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W. J. McGuire
Cloud County Community College

K. Imakawa
University of Tokyo

K. Tamura
Tokyo University of Pharmacy and Life Sciences

C. S. R. Meka
Oklahoma Medical Research Foundation

R. K. Christenson
USDA-ARS

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Regulation of endometrial granulocyte macrophage-colony stimulating factor (GM-CSF) in the ewe☆

W.J. McGuire[a,1], K. Imakawa[b,2], K. Tamura[b,3], C.S.R. Meka[b,4], R.K. Christenson[a,*]

[a] *USDA, Agricultural Research Service, Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, NE 68933-0166, USA*

[b] *Department of Pathology and Laboratory Medicine, University of Kansas Medical Center, Kansas City, KS 66160-7410, USA*

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Abstract

Granulocyte macrophage-colony stimulating factor (GM-CSF) increases ovine interferon-tau (oIFNτ) secretion by ovine conceptuses, but endometrial production of GM-CSF has not been characterized. Endometrial GM-CSF expression was evaluated in ovariectomized ewes implanted with estradiol-17β (E_2) and/or progesterone (P_4) for 14 days, in day 14 cyclic and day 14 pregnant ewes. Relative levels of endometrial GM-CSF mRNA were 3-fold higher in E_2- and E_2/P_4-treated ewes than that of control or P_4-treated ovariectomized ewes. Levels of endometrial GM-CSF mRNA for cyclic ewes were similar to E_2- and E_2/P_4-treated ewes, but amounts of GM-CSF mRNA in pregnant ewes were 2-fold higher. GM-CSF concentrations in endometrial culture media, determined by GM-CSF bioassay, for cyclic and E_2/P_4-treated ovariectomized ewes were 3-fold higher than those of control, E_2- and P_4-treated ovariectomized ewes; however, amounts of GM-CSF in pregnant ewes were 2-fold higher. Immunoreactive GM-CSF, examined by western blot, was detected in the culture medium from E_2/P_4-treated ovariectomized, cyclic and pregnant ewes. Luminal and glandular epithelia and stromal regions were

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* Corresponding author. Tel.: +1-402-762-4192; fax: +1-402-762-4382.

E-mail address: christenson@email.marc.usda.gov (R.K. Christenson).

[1] Present address: Cloud County Community College, 2221 Campus Drive, P.O. Box 1002, Concordia, KS 66901-1002, USA.

[2] Present address: Laboratory of Animal Breeding, University of Tokyo, Tokyo 113-8657, Japan.

[3] Present address: Department of Pharmacology, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo 192-03, Japan.

[4] Present address: Immunobiology and Cancer Research, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104, USA.

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determined to be sites of GM-CSF expression by immunohistochemistry and *in situ* hybridization techniques. Data indicate that combined E_2 and P_4 treatment of ovariectomized ewes is sufficient to restore GM-CSF expression to the level found in cyclic ewes; however, GM-CSF mRNA and protein in pregnant ewes is 2-fold greater than in ovariectomized or cyclic ewes. These data suggest that the conceptus, in addition to steroids, may play a role in the regulation of endometrial production of GM-CSF.
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1. Introduction

Ovine interferon-tau (oIFNτ) is a conceptus signal that is implicated in the process of maternal recognition of pregnancy [1–4]. Accumulating data shows that oIFNτ production by the conceptus of ruminant ungulates is positively regulated by endometrially derived cytokine/growth factors [5,6]. One of these factors, granulocyte macrophage-colony stimulating factor (GM-CSF) [7], increases conceptus production of oIFNτ *in vitro* [8,9]. GM-CSF mRNA has been localized by *in situ* hybridization to luminal and glandular epithelia of the ovine uterus on day 14–16 of gestation [8]. In order for GM-CSF to manifest an effect on conceptus production of oIFNτ, GM-CSF needs to be available for the conceptus *in utero*. The production of this protein *in utero* has not been well characterized.

GM-CSF production has been extensively studied in hematopoietic cells. Activation of T-cells by recognition of antigens on antigen responding cells leads to rapid induction of a nuclear protooncogene, c-*fos*, followed by induction of a battery of lymphokine genes including interleukin-2 and GM-CSF [10,11]. Major events associated with T-cell activation that upregulate GM-CSF production are the activation of protein kinase C second messenger system and increased concentrations of free intracellular calcium [12,13]. This stimulation of GM-CSF levels appears to be the result of increased transcription, as the promoter region of GM-CSF contains two cis-acting elements, GM-κB/GC-box and conserved lymphokine element O (CLEO), that are required for maximal induction of the GM-CSF gene following stimulation with phorbol-12-myristate-13-acetate (PMA) or calcium ionophore (A23187) [14]. In addition, uterine epithelial cells were found to be another source of GM-CSF expression [15,16]. However, GM-CSF expression in cell types other than T-cells and an endocrine or paracrine factors(s) that directly regulates endometrial GM-CSF production have not been well characterized.

