Expression of membrane progesterone receptors on human T lymphocytes and Jurkat cells and activation of G-proteins by progesterone

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Abstract

Although there is significant evidence for progesterone's role as an immunomodulator, nuclear progesterone receptors have not been consistently identified in immune cells. Recently, three new putative membrane progesterone receptors (mPRs), mPR α , mPR β , and mPR γ have been described. The objective of this study was to examine whether mPRs are expressed in peripheral blood leukocytes (PBLs) in women of reproductive age, and to further characterize them in T lymphocytes and immortalized T cells (Jurkat cells). Transcripts for mPR α and mPR β but not mPR γ , were detected by RT-PCR in PBLs, T lymphocytes, and Jurkat cells. Western blot analysis showed the presence of the mPR α and mPR β proteins on cell membranes of T lymphocytes

and Jurkat cells. Expression of the mPR α mRNA was upregulated in the luteal phase of the menstrual cycle in cluster of differentiation (CD)8⁺, but not in CD4⁺, T lymphocytes. Radioreceptor assays revealed specific [³H]progesterone binding to T- and Jurkat cell membranes (K_d 4.25 nM) characteristic of steroid membrane receptors. Progesterone activated an inhibitory G-protein (G_i), suggesting that mPRs are coupled to G_i in Jurkat cells. These results suggest a potential novel mechanism for progesterone's immunoregulatory function through activation of mPRs.

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Introduction

Modulation of the immune system by progesterone in women has been suggested by a number of observations. First, the course and symptoms of autoimmune diseases change during pregnancy and different phases of the menstrual cycle. For example, the rate of relapse in patients with multiple sclerosis decreases from 0.7/woman per year before pregnancy to 0.2/woman per year in the third trimester (Confavreux *et al.* 1998). Similarly, in prospective studies of rheumatoid arthritis, arthritis symptoms improve in 71–86% of pregnancies, and in more than half of the cases, remission is complete (Nelson & Ostensen 1997). Secondly, the cellular immune response to infection varies during the menstrual cycle. *Candida* vaginitis is most prevalent in pregnancy and in the late luteal phase of the menstrual cycle (Kalo-Klein & Witkin 1989). Lymphocyte proliferation in response to *Candida albicans* has been shown to be the greatest in the second week of the menstrual cycle and the lowest in the fourth week of the menstrual cycle (Kalo-Klein & Witkin 1989). In addition, interferon (IFN)-related genes are downregulated in peripheral blood leukocytes (PBLs) in women in the luteal phase compared with the follicular phase of the cycle (Dosiou *et al.* 2004).

In vitro studies of hormonal effects on cytokine production provide additional support for a possible immunomodulatory role for progesterone. The immune response has been classified as Th1 or Th2, depending on the cytokines produced. A Th1 response involves IFN- γ , interleukin-2 (IL-2), tumor necrosis factor- α (TNF- α), and the generation of cell-mediated immunity, while a Th2 response involves IL-4, IL-5, IL-6, IL-10, IL-13, and the stimulation of humoral immunity (Abbas *et al.* 1996). Progesterone favors T-cell differentiation along the Th2 pathway by human Th1 T cell clones *in vitro* (Piccinni *et al.* 1995). Lymphocytes from pregnant *Leishmania*-infected C57BL/6 mice produce less IFN- γ and more IL-4, IL-5, and IL-10 after stimulation *in vitro* compared with lymphocytes from nonpregnant mice (Krishnan *et al.* 1996). Finally, progesterone directly enhances differentiation of mouse double-positive thymocytes as well as mouse splenic naïve cluster of differentiation (CD)4⁺T cells along the Th2 pathway while inhibiting Th1 cell development (Miyaura & Iwata 2002).

Even though the above evidence clearly suggests an immunomodulatory role for progesterone, the exact mechanism of such action is unknown. The presence of traditional progesterone receptors in immune cells has been a matter of dispute (Szekeres-Bartho *et al.* 1990, Mansour *et al.*

1994, Chiu *et al.* 1996, Bamberger *et al.* 1999), with most studies showing an absence of nuclear PRs in lymphocytes from nonpregnant women (Szekeres-Bartho *et al.* 1990, Mansour *et al.* 1994, Bamberger *et al.* 1999). A number of alternatives to nuclear PR have been suggested in the literature for progesterone signaling in immune cells, including the glucocorticoid receptor (Kontula *et al.* 1983), voltage-gated and Ca^{2+} -activated K^{+} channels (Ehring *et al.* 1998), or another yet unidentified membrane delimited mechanism (Gamberucci *et al.* 2004). Recently, a novel membrane progesterone receptor (mPR α) was identified in sea trout oocytes, where it was demonstrated to play a role in oocyte maturation (Zhu *et al.* 2003b). Three homologous genes have been subsequently identified in humans, mPR α , mPR β , and mPR γ (Zhu *et al.* 2003a). The objective of the current study was to examine whether mPR α , mPR β , and mPR γ are expressed in PBLs in women of reproductive age, and to further characterize these receptors in T lymphocytes and in an immortalized T cell line, Jurkat cells. We studied the expression of mPR α in greater detail, because it is the mPR whose function and regulation have been best characterized in other tissues (Zhu *et al.* 2003b, Thomas *et al.* 2005b).

