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Modulation of TNFα and Nitric Oxide Production by Macrophages and Uterine Protein Expression in Mice Chronically Treated with Sex Steroids

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Abstract

The objective of the current studies was to analyze the relation of sex steroid treatment with the pattern of uterine secretory proteins and production of tumor necrosis factor α(TNFα) and nitric oxide (NO) by murine macrophages. Specific pathogen free (SPF) CD1 mice were ovariectomized and divided into 6 groups which were treated with different sex steroids: progesterone (group 2), estradiole (group 3), progesterone plus estradiole (group 4), estradiole plus progesterone (group 5), testosterone (group 6) and placebo (group 1). The treatments were continued by subcutaneous embedding of hormone pellets for 30 days. Total white blood cell counts in groups 4 and 5 showed statistically higher total leukocyte count ($P \leqq 0.05$) than that in groups 2 and 6 ($p \leqq 0.05$). Lipopolysaccharide (LPS)-stimulated peritoneal macrophages from estrogen-treated mice produced a significantly higher of NO than those in the other groups ($p \leqq 0.05$). TNFα production by the macrophages significantly increased by estrogen or testosterone treatment compared with the control ($p \leqq 0.05$). LPS induced a 2 fold production of both TNFαand NO by peritoneal macrophages. It was observed that steroid treatment induced a de novo synthesis of products with patterns specific to uterine proteins. Our results showed that sex steroids affect the macrophage function and modulate the pattern of secretory uterine proteins. The usefulness of the mouse model with subcutaneous embedding of hormone was also demonstrated.

Discipline: Animal health / Animal industry
Additional key words: LPS, uterus, NO

Introduction

The sex steroids exert various actions on the reproductive tract and they modulate the immune system[18,31]. It is known that estrogen stimulates, whereas progesterone and testosterone suppress systemic[12] and local[33] production of immunoglobulins, respectively. The natural killer cell activity was inhibited by estradiole[1,2] and progesterone[2]. Both estrogen and progesterone show an enhancing effect while Danzol, a synthetic testosterone, an inhibitory effect on interleukin-1β (IL-1β), serum proteins and TNFα production in cultured human monocytes[19]. Macrophages of the uterus show a change in number and functional ability in the production of cytokines, reactive oxygen intermediates and bioactive lipids depending on the reproductive cycle[9]. Although data on the influence of sex steroids on macrophages have accumulated, many aspects remain to be elucidated[3,4,9,12,15]. Characteristic pattern of uterine secretory proteins which has been analyzed in relation to the host reproductive cycle and host defense mechanisms in some animals, has not been reported in mice[14,20,24,25]. The purpose of this study was to develop a mouse model for chronic sex steroid treatment with estradiole, progesterone, testosterone and their combination and to determine the effects on the modulation of TNFα and NO production by macrophages, as well as the pattern of uterine secretory proteins.

Materials and methods

1) Animals

Forty specific pathogen free (SPF) mice (strain CD1), nulliparous (CLEA Inc., Japan), 6–8 weeks of age and weighing 30–40 g were used. The mice were kept in micro-isolation units in a laminar flow rack housing system (ICM Inc., Tsukuba, Japan). All the mice underwent an ovariectomy operation under general anesthesia with intraperitoneal injection of 50 μg/mice of pentobarbital

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sodium (ABBOT Laboratories, USA). Mice were given proper postoperative care and were maintained under SPF housing system.

2) Treatment with sex steroids

Two weeks after the ovariectomy, the mice were divided into 6 groups and treated as shown in Table 1. In the combined treatment, implants were applied at 2 different sites. The treatment continued for 30 days in the SC implants. Progesterone (C_{21} H_{30} O_2), estradiole (C_{18} H_{24} O_2) and testosterone (C_{19} H_{28} O_2) were purchased from NACALAI TESQUE Inc., Kyoto, Japan. The hormone pellets were prepared by mixing the sex steroid with 0.2 g cholesterol and 0.1 g sesame oil[6]. This mixture was injected subcutaneously in the hind back with a syringe with a wide gauge needle. The controls received only the vehicle.

3) Hematology

At the end of the treatment period, heparinized and non-heparinized blood was collected under general ether anesthesia. Serum was stored at –20°C until the assay. Differential leukocyte count was performed using May-Grunwald-Giemsa stained blood smears. White blood cell count (WBC) was performed using a Coulter counter (Coulter Electronics Inc., Florida), with LYSES II (K-CN) reagent for the simultaneous quantitative determination of hemoglobin and leukocytes (Japan Scientific Instrument Co., Ltd.).