The steroid regulation of GM-CSF production from the ovine uterine endometrium has not been investigated. Ovine GM-CSF (oGM-CSF) production from the endometrium may be regulated partially by steroid hormones as demonstrated by Robertson *et al*. [16,17] when uterine epithelial production of GM-CSF was increased in ovariectomized mice by exogenous estrogens. Because estrogens elicit considerable uterine edema [18] and alter the cellular composition of the uterus [19], *in situ* techniques are required to determine the uterine source of growth factors/cytokines; resident endometrial cells, cells recruited to synthesize specific growth factors/cytokines and/or the influx of serum growth factors into the uterus. To ascertain endometrial production of GM-CSF, in addition to the presence of GM-CSF in uterine flushing medium, endometrial tissues need to be cultured *in vitro* and the culture media must be examined for the presence of GM-CSF. Therefore, using endometrial culture,

immunohistochemistry and *in situ* hybridization methods, this study was undertaken to (1) determine the effects of estradiol-17β (E_2) and/or progesterone (P_4) on the expression of endometrial GM-CSF in ovariectomized ewes, and (2) characterize endometrial expression of GM-CSF in cyclic and pregnant ewes. A newly developed antibody against oGM-CSF was used to detect and neutralize endometrial GM-CSF.

2. Materials and methods

2.1. Animals

Thirty mature, whiteface crossbred ewes were maintained in drylot pens and assigned to three experimental groups: (I) ovariectomized ($n = 15$), (II) day 14 cyclic ($n = 7$), and (III) day 14 pregnant ($n = 8$) ewes. Estrus was synchronized in all ewes using a 14-day treatment with an intravaginal sponge containing 40 mg of 17α-acetoxy-9α-fluoro-11β-hydroxy P_4. On the day of sponge removal, ewes were given an intramuscular injection of 10 mg P_4. The following day, a second intramuscular injection of 10 mg P_4 and a single subcutaneous injection of 500 IU PMSG were administered [6].

On day 14 after the synchronized estrus (day 0 = day of estrus), 12 group I ewes were anesthetized with xylazine hydrochloride (0.1 mg/lb) and lidocaine [6] and both ovaries were removed through a midventral laparotomy. After a 4-week recovery, ovariectomized ewes were aseptically implanted subcutaneously with the following treatments: control-blank silastic implants ($n = 3$), two E_2 silastic implants ($n = 3$), three P_4 elastomer implants ($n = 3$), or two E_2 silastic and three P_4 elastomer implants ($n = 3$), based on preliminary dose response results. Silastic implants were prepared from silastic medical grade tubing (3.35 mm × 4.65 mm OD; 8 cm total length; Dow Corning, Midland, MI, USA) that were filled with E_2 [20]. Silicone elastomer implants (Sil-Estrus, Abbott Laboratories, North Chicago, IL, USA), each containing 375 mg P_4, were used to provide P_4. Blood samples from ovariectomized ewes were collected *via* jugular venipuncture prior to implant placement (day 0) and before hysterectomy on day 14. Blood samples were allowed to clot at 4°C, and serum was collected by centrifugation and stored at −20°C until assayed for concentrations of P_4 and E_2. P_4 was assayed using a rabbit antiprogesterone-11α-bovine serum albumin (Cambridge medical Technology, Billerica, MA, USA) in one assay [21]. Estradiol-17β was assayed using an antiserum provided by N. R. Mason (Lilly Research Laboratories, Indianapolis, IN, USA) in one assay [22]. On day 14 of implant treatment, group I ewes were anesthetized as previously described and hysterectomy was performed. Group II ewes were monitored for estrus with vasectomized rams fitted with marking harnesses, and estrus was recorded twice daily. Group III ewes were monitored for estrus and mated by two fertile rams fitted with marking harnesses, and estrus and mating were recorded twice daily. Hysterectomies were performed on group II ewes on day 14 of the estrous cycle and on group III ewes on day 14 after mating.

2.2. Tissue culture

After hysterectomy, uteri were immediately placed on ice and uteri from all ewes were transported to the laboratory and processed within 15 min. Uteri of pregnant ewes were flushed

with 20–30 mL sterile phosphate-buffered saline (PBS, pH 7.2) to recover conceptuses. Conceptuses were removed from the flushing media and protease inhibitor (PMSF, 1 mM) was added to each uterine flush before freezing (liquid nitrogen) and storing at −80°C for later GM-CSF analysis by bioassay (described later). Endometrial tissue was stripped from the uterus, minced, and an equal mass of tissue (300 mg wet weight, three culture dishes per ewe) from each implant-treated ovariectomized, cyclic and pregnant ewe was cultured in 10 mL Eagle's minimum essential medium (Sigma Chemical Co., St. Louis, MO, USA). All samples were cultured at 37°C on a rocking platform under 50% N_2, 45% O_2, and 5% CO_2. Following a 24 h culture period, endometrium was collected by centrifugation at 2500 rpm for 5 min. Culture medium was collected and stored at −20°C, and cultured endometrial samples were frozen in liquid nitrogen and stored at −80°C until RNA extraction was performed.

