Table 1). Purified or recombinant CSF-1 does not suppress antigen-induced T cell proliferation (Table 1), and neutralizing antibodies against CSF-1 have no effect on MAF immunosuppression. Antisera to AFP, albumin, IFN-α, -β, and -γ, and TNF-α also have no effect on suppression by MAF or TGF-β when added at the initiation of the T cell assay (data not shown). The immunosuppressive factor in MAF is stable to lyophilization and acidification but sensitive to pronase.

Chromatographic Characterization of MAF Immunosuppressive and TGF-β-like Activities. Size exclusion chromatography of MAF on Sephadex G-150 at neutral pH demonstrates (see Fig. 7 *A*) that the immunosuppression is associated with fractions eluting at ~70 kD (containing mainly albumin and AFP). The suppressive effect of these fractions is completely neutralized by rabbit anti-TGF-β2 but not by antisera to TGF-β1. However, when MAF is chromatographed under acidic conditions (pH 2.3) on BioGel P 100 (see Fig. 7 *B*), the suppressive activity (assessed by inhibition of both antigen-induced T cell proliferation and mink cell growth) elutes in a low molecular weight region in the same location as human platelet-purified TGF-β1 (pTGF-β), demonstrating the retarded and anomalous elution reported for the mature 25-kD TGF-β molecule (38). The immunosuppressive and mink cell inhibition activities eluted from BioGel acid chromatography, in the low molecular weight region, are both completely neutralized by the rabbit anti-TGF-β2-specific antibody. Further, when the 70-kD fractions obtained from chromatography of MAF at neutral pH are subjected to chromatography at acid pH, the anti-TGF-β2-neutralizable, immunosuppressive and mink cell inhibitory activities elute in the same location as pTGF-β (data not shown). We have previously reported (8) that the immunosuppressive activity in MAF can be partially depleted after pretreatment with antibody to mouse AFP. Pretreatment of native amniotic fluid with anti-AFP-

Table 1. *Differentiation of Immunosuppressive and CSF-1 Activities in Amniotic Fluid*

Addition	Percent suppression of antigen-induced T cell proliferation*	Bone marrow soft agar colonies/well‡
Normal sera control (10%)	0	0
Amniotic fluid (10%)	92	29
Heat-treated amniotic fluid (10%)	96	0
rCSF-1 (100 CFU/ml)	8	24
TGF-β2 (2 ng/ml)	91	0

* OVA-primed CBA mouse LN cells were cultured, in the presence or absence of OVA, with the indicated addition. Plates were incubated for 72 h, and [^{3}H]TdR incorporation was measured as described in Materials and Methods.

‡ Bone marrow cells were obtained from DBA/2Ha mice and cultured in soft agar in the presence of the indicated additions as detailed in Materials and Methods. Colonies of >50 cells were counted at 7 and 14 d. The experiements were repeated at least three times with equivalent results.