4) Peritoneal macrophages

Peritoneal macrophages were collected aseptically by injection with 5 mL of cold RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Japan) and the back of the mice was agitated for 30 s. Thereafter, the fluid was aspirated. Peritoneal cells were harvested with 2 successive washings in RPMI 1640 medium by centrifugation at 300 ×g for 5 min at 4°C. The number of macrophages was counted and the macrophages were seeded into the wells of 96 well plates (Becton Dickinson) at the rate of 10^4 macrophages/ well. They were incubated for 2 h at 37°C in 5% CO_2 to allow them to adhere and those that did not adhere were removed by washing twice in RPMI 1640.

Table 1. Type of treatment and doses used

Group	No. of animals	Treatment	mg/Implant
1	12	Placebo	20
2	7	Progesterone	20
3	6	Estradiole	20
4	7	Progesterone	20
		+	
		Estradiole	5
5	5	Estradiole	20
		+	
		Progesterone	5
6	7	Testosterone	20

5) Analysis of TNFα and NO

The macrophages of each group were stimulated by lipopolysaccharide (LPS, *Escherichia coli* 0111: B4, Sigma, USA) for 4 h and 20 h. Supernatants were collected at 4 h for the TNFα assay and 20 h for the NO assay after the stimulation and stored at –20°C until the assay. Two sets of wells were prepared: LPS+ and 2 other sets as LPS– or controls during the assays.

The NO synthesis of the macrophages was determined by Griess assay[7]. Cell culture supernatants were assayed by the addition of 1:1 (v/v) 50 µL to each well of a 96-well plate (in triplicate), and 50 µL Griess reagent (N-1-naphthyl ethylene diamine dehydrochloride 0.1% in H_2O, sulphonil amide 1% in 5% H_3PO_4, Sigma), then immediately mixed and incubated at room temperature for 5 min. Absorbance at 550 nm was measured by using the plate reader Model 450 (Bio Rad, USA). The NO concentration was calculated from the standard curve produced during each assay by using $NaNO_2$ (NACALAI TESQUE Inc., Kyoto, Japan) dissolved in 15 mM Hepes, pH 7.5 and expressed as nmol /mL as mean values ±SD.

The level of TNFα in the supernatant of the macrophage culture was measured by the sandwich ELISA method. Rat anti-mouse TNFα monoclonal antibody (mAb) (generously provided by Dr. Nakane of Hirosaki University) was diluted to 2 µg/mL in 0.1 M $NaHCO_3$, pH 8.2. Wells of a 96 flat-bottomed probind assay plate (Becton Dickinson) were coated with 50 µg/well of the capture antibody and incubated overnight at 4°C. The plates were washed 2 times in 350 µL of PBS Tween-20 (Bio Rad) using an immuno-wash 450 (Bio Rad). Three hundred fifty µL of blocking agent (Snow Brand, Japan) was added per well and incubated for 2 h at room temperature and washed 2 times. Standard recombinant mouse TNFα (Genzyme) diluted serially in the blocking agent to generate a standard curve and samples were added at the rate of 100 µL/well in triplicate and incubated at 4°C overnight and washed 4 times. Biotinylated anti-mouse TNFα clone mp6-XT3 (Pharmingen, USA) diluted to 1 µL/mL in the blocking agent was added and washed 6 times. Then 4×10^3/mL of the peroxidase-conjugated streptavidin (Biogenx Lab. USA) diluted 1:3 v/v in the

blocking agent was added at 100 µL/well, incubated for 30 min at room temperature and washed 8 times. Finally 20 mg of a substrate, phenylene diamine (NACALAI TESQUE), in 25 mL of 0.1 M citric acid, 25 mL of 0.2 M $NaHPO_4/2H_20$ and 1 µL of 30% H_2O_2 was added at the rate of 100 µL/ well and the color was allowed to develop at room temperature for 90 min. The color reaction was stopped using 3N H_2SO_4 and read at OD 450 nm using a microplate reader 450 (Bio Rad). TNFα values were calculated from the standard curve as mean ±SD. The sensitivity of the assay was 50 pg/mL.

6) Examination of uterine fluid

Uterine secretion was washed out aseptically in 200 µL of 0.9% NaCl solution. Individual and pooled samples from the same group were designated as uterine luminal proteins 1 to 6 (ULP1 to ULP6) and stored at –20°C until assay. Uterine fluid was cultured in DHL and B. H. I agar medium (Eiken Chemical Co., Ltd., Japan) for bacterial examination.