2.3. Quantitation of GM-CSF mRNA

Total RNA was extracted from 300 mg of cultured endometrial tissue samples according to the method of Chomzynski and Sacchi [23]. After spectrophotometric quantitation of samples, integrity of extracted RNA was confirmed by the presence of distinct 18s and 28s rRNA bands through an agarose gel electrophoresis containing ethidium bromide (2 μg/mL) to visualize the RNA [24]. Relative levels of GM-CSF mRNA were quantitated by reverse transcription–polymerase chain reaction (RT–PCR) method as described by Nephew *et al.* [25]. Preliminary experiments were performed to validate the primer specificity and PCR conditions under which each amplification reaction was confined to the exponential phase. For the determination of primer specificity, endometrial RNA samples (5 μg each) from group I, II and III ewes were treated with RNase free DNase (Stratagene, South San Francisco, CA, USA) and then reverse transcribed with AMV reverse transcriptase (Promega, Madison, WI, USA) in the presence of a 3′-downstream primer corresponding to bp 340–361 (5′-CTG GGT TTC ACA GGA AGT TTC C-3′) [26]. The resulting cDNA was amplified through 30 cycles (94:60:74°C for 1, 1, and 2 min, respectively) of PCR using a 5′-upstream primer corresponding to bp 14–33 (5′ATG TGG CTG CAG AAC CTG CT-3′). The amplification products were electrophoresed on a 1.5% agarose gel containing ethidium bromide (10 μg/mL) to visualize a 348 bp band. The PCR products were eluted from the agarose gel, cloned into pBS vector (Stratagene, South San Francisco, CA, USA) and nucleotides were determined by dideoxy sequencing method (Sequenase, Amersham Life Science, Cleveland, OH, USA) [27]. Nucleotide sequences confirmed that these primers were specific for the detection of oGM-CSF mRNA. For cRNA standard preparation, oGM-CSF cDNA (500 bp; C.J. McInnes, Moredun Research Institute, Edinburgh, UK) was appropriately linearized and sense strand oGM-CSF cRNA was produced by an *in vitro* transcription [24,25]. cRNA standards, ranging from 0.01 to 60 pg, were reverse transcribed using the 3′-downstream primer as aforementioned. Resulting cDNAs were amplified with the upstream primer labeled with ^{32}P-γ-dATP (specific activity 6000 Ci/mmol; Dupont NEN, Boston, MA, USA). PCR was carried out 20–25 cycles to yield PCR products proportional to the initial concentrations of cRNA. Amplified products were examined on an agarose gel and incorporated radioactivity was determined by liquid scintillation counting [25,28,29]. Levels of cRNA standard were plotted against radioactivity (cpm); data were also plotted against PCR cycle number, and a linear line was fitted to each

curve [30]. RNA samples along with cRNA standards ranging from 0.1 to 30 pg were reverse transcribed with the downstream primer. The resulting cDNA was amplified for 22 cycles using the upstream primer, which had been validated to be proportional to the range (0.1–30 pg) of cRNA standard. Control reactions, RNA without reverse transcriptase and PCR without cDNA template were run in parallel, which resulted in no PCR product. Levels of GM-CSF mRNA for RNA samples were then calculated from the standard curve.