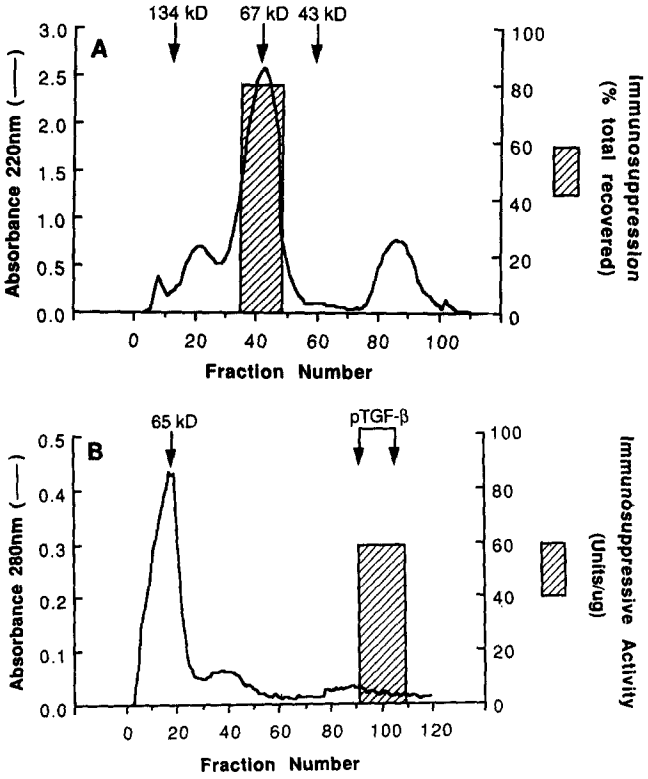

Figure 7. Separation of the amniotic fluid TGF-β2-like immunosuppressive activity by size exclusion chromatography. Immunosuppression was evaluated by the inhibition of antigen-induced T cell proliferation. TGF-β2 activity was evaluated by the suppression of Mv1Lu cell growth and the anti-TGF-β2-specific neutralization of suppression in both assays. The suppressive fractions are indicated by the hatched bars. (*A*) Murine amniotic fluid chromatographed on Sephadex G-150 with PBS at pH 7.1. (*B*) Amniotic fluid chromatographed on BioGel P 100 with 1 M acetic acid at pH 2.3.

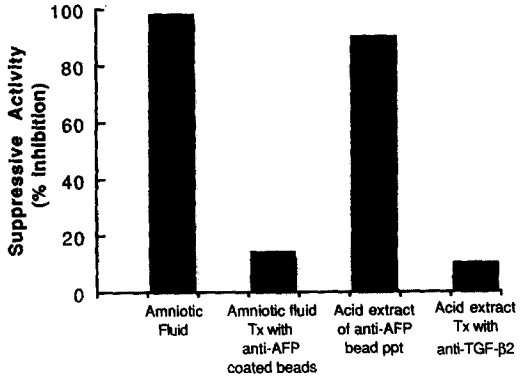

Figure 8. Anti-AFP-coated glass bead removal of the amniotic fluid TGF-β2-like immunosuppressive activity. The suppressive activity is eluted from the anti-AFP beads exposed to MAF by glycine, pH 3, and is fully neutralized solely by anti-TGF-β2-specific antibodies. Control beads coated with normal serum, anti-mouse IgG, or anti-normal mouse serum have no or minimal effect.

coated glass beads removes suppressive activity (see Fig. 8), while beads coated with normal sera, anti-IgG, or anti-NMS have minimal effect. Immunosuppressive activity is eluted from the anti-AFP-coated beads by low pH (glycine, pH 3), and this activity is fully neutralized by antibodies to TGF-β2 (Fig. 8). In addition, after chromatography of the acid extract on BioGel P100 at pH 2.3, the immunosuppressive activity elutes in a position similar to pTGF-β (data not shown).

Northern Analysis of TGF-β mRNA Expression in Maternal and Fetal Tissues. To determine the site(s) of synthesis of the TGF-β2-like immunosuppressive factor in MAF, we have examined the expression of TGF-β2 and TGF-β1 mRNA in various tissues during mouse gestation. Northern analysis of mRNA prepared from 9.5–19-d fetuses, placental, and maternal tissues was performed using a cDNA probe specific for TGF-β1 and another specific for TGF-β2. We find that TGF-β1 mRNA is expressed at low levels in many tissues without significant gestational change and is not detected in the pregnant uterus by the methods we used. Low levels of TGF-β2 mRNA were expressed in the fetus, placenta, and the decidua with little variation during gestation. TGF-β2 mRNA was present at low levels in the uterus on day 9.5 of gestation. However, strikingly high levels of TGF-β2 mRNA were found in the day 12.5 uterus, reaching a maximum on day 15.5, as illustrated in Fig. 9. Between days 15.5 and 19 of gestation, there is a precipitous fall in the levels of mRNA for TGF-β2, which essentially disappears from the uterus by day 19, immediately preceding birth (22). Nonpregnant uterus contains little if any TGF-β2 mRNA (data not shown). The expression of TGF-β2 mRNA in the pregnant uterus was seen coincidentally with CSF-1 mRNA, while c-*fms* mRNA was essentially undetectable in the uterus with significant levels appearing in the fetal placenta (data not shown), as previously reported by Stanley and colleagues (39). AFP mRNA was localized in the yolk sac, fetal liver, and to a lesser extent, the fetal gut, in agreement with others (40) (data not shown).

