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Seminal Transforming Growth Factor β_1 Stimulates Granulocyte-Macrophage Colony-Stimulating Factor Production and Inflammatory Cell Recruitment in the Murine Uterus[1]

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ABSTRACT

Mating in rodents evokes an inflammatory-like reaction within the uterine endometrium, characterized by extensive infiltration and activation of macrophages, dendritic cells, and granulocytes. This response is initiated when seminal vesicle gland-derived factors in the ejaculate stimulate uterine epithelial cells to release proinflammatory cytokines including granulocyte-macrophage colony-stimulating factor (GM-CSF). Experiments in which seminal vesicle secretions were fractionated by Sephacryl S-400 chromatography and assayed in vitro for GM-CSF-stimulating activity revealed that the seminal moiety coeluted with transforming growth factor β_1 (TGFβ_1) in the 150–440-kDa range and was neutralized by anti-TGFβ_1 antibodies. Comparable amounts of recombinant TGFβ_1 stimulated GM-CSF release in cultures of uterine epithelial cells from estrous mice and, when instilled into the uterine lumen, caused an increase in GM-CSF content and an infiltration of leukocytes into the endometrium similar to the postmating response. These results show that seminal vesicular fluid contains TGFβ_1 at levels sufficient to be the primary causative agent in the postmating inflammatory cascade through induction of GM-CSF synthesis by uterine epithelial cells. Seminal TGFβ_1 is thus implicated as a key factor in initiation of the remodeling events and immunological changes that occur in the uterus during the preimplantation period of pregnancy.

INTRODUCTION

In mice, as in other mammals, deposition of semen in the female reproductive tract at mating provokes a cascade of cellular and molecular changes that in many respects resemble a classic inflammatory response [1, 2]. Within hours after mating, a striking leukocytic infiltration occurs in the murine endometrium: neutrophils, eosinophils, and large numbers of activated macrophages and dendritic cells expressing interleukin (IL)-1, tumor necrosis factor (TNF)-α, and major histocompatibility complex (MHC) class II and CD86 antigens accumulate in the stromal tissue subjacent to the luminal and glandular epithelium ([3–5]; S.A.R., unpublished data). Enlargement of the lymph nodes draining the uterus becomes evident over the ensuing days [6, 7]. This inflammatory response is transient and fully dissipates by the time of embryo implantation on Day 4 of pregnancy [3–5], when the leukocytes that persist in the endometrium are predominantly macrophages with an immunosuppressive phenotype [8].

The temporal changes in the trafficking and phenotypic behavior of endometrial leukocytes during the estrous cycle and preimplantation period appear to be orchestrated largely by steroid hormones and seminal factors acting through cytokines that emanate from epithelial cells that line the uterine lumen and comprise the endometrial glands [9]. Of particular importance are granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-6, the synthesis of which is up-regulated at least 20-fold and 200-fold, respectively, in response to specific proteinaceous factors that are present in semen [10, 11] and originate in the seminal vesicle gland [12]. Previous studies have implicated the surge in epithelial GM-CSF release as a key mediator in the postmating inflammatory response since instillation of recombinant GM-CSF into the uterus at estrus evokes cellular changes resembling those seen to follow natural mating [13]. However, a GM-CSF surge is not wholly responsible, since the numbers and activation states of leukocytes infiltrating the endometrium after mating in genetically GM-CSF-deficient mice are not substantially different from those in cytokine-replete mice [14]. Any activity of GM-CSF can therefore be compensated for, or augmented by, an array of C-X-C and C-C chemokines, the expression of which is also up-regulated after mating [14], and other cytokines synthesized by epithelial cells and/or activated endometrial macrophages including IL-6, IL-1, and TNFα [5, 11].

In the study reported here, we investigated the nature of the seminal factor that acts to stimulate GM-CSF release from the uterine epithelium. Previous experiments have shown that the increase in uterine GM-CSF content is not due to the introduction of GM-CSF contained within the ejaculate, is not provoked by cervical stimulation, and is independent of both the presence of sperm in the ejaculate and any MHC disparity between the male and female [10]. Significantly, semen from seminal vesicle-deficient males failed to induce GM-CSF synthesis or the postmating inflammatory response. It was subsequently shown that the stimulating factor is trypsin-sensitive, of high molecular weight, and present in seminal vesicular fluid [12]. In this study, we identify this factor as transforming growth factor β_1 (TGFβ_1) and show that TGFβ_1 instilled into the uterus can induce GM-CSF release and initiate an influx of leukocytes into the endometrium similar to that seen at mating. This activity suggests a key role for seminal TGFβ_1 in initiating the cascade of molecular and cellular events that precede successful implantation in the mouse.

MATERIALS AND METHODS

Cell Lines, Media, Cytokines, and Antibodies

RPMI-1640 and low-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) were

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supplemented with 10% fetal calf serum (FCS; Commonwealth Serum Laboratories, Melbourne, Australia), 10 mM HEPES (pH 7.2), 5×10^{-5} M β-mercaptoethanol, 2 mM L-glutamine, and antibiotics (RPMI-FCS and DMEM-FCS). FD 5/12 cells [15] and mouse fibroblast cells (L-cells, CCL 1.1; American Type Culture Collection [ATCC], Rockville, MD) were cultured in RPMI-FCS. Mink lung cells (Mv-1-Lu, CCL 64; ATCC) and uterine epithelial cells were cultured in DMEM-FCS. Recombinant human (rh) $TGF\beta_1$ was from R & D Systems (Minneapolis, MN), recombinant murine GM-CSF was provided by N. Nicola, The Walter and Eliza Hall Institute for Cancer Research (Melbourne, Australia), and recombinant human activin and recombinant human inhibin were provided by J. Findlay, Prince Henry's Institute for Medical Research (Melbourne, Australia). Monoclonal antibodies (mAb) used for immunohistochemistry were anti-CD45 (ATCC TIB 122; reactive with all leukocytes), anti-Mac-1 (CD11b/CD18, ATCC TIB 128; reactive with macrophages and neutrophils), anti-MHC class II (Ia antigen, ATCC TIB 120; reactive with activated macrophages/dentritic cells), F4/80 (macrophages [16]), and RB6–8C5 (neutrophils [17]). Mouse anti-bovine $TGF\beta_{1,2,3}$ mAb (which neutralizes all three mammalian TGFβ isoforms) was from Genzyme (Cambridge, MA), and chicken anti-bovine $TGF\beta_1$ mAb (which neutralizes $TGF\beta_1$, < 2% cross-reactivity with $TGF\beta_2$ and β_3) was from R & D Systems.