2.4. Bioassay of GM-CSF

After endometrial culture, whole medium samples (10 mL/culture) along with oGM-CSF enriched conditioned media prepared from COS-1 monkey kidney cells (oGM-CSF CM, C.J. McInnes) were dialyzed in the presence of protease inhibitors, pepstatin A (0.7 mg/mL) and leupeptin-propionyl (0.35 mg/mL). A total of 500 μL endometrial culture media was used for a bioassay and the remaining medium (9.5 mL) and oGM-CSF CM upon concentration were subjected to western blot analysis. Concentrations of oGM-CSF in endometrial culture and uterine flushing media were quantified by a proliferation bioassay using GM-CSF dependent TF-1 cells [31] supplied by L. Guilbert (Department of Immunology, University of Alberta, Alberta, Canada). TF-1 cells were grown in RPMI medium 1640 (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) and 5 ng/mL human recombinant GM-CSF (hrGM-CSF; Genetics Institute, Cambridge, MA, USA). Before GM-CSF assay, these cells were deprived of hrGM-CSF for 24 h. Four thousand TF-1 cells, prepared in 50 μL RPMI 1640 medium, were added to hrGM-CSF standard (5, 10, 20, 40, 80, 160, 320, and 660 pg/mL), endometrial culture samples, and uterine flushing samples in triplicate. The final volume (150 μL, which contained 10% FBS) was cultured at 37°C under the conditions of 95% air and 5% CO_2 for 3 days. On day 4 of the culture, 5 μL RPMI 1640 containing 1 μCi ^{3}H-thymidine (specific activity 70–90 Ci/mmol; DuPont NEN, Boston, MA, USA) and 10% FBS (Sigma Chemical Co., St. Louis, MO, USA) were added to each well and incubated for 4 h. The cells were harvested in a cell-harvester fitted with filter paper (DE81, Whatman International, Ltd., Maidstone, UK) to measure ^{3}H-thymidine incorporation. A total of eight standards were placed at the beginning, middle, and end of each plate. In order to assess assay variation, oGM-CSF CM and pooled endometrial culture media in triplicate were included in each assay. Assay sensitivity was 23 pg per well and the intra and interassay coefficient of variation, calculated from the values of GM-CSF CM and pooled samples within and between assays, were 6.2 and 9.1%, respectively. To determine the specificity of the bioassay, an antibody against oGM-CSF (described later) was prepared and used to neutralize hrGM-CSF, oGM-CSF CM and oGM-CSF from endometrial culture media. More than 95% of GM-CSF, regardless of species, was neutralized with this azide-free antiserum.

2.5. oGM-CSF antibody production and western blot analysis

An antibody to a synthetic peptide representing a portion of oGM-CSF was developed using the following strategy: an antigenic site along the deduced amino acid sequence of oGM-CSF [26] was determined by assigning each amino acid hydrophilicity values. The point of the

highest local average hydrophilicity is invariably located in, or immediately adjacent to, an antigenic determinant [32,33]. An antiserum to that domain prepared by Research Genetics (Huntsville, AL, USA) is ETQIITFKSFKENLKD, which is equivalent to a region found in hrGM-CSF [34].

After dialysis, 9.5 mL samples of endometrial culture media or 3 mL samples of oGM-CSF CM were concentrated by Centricon (Amicon, Beverly, MA, USA), separated by 12.5% SDS–PAGE and then electrotransferred to Immobilon (Millipore, Bedford, MA, USA) [6]. The filters were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST). The filters were then incubated with rabbit preimmune serum or anti-oGM-CSF antibody at a dilution of 1:1000 in TBST. The immunoreactive proteins were detected using alkaline-phosphatase conjugated to rabbit IgG antibody.

2.6. In situ hybridization and immunohistochemistry

In addition to endometrial tissues subjected to *in vitro* culture study, a 0.125 cm^3 (0.5 cm × 0.5 cm × 0.5 cm) section of uterus from all treatment groups was fixed, paraffin embedded, sectioned and evaluated for the presence of GM-CSF mRNA and its protein. *In situ* hybridization was performed as previously described [6,8]. Briefly, oGM-CSF cDNA (C.J. McInnes) of which a portion of the 3′ region had been subcloned into pBS M13 [8,26], was appropriately linearized and sense and antisense GM-CSF cRNA probes were produced using T3 and T7 RNA polymerases, respectively. Digoxigenin-11 UTP (Boehringer Mannheim, Indianapolis, IN, USA) was incorporated into the probes during *in vitro* transcription. Binding of oGM-CSF cRNA to endometrial tissues was detected by antidigoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN, USA). Sections were then counterstained with methyl green.

Immunohistochemistry was performed with the oGM-CSF antiserum described previously. Briefly, a rabbit polyclonal antibody to the oGM-CSF oligopeptide was used at a dilution of 1:250 in PBS, and binding was detected by means of an avidin–biotin immunoperoxidase staining kit [6]. 3-amino-9-ethylcarbazole in *N*,*N*-dimethylformamide (Zymed Laboratories, Inc., South San Francisco, CA, USA) was used as substrate for the peroxidase, and positive cells were identified by the presence of a reddish-brown reaction product. An equal concentration of preimmune serum was used on semiserial sections of the same tissue to evaluate nonspecific binding of the avidin–biotin immunoperoxidase. Tissue sections were then counterstained with hematoxylin.

2.7. Statistical analysis

Data obtained from ovariectomized, cyclic and pregnant ewes were analyzed as a one-way analysis of variance [30] of six treatment groups (i.e., ovariectomized control (1), ovariectomized plus E_2 implant (2), ovariectomized plus P_4 implant (3), ovariectomized plus E_2 and P_4 implants (4), cyclic (5), and pregnant (6)). The sums of squares were partitioned into single degree of freedom contrasts ((1) versus (2), (1) versus (3), (1) versus (4), (4) versus (5), (5) versus (6), and (4) versus (6)) that had been selected *a priori* to compare the treatment groups.