Materials and Methods

Human subjects

For the studies of progesterone receptor expression on PBLs and T lymphocytes, subjects were healthy female volunteers ($n=4$ for PBL studies, $n=7$ for T lymphocyte studies), 20–40 years old, with either regular menstrual cycles of 26–33 days or a history of regular menstrual cycles but on a hormonal form of contraception at the time of study. For the studies of progesterone receptor expression on CD4^{+} and CD8^{+} T cells at different times of the menstrual cycle, all subjects had regular menstrual cycles of 26–33 days ($n=3$). Subjects had no history of recent infection and were not taking any other medications. Informed consent was obtained under an approved protocol by Stanford University Committee in the Use of Human Subjects in Medical Research.

Buffy coat preparations were obtained from whole blood donors through the Stanford Medical School Blood Center's Research Products and Services Division. Specimens from a total of nine female donors 20–40 years old were used. All specimens collected through the Blood Center for buffy coat preparation are by-products from transfusable donor units, and meet all of the Blood Center donor requirements.

Cell collection and isolation

For the PBL experiments, peripheral blood (5 ml) was obtained by venipuncture from healthy female volunteers ($n=3$ in the luteal phase of the menstrual cycle, $n=1$ taking oral contraceptives) and collected into Paxgene RNA tubes (PreAnalytiX, Qiagen).

For the T lymphocyte isolations, ~ 25 ml peripheral blood were collected in tubes containing EDTA as anticoagulant from women either receiving hormonal contraception ($n=3$), with regular cycles in the follicular phase of the menstrual cycle ($n=2$), or in the luteal phase ($n=3$). Peripheral blood mononuclear cells (PBMCs) were then isolated on Accuprep (Accurate Chemical & Scientific Corporation, Westbury, NY, USA), and then T lymphocytes were negatively selected through magnetic bead depletion using the Dynal T Cell Negative Isolation Kit (Dynal Biotech Inc., Lake Success, NY, USA).

CD3^{+} T cells were isolated from the buffy coat preparation using the RosetteSep T cell Enrichment Cocktail (Stem Cell Technologies, Vancouver, Canada). In order to ensure proper separation of the buffy coat, the total number of white cells must not exceed 5×10^7 cells/ml. Therefore, the number of white cells was counted by incubation with a 3% acetic acid solution (in order to lyse red blood cells (RBCs) and count only leukocytes). After adjusting the concentration if needed, the cells were incubated with the cocktail for 20 min at room temperature. A 2:1 ratio of $1 \times \text{PBS}/2\%$ fetal bovine serum (FBS) was added and the cells were layered on Ficoll density medium and spun for 20 min at 1200 *g* with the brake off. The cell layer was then removed by Pasteur pipette and washed twice or thrice. After cell collections, samples underwent RBC lysis by mixing in a 1:5 ratio with ammonium chloride for 5 min at room temperature. T-cell purity was assessed by flow cytometry.

For the CD4^{+} and CD8^{+} T-cell isolations, paired peripheral blood samples (about 28 ml) were drawn from $N=3$ donors in both the proliferative (days 10–13) and mid-secretory (days 20–24) phase of the menstrual cycle, into EDTA-containing tubes as described above. CD4^{+} and CD8^{+} T cells were isolated using the RosetteSep CD4^{+} and CD8^{+} Selection Cocktails respectively (Stem Cell Technologies). Briefly, cells were incubated with the appropriate amount of selection cocktail for 20 min at room temperature. Cells were then mixed in a 1:1 ratio with $1 \times \text{PBS}/2\%$ FBS, layered on Ficoll density medium, and spun as described above. The remaining steps of the isolation were as described previously for T cells. CD4^{+} and CD8^{+} T-cell purity was assessed by flow cytometry.

Cell culture

Jurkat cells (clone E6-1, a human acute T-cell leukemia cell line) were purchased from ATCC (Manassas, VA, USA) and cultured in suspension in RPMI 1640 medium with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% FBS, at 37 °C and in 5% CO_2 . Fresh medium was added every 2–3 days to maintain a cell concentration between 1×10^5 and 1×10^6 viable cells/ml.

Assessment of cell purity by flow cytometry

In order to assess cell purity after different cell isolations, flow cytometry was performed as per the standard protocol. Briefly, 0.5×10^6 cells were incubated with the appropriate

fluorescein isothiocyanate (FITC)-labeled antibody diluted in PBS/2% FBS/0.1% azide in a total volume of 200 µl for 30 min at 4 °C. Cells were washed twice with 1 ml PBS/0.1% azide, centrifuged for 5 min at 162 g at 4 °C, resuspended in 200 µl PBS/0.1% azide, and taken to the Stanford Flow Cytometry facility for analysis. We obtained the anti-CD3-FITC, anti-CD4-FITC, and anti-CD8-FITC antibodies along with the appropriate isotype controls from BD Pharmingen (San Diego, CA, USA).