For electrophoresis, uterine fluid was adjusted to 200 µg/mL protein, and added to the 2X SDS sample buffer 2: 1 (v/v) and to 5% 2-mercaptoethanol. The sample cocktail was then denatured by incubation for 5 min at 100°C. The samples and molecular markers were loaded on Phast gel gradient 10–15 (Pharmacia Biotech, USA) using an 8/1 sample applicator and analyzed by Phast system SDS-PAGE (Pharmacia KLB). Electrophoresis was run for 65 Vh at 250 V, 10.0 mA, 3.0 W and 15°C. Gels were then stained using a silver staining kit (Pharmacia Biotech). The gels were analyzed using Phast image software (Pharmacia LKB); gels were scanned in transmittance mode. Lanes were examined slice by slice with calibration curves.

7) Statistical analysis

Data were analyzed by the Student T test ($p \leq 0.05$ indicated statistical significance).

Results

The concentration of NO was compared in the 20 h culture supernatant of resident peritoneal macrophage cell culture obtained from the *in vivo* sex steroid-treated mice and *in vitro* macrophages exposed to LPS (Table 2). The peritoneal macrophages from the group treated with estradiole alone showed a significantly higher concentration of NO than those in the other groups ($p \leq 0.05$) (Table 2). No statistical difference was observed in the production by macrophages without LPS stimulation (Table 2). NO values were always higher in the LPS+ than LPS– macrophage culture supernatants.

TNFα production in the 4 h culture supernatants with or without LPS is shown in Table 2. Peritoneal macrophages from the estradiole or testosterone-treated groups showed a higher level of TNFα production without LPS stimulation ($p \leq 0.05$) than those in groups 1, 2 and 4. LPS treatment stimulated TNFα production by the macrophages in all the groups. However, the groups treated with estradiole or testosterone showed a significantly higher TNFα production than group 1 ($p \leq 0.05$).

Mouse uterine fluid and serum samples were subjected to SDS-PAGE. There were many similar bands in both samples, but unique bands were also detected. After the samples were scanned, the bands common to serum and uterine proteins were deleted to determine whether the treatments with each of the sex steroids had induced specific uterine protein bands. The treatments induced several proteins as shown in Fig. 1. After the samples were scanned, bands common to serum and uterine proteins, as well as specific bands in the uterine fluid or serum were obtained as shown in Table 3.

The effect of the chronic sex steroid treatment on the total and absolute number of leukocytes is shown in Table 4. The total number of white blood cells (WBC) and subsets of peripheral leukocytes in the groups 4 or 5

Table 2. Production of NO and TNFα by peritoneal macrophages (mean±D)

	NO production (nmol/well)		TNFα production (pg/well)	
Group	LPS–	LPS+	LPS–	LPS+
1	34.7+25.4	71.6+24.9 [a)]	43.0± 51.5 [a)]	92.5± 61.0 [a)]
2	37.3+31.0	64.2+17.0 [a)]	69.0± 50.0 [a)]	180.0± 91.5
3	41.3+38.6	89.5+37.5 [b)]	106.0±102.0 [b)]	225.5±171.5 [b)]
4	37.0+12.5	63.2+39.0 [a)]	97.0± 61.0	212.5±188.5
5	38.2+ 8.7	68.5+10.2 [a)]	52.0± 33.5 [a)]	178.0±162.0
6	39.8+23.3	61.6+ 2.0 [a)]	105.0±118.5 [b)]	220.0±152.5 [b)]

a) vs b) in each test was $p \leq 0.05$.