Table 1
Serum concentrations of E_2 and P_4 before and after E_2 and/or P_4 treatment in group I ovariectomized ewes

Treatment	E_2 (pg/mL)		P_4 (ng/mL)	
	Day 0[a]	Day 14[a]	Day 0[a]	Day 14[a]
Control	4.6 ± 0.4	6.4 ± 1.5	0.04 ± 0.01	0.21 ± 0.15
E_2	5.7 ± 1.0	40.5 ± 6.2	0.07 ± 0.02	0.08 ± 0.03
P_4	4.1 ± 0.3	5.4 ± 0.24	0.07 ± 0.04	2.27 ± 0.31
E_2/P_4	6.2 ± 0.9	46.4 ± 10.3	0.09 ± 0.01	1.51 ± 0.52

Mean ± SEM.
[a] Blood samples were collected before implanting ewes on day 0 and before hysterectomy on day 14.

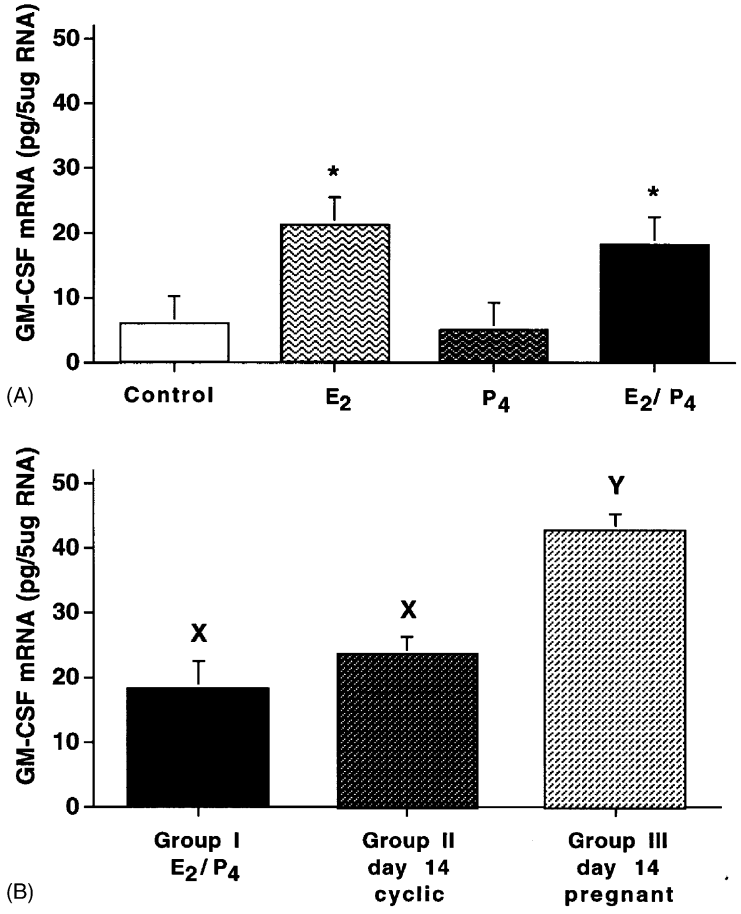

Fig. 1. Mean (±SEM) levels of GM-CSF mRNA in cultured endometrium from group I, II and III ewes. RNA was isolated from cultured endometrium and subjected to quantitative RT–PCR. (A) Depicts group I, ovariectomized ewes assigned to the following treatments: controls, E_2, P_4 and E_2/P_4. (B) Depicts group I (E_2/P_4), group II (day 14 cyclic) and group III (day 14 pregnant) ewes. (∗) Indicates that the mean is different from control ($P < 0.05$). Means having different superscripts (x, y) differ ($P < 0.01$).

3. Results

Mean serum concentrations of E_2 and P_4 in group I ovariectomized ewes, as determined by specific RIA on days 0 and 14, are shown in Table 1. Serum concentrations of E_2 and P_4 did not differ between day 0 and 14 in ovariectomized ewes that did not receive E_2 or P_4 implants. Serum concentrations of E_2 in the E_2- and E_2/P_4-treated ovariectomized ewes on day 14 were 40.5 ± 6.2 and 46.4 ± 10.3 pg/mL, respectively. Similarly, serum concentrations of P_4 in the P_4- and E_2/P_4-treated ewes on day 14 were 2.27 ± 0.31 and 1.51 ± 0.52 ng/mL, respectively.