RT-PCR

For the PBL experiments, total RNA was extracted using the Paxgene Blood RNA Kit (PreAnalytiX, Qiagen). RNA was subsequently DNase treated using Qiagen's RNase-free DNase digestion kit. Samples were then purified using the Qiagen RNeasy Kit (Qiagen). For the T lymphocyte RNA isolation, cells were lysed in RLT buffer containing β-mercaptoethanol from Qiagen's RNeasy Kit. We then followed the RNA Isolation protocol, where we included on-column DNase digestion in the preparation (Qiagen). Total RNA (1.0 µg) was reverse transcribed using the Omniscript Kit (Qiagen), according to the manufacturer's instructions with a 1:1 ratio of oligo (dT)_{16–18} and random hexamers (Invitrogen). PCR was performed using HotStarTaq Master Mix (Qiagen) in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany). Primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH; F: 5'-cacagtccatgccatcactgc and R: 5'-ctcaccagttgcatgtagacc), mPRα (F: 5'-cctgctgtgtgatcttag and R: 5'-cggaaatagaagcgccag), mPRβ (F: 5'-cacg aaggaccacaaaaa and R: 5'-caatcccaagcaccacctat), mPRγ (F: 5'-agccctggagcgt ctttga and R: 5'-ggctctgagtcagtgttct), and nuclear PR A and B (F: 5'-gctacgaagtcaaacccagt and R: 5'-cacc atccctgccaatc) were designed from public sequence databases (GenBank accession numbers AF313620 for mPRα, AF313619 for mPRβ, AK000197 for mPRγ J04038 for GAPDH, and NM_000926 for nuclear PR A and B). Primers were synthesized at the Protein and Nucleic Acid Facility of the Stanford University School of Medicine. Intron-spanning primers were used for mPRα, mPRγ, nuclear PR, and GAPDH, and non-intron-spanning primers for mPRβ. Positive controls for nuclear PR, mPRα, mPRβ, and mPRγ expression were amplified cDNAs synthesized from RNA from decidualized human endometrial stromal cells (NIH SCCPRR Tissue Bank at the Stanford University), human testes (BD Biosciences Clontech), human brain (BD Biosciences Clontech), and HeLa cells (ATCC) respectively. Negative controls comprised either no template or no reverse transcriptase (no RT) samples. No RT controls were used for non-intron-spanning primers, as for mPRβ, to ensure negligible genomic DNA contamination. The PCR amplification temperatures were 94 °C for 45 s, 56 °C for 45 s, and 72 °C for 45 s, for 35 cycles with a final extension at 72 °C for 10 min. Agarose gel electrophoresis of the PCR products was performed for visualization. In addition, RT-PCR products corresponding to mPRα and mPRβ were purified using the

QIAquick gel extraction kit (Qiagen) and submitted for sequencing to the Stanford Protein and Nucleic Acid facility.

Quantitative RT-PCR

Total RNA was isolated from PBLs, T lymphocytes, CD4⁺ cells, and CD8⁺ cells using the RNeasy Mini Kit (Qiagen), and 0.5 µg was used to generate first-strand cDNA using the Omniscript Reverse Transcription Kit (Qiagen), with a 1:1 ratio of oligo (dT)_{16–18} and random hexamers (Invitrogen). The housekeeping gene RPL19 was used as a normalizer, as it has been shown to be an adequate normalizer by others in our laboratory, and displayed minimal variation between all samples (1 C_t value or less). The cDNA template was diluted 1:2 for mPRα and mPRβ analysis, and 1:200 for ribosomal protein L19 (RPL19). Real-time PCR was performed in triplicate in 25 µl reactions using the QuantiTect SYBR Green PCR Kit (Qiagen), according to the manufacturer's instructions. PCR primers were designed to be intron spanning to serve as a control for contamination of genomic DNA, and to have an optimal amplicon range of 150–200 bp (primer sequence shown above). In the case of mPRβ, where the primers were not intron spanning, a no RT control was run to make sure there was no genomic contribution to the fluorescence signal. The efficiencies of amplification for each gene were calculated by the equation $EFF = 10^{(-1/\text{slope})} - 1$. Standard curves using serially diluted template were run for mPRα, mPRβ, and RPL19. All three genes had sufficient amplification efficiency (100% ± 5%) to be considered comparable. For all genes, a dissociation curve was generated by taking fluorescence measurements at 1 °C increments from 55 to 95 °C and checked for a single melting product. In addition, PCR product was run on agarose gel to ensure amplification of a single product (data not shown). We calculated the relative expression ratio of the normalized C_t values for mPRα and mPRβ in the mid-secretory (days 20–24) compared with the proliferative (days 10–13) phase for both CD4⁺ and CD8⁺ T cells using the following mathematical model:

$$R = \frac{(1 + EFF)^{C_{t\text{proliferative}} \text{mPR}\alpha/\beta} / (1 + EFF)^{C_{t\text{proliferative}} \text{RPL19}}}{(1 + EFF)^{C_{t\text{mid-secretory}} \text{mPR}\alpha/\beta} / (1 + EFF)^{C_{t\text{mid-secretory}} \text{RPL19}}}$$

Analysis was performed on an N = 3 paired patient samples, and a paired *t*-test was used to determine statistical significance.

Membrane preparation

Plasma membrane fractions of human T lymphocytes and Jurkat cells were obtained by sonicating the cell suspension for ~15 s followed by sequential centrifugation as described previously (Patino & Thomas 1990, Thomas *et al.* 2007). The nuclear pellet was obtained from a 7-min 1000 g spin, the plasma membrane fraction from a 20-min 20 000 g spin, the microsomal fraction from a 1-h 100 000 g spin, and

the cytoplasm from the remaining supernatant. The plasma membrane fraction was further purified for some analyses by centrifugation one to three times with a sucrose pad (1.2 M sucrose) at 6500 *g* for 45 min (Zhu *et al.* 2003b).