Mice and Surgical Procedures

This study was carried out in accordance with the Guiding Principals for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction.

Adult (8–12 wk) Balb/c × C57B1 F_1 female mice and CBA × C57B1 F_1 male mice obtained from the University of Adelaide Central Animal House were housed in a minimal security-barrier, specific pathogen-free facility on a 12L:12D cycle with food and water available ad libitum. Females were synchronized into estrus using the Whitten effect [18], and cycle stage was confirmed by analysis of vaginal smears. For natural mating, females were placed 2 per cage with individual intact or vasectomized males, and the day of sighting of a vaginal plug was designated Day 1 of pregnancy or pseudopregnancy. For matings with seminal vesicle-deficient males, vaginal smears were made each morning and checked for the presence of sperm, since a copulatory plug was not formed.

All surgical procedures were performed under anesthesia using 1 mg/ml tribromoethyl alcohol in tertiary amyl alcohol (Sigma, St. Louis, MO) diluted to 2.5% v:v in saline (15 μl/g BW injected i.p.). Vasectomized mice were prepared by bilateral ligation of the vas deferens through a transverse incision in the abdomen [19], and seminal vesiculectomized mice were prepared by removal of the seminal vesicles through a transverse incision in the abdomen after ligation and severing of the proximal tubule at the base of the gland. The peritoneal wall and skin were sutured, and the mice were allowed to recover for at least 2 wk before mating.

For intrauterine injections, uterine horns of estrous females were exteriorized through a dorsal paravertebral incision and injected close to the oviductal junction, using a 30-gauge needle, with 0.4–40 ng $rhTGF\beta_1$ in 50 μl of PBS/1% BSA, or vehicle only. The uterus was returned to the retroperitoneal space, and the skin was closed with a stainless steel wound clip (Becton Dickinson, Sparks, MD). Mice recovered from the anesthesia within 30 min after surgery and were killed 16 h later for assessment of luminal cytokine content or collection of uterine tissue for immunohistochemistry.

Collection of Reproductive Tract Fluids

Male studs used for collection of accessory gland secretions were all of proven fertility and were rested for 1 wk before use. Seminal vesicle secretions were extruded by gentle compression from intact glands (50–100 μl fluid recovered per gland) and solubilized in 6 M guanidine HCl (Sigma; 1:4 v:v), then desalted into DMEM using 5 ml Sephadex G-25 desalting columns (Pharmacia, Uppsala, Sweden) before application to epithelial cell cultures. Prostate and coagulating gland secretions were extracted by homogenization of intact glands in 0.5 ml of PBS/1% BSA, followed by sedimentation of debris at 5000 × *g*.

In experiments in which uterine GM-CSF was measured, uterine luminal fluid was collected 16 h after mating or instillation of $rhTGF\beta_1$ into the uterus by flushing each horn with 500 μl of RPMI-FCS. Debris was sedimented at 2000 × *g*, and the supernatant was stored at −80°C before cytokine assay. In experiments in which uterine $TGF\beta_1$ was measured, mice were killed 1 h after mating, and flushings of the right horn were made with 6 M guanidine HCl/0.1% BSA and desalted into PBS/0.1% BSA before cytokine assay. For matings with intact and seminal vesicle-deficient males, the left horn was flushed with DMEM, and the sperm content was determined to confirm that insemination of the uterus had occurred (> 1×10^6 sperm per uterine horn).

Chromatography

One milliliter of seminal vesicle fluid diluted into 6 M guanidine HCl (pooled from 4–6 mice) was applied to a Sephacryl S-400 column (40 cm × 16 mm; Pharmacia) equilibrated in 6 M guanidine HCl/0.05 M HEPES, pH 7.4. Fractions of 1 ml were collected, desalted into DMEM, and assayed for TGFβ content or GM-CSF-stimulating activity by addition to uterine cultures. Before desalting, half of each fraction was transiently acidified to pH 2.0. This "acid-activation" was achieved by addition of 2 μl of 5 M HCl to each 0.5-ml fraction for 10 min at room temperature, then neutralization by addition of 2 μl of 5 M NaOH, as previously described [20].

Uterine Epithelial Cell Cultures

Uterine epithelial cells prepared as previously described [11] were pooled from 4–6 estrous mice and plated in 1-ml culture wells (Nunc, Roskilde, Germany) at $1–2 \times 10^5$ cells per ml in 500 μl of DMEM-FCS. After a 4-h incubation at 37°C in 5% CO_2 to allow cell adherence, a further 500 μl of desalted seminal vesicle fluid, cytokines or antibodies in DMEM-FCS, or DMEM-FCS alone was added. Culture supernatants were collected and replaced with fresh medium at 16 h, then collected again 24 h later, at which time adherent cells were quantified as previously described [11]. All treatments were performed in duplicate or triplicate.