After RT–PCR of GM-CSF cRNA standards (0.1–30 pg), levels of cRNA were plotted versus cpm producing a linear standard curve with an r^2 of 0.993 ($y = -7091.7 + 1.6876x$). The incorporated cpm (3000–65,000 cpm) resulting from RT–PCR of RNA samples yielded values that fell within the standard curve (0.1–30 pg). Levels of GM-CSF mRNA extracted from cultured endometrial tissues obtained from E_2- and E_2/P_4-treated ovariectomized ewes were

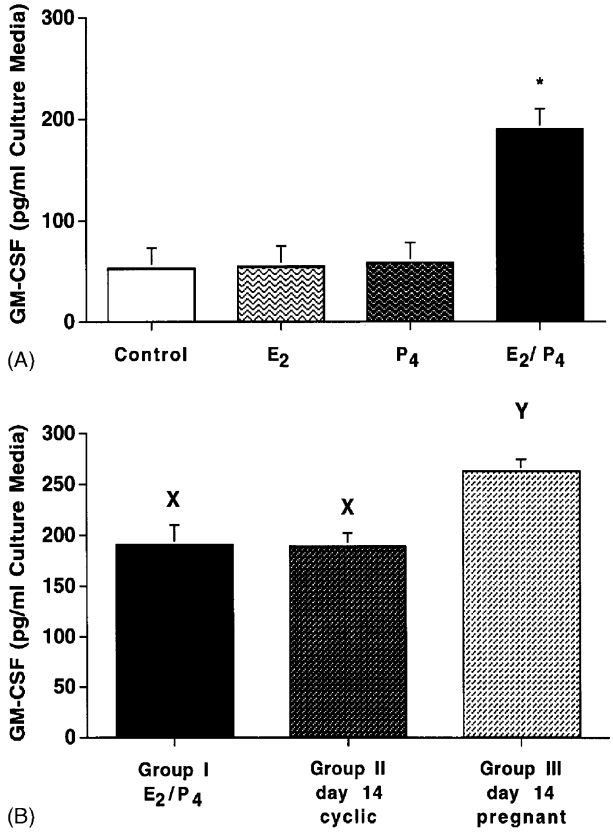

Fig. 2. Mean (±SEM) concentrations of bioactive GM-CSF in medium from cultured endometrium from group I, II and III ewes. GM-CSF was determined by bioassay based on proliferation of GM-CSF dependent TF-1 cells. (A) Depicts group I, ovariectomized ewes assigned to the following treatments: control, E_2, P_4 and E_2/P_4. (B) Depicts group I (E_2/P_4), group II (day 14 cyclic) and group III (day 14 pregnant) ewes. (∗) Indicates that the mean is different from control ($P < 0.01$). Means having different superscripts (x, y) differ ($P < 0.01$).

higher ($P < 0.05$) than those seen in controls (Fig. 1A). GM-CSF mRNA levels extracted from cultured endometrial tissues from P_4-treated ovariectomized ewes were not different from controls. GM-CSF mRNA levels from cultured endometrial tissues from E_2/P_4-treated ovariectomized and cyclic ewes were less ($P < 0.01$) than that of pregnant ewes (Fig. 1B).

GM-CSF concentrations, determined by a bioassay, in endometrial culture media obtained from either E_2- or P_4-treated ovariectomized ewes were not different from those seen in controls (Fig. 2A). The combined treatment of E_2/P_4 resulted in a 3-fold increase ($P < 0.01$) in GM-CSF secreted into culture media as compared to that of control ovariectomized ewes. However, GM-CSF concentrations in endometrial culture media obtained from E_2/P_4-treated ovariectomized and cyclic ewes were similar and both of these were less ($P < 0.01$) than that of pregnant ewes (Fig. 2B). In addition, GM-CSF detected in the uterine flushing media (23 mL) obtained from pregnant ewes was 86 ± 21 pg/mL, resulting in approximately 2 ± 0.5 ng *in utero* at the time of hysterectomy.

Results of GM-CSF western blot analysis are presented in Fig. 3. The anti-oGM-CSF antibody detected a band approximately 23 kDa in size in endometrial culture medium, which is in agreement with the value for mouse native GM-CSF reported in a previous study [7]. Immunoreactive GM-CSF was detected in samples of endometrial culture media from the E_2/P_4-treated ovariectomized, cyclic and pregnant ewes and not in endometrial culture media from control, E_2- or P_4-treated ovariectomized ewes.