Western blot analysis

Polyclonal antibodies generated against synthetic 15 oligo peptides in the N-terminal extracellular domains of the human mPR α (TVDRAEVPPLFWKPC) and human mPR β (KILEDGLPK MPCTVC), which have been extensively validated for the detection of the mPR α and mPR β proteins (Karteris *et al.* 2006), were used for western blot analysis. T lymphocyte or Jurkat cell membrane proteins (16 μ g/lane) were boiled in loading buffer (Pierce, Rockford, IL, USA) for 10 min. The solubilized proteins were resolved on 12% SDS-PAGE gels and subsequently transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in TBST (50 mM Tris/100 mM NaCl/0.1% Tween 20, pH 7.4) buffer for 1 h and incubated with the anti-mPR α antibody (1:3000) or anti-mPR β antibody (1:2000) overnight at 4 °C. The membranes were subsequently washed thrice with TBST buffer and incubated for 1 h at room temperature with horseradish peroxidase conjugated to goat anti-rabbit antibody (Cell Signaling Technology, Danvers, MA, USA), followed by three final washes for 15 min each with TBST buffer. Blots were then treated with enhanced chemiluminescence substrate (SuperSignal, Pierce) and exposed to X-ray film (Zhu *et al.* 2003b). The specificity of the immunoreactions was confirmed using the peptides used to generate the antibodies to block mPR-antibody binding. The peptides (3 μ g in 60 μ l deionized water) were incubated with the primary antibody (at a dilution of 1:50) for 1.5 h at room temperature. The blocked antibody solution was then incubated with the protein membrane at the same dilution factor as the primary antibody (1:3000).

Localization of mPR α by flow cytometry

Localization of the mPR α protein on the surface of Jurkat cells was examined by flow cytometry using the N-terminal mPR α antibody as described by Thomas *et al.* (2007). Briefly, the cells were scraped from the culture plates and washed with PBS followed by low-speed centrifugation (250 *g*) to remove any cellular debris and damaged cells. Cells were first incubated in blocking solution (0.5% BSA in PBS) for 1 h and then incubated in blocking solution containing the mPR α antibody ($\sim$ 1:1000) at room temperature for an additional 1 h. The cells were washed twice with PBS and incubated for 30 min with Alexa Fluor 488 goat anti-rabbit IgG antibody (Molecular Probes, Carlsbad, CA, USA) at room temperature in the darkness. The cells were washed twice with blocking solution, resuspended in 1 ml PBS, and stored at 4 °C for up to 24 h until they were analyzed on a flow cytometer (Becton and Dickinson, Franklin Lakes, NJ, USA, FACS Calibur, University of Texas at Austin, Institute for Cellular and Molecular Biology (ICMB)). Data were

analyzed with CellQuest Pro software (BD Biosciences, Franklin Lakes, NJ, USA).

Progesterone binding to T-cell plasma membranes

Binding of radiolabeled progesterone to T-lymphocyte plasma membranes was determined by single-point assays (Patino & Thomas 1990, Karteris *et al.* 2006). Membrane fractions (0.15 mg/ml) were incubated for 30 min at 4 °C with 1.0 nM [2,4,6,7-³H]progesterone (Amersham; specific activity 102.1 Ci/mmol) in the absence (total binding) or presence of 900 nM non-radiolabeled progesterone (nonspecific binding). At the end of the incubation period, the membrane-bound radiolabeled progesterone was separated from free steroid by filtration (Whatman GF/B filters, presoaked in assay buffer (25 mM HEPES, 10 mM NaCl, 1 mM dithioerythritol, 1 mM EDTA, pH 7.6)). The filters were subsequently washed twice with 25 ml ice-cold wash buffer (25 mM HEPES, 10 mM NaCl, 1 mM EDTA, pH 7.6) and bound radioactivity on the filters measured by scintillation counting. Receptor binding was calculated as the mean of three separate estimations and the assays repeated utilizing T lymphocytes from three different subjects. Saturation analysis was used to investigate radiolabeled progesterone binding to plasma membranes of Jurkat cells. One set of tubes contained a range (0.5–8.0 nM) of [2,4,6,7-³H]progesterone alone (total binding) and another set also contained 100-fold excess (50–800 nM) progesterone competitor (nonspecific binding). Other assay conditions were identical to those used in the single-point assay. The entire assay was repeated thrice with different batches of Jurkat cells.

G-protein activation assay

G-protein activation after progesterone treatment was assayed by measuring the increase in specific binding of [³⁵S]GTP γ -S to plasma membranes ($\sim$ 50 μ g protein) following the procedure of Liu & Dillon (2002) with a few modifications. T-lymphocyte plasma membranes were incubated at 25 °C for 15 min in the presence of 200 nM progesterone with 10 μ M GDP and 0.5 nM [³⁵S]GTP γ -S ($\sim$ 12 000 c.p.m., 1 Ci/mol) in 320 μ l assay buffer (100 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂, 0.6 mM EDTA, 0.1% BSA, 50 mM Tris-HCl, pH 7.4). Jurkat cell membranes were incubated under the same conditions with progesterone (20 or 100 nM), RU486 (100 nM), or progesterone (100 nM) and RU486 (100 nM) in combination. Nonspecific binding was determined by the addition of 100 nM GTP γ -S. The reaction was stopped at the end of the incubation period, by adding 300 μ l of stop solution (100 μ M GDP, 100 μ M GTP γ -S in assay buffer). Two hundred microliter aliquots of the reaction mixtures were filtered through Whatman GF/B glass fiber filters, followed by several washes and subsequent scintillation counting of the filters. The experiment was repeated thrice, using T lymphocytes from three different subjects.