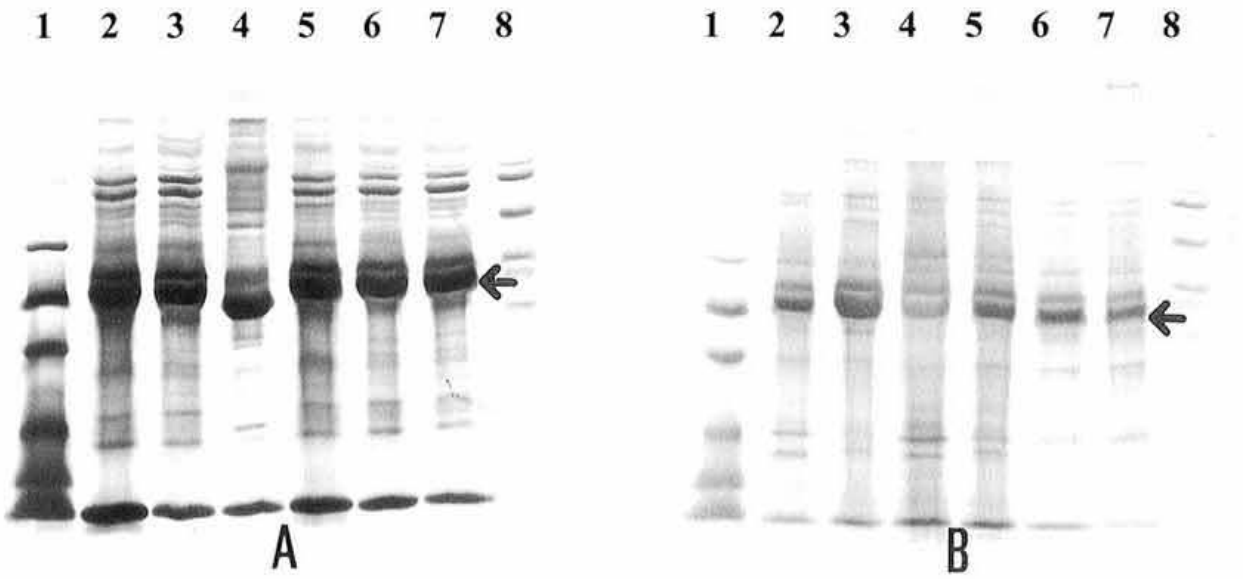

Fig. 1. SDS-PAGE analysis of total serum proteins (A) and (B) uterine proteins in response to sex steroid treatments
Lane 1: low molecular weight marker (94,000, 67,000, 43,000, 30,000, 20,100, 14,4000).
Lanes 2–7: samples from treatment groups 1, 2, 3, 4 and 5, as described in the materials and methods.
Lane 8: high molecular weight marker (212,000, 170,000, 116,000, 76,000, and 53,000).
Arrows indicate bands of 70 KDa protein.

were higher compared to the groups 2 and 6. WBC numbers and their subsets were affected by the treatment ($p \leq 0.05$) as shown in Table 4. The total number of red blood cells (RBC) and mean corpuscular hemoglobin concentration (MCHC) were not different among the treated and control groups (data not presented here). Bacterial culture of uterine fluid for *E. coli* was negative.

Discussion

In the present study, the effects of chronic subcutaneous treatment with embedded sex steroid pellets in mice on the modification of the macrophage activities, hematological characteristics, and pattern of uterine secretory protein were investigated. LPS is a well-known stimulator of macrophages[17,23,29]. Priming effects for the up- and down-regulation of TNFα and NO production by different concentrations of LPS and other substances were reported[22,26,34]. The present results showed that there were no significant differences in the background level of NO production by peritoneal macrophages obtained from mice treated with different sex steroids. However, LPS stimulation up-regulated NO synthesis by the peritoneal macrophages. Schneemann et al. (1993) reported a 2.5–30 fold increase of NO in the supernatants from murine peritoneal macrophages after stimulation with LPS[22]. Furthermore, a single estrogen treatment led to the priming of macrophages to produce higher levels of NO than those attained with other steroids[22]. This observation suggests that estrogen plays an immunopotentiating role. In contrast to the present results, rat alveolar macrophages treated *in vitro* with estradiol or progesterone showed a down-regulation of NO production[21]. In other cells than macrophages, it was reported that estradiole up-regulated NO production in cultured human aortic endothelial cells[8]. No significant calcium-independent NO activity was reported either during the pregnancy or after administration of sex hormones[32]. These data suggest that the effect of female sex steroids varies depending on the tissues, or duration of the treatments, and under *in vivo* and *in vitro* conditions. Sex steroids affect the host antimicrobial function via NO production[13,32].