Cytokines and Cytokine Assays

GM-CSF was assayed using the GM-CSF-dependent cell line FD 5/12, essentially as previously described [11]. Cell proliferation was determined by the addition of Alamar

Blue dye (Astral Scientific, Gymea, Australia) for the last 24 h of the assay or by pulsing with 1 μCi of [^{3}H]thymidine per well for the last 6 h of the assay. The minimal detectable amount of GM-CSF was 1 U/ml (50 U/ml defined as that producing half-maximal FD 5/12 proliferation). The identity of the bioactivity in in vitro uterine epithelial cell cultures and in uterine luminal fluid was confirmed using a goat polyclonal antibody to murine GM-CSF kindly provided by J. Schreurs (DNAX, Palo Alto, CA) as previously described [11]. Seminal vesicle fluid, rhTGFβ$_1$, activin, and inhibin did not have any bioactivity in the FD 5/12 assay ([10], data not shown).

TGFβ bioactivity was measured using Mv-1-Lu cells as previously described [21], except that cell numbers were quantified by the addition of Alamar Blue dye for the last 24 h of the assay. The specificity of the Mv-1-Lu bioassay for TGFβ was confirmed using TGFβ-specific neutralizing antibodies (described above). The minimal detectable amount of TGFβ in this assay was 15 pg/ml. TGFβ$_1$ immunoactivity was measured in a specific ELISA (R & D Systems) according to the manufacturer's instructions. For measurement of "total" (active + latent) TGFβ, reproductive tract fluids or homogenates (other than seminal vesicle chromatography fractions; see above) were transiently acidified by addition of 200 μl of 1 M HCl to each 1-ml sample for 10 min at room temperature, and neutralized by addition of 200 μl 1 M NaOH/0.05 M HEPES. For measurement of "active" TGFβ, reproductive tract fluids and extracts were left untreated.

TGFβ$_1$ mRNA expression was evaluated by reverse transcription-polymerase chain reaction (RT-PCR). Extraction of seminal vesicle mRNA, reverse transcription into cDNA, and PCR were performed as previously described [12]. The PCR primers were 5′-GGAGCAACATGTGGAACT-3′ (nucleotides [nt] 844–861) and 5′-GGTTCATGTCA-TGGATGG-3′ (complementary to nt 1098–1115), which generate a 271-base pair product.

Immunohistochemistry

Uterine tissue was embedded in O.C.T. Tissue Tek (Miles Scientific, Elkhart, IN) and frozen in isopropanol cooled by liquid N_2, then stored at −80°C until use. Six-micrometer semi-serial sections were cut from uteri collected at 1400 h on the day of estrus or on Day 1 of pregnancy, or from mice that had received intrauterine injections with rhTGFβ$_1$, and fixed in 96% ethanol (4°C/10 min). Sections were taken from approximately halfway between the cervical and oviductal junctions of the uterus, e.g., at least 3–4 mm proximal to the injection site. For mAb staining, duplicate sections were incubated with mAbs (neat hybridoma supernatant containing 10% normal mouse serum [NMS]) and goat anti-rat-horseradish peroxidase (HRP; Dako, Carpinteria, CA; 1:20 in PBS containing 10% NMS) as detailed previously [9]. Negative control sections were incubated with an irrelevant, isotype-matched rat mAb or without mAb. To visualize HRP or endogenous peroxidase (to detect eosinophils), slides were incubated in diaminobenzidine (Sigma; 5 mg/ml in 0.05 M Tris-HCl [pH 7.2]) plus 0.02% hydrogen peroxide for 10 min at room temperature. After being counterstained in hematoxylin, the sections were analyzed using video image analysis software (Video Pro, Faulding Imaging, Adelaide, Australia), in which the mean area of positive staining in ten medium-power fields (×20 objective) of endometrial stroma was expressed as a percentage of total staining.

RESULTS

GM-CSF-Stimulating Activity and TGFβ$_1$ Were Copurified from Seminal Vesicle Fluid

Our previous studies have demonstrated the capacity of seminal vesicle fluids to induce expression of GM-CSF mRNA and protein in uterine epithelial cells in vivo and in vitro [11, 12]. We have shown that the epithelial cell GM-CSF is biologically and immunologically indistinguishable from T cell-derived GM-CSF [11]. Characterization of the molecular weight of the GM-CSF-stimulating moiety in seminal vesicle fluid by Sephacryl S-400 chromatography has identified two proteinaceous fractions of high molecular weight with the capacity to stimulate release of GM-CSF bioactivity from uterine epithelial cells in vitro. The major proportion of the activity was found to elute in a peak corresponding to approximately 660 kDa with a lesser and more heterogenous peak of activity eluting in the 100- to 440-kDa range [12]. To further characterize the nature of the active moieties, the stability of the active fractions at acid pH was investigated. Unexpectedly, the GM-CSF-stimulating activity of material eluting at less than 440 kDa was consistently found to be markedly enhanced by transient acidification to pH 2.0, whereas the activity of the 660-kDa fraction was abolished by acid treatment (Fig. 1A).

Since acid activation is characteristic of the TGFβ family of cytokines [22], and TGFβ is known to be present in human seminal plasma [23], we then examined the TGFβ content of fractionated seminal vesicle extracts. Maximal TGFβ$_1$ immunoactivity was found to coelute from Sephacryl S-400 in the same molecular weight range as the GM-CSF-stimulating activity (Fig. 1B). When the TGFβ content of fractions was assessed using TGFβ-sensitive Mv-1-Lu cells, bioactivity was clearly detectable in the 250- to 440-kDa fractions (> 300 pg/ml) but was low (< 50 pg/ml) or undetectable in fractions of higher or lower molecular mass. The specificity of this bioactivity was confirmed by the findings that these fractions did not affect the growth of TGFβ-resistant mouse fibroblasts (L-cells), and that anti-TGFβ$_{1,2,3}$ mAb neutralized the capacity of vesicular fluid fractions to inhibit Mv-1-Lu cell growth (data not shown).