Immunoprecipitation of [35 S]GTP γ -S-labeled G-protein α -subunits

The activated G-protein α -subunits were immunoprecipitated following the procedures described by Thomas *et al.* (2005a) and Karteris *et al.* (2006). Briefly, Jurkat cell plasma membranes (~ 100 μ g protein) were incubated in 300 μ l Tris buffer containing 4 nM [35 S]GTP γ -S, 10 μ M GDP, and protease inhibitors with 200 nM progesterone for 30 min at 25 $^{\circ}$ C. At the end of the incubation period, the reaction was stopped by the addition of 750 μ l ice-cold stop solution containing 100 μ M GDP and 100 μ M unlabeled GTP γ -S, and the samples were centrifuged at 20 000 g for 15 min. The resulting pellet was resuspended in immunoprecipitation buffer containing 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 25 mM Tris-Cl, and protease inhibitors, and incubated with specific antisera to the α -subunits of G-proteins (G_i and G_s , 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at

4 $^{\circ}$ C with gentle shaking for 6 h. A suspension of protein A Sepharose beads was added and the mixture was incubated for an additional 12 h at 4 $^{\circ}$ C. The immunoprecipitates were collected by centrifugation, washed, boiled in wash buffer containing 0.1% SDS, and the radioactivity in the immunoprecipitated [35 S]GTP γ -S-labeled G-protein α -subunits counted.

Statistical analysis

A paired *t*-test was used to compare the levels of expression of mPR α in CD4 $^{+}$ and CD8 $^{+}$ T cells at different phases of the menstrual cycle, and to assess statistically significant differences in progesterone binding and [35 S]GTP γ -S-binding studies. A two-tailed paired *t*-test was used, with $\alpha=0.05$. Linear and nonlinear regression analyses for all receptor-binding assays and calculations of K_d and binding capacity were performed using GraphPad Prism for Windows (version 3.02; Graph Pad Software, San Diego, CA, USA).

Figure 1 (A and B) Expression of nuclear PR, mPR α , mPR β and mPR γ by PBLs and T lymphocytes. RT-PCR was performed on RNA prepared from PBLs (A) or T lymphocytes (B) of female subjects, using mPR α , mPR β , mPR γ , GAPDH, and nuclear PR-specific primers. cDNAs for human testes, human brain, HeLa cells, and decidualized human endometrial stromal cells were used as positive controls for mPR α , mPR β , mPR γ , and nuclear PR expression respectively. Agarose gel electrophoresis was performed for visualization. The results from a representative sample (of four subjects for PBLs and of seven subjects for T lymphocytes) are shown. Lanes 1–4: mPR α . Lane 1: 100 bp ladder, lane 2: PBLs (A) or T lymphocytes (B), lane 3: human testes, lane 4: no template control. The two bands in lane 3 represent the two transcripts of mPR α that are known to be expressed in human testes (Zhu *et al.* 2003a). Lanes 5–9: mPR β . Lane 5: 100 bp ladder, lane 6: PBLs (A) or T lymphocytes (B), lane 7: human brain, lane 8: no template control, lane 9: PBLs (A) or T lymphocytes (B), no RT control. Lanes 10–13: mPR γ . Lane 10: 100 bp ladder, lane 11: PBLs (A) or T lymphocytes (B), lane 12: HeLa cells, lane 13: no template control. Lanes 14–16: GAPDH. Lane 14: 100 bp ladder, lane 15: PBLs (A) or T lymphocytes (B), lane 16: no template control. Lanes 17–20: Nuclear PR. Lane 17: 100 bp ladder, lane 18: PBLs (A) or T lymphocytes (B), lane 19: decidualized human endometrial stromal cells, lane 20: no template control. Expected transcript size for mPR α : 228 bp, mPR β : 232 bp, mPR γ : 276 bp, GAPDH: 609 bp, nPR: 443 bp.

Figure 2 Membrane PRα and mPRβ expression in CD4⁺ and CD8⁺T lymphocytes in the luteal versus follicular phase of the menstrual cycle. Real-time PCR was performed on CD4⁺ and CD8⁺T lymphocytes, collected either during the late follicular phase (days 10–13) or mid-luteal phase (days 20–24) of the menstrual cycle. The relative expression ratio (fold change) of mPRα and mPRβ mRNA expression in the luteal versus the follicular phase is depicted. Average results for N=3 subjects are shown; bars represent standard error. P values reflect statistical significance of the fold change in mRNA expression levels between the luteal phase and the follicular phase. NS, not significant.

Results

Expression of mPRs in PBLs and T lymphocytes

Expression of mPRα and mPRβ mRNAs was detected in PBLs by RT-PCR (Fig. 1A). In contrast, the nuclear PR and mPRγ mRNAs were not detected (Fig. 1A). Sequencing confirmed the identity of the bands observed in gel electrophoresis corresponding to mPRα and mPRβ (data not shown).