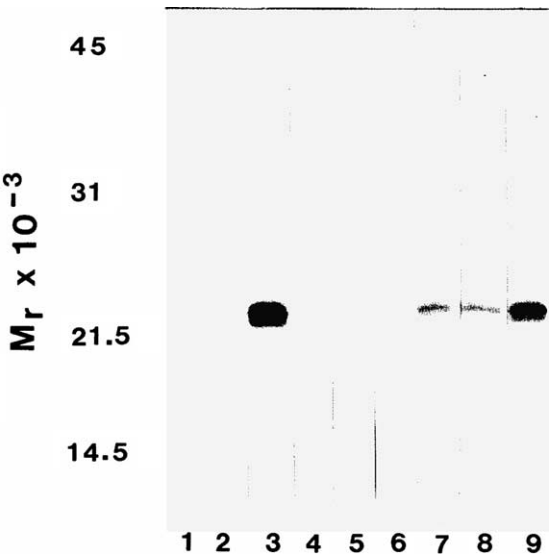

Fig. 3. Western blot analysis of GM-CSF released into culture medium from endometrium of ovariectomized, cyclic and pregnant ewes. Endometrial culture media (9.5 mL) and oGM-CSF enriched conditioned media (oGM-CSF CM) were dialyzed, concentrated, separated by 12.5% SDS–PAGE and transferred to filters. Filters were incubated with rabbit preimmune serum or anti-oGM-CSF antiserum, and immunoreactive proteins detected by alkaline phosphatase conjugated to rabbit IgG antibody. Lane (1) Mr markers, (2) oGM-CSF CM with preimmune serum, (3) oGM-CSF CM with the anti-oGM-CSF antiserum, (4) control-ovariectomized ewe, (5) E_2-treated ovariectomized ewe, (6) P_4-treated ovariectomized ewe, (7) E_2/P_4-treated ovariectomized ewe, (8) day 14 cyclic ewe, and (9) day 14 pregnant ewe.

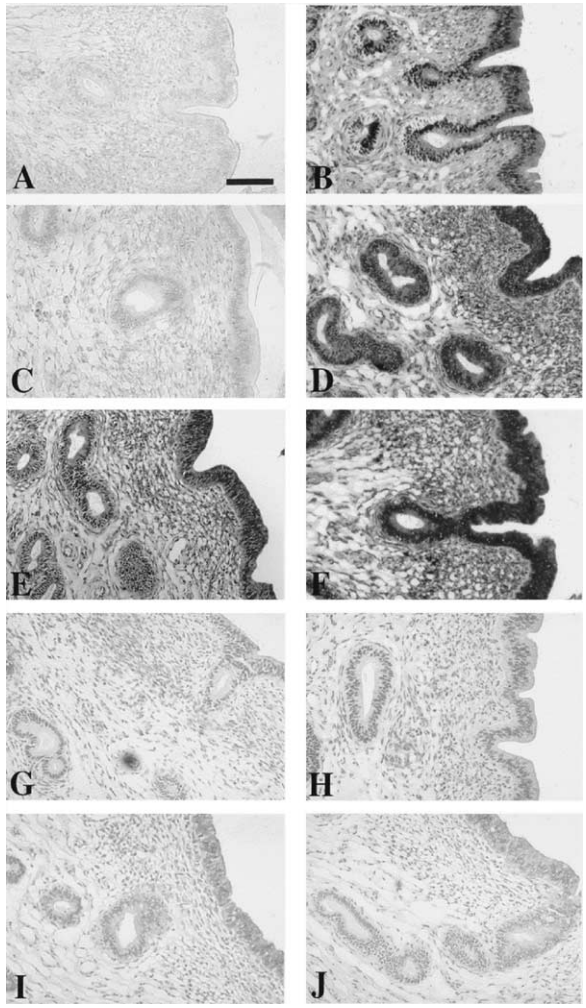

Fig. 4. Localization of oGM-CSF mRNA and its protein to uterine endometria from ovariectomized, cyclic, and pregnant ewes. Bar = 100 μm; all micrographs are same magnification. Cellular localization of oGM-CSF mRNA by: (A) oGM-CSF sense cRNA probe to semiserial section from day 14 pregnant ewe (no hybridization), (B) oGM-CSF antisense cRNA probe to section from E_2-treated ovariectomized ewe (hybridization), (C) oGM-CSF antisense cRNA probe to section from P_4-treated ovariectomized ewe (no hybridization), (D) oGM-CSF antisense cRNA probe to section from E_2/P_4-treated ovariectomized ewe (hybridization), (E) oGM-CSF antisense cRNA probe to section from a day 14 cyclic ewe (hybridization), (F) oGM-CSF antisense cRNA probe to semiserial section (see (A)) from a day 14 pregnant ewe (hybridization). Because no hybridization was observed, endometrial section from control-ovariectomized ewe are not shown. Immunohistochemical localization of oGM-CSF by: (G) preimmune rabbit serum (negative control) to semiserial section from day 14 pregnant ewe (no staining), (H) anti-oGM-CSF antiserum to section from E_2-treated ovariectomized ewe (no staining), (I) anti-oGM-CSF antiserum to section from E_2/P_4-treated ovariectomized ewe (staining), (J) anti-oGM-CSF antiserum to semiserial section (see (G)) from day 14 pregnant ewe (staining). Because no staining was observed, endometrial sections from control or P_4-treated ovariectomized ewe are not shown.