The amount of active and total TGFβ$_1$ in whole seminal vesicle fluid was also measured by ELISA of untreated and acid-activated vesicular secretions, respectively, and compared with aqueous extracts from other male accessory glands. High concentrations of TGFβ$_1$ immunoactivity were detected in seminal vesicle fluid and were elevated 3.2-fold by transient acidification (median [range] = 74 [36–104] ng/ml), indicating that > 70% of the TGFβ$_1$ in the seminal vesicle exists in the latent form. The seminal vesicle gland was found to be a far more potent source of TGFβ$_1$ than the prostate or coagulating gland (mean values for acid-activated tissue homogenates were 1.9 and 1.7 pg per mg tissue, respectively). Additional evidence that TGFβ$_1$ mRNA is synthesized in the seminal vesicle gland was obtained by RT-PCR (data not shown).

Anti-TGFβ$_1$ Neutralized Seminal GM-CSF-stimulating Activity

To determine whether TGFβ$_1$ accounted for the seminal vesicular GM-CSF-stimulating activity, the effect of anti-TGFβ mAbs on the capacity of dilute seminal vesicle secretions to stimulate GM-CSF production from uterine epithelial cell cultures was determined. The GM-CSF output

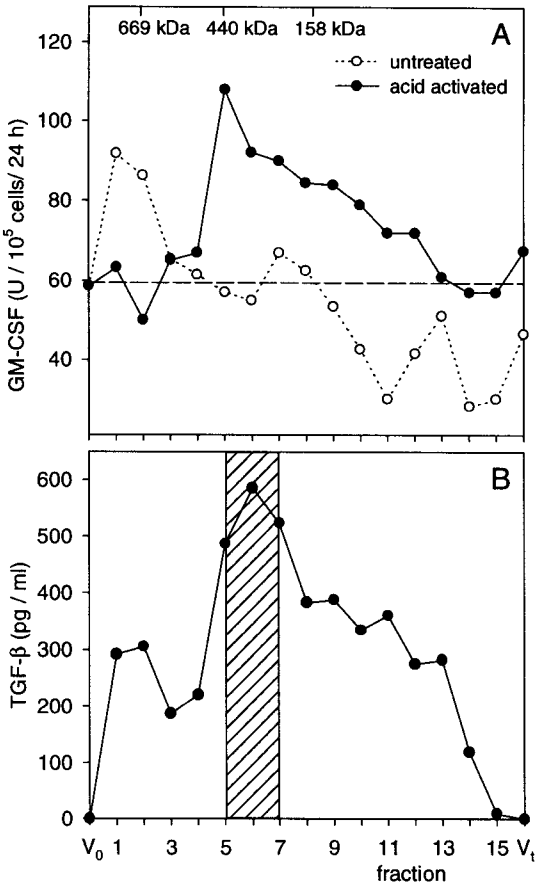

FIG. 1. Sephacryl S-400 size exclusion chromatography of **A**) GM-CSF-stimulating activity and **B**) TGFβ$_1$ immunoactivity and bioactivity in murine seminal vesicle fluid. **A**) Uterine epithelial cells from estrous mice were incubated for 16 h with untreated (open circles = active TGFβ) or acid-activated (solid circles = active + latent TGFβ) fractions of seminal vesicle fluid. After a further 24-h culture, the GM-CSF content of supernatants was determined by FD 5/12 bioassay. Values are means of triplicate cultures, and the horizontal dashed line is GM-CSF production by epithelial cells cultured with DMEM-FCS alone. **B**) The content of immunoactive TGFβ$_1$ (solid circles) in fractions of seminal vesicle fluid was determined by ELISA. TGFβ bioactivity was detected by Mv-1-Lu cell bioassay. Fractions depicted by the hatched area contained > 300 pg/ml, and other fractions contained < 50 pg/ml. Data are representative of similar results obtained from three replicate experiments.

of epithelial cells from estrous mice was elevated 2.5-fold by coculture with 2% crude seminal vesicle fluid, but this effect was completely neutralized in the presence of anti-TGFβ$_{1,2,3}$ mAb and diminished by > 80% in the presence of anti-TGFβ$_1$ mAb (Fig. 2). Irrelevant, isotype-matched mAbs had no effect on epithelial cell GM-CSF release (data not shown). This experiment confirmed the identity of the seminal GM-CSF-stimulating factor as TGFβ, primarily in the TGFβ$_1$ isoform.

Seminal Vesicle-Derived TGFβ$_1$ Was Deposited in the Uterus After Mating

To determine the effect of mating on the TGFβ$_1$ content of the uterus at mating, uterine luminal fluids were collected by lavage from the uteri of unmated estrous mice and of mice 1 h after mating. The content of both active and total TGFβ$_1$ immunoactivity was measured by ELISA. The median total TGFβ$_1$ content of fluids harvested after mating with intact males was 3.3-fold higher than that of fluids from unmated estrous mice (median [range] = 630

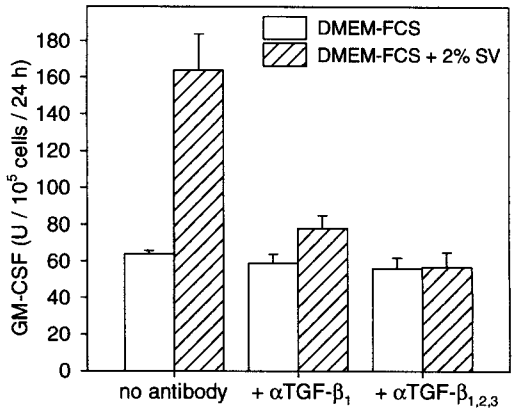

FIG. 2. The effect of neutralizing antibodies specific for TGFβ$_{1,2,3}$ and TGFβ$_1$ on GM-CSF-stimulating activity in murine seminal vesicle fluid. Uterine epithelial cells from estrous mice were incubated for 16 h with 2% crude seminal vesicle fluid or DMEM-FCS alone or in the presence or absence of mouse anti-bovine TGFβ$_{1,2,3}$ (20 μg/ml) or chicken anti-bovine TGFβ$_1$ (10 μg/ml). After a further 24-h culture, the GM-CSF content of supernatants was determined by FD 5/12 bioassay. Values are mean ± SD of triplicate cultures. Data are representative of similar results obtained from three replicate experiments.