In the T-cell isolation experiments, flow cytometry using anti-CD3 antibody verified high T-cell purity following magnetic bead depletion (>95%; data not shown). Also, in

purified T lymphocytes, expression of mPRα and mPRβ was detected, whereas the nuclear PR and mPRγ were not (Fig. 1B). There was no variation in expression evident in RT-PCR experiments between samples obtained by women in the follicular phase of the cycle, in the luteal phase, or women taking hormonal contraceptives (data not shown).

Flow cytometry verified high purity of the CD4⁺ and CD8⁺T lymphocyte populations following magnetic bead depletion (>90%; data not shown). Real-time PCR analysis showed that the levels of expression of mPRα did not change significantly during the menstrual cycle in paired samples of CD4⁺T lymphocytes (Fig. 2). In contrast, expression of mPRα increased by about 2.5-fold in CD8⁺T cells in the luteal phase compared with the follicular phase of the cycle (Fig. 2). Expression of mPRβ did not change significantly during the menstrual cycle in either CD4⁺ or CD8⁺T lymphocytes (Fig. 2). We verified that the follicular samples were characterized by low progesterone levels and the mid-luteal samples by high progesterone levels by measuring serum progesterone levels (mean values: 0.607 vs 10.7 ng/ml respectively, P<0.005).

Western blot experiments designed to examine mPRα protein expression showed the presence of a protein of about 40 kDa in size in T-cell plasma membranes (Fig. 3A). In addition, a second band of about 80 kDa was observed; this is likely the result of SDS-resistant homodimers of the 40 kDa mPRα protein that have been described (Thomas *et al.* 2005b, Karteris *et al.* 2006). The relative abundance of the 40 and 80 kDa bands in different membrane preparations varied, probably as a result of minor differences in membrane preparation, storage, and solubilization conditions (Thomas *et al.* 2007), and the 40 kDa band was absent in some T-cell membrane samples (Fig. 3B). Binding of the anti-mPRα

Figure 3 Western blot analyses of mPRα and mPRβ expression by T lymphocytes. Western blots of T-cell membranes (16 µg protein/lane) obtained from buffy coats of female blood donors using the mPRα antibody (1:3000, A and B) and the mPRβ antibody (1:2000, C). The Western blots were repeated thrice and similar results were obtained with each analysis. pep, blocked with mPRα or mPRβ peptide; M, molecular weight marker.

Figure 4 Progesterone binding to plasma membranes prepared from peripheral blood T lymphocytes and G-protein activation. (A) Progesterone binding to T-cell membranes assessed using a single-point [^3H]progesterone-binding assay. Mean values of three different experiments are shown; bars represent standard error. TB, total binding; NSB, nonspecific binding. $*P < 0.0005$. (B) Specific [^{35}S]GTP- γ -S binding to T-cell plasma membranes treated with 200 nM progesterone (P4) or vehicle control (CTL). Bars represent means ($\pm$ S.E.M.) from three different experiments. $*P < 0.005$ compared with vehicle alone.

Figure 5 Expression of mPRs by Jurkat cells. (A) Expression of nuclear PR, mPR α , mPR β and mPR γ by Jurkat cells. RT-PCR was performed on RNA prepared from Jurkat cells, using mPR α , mPR β , mPR γ , GAPDH, and nuclear PR-specific primers. cDNAs for human testes, human brain, HeLa cells, and decidualized human endometrial stromal cells were used as positive controls for mPR α , mPR β , mPR γ and nuclear PR expression respectively. Agarose gel electrophoresis was performed for visualization. Lanes 1–4: mPR α . Lane 1: 100 bp ladder, lane 2: Jurkat cells, lane 3: human testes, lane 4: no template control. The two bands in lane 3 represent the two transcripts of mPR α , which are known to be expressed in human testes (Zhu *et al.* 2003a). Lanes 5–9: mPR β . Lane 5: 100 bp ladder, lane 6: Jurkat cells, lane 7: human brain, lane 8: no template control, lane 9: Jurkat cells, no RT control. Lanes 10–13: mPR γ . Lane 10: 100 bp ladder, lane 11: Jurkat cells, lane 12: HeLa cells, lane 13: no template control. Lanes 14–16: GAPDH. Lane 14: 100 bp ladder, lane 15: Jurkat cells, lane 16: no template control. Lanes 17–20: Nuclear PR. Lane 17: 100 bp ladder, lane 18: Jurkat cells, lane 19: decidualized human endometrial stromal cells, lane 20: no template control. Expected transcript size for mPR α : 228 bp, mPR β : 232 bp, mPR γ : 276 bp, GAPDH: 609 bp, nPR: 443 bp. (B–D) western blot analyses of mPR α and mPR β expression by Jurkat cells. (B and C) Western blots of Jurkat cell membranes with the mPR α antibody (1:3000, B) and with the mPR β antibody (1:2000, C). (D) Analysis of subcellular fractions of Jurkat cells with the mPR α antibody. The western blots were repeated thrice with different batches of cells and similar results were obtained with each batch. pep, blocked with mPR α or mPR β peptide. M, molecular weight marker; Mem, plasma membrane fraction; Mem(sp), plasma membrane fraction purified with sucrose pad; Nuc, nuclear fraction; Ms, microsomal fraction; Cyt, cytoplasmic fraction.