Figure 9. Northern analysis of TGF-β2 and TGF-β1 mRNA expression in fetal and maternal tissues during gestation. RNAs from day 15.5 of gestation fetus (*F*), placenta (*P*), decidua (*D*), and uterus (*U*), and the PC-3 adenocarcinoma control (*C*), were prepared, subjected to electrophoresis, and hybridized with a cDNA probe specific for TGF-β2 and another specific for TGF-β1, as described in Materials and Methods. The predominant pregnant uterine TGF-β2 mRNA band is 3.5 kb with minor bands of 5.0 and 4.1 kb. PC-3 shows a major TGF-β2 band of 3.25 kb with minor bands of 5.0 and 4.1 kb, and a major TGF-β1 band of 2.3 kb. The lower band in each lane is the mouse pL-7 rRNA probe, the internal hybridization intensity loading control.

Discussion

The immunosuppressive activity in MAF is novel in that it appears to be due to the presence of a specific TGF-β2-like factor. Although TGF-β1 and β2 share many bioactivities, such as inhibiting epithelial cell and keratinocyte proliferation and stimulating myocyte and adipocyte differentiation (10, 11), some functional differences have been described. For example, TGF-β1 has been reported to be much more effective in inhibiting multipotential hematopoietic progenitor cell responses to IL-3 (41, 42) and in inhibiting vascular endothelial cell proliferation (43), while TGF-β2, but not TGF-β1, exhibits mesoderm-inducing activity in *Xenopus* embryos (24). Both TGF-β1 and TGF-β2 exhibit immunosuppression, however, the immunosuppressive factor in MAF is structurally and functionally related specifically to TGF-β2. From our data, the concentration of TGF-β2-like activity in native MAF appears to be very high, equivalent to ~40 ng/ml (1.6 nM) of TGF-β2. We cannot exclude the possibility that some TGF-β1 activity is present in MAF, but the antibody inhibition studies suggest that there is <0.07 ng/ml, which would yield a ratio of TGF-β2 to TGF-β1 activities in MAF of >500:1. It is important to note that our data do not exclude the possibility that the amniotic fluid immunosuppressive factor is more closely related to another member of the TGF-β family, such as TGF-β3 (44, 45), than to TGF-β2. TGF-β3 has been shown to possess activities common to TGF-β1 and TGF-β2 (46), and the TGF-β2-specific antisera used could possibly crossreact with TGF-β3, although they do not react appreciably with TGF-β1. It is unlikely that we are detecting TGF-β3 mRNA in the tissue studies, because the TGF-β2 specific cDNA probe used in the Northern analysis of gestational tissues was designed to eliminate the 3′ regions, which have greater sequence conservation in the TGF-β family, and our studies detect the transcript sizes previously identified as TGF-β2 specific. Also, we used high stringency hybridization conditions that would reduce crosshybridization. Nevertheless, further work, including amino acid sequence analysis and gene cloning, is necessary to establish whether the MAF TGF-β2-like factor is: (*a*) identical to TGF-β2; (*b*) a modified form of TGF-β2; (*c*) an already described member of the TGF-β family; or (*d*) a unique new family member.