[310–760] and 200 [150–270] pg/uterus, respectively; p = 0.004; Fig. 3). Comparable increases in uterine TGFβ$_1$ content were observed in mice mated with vasectomized males (median [range] = 680 [540–1000] pg/uterus). In both cases, the majority of the TGFβ$_1$ recovered from the uterus after mating was found to be biologically active before acid activation (58% and 68% in females mated with intact and vasectomized males, respectively). In contrast, the TGFβ$_1$ content of mice mated with seminal vesicle-deficient males (median [range] = 100 [90–180] pg/uterus) was not significantly higher than that of unmated mice, in fact it was less. These data show that the increase in uterine luminal TGFβ$_1$ content after mating is not due to the neuroendocrine stimulus provided by the mating act, nor by the presence of sperm in the uterus, but rather is a consequence of introduction of seminal vesicle-derived components of the seminal plasma into the female reproductive tract.

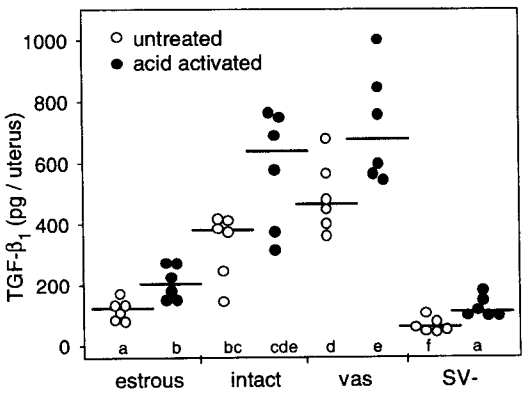

FIG. 3. The effect of seminal composition on the TGFβ$_1$ content of uterine luminal fluid after mating. TGFβ$_1$ immunoactivity was determined by ELISA in untreated (open circles = active TGFβ) or acid-activated (solid circles = active + latent TGFβ) uterine luminal fluids collected from estrous mice, or from mice 1 h after mating with intact, vasectomized (vas) or seminal vesicle-deficient (SV−) males. Symbols represent data from individual mice, and median values for treatment groups are scored. Data were compared by Kruskal-Wallis one-way ANOVA and the Mann Whitney Rank Sum test. Data sets labeled on the x-axis with different lowercase letters denote statistical significance between treatment groups (p < 0.01).

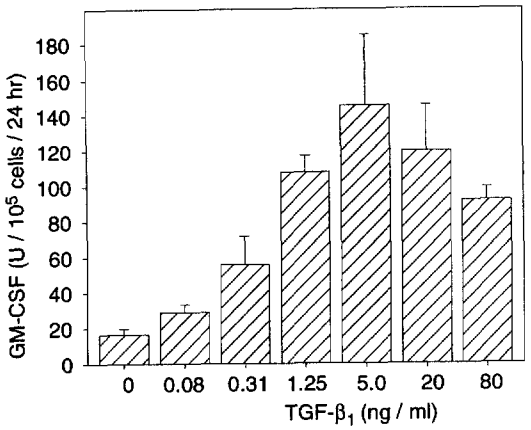

FIG. 4. The effect of TGFβ$_1$ on GM-CSF production by uterine epithelial cells in vitro. Uterine epithelial cells from estrous mice were incubated for 16 h with 0.08–80 ng/ml recombinant human TGFβ$_1$. After a further 24-h culture, the GM-CSF content of supernatants was determined by FD 5/12 bioassay. The mean ± SD of triplicate wells is shown. Data are representative of similar results obtained from four replicate experiments.

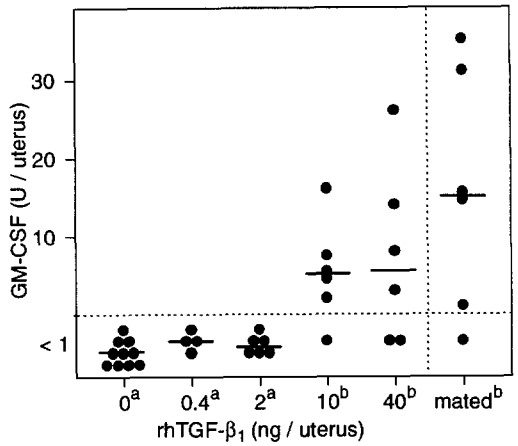

FIG. 5. The effect of intrauterine TGFβ$_1$ on the GM-CSF content of uterine luminal fluid. Fluids were collected 16 h after natural mating with intact males, or after administration of 0.4–40 ng recombinant human TGFβ$_1$ in 50 μl PBS/1% BSA, or vehicle only, to the uterine luminal cavity of estrous mice. Symbols represent data from individual mice, and median values for treatment groups are scored. Data were compared by Kruskal-Wallis one-way ANOVA and the Mann Whitney Rank Sum test. Data sets labeled on the x-axis with different superscripts denote statistical significance between treatment groups ($p < 0.01$).

In view of the rapidity of the increase in uterine TGFβ$_1$ content after mating and the occurrence of TGFβ$_1$ in the seminal vesicular fluid, we propose that the postmating increase in uterine TGFβ$_1$ is a direct consequence of the deposition of semen and is not due to induction of cytokine expression in the female reproductive tract. The median increase in the TGFβ$_1$ content of uterine fluids induced by mating with intact or vasectomized males (approximately 400 pg/uterus, Fig. 3) closely approximates that expected due to deposition of semen, given our finding that the median concentration of TGFβ$_1$ in seminal vesicular fluid is 74 ng/ml, and assuming that approximately 50% of a 10-μl volume of vesicular fluid is transferred to the uterine lumen during a typical mating episode comprising 2–3 ejaculations [24].

rhTGFβ$_1$ Stimulated Uterine Epithelial Cell GM-CSF Production In Vitro and In Vivo

To determine the capacity of TGFβ$_1$ to stimulate GM-CSF production from epithelial cells in vitro, rhTGFβ$_1$ was cultured with uterine epithelial cells harvested from estrous mice. A dose-dependent effect of TGFβ$_1$ on GM-CSF production was consistently observed, with a maximal increase elicited at a concentration of 5 ng/ml (median [range] = 1300% [800–1440%] increase; n = 4 experiments). At concentrations exceeding 5 ng/ml, the increase in GM-CSF production declined in a dose-dependent manner (Fig. 4).