Results of *in situ* hybridization and immunohistochemical analyses for GM-CSF are presented in Fig. 4. Results from *in situ* hybridization studies agreed with GM-CSF mRNA levels detected by RT–PCR (Fig. 1), with the positive staining being observed in sections of uteri obtained from pregnant, cyclic and E_2- and E_2/P_4-treated ovariectomized animals. Similar to the previous results [8], GM-CSF mRNA was localized to the luminal and glandular epithelial and stromal regions. Minimal to nondetectable staining was observed in tissues obtained from the control and P_4-treated ovariectomized ewes.

GM-CSF protein was detected at the luminal and glandular epithelia in endometrial sections obtained from ewes in the E_2/P_4-treated ovariectomized, cyclic, and pregnant animals (Fig. 4). No staining was associated with the luminal and glandular epithelia of endometrial tissues from the control or P_4-treated ovariectomized ewes. However, minor staining of GM-CSF was noted at the edge of luminal epithelium in one of three E_2-treated ovariectomized animals while no staining was observed in the endometrium of the remaining E_2-treated ovariectomized ewes. These results were in close agreement with the results of the bioassay (Fig. 2) and western blot analysis (Fig. 3), which indicated that GM-CSF protein was present in appreciable quantities in the E_2/P_4-treated ovariectomized, cyclic and pregnant ewes.

4. Discussion

In ovariectomized ewes, the implants produced serum concentrations of P_4 that mimicked those seen *in vivo* during the late luteal phase or early pregnancy in ewes [35]. Concentrations of E_2 in serum of ovariectomized ewes with E_2 implants were higher than previously reported. However, the magnitude of increase in serum concentrations of E_2 produced by the E_2 implants was similar to that shown by Karsch *et al.* [20] and Goodman *et al.* [35]. The steroid environment produced by the implants was maintained for 14 days, which should have adequately allowed for any necessary steroid priming of the endometrium. It should be noted that by pathological examination, the E_2/P_4 regimen utilized in this study were not found to cause uterine edema or to alter the cellular composition of the uterus (I. Damjanov, University of Kansas Medical Center, Kansas City, KS, USA). Therefore, GM-CSF expression in E_2- or E_2/P_4-treated ovariectomized ewes observed in the present study did not result from pharmacological or confounding effects of E_2.

Treatment of ovariectomized ewes with E_2 implants stimulated increases in levels of endometrial GM-CSF mRNA, presumably through increased transcription and/or message stability [36]. Although P_4 diminishes GM-CSF mRNA synthesis in the mouse [16], P_4 treatment alone, when implanted for 14 days in sheep, did not stimulate or inhibit endometrial GM-CSF mRNA or protein levels. Although GM-CSF mRNA levels were elevated in endometrium obtained from E_2-treated ewes, no additional increase in GM-CSF protein was detected in these animals and, therefore, GM-CSF protein levels remained similar to controls. P_4 treatment along with E_2 in the ovariectomized ewes increased GM-CSF protein to the level detected in cyclic ewes. However, the degree of GM-CSF expression in E_2/P_4-treated ovariectomized and cyclic ewes was still less than that of pregnant animals. These data suggest that the combined E_2 and P_4 treatment of ovariectomized ewes sufficiently restores GM-CSF expression to the level of cyclic ewes and that the conceptus may play a role in the regulation of GM-CSF expression *in*

utero. Higher GM-CSF levels detected in pregnant animals agree with the recent observations in which oIFNτ enhanced endometrial GM-CSF [37]. Additionally, detection of GM-CSF protein in the uterine flushing indicates that bioactive protein, at least by the bioassay of the TF-1 cells, is present *in utero*. The fact that GM-CSF is not detected in the ovine conceptuses [6] further confirms that the source of GM-CSF production is the uterine endometrium. The present study also confirms the results by Giacomini *et al.* [15] and Robertson *et al.* [16] in which GM-CSF expression *in utero* occurs at the luminal and glandular epithelial cells, not by lymphocytes recruited by the endometrium.

These results indicate that the steroidogenic environment of early pregnancy should be capable of inducing the expression of endometrial GM-CSF, which in turn has been demonstrated to increase oIFNτ production from the ovine conceptus [6,8]. The molecular mechanism(s) by which the conceptus upregulates endometrial expression of GM-CSF during early conceptus development is still unclear. However, oIFNτ could be a factor that enhances endometrial GM-CSF production [37]. To study the effect of conceptus and/or conceptus secretory protein(s) on endometrial production of GM-CSF is beyond the scope of the present investigation. However, this localized production of bioactive GM-CSF that is readily available to the conceptus provides further evidence for the existence of a biochemical network of embryo-maternal communication.

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