The observation that at neutral pH the active suppressive factor chromatographs in the 70-kD region peak suggested that TGF-β could possibly be complexed to a protein(s) in amniotic fluid. This possibility is suggested by our data showing that pretreatment of MAF with beads coated with antibodies to AFP removes suppressive activity, while beads coated with other antibodies do not significantly affect activity. Moreover, the immunosuppressive TGF-β activity can be eluted from the complexes and is neutralizable by anti-TGF-β2. Thus, the presence of AFP in amniotic fluid presents the potential for a unique carrier protein for TGF-β2, and, although additional and more definitive binding studies are necessary, we speculate that the TGF-β2 or a closely related molecule in amniotic fluid can bind to AFP in a reversible, noncovalent manner. AFP is synthesized largely in the fetal liver and "back diffuses" across the placenta, where it could potentially play an important role in interacting with a potent immunosuppressive TGF-β-like agent at the placental site. It is also possible that TGF-β is carried into the maternal circulation in an AFP complex, although we have not yet determined whether the anti-TGF-β2 neutralizable factor is present in pregnant serum. TGF-β2 or a closely related factor bound or released from AFP could affect immune suppression locally at the level of the maternal-fetal interface, and possibly in the mother, as well as the fetus, where immune deficiencies have been described (1, 2). Copurification, or "contamination" of AFP preparations with the TGF-β2-like amniotic fluid factor, may account for the variable immunosuppression reported for AFP. For example, we have previously observed (5) that methods of isolating AFP that involve chromatography with acidic buffers (which separates the TGF-β2-like factor from AFP), or high concentrations of chaotropic/denaturing salts, produce nonsuppressive AFP preparations. Further, since AFP suppression in vitro is seen with microgram/milliliter concentrations, while TGF-β2 is immunosuppressive at picogram/milliliter levels, even minute quantities of TGF-β2 (one molecule per 10^6 of AFP) could account for suppression in apparently "pure" AFP preparations. It is possible that the suppressive specificities of AFP reported by van Oers et al. (9) are due to a particular structure that is capable of binding TGF-β2 or the closely related amniotic fluid factor.

The TGF-β2-like MAF factor is active in immunosuppression and mink cell inhibition without acidification or apparent proteolysis, which is usually necessary to activate the latent form of TGF-β present in bone or secreted by platelets or cultured cell lines. This suggests that the MAF immunosuppressive factor may represent a developmentally specific species of TGF-β that accumulates in a physiologically active form in amniotic fluid. It may also have a unique responsiveness to the hormones involved in pregnancy, since there is a striking accumulation of TGF-β2 mRNA in the uterus during pregnancy. However, whether the TGF-β2-like molecule in MAF is derived entirely or in part from synthesis in the pregnant uterus remains to be proven. Recent studies by Moses and colleagues (47, 48) have shown TGF-β2 mRNA in the mid to late gestation mouse fetus and placenta using a murine TGF-β2 cDNA probe. Using our human TGF-β2 cDNA probe, hybridized under high stringency, we detected high TGF-β2 mRNA levels in the 12.5–15.5 d of gestation uterus. In comparison, however, we did not detect significant levels in the fetal placenta, yolk sac, amnion, or decidua. Discrepancies between those reports and our data could be due to differences in the probe used and/or the conditions of hybridization. However, very recent data using the same cDNA probe and hybridization conditions as those reports continue to demonstrate the same localization depicted in Fig. 9 (data not shown). Further purification of the TGF-β2-like factor from MAF with the objective of biochemical characterization (including sequencing) and cloning of the TGF-β2 gene from pregnant uterus are currently in progress.

A study by Clark et al. (49) suggests that suppressor cells from the murine decidua release a soluble 13-kD suppressor

antigenically related to TGF-β. A recent report (50), which appeared while this manuscript was in preparation, describes a suppressive factor functionally and antigenically related to TGF-β2 that is produced in decidual cell preparations. However, we detect only very small amounts of TGF-β2 mRNA in carefully dissected decidual preparations. In comparison, we find very high levels of TGF-β2 mRNA in the uterus appearing after day 9.5 of gestation with little or no TGF-β1 mRNA, despite the predominance of TGF-β1 in most adult tissues. One possible explanation is that the decidual preparations studied by Clark et al. (50) were contaminated with uterine cells and/or TGF-β2 protein produced in the maternal uterine cells.

The uterine TGF-β2 mRNA reaches peak levels at about day 15.5, followed by a rapid fall of mRNA levels, and by day 19 of gestation, TGF-β2 message is essentially undetectable (22). The dramatic fall of a potent immunosuppressive factor as birth approaches raises interesting speculations concerning the role of the TGF-β2-like factor in the maintenance of pregnancy and its relationship to the onset of normal birth, as well as abortions and miscarriages. Additional data on the complete time course and tissue distribution of TGF-β2 expression during gestation, using Northern analysis, as well as in situ hybridization, is currently in progress.