To determine the effect of estrous cycle stage on the responsiveness of uterine epithelial cells to TGFβ, epithelial cells were harvested from groups of five diestrous and six estrous mice and cultured individually with 1 ng/ml rhTGFβ$_1$. In accordance with previous findings [25], unstimulated epithelial cells from diestrous mice secreted significantly less GM-CSF than did cells from estrous mice (median [range] = 19.4 [11.1–23.3] and 29.9 [24.9–61.6] U/10^5 cells/24 h, respectively; $p = 0.004$). Responsiveness to TGFβ$_1$ was diminished in epithelial cells from diestrous mice compared to estrous mice (median [range] increase in GM-CSF output = 134% [100–199%] and 237% [119–406%] of untreated cells, respectively; $p = 0.082$).

Inhibin and activin, which are members of the TGFβ cytokine family known to be present in human semen [26], were also tested for their capacity to induce GM-CSF release from uterine epithelial cells in vitro. Recombinant human inhibin had no effect on the release of GM-CSF when added to epithelial cells at concentrations ranging from 0.5 to 50 ng/ml. In contrast, recombinant human activin was found to have moderate GM-CSF-stimulating activity, but only at high concentrations; GM-CSF release from epithelial cells was not affected by culture with activin at 5 ng/ml or less but was increased to 300% of the control value by incubation with 50 ng/ml activin. Since comparable increases in GM-CSF output were achieved with 0.5 ng/ml TGFβ$_1$, the GM-CSF-stimulating activity of activin was considered to be approximately 100-fold less potent than that of TGFβ$_1$.

To determine whether TGFβ could elicit an increase in uterine epithelial cell GM-CSF output in vivo, rhTGFβ$_1$ was injected into uteri of estrous mice, and the GM-CSF content of the uterine fluids harvested 16 h later was determined. A dose-dependent increase in uterine luminal flu-

TABLE 1. The effect of intra-uterine injection with TGF-β$_1$ on endometrial leukocyte parameters.*

Treatment	n†	CD45	F4/80	Mac-1	Ia	RB6-8C5	Peroxidase
Vehicle	5	15 (8–19)^a	15 (12–25)^a	9 (7–21)^a	20 (8–23)^a	11 (5–15)^a	4 (4–7)^a
rhTGF-β$_1$	4	28 (13–39)^{ab}	37 (30–48)^b	23 (18–42)^a	25 (15–35)^a	15 (4–20)^a	15 (11–19)^b
Mated	4	41 (30–60)^b	31 (21–49)^b	48 (46–56)^b	32 (26–57)^b	36 (15–41)^b	13 (10–20)^b

* The number of mAb reactive cells in endometrial tissues from naturally mated mice, or estrous mice after intrauterine administration of rhTGFβ$_1$ or vehicle, are expressed as the median (range) percentage positivity; data were compared by Kruskal-Wallis one-way ANOVA and Mann Whitney Rank Sum test; data sets labeled with difference lowercase letters within columns denote statistical significance between treatment groups ($p < 0.01$).
† n = The number of mice in each experimental group.