Figure 6 Flow cytometry of mPR α expression in Jurkat cells. Representative histograms depict staining with the appropriate isotype control antibody (top) and staining with the mPR α antibody (bottom). The x-axis depicts fluorescence intensity. CTL, control; IgG, immunoglobulin G; hmPR α , human mPR α .

antibody to the 80 kDa band in the plasma membrane was effectively blocked by the addition of the mPR α peptide, indicating that binding is specific (Fig. 3B). The mPR β protein was also detected in plasma membranes of T cells by western blotting. A single band at 80 kDa was detected, which could be blocked with the mPR β peptide (Fig. 3C).

Progesterone binding to T lymphocytes and G-protein activation

Radiolabeled progesterone binding studies demonstrated a significant difference between total binding and nonspecific binding of [3 H]progesterone to T-lymphocyte cell membranes, indicating the likely presence of specific progesterone receptors (Fig. 4A). Progesterone increased specific [35 S]GTP γ -S binding to the plasma membranes of T lymphocytes (Fig. 4B), indicating activation of G-proteins.

Expression of mPRs in Jurkat cells

In order to study mPR expression and function in T cells more extensively, we used a human immortalized T cell line, Jurkat cells. Jurkat cells had a similar pattern of mPR expression, as detected by RT-PCR, to peripheral blood T lymphocytes (Fig. 5A). In western blot analysis, both the 40 and 80 kDa bands

were detected with the mPR α antibody in Jurkat cell plasma membranes and both the bands could be blocked with the mPR α peptide (Fig. 5B). Plasma membranes from Jurkat cells also expressed the mPR β protein. The 80 kDa band was blocked by preincubation with the mPR β peptide, indicating the specificity of the immunoreaction (Fig. 5C). Lower amounts of the mPR α protein were also detected in cytosolic and microsomal fractions of Jurkat cells, but were absent in the nuclear fraction (Fig. 5D). Flow cytometry of nonpermeabilized Jurkat cells using the N-terminal mPR α antibody (rabbit IgG) resulted in a marked increase in immunofluorescence compared with the rabbit IgG controls (Fig. 6), indicating the presence of the mPR α protein on the cell surface and its orientation, with an extracellular N-terminal.

Progesterone binding to Jurkat cells and inhibitory G-protein activation

High-affinity ($K_d = 4.25$ nM), saturable, limited capacity ($B_{max} = 0.059$ nM), specific [3 H]progesterone-binding characteristic of mPRs was identified on Jurkat cell plasma membranes by saturation analysis (Fig. 7A), while Scatchard analysis showed a single progesterone-binding site (Fig. 7B). Progesterone treatment also caused G-protein activation in Jurkat cells, and the response was concentration dependent (Fig. 7C). This response was progesterone-specific, since treatment of the cells with 100 nM of either testosterone or 17 β -estradiol did not induce G-protein activation (data not shown). Co-treatment with the nuclear glucocorticoid receptor and progesterone receptor antagonist, RU486, did not impair progesterone-induced G-protein activation in Jurkat cell membranes (Fig. 7D), which suggests this action of progesterone is not mediated by either of these nuclear receptors. Immunoprecipitation of Jurkat cell membrane-bound [35 S]GTP γ -S with G $_s$ and G $_i$ α -subunit specific antibodies showed that progesterone treatment leads to activation of G $_i$ (Fig. 7E).

Discussion

In this study, we show that the newly characterized mPR α and mPR β are expressed by PBLs and specifically by T lymphocytes in women of reproductive age, as well as by the Jurkat T cell line. Western blot analysis of subcellular fractions of Jurkat cells and flow cytometry studies showed that the mPR α protein is localized on the cell membrane and the N terminus is extracellular, in agreement with recent results for the recombinant human mPR α protein expressed in breast cancer cells (Thomas *et al.* 2007). We demonstrated specific binding of progesterone to the plasma membranes of T cells, as well as activation of G-proteins upon progesterone exposure. More extensive characterization of progesterone binding to Jurkat cell plasma membranes by saturation analysis showed the presence of a single, high-affinity, saturable binding site that has the characteristics of a mPR. The affinity of the putative progesterone membrane receptor identified here on Jurkat

Figure 7 Progesterone binding to plasma membranes prepared from Jurkat cells and G-protein activation. (A) Representative saturation plot of specific [^3H]progesterone binding to Jurkat plasma cell membranes. The reported K_d and B_{max} values are the means of three separate saturation analyses. (B) Scatchard analysis of specific [^3H]progesterone binding to Jurkat plasma membranes. (C) Specific [^{35}S]GTP γ -S binding to Jurkat cell plasma membranes treated with 20 or 100 nM progesterone. Bars represent means ($\pm$ S.E.M.) from three different experiments. * $P < 0.005$ compared with vehicle alone. CTL, control; P4, progesterone. (D) Specific [^{35}S]GTP γ -S binding to Jurkat cell plasma membranes treated with 100 nM progesterone and 100 nM RU486, alone and in combination. Bars represent means ($\pm$ S.E.M.) from multiple experiments. * $P < 0.005$ compared with vehicle alone. (E) Immunoprecipitation of the Jurkat cell membrane-bound [^{35}S]GTP γ -S with specific G_i and G_s α -subunit G-protein antibodies. Mean value of three experiments is shown; bars represent standard error. ** $P < 0.05$ compared with vehicle alone. P4, progesterone.

cells (K_d 4.25 nM) is very similar to that of the recombinant human mPR α (K_d 4.17 nM; Thomas *et al.* 2007).