Reports of additional immunomodulatory roles of TGF-β have appeared recently that expand the role of this factor in immune regulation. TGF-β has been identified as a cofactor in stimulating IgA production in B cells (51) and as an inhibitor of the generation of cytotoxic T cells directed against virally infected cells (52). Immunogenic tumors expressing TGF-β (53), either as a result of transfection of the TGF-β gene or spontaneous production, and human glioblastomas that produce TGF-β2 (54), have been shown to suppress and evade host immune responses. These studies suggest that TGF-β, produced either by the tumor or by host cells infiltrating the tumor, may play an important role in regulating tumor rejection. Recent studies showing that TGF-β suppresses NK cell activity (19) and the production of lymphokine-activated killer cells (55, 56) are consistent with this hypothesis.

TGF-β treatment has been reported to promote the acceptance of rat to mouse pancreatic islet cell xenografts when given with anti-IFN-γ (57). This is consistent with our work (7) demonstrating that amniotic fluid administered intraperitoneally prevents skin graft rejection across minor histocompatibility barriers in mice. In these studies, MAF, containing the TGF-β2-like factor, was given in large amounts without apparent toxicity and resulted in permanent engraftment that in most animals persisted after cessation of the treatment. These studies provide an impetus to further explore the use of the TGF-β2-like factor (as well as rTGF-β) in transplantation, as well as in GVHD and autoimmunity.

The precise mechanism(s) by which TGF-β suppresses immune responses is uncertain. Previous studies have reported various effects on the growth, cell cycling, and function of T cells (and possibly IL-2Rs) (17, 56, 58–60), and of B cells (61, 62). Modulations of cell adhesion receptors such as LFA-1 (63), and of MHC class II expression (62, 64, 65), have also been reported. With regard to MHC expression, prior reports (66, 67) have suggested an inhibition of macrophage cell surface Ia by AFP, an observation consistent with our previous finding (68) that MAF and AFP suppress antigen-induced T cell proliferation at the level of the APC. Whether this effect is related to the binding of TGF-β to AFP, as discussed above, is unknown, but deserves further exploration. It will be of particular interest to determine whether the TGF-β2-like factor is involved in the deficiencies in MHC antigen expression on fetal and placental tissues that have been reported and postulated to be important in fetal survival (1, 2). We have recently shown that TGF-β and the amniotic fluid TGF-β2-like factor modulate macrophage and B cell MHC class II gene expression and surface Ia induced by certain cytokines (23). However, the effects of TGF-β and the TGF-β2-like amniotic fluid factor on Ia expression are complex and depend on the cell type, state of differentiation, and the nature of Ia-inducing cytokines present (23).

We thank Drs. Linda Dart and Michael Sporn (National Cancer Institute, Bethesda, MD) for the kind gift of anti-TGF-β1 and anti-TGF-β2 turkey antibodies; Dr. Anthony Purchio (Oncogen, Seattle, WA) for kindly providing the cDNA probes for TGF-β1 and TGF-β2; Dr. Harold Moses (Vanderbilt University School of Medicine, Nashville, TN) for the AKR-2B cells; Dr. Robert Perry (Fox Chase Cancer Center, Philadelphia, PA) for providing the plasmid pL7; Dr. Anne Croy (University of Guelph, Guelph, Ontario, Canada) for helpful discussions and demonstrations on the identification, and separation of maternal and fetal tissues. We also thank Mrs. Carolyn Budzinski for typing the manuscript and Mrs. Cathy Grande for technical assistance.

This work was supported by National Institutes of Health grants HD-17013, CA-22105, and AR-17554. David J. Altman is a Medical Scientist Scholar of the Life and Health Insurance Medical Research Fund.

Address correspondence to Thomas B. Tomasi, Department of Molecular Medicine and Immunology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263.

Received for publication 10 August 1990.

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