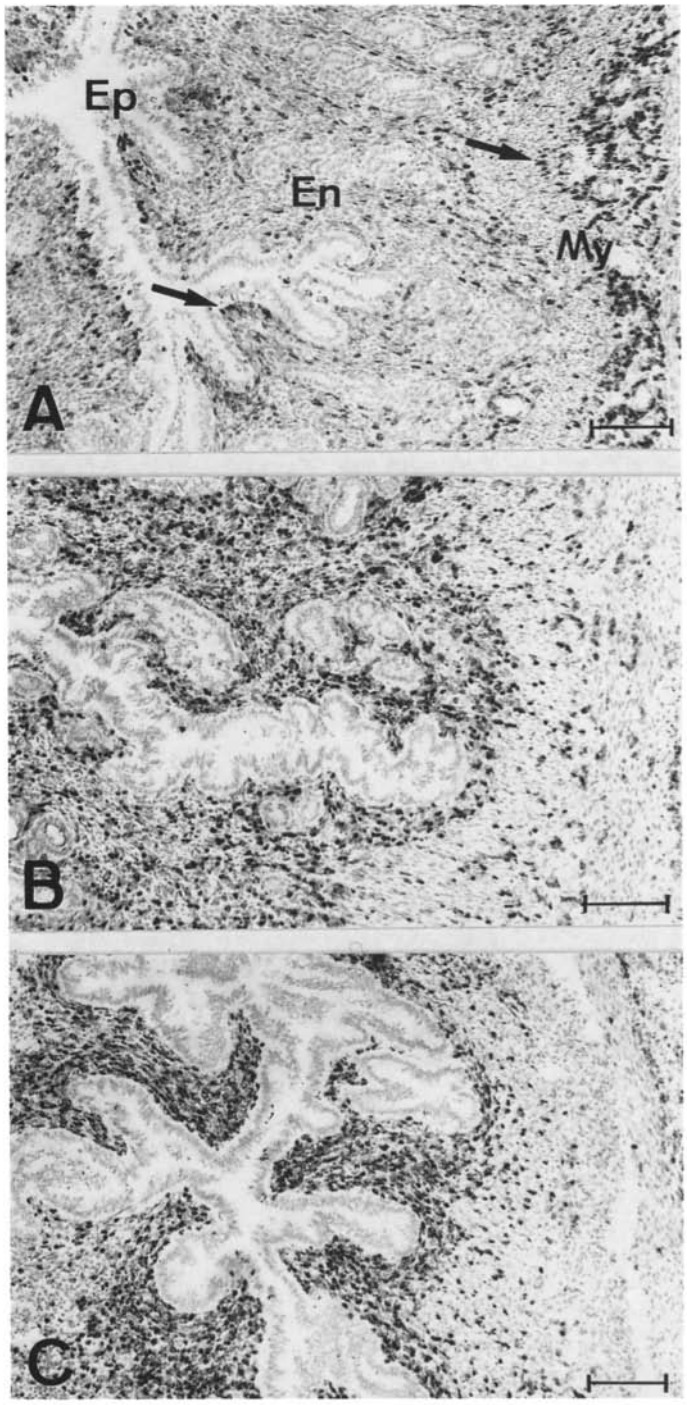

FIG. 6. The effect of intrauterine injection with TGFβ$_1$ on uterine leukocytes. Leukocytes reactive with anti-CD45 mAb and endogenous peroxidase positive eosinophils were localized immunohistochemically in the uteri of mice killed at 1000–1200 h on the day of estrus (**A**), 16 h after administration of 20 ng recombinant human TGFβ$_1$ in 50 μl PBS/1% BSA (**B**), or at 1000–1200 h on the day after mating with intact males (**C**). Endogenous peroxidase-positive eosinophils (small intensely stained cells [arrows]) predominate at the endometrial/myometrial junction at estrus, and other leukocytes (shown on the basis of reactivity with other mAbs to be predominantly macrophages [Table 1]; large stained cells) are evident in the endometrial stroma and beneath the epithelial surface (**A**). After administration of rhTGFβ$_1$, both eosinophils and other leukocytes are more plentiful in the endometrial stroma, particularly in areas immediately subjacent to the luminal and glandular epithelium (**B**). This pattern of leukocyte distribution is comparable to that seen after natural mating (**C**). Ep, epithelium; En, endometrium; My, myometrium. Scale bar = 150 μm.

id GM-CSF content was elicited, with maximal GM-CSF output occurring after the administration of 40 ng rhTGFβ$_1$. The extent of the increase in uterine GM-CSF content, and the variability between the responsiveness of individual mice, was comparable to that observed in a group of naturally mated mice (Fig. 5).

rhTGFβ$_1$ Elicited an Inflammatory Response in the Endometrium In Vivo

To investigate the effect of TGFβ$_1$ on uterine leukocyte parameters, an immunohistochemical analysis was made of the endometrial leukocyte populations in estrous mice 16 h after intraluminal injection of 20 ng rhTGFβ$_1$. The numbers of endometrial leukocytes reacting with F4/80 mAb specific for macrophages, or containing endogenous peroxidase (eosinophils), were significantly increased by administration of TGFβ$_1$ (Table 1). The extent of infiltration with macrophages and eosinophils after cytokine treatment, and the spatial distribution of these cells in the uterus (particularly their prominent accumulation beneath the luminal epithelium and adjacent to glands), were indistinguishable from the pattern seen in uteri harvested from mice 16 h after natural mating (Fig. 6). A trend towards increased numbers of endometrial leukocytes reacting with anti-CD45 (all leukocytes), anti-Ia (activated macrophages and dendritic cells), and anti-Mac-1 (neutrophils and newly recruited macrophages) was also evident in mice treated with rhTGFβ$_1$. In contrast, there was no significant effect of TGFβ$_1$ treatment on the number of neutrophils detected by reactivity with RB6–8C5.

DISCUSSION

Seminal plasma is thought to function primarily as a transport and support medium for spermatozoa traversing the female reproductive tract [27]. However, emerging studies indicate that this complex fluid also contains cytokines and other bioactive moieties that can interact with specific target cells within the reproductive tract and may enhance the survival of the conceptus and pregnancy outcome. In previous studies we and others have shown that factors in semen act on uterine epithelial cells to induce a massive release of GM-CSF, IL-6, and a range of other proinflammatory cytokines and chemokines [5, 11, 14] implicated in the postmating inflammatory response in mice. Induction of GM-CSF release is regulated at the transcriptional level by a proteinaceous factor in seminal plasma, originating in the seminal vesicle gland [12]. In this study, we have identified this factor as TGFβ$_1$, produced in the latent form in the seminal vesicle and activated in the female reproductive tract after mating.

The TGFβ$_1$ content of murine seminal vesicle secretions, like that previously reported for human seminal plasma [23], exceeds that reported for all other tissues and biological fluids, and rivals the content of platelet distillate [28]. In mammalian species, the TGFβ family comprises at least three closely related polypeptides—TGFβ$_1$, β$_2$, and β$_3$ [22]—which exhibit 70–80% sequence homology and share many biological actions. Although we have not specifically quantified TGFβ$_2$ or β$_3$ in murine seminal vesicular secretions, these isoforms did not contribute substantially to the GM-CSF-stimulating activity of vesicular secretions on uterine epithelial cell cultures since this activity was almost totally neutralized by antibodies specific for the TGFβ$_1$ isoform. The molecular mass range encompassed by the GM-CSF-stimulating activity in seminal vesicle fluid (150–440

kDa) is consistent with that of the latent form of $TGF\beta_1$, a complex of 230–290 kDa made up of the mature TGFβ dimer (25 kDa) noncovalently associated with a 75- to 80-kDa latency-associated protein and a 130- to 190-kDa binding protein [28]. The heterogeneity in size of the activity present in murine seminal vesicle fluid and human seminal plasma [23] may reflect the presence of incomplete complexes and complexes comprising other carrier proteins such as the 250- to 300-kDa binding protein betaglycan [29] and/or additional members of the TGFβ family.