In the present study, the level of TNFα increased by treatment with both estradiole and testosterone. It was reported that female sex steroid hormones enhance the production of colony-stimulating factor-1 (CSF-1), transforming growth factor (TGFα), granulocyte monocyte-colony stimulating factor (GM-CSF) and TNFα[9]. Since the NO synthesis induced by LPS was mediated by IFN and not by TNFα, it is suggested that TNFα and NO had different regulatory pathways[5]. In the LPS stimulated mouse macrophage cell lines (RAW264, ANA cells) estrogen up-regulated the TNFα production and progesterone down-regulated it[16]. Chao et al. (1995) reported that estrogen treatment at a rate of 10^{-2} and 10^{-3} g/mL significantly up-regulated TNFα expression in male rat peritoneal macrophages, but down-regulated it at a rate of 10^{-1}

Table 3. Protein identification in uterine fluid and serum

Peak Mr Wt	Source*											
KDa	serum						uterus					
251–260	g1	g2	g3	g4	g5	g6	g1	g2	g3	g4	g5	g6
241-250												
231–240			g3	g4								
221–230	g1	g2			g5							
191–200	g1	g2										g6
181–190				g4	g5		g1		g3	g4	g5	g6
171–180	g1	g2	g3			g6		g2			g5	
161–170									g3	g4		
151–160	g1			g4	g5		g1	g2				g6
141–150		g2	g3			g6			g3	g4	g5	
131–140	g1		g3		g5		g1	g2				
121–130	g1	g2		g4		g6	g1					
111–120					g5							
91–100	g1	g2	g3	g4		g6			g3			
81–90	g1				g5				g3		g5	g6
71–80	g1	g2	g3	g4			g1	g2	g3	g4	g5	g6
61–70	g1	g2	g3	g4	g5	g6	g1	g2	g3	g4	g5	g6
51–60				g4	g5					g4		
41–50	g1	g2	g3	g4	g5		g1	g2	g3	g4	g5	g6
31–40	g1	g2	g3	g4	g5	g6		g2	g3			g6
21–30	g1	g2	g3	g4	g5	g6	g1	g2	g3	g4	g5	g6
11–20	g1	g2	g3	g4	g5	g6	g1	g2	g3	g4	g5	g6

*Bands in each group were represented as: g1 =Pc, g2= P_4, g3= E_2, g4= P_4E_2, g5= E_2P_4 and g6= Te.

or less than 10^{-4} μg/mL[3]. These results also indicate that the effects of sex steroids on the macrophage function are complex.

Characteristic pattern of uterine fluid depending on the reproduction cycle was reported and the role of the proteins in host defense mechanisms was examined[18-20,24,25,28,30,33]. However, since no report on the pattern in mice had been reported previously, we observed the expression patterns of protein in the uterine fluid in the present study. Treatment of ovariectomized ewes with progesterone F2α induced the production of an immunosuppressive protein with a molecular weight of 55,000 in the uterus[24]. Chronic estrogen exposure was reported to lead to the development of complex endometrial hyperplasia in women and was related to the over-expression of lactoferrin[20]. Lactoferrin (LF) is a 70 KDa protein that is not induced by either progesterone or testosterone[28,30]. However, in our study in all the treated groups, a 70 KDa protein was induced, presumably due to the longer treatment period. However, further determination using Western blotting should be carried out.

The uterus is a sex steroid-responsive organ[27]. Treatment with steroids induced the formation of several proteins, which were identical with serum proteins, presumably due to the uterine edematous reaction, serum transudation by vasodilation[10,27], or secretion by uterine epithelial cells[27]. The patterns specific to uterus protein, in the present study, corresponded to *de novo* synthesized products as a result of the steroid treatment.

Compared to previous reports[11], in our study, the number of WBC was lower, which may be due to the fact that we used SPF mice.

In conclusion, we demonstrated the modulation of macrophage function and the expression of uterine proteins by sex steroid treatment. Further studies are required to characterize fully the uterine proteins and analyze their relationship with the function of macrophages.

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Table 4. Total and absolute leukocyte Nos/μL blood (mean±SD)

Group	WBC	Neutrophils	Lymphocytes	Monocytes	Eosinophils
1	4.3±2.2	0.046±0.064[b]	4.2±2.2	0.023±.036[b]	0.003±.008[b]
2	3.3±1.2[b]	0.092±0.149	3.1±1.2[b]	0.050±.080[b]	0.010±.017[b]
3	4.2±1.7	0.047±0.085[b]	4.0±1.6	0.023±.042[b]	0.008±.021[b]
4	5.4±3.1[a]	0.204±0.223[a]	5.0±2.6[a]	0.145±.310[a]	0.026±.046[a]
5	5.8±2.5[a]	0.093±0.075[a]	5.7±2.6[a]	0.018±.025[b]	0.012±.026[b]
6	3.3±1.6[b]	0.065±0.082[b]	3.2±1.6[b]	0.013±.023[b]	0.015±.027[b]

($p \leq 0.05$) between groups for total WBC and absolute number of leukocytes.
a) vs b) in each test was $p \leq 0.05$.

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