Our results also confirm previous findings of the absence of traditional nuclear PRs in lymphocytes from nonpregnant women (Szekeres-Bartho *et al.* 1990, Mansour *et al.* 1994, Bamberger *et al.* 1999) in both PBLs and specifically in T lymphocytes. One previous study had detected nuclear PR expression by immunoperoxidase staining in lymphocytes from

pregnant women, but not in lymphocytes of nonpregnant subjects (Szekeres-Bartho *et al.* 1990), while a second study, using a different monoclonal antibody, detected expression of PRs by flow cytometry in about 21% of lymphocytes of nonpregnant women (Chiu *et al.* 1996). On the other hand, a third study using an enzyme immunoassay, detected very low levels of the nuclear PR in lymphocytes from women and men (which they hypothesize could just be the positive background

of the method), with no increased expression in pregnancy (Mansour *et al.* 1994). Finally, Bamberger and colleagues failed to detect the nuclear PR in lymphocytes from nonpregnant women, men, or Jurkat cells using RT-PCR and western blotting (Bamberger *et al.* 1999). Our results together with the results of the Bamberger study raise the question of whether the previous positive findings using monoclonal antibodies were due to binding of the monoclonal antibodies to proteins other than the nuclear PR, with cross-reactive epitopes.

The finding that progesterone causes activation of an inhibitory G-protein in Jurkat cells is consistent with recent studies with wild-type mPRs in human myocytes and fish oocytes and recombinant mPR α s in transfected breast cancer cells showing that mPRs are directly coupled to an inhibitory G-protein (Pace & Thomas 2005, Karteris *et al.* 2006, Thomas *et al.* 2007). The presence of G_i-coupled mPRs in T cells provides an attractive potential mechanism for progesterone's immunomodulatory effects.

The expression of mPR α appears to be hormonally regulated in CD8⁺T lymphocytes, but not in CD4⁺T lymphocytes, with increased expression in the mid-luteal phase of the cycle, a high progesterone state (Fig. 2). In contrast, the relative expression of mPR β does not change in either T-cell subset between the follicular and mid-luteal phase. A direct stimulatory effect of progesterone on mPR α mRNA and protein expression in human myocytes has been demonstrated in *in vitro* experiments (Karteris *et al.* 2006). The upregulation of mPR α expression in the luteal phase in CD8⁺ cells is particularly interesting, since CD8⁺T lymphocytes have been recently shown to mediate the protective effects of progesterone derivatives against stress-induced abortion in mice by altering the Th1/Th2 cytokine profile in favor of Th2 cytokines (Blois *et al.* 2004). The increased expression of mPR α in CD8⁺T cells in the luteal phase in women could therefore contribute to the immunomodulatory effects of progesterone in the second half of the menstrual cycle and in pregnancy.

Nongenomic actions of progesterone, as well as other steroids, have been well described in a variety of tissues outside the immune system, and are reviewed in recent publications (Losel & Wehling 2003, Norman *et al.* 2004). Some examples of such nongenomic progesterone action include facilitation of female mouse sexual behavior (Frye & Vongher 1999), oocyte maturation in *Xenopus* (Masui & Markert 1971), and spotted sea trout (Zhu *et al.* 2003b), as well as the acrosome reaction in sperm (Meizel & Turner 1991). Putative mPRs have been identified in tissues such as human sperm (Luconi *et al.* 1998, Buddhikot *et al.* 1999, Falkenstein *et al.* 1999), rat brain (Krebs *et al.* 2000), rat granulosa cells (Peluso *et al.* 2004), porcine liver (Meyer *et al.* 1996), human liver, kidney, and placenta (Meyer *et al.* 1998). Members of the mPR family described in this study have previously been identified in fish oocytes (Zhu *et al.* 2003b), fish sperm (Thomas *et al.* 2005b), various human tissues (Zhu *et al.* 2003a, Chapman *et al.* 2006, Karteris *et al.* 2006, Dressing & Thomas 2007, Nutu *et al.* 2007), rat corpus luteum (Cai & Stocco 2005), and sheep ovary (Ashley *et al.* 2006). This is the first report showing that mPRs are expressed in the human immune system and mediate progesterone activation of G_i-proteins.

Further studies, involving mPR siRNA experiments or knockout animals, are required to address definitively the functionality of mPRs in the immune system. To date, we have only examined expression of mPRs in immune cells of nonpregnant women. It would be interesting to examine whether mPR expression in immune cells changes in pregnancy, when levels of progesterone are at their peak. If future studies on lymphocytes from pregnant women document the presence of both mPRs and nuclear PRs on these cells, the potential involvement of mPRs in the regulation of nuclear PR activity, as shown recently in the case of human myometrium (Karteris *et al.* 2006), would be a very interesting area of investigation.

In summary, we have described the expression of mPRs in PBLs and T lymphocytes of nonpregnant women, and have shown that progesterone results in G_i-protein activation in an immortalized T cell line. From a clinical perspective, if progesterone has an immunomodulatory function through interaction with these mPRs on T lymphocytes, progestins used in oral contraceptive or hormone replacement regimens may have similar actions. These effects may be desirable in some women (such as women with autoimmune diseases involving a Th1 immune response) and undesirable in women suffering from disorders caused by a Th2 immune response. New progestational agents could similarly be developed with either enhanced or diminished immunoregulatory activity. This study provides a novel potential mechanism for progesterone's action as a regulator of the immune response.

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