The secretion of TGFβ as a latent complex is believed to help stabilize the cytokine [30] and focus its activity at the target site by binding to extracellular matrix [31]. The majority of the $TGF\beta_1$ found in the uterine luminal fluid after mating was in the active form, and we speculate that plasmin or other proteolytic enzymes derived from uterine cells or other male accessory organs [32, 33] may contribute to the activation of TGFβ after ejaculation. Furthermore, it is clear that seminal TGFβ would be augmented by TGFβ derived from uterine epithelial cells [34, 35], and seminal factors could act to promote the release or activation of cytokine produced endogenously. However, induction of TGFβ synthesis at the transcriptional level is unlikely to contribute significantly to the increase in uterine TGFβ content, since $TGF\beta_1$ mRNA levels were not found to fluctuate after mating when measured by in situ hybridization [34] or quantitative RT-PCR (SAR, unpublished data).

The causal sequence between instillation of seminal TGFβ and downstream events in the postmating inflammatory cascade remain to be fully elucidated. The experiments reported herein provide a clear link between seminal TGFβ and the induction of GM-CSF expression in endometrial epithelial cells. Interestingly, TGFβ responsiveness was higher in epithelial cells from estrous mice than from diestrous mice, suggesting that estrogen may attenuate expression of TGFβ receptors, binding proteins or other components of the signalling network leading to induction of GM-CSF. Endogenous TGFβ synthesized by uterine epithelial cells [34] could further modulate this axis; and, since its expression is estrogen-regulated [36], autocrine activation by TGFβ may also contribute to the estrous cycle-dependent variations in GM-CSF release reported previously [25].

GM-CSF is sufficient to induce many of the cellular changes seen to ensue after mating [13]. However, experiments in knockout mice demonstrated redundancy of GM-CSF in the postmating inflammatory response [14], indicating the existence of GM-CSF-independent pathways for the recruitment and activation of inflammatory cells. TGFβ would almost certainly have direct effects on the inflammatory response through its potent effects on the migration and synthesis of effector molecules in macrophages [37, 38]. Additional chemotactic cytokines including IL-6, TNFα, and IL-1, as well as a range of chemokines, are also expressed in the uterus after mating [5, 11, 14] and could supplement or replace the proinflammatory activities of GM-CSF. Whether TGFβ has a role in induction of these agents in epithelial or other somatic uterine cells is not known but is entirely possible given the highly pleiotropic nature of TGFβ and the ubiquitous expression of TGFβ receptors in uterine tissues [36].

Factors present in seminal fluid other than TGFβ or TGFβ-activating factors (for example, a 650-kDa protein described previously [12]) could also contribute to eliciting the full range of molecular and cellular changes seen in the endometrium after mating. In our experiments, the infiltration of neutrophils into the uterus typically following natural mating was not elicited by intrauterine injection of rhTGFβ. This could be due to failure of the treatment to induce specific components of the inflammatory cascade or, alternatively, may reflect a dosage effect related to the amount of GM-CSF induced. Indeed, our previous studies in naturally mated mice [11, 12] indicate that the extent of neutrophil recruitment into the endometrium and the quantity of GM-CSF synthesized by epithelial cells are highly variable and may be interdependent.

The full physiological significance of the series of orchestrated changes occurring in the endometrial cytokine-leukocyte axis during the preimplantation period of early pregnancy remains to be evaluated. The dramatic influx of leukocytes into the uterine endometrium after mating may simply serve to remove seminal debris and phagocytose bacteria and protect the female from infectious disease. However, we speculate that these cellular changes may also contribute to the immunological changes and tissue remodeling processes required to accommodate the semi-allogeneic conceptus during implantation and placental development.

One critical determinant of pregnancy success is the need for the maternal immune system to respond appropriately to antigens expressed by sperm and the conceptus. The recruitment and activation of antigen-presenting cells, including macrophages and dendritic cells, after mating would be expected to facilitate priming to sperm-associated or soluble antigens in semen, many of which are shared by the conceptus [39]. The role of TGFβ in this environment could extend beyond that of induction of the inflammatory cascade, since in other sites TGFβ is recognized as a potent immune-deviating agent associated with the induction of tolerance and Th2-type immune responses [40–42]. Seminal plasma TGFβ may therefore have additional roles in protecting against anti-sperm immunity and facilitating induction of the hyporesponsiveness to paternal MHC antigens [43], possibly through preferential induction of Th2-type responses [44], upon which maternal acceptance of the semi-allogeneic conceptus is believed to be founded. This postulate is consistent with our recent finding that exposure to semen at mating is sufficient to induce transient hyporesponsiveness to paternal MHC class 1 antigens [45], and with other studies that provide further evidence for a potent immune-deviating activity of seminal plasma [46, 47]. Of particular significance in this regard are Beer and Billingham's [6] data illustrating the capacity of seminal plasma to skew towards tolerance the transplantation immunity incited by intrauterine inoculation of washed allogeneic spermatozoa.

Although it is clear that pregnancy can proceed in the absence of exposure to semen, studies in various species suggest a role for both sperm and seminal plasma in optimizing pregnancy success. In mice, embryos transferred to recipients prepared by mating to vasectomized males usually have no more than a 50% survival rate to term [48], and fetal loss is even higher when pseudopregnancy in the recipient is achieved by artificial means [49]. Studies in rats show that to some extent this deficiency can be restored by insemination of females before embryo transfer [50]. The importance of seminal plasma, particularly secretions from the seminal vesicle, in optimizing pregnancy success is shown by studies in accessory gland-deficient mice [51]. In livestock species, the dysregulated fetal and/or placental growth that can occur after embryo transfer, or during the

first pregnancy, can be partially ameliorated by prior exposure to semen [52–54]. Studies in humans also clearly identify lack of exposure to semen due to limited sexual experience, use of barrier methods of contraception, or in vitro fertilization pregnancies with increased risk of implantation failure, spontaneous abortion, and pre-eclampsia [55–57].

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