Signal regulatory protein $\alpha 1$ is involved in the inhibitory effect of glucocorticoid receptor on the proliferation of murine macrophage RAW264.7 cell and mouse peritoneal macrophage

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Abstract

Glucocorticoid (GC) effectively suppresses immune and inflammatory responses and inhibits the growth of several types of cells, but the role of GC and its receptor on macrophage proliferation is unclear. In our previous work, we found RAW-GR(–) cells (murine macrophage RAW264.7 cells stably transfected with GR-siRNA expression vector by RNA interference) grew faster by about twofold. In this study, we further explored the role and mechanisms of GC/GR on the proliferation of macrophage. We found that the growth of RAW264.7 cells was inhibited by dexamethasone (Dex) in a concentration-dependent manner. The mRNA and protein levels of signal regulatory protein $\alpha 1$ (SIRPA) were induced by GC/GR in RAW264.7 cells and SIRPA expression was decreased remarkably in RAW-GR(–) cells. Overexpression of SIRPA negatively regulated the proliferation of RAW-GR(–) cells, and inhibition of SIRPA expression by a small from RNA interference attenuated Dex-induced proliferation inhibition in RAW264.7 cells. The proliferation inhibition of GC/GR was also found in mouse peritoneal macrophage, which was associated with the increase in SIRPA induced by GC/GR as well. In addition, elevation of the expression of CDK2, cyclinD1, and cyclinB1, but not phosphorylated ERK1/2 and p38, was found in RAW-GR(–) cells. In conclusion, we provided the novel evidences that GC/GR inhibited the growth of RAW264.7 cells and mouse peritoneal macrophage, and the antiproliferative effect of GC/GR on these cells was at least in part a result from GC/GR-induced SIRPA expression. Up-regulation of CDK2, cyclinD1, and cyclinB1 was also related to the increased proliferation of RAW-GR(–) cells.

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Introduction

It is well known that glucocorticoid (GC) plays a key role in regulating diverse physiological processes including immune and inflammatory responses, cell proliferation, differentiation, apoptosis, and development. These actions are mediated by glucocorticoid receptor (GR) that belongs to the nuclear receptor superfamily and is normally localized in the cytoplasm in an inactive state. On hormone binding, GR translocates to the nucleus where it binds to glucocorticoid response elements (GREs) on target genes and recruits coactivators or corepressors to regulate the expression of target genes (Heitzer *et al.* 2007).

GC is commonly used in the treatment of autoimmune and inflammatory diseases. It has been well documented that the inhibition of proliferation and induction of lymphocytic apoptosis are involved in the mechanisms of immunosuppressive effects of GCs (Distelhorst 2002, Frankfurt & Rosen 2004). Macrophages also play a key role in the immune and inflammatory processes, mainly acting as antigen-

processing/presenting cells and sources of inflammatory cytokines. Although GCs have been reported to inhibit macrophage killing activity and cytokine production in response to proinflammatory stimuli (Brummer *et al.* 2005), the effect of GCs on macrophage proliferation is controversial. Pagniello *et al.* (2002) found that dexamethasone (Dex) inhibited proliferation of the rainbow trout monocyte/macrophage cell line, RTS11. However Lloberas *et al.* (1998) reported that Dex itself had no effect on the proliferation of differentiated or undifferentiated bone marrow-derived macrophages and isolated peritoneal macrophages. In our previous study, we found that inhibition of GR expression in murine macrophage cell line RAW267.4 by RNA interference (RNAi) significantly promoted cell proliferation (Zhu *et al.* 2004). Cole *et al.* (2004) also reported that the number of undifferentiated epithelial cells increased in the fetal lung of GR null mice. These results suggest that the expression of functional GR may play an important role in making cells a more differentiated and less proliferative phenotype.

SIRPA (also known as SHPS-1, BIT, MFR, CD172a, or p84) is the first and best characterized member of the signal regulatory protein (SIRP) family and is a plasma membrane protein relatively ubiquitously expressed on myeloid cells, including macrophages (van Beek *et al.* 2005, Barclay & Brown 2006). The cytoplasmic region of SIRPA contains four inhibitory immunoreceptor tyrosine-based inhibitory motifs which become phosphorylated upon ligand binding, and mediates recruitment and activation of tyrosine phosphatases of PTPN6 (SH2 domain-containing protein phosphatase-1) and PTPN11, which in turn, dephosphorylate specific protein substrates and thereby regulate cellular function (Veillette *et al.* 1998, Oshima *et al.* 2002, van Beek *et al.* 2005, Barclay & Brown 2006). SIRPA plays important roles in immune regulation, generally in a negative fashion. It was also reported that the expression level of SIRPA decreased significantly in several types of tumor cells, including hepatocarcinoma cells, breast cancer cells, astrocytoma cells, glioblastoma cells, and most osteocarcinoma cells (Li *et al.* 1999, Ruhul Amin *et al.* 2002, Chen *et al.* 2004, Yan *et al.* 2004). Increasing SIRPA expression led to a reduction in tumor cell growth, migration, and cell transformation (Li *et al.* 1999, Ruhul Amin *et al.* 2002, Chen *et al.* 2004, Yan *et al.* 2004), suggesting that SIRPA is a negative regulator of cell growth. The signal transduction pathway involving the antiproliferative effect of SIRPA is unclear. Yan *et al.* (2004) reported that SIRPA negatively regulated MAPK3 activation in response to growth factors in human hepatocellular carcinoma cells. It was also reported that transfection with SIRPA plasmid decreased the expression of cyclinD1, a positive regulator of cell cycle, in hepatocarcinoma cells (Qin *et al.* 2005).

Cyclin/cyclin-dependent kinase (CDK) complexes are positive regulators in cell cycle, and CDKs activation induced by cyclins results in cells moving from one phase of the cell cycle to the next. CyclinD1, cyclinB1, and CDK2 play pivotal roles in cell cycle, especially promoting G1/S phase transition (Kaldis & Aleem 2005, Schwartz & Shah 2005, Walker & Assoian 2005). It has been reported that MAPKs play important roles in the regulation of cellular proliferation. The signals transmitted through this cascade, especially ERK1/2 and p38 can cause an activation of diverse molecules that regulate cell growth, survival, and differentiation (Robinson & Cobb 1997, Garrington & Johnson 1999).

The present study was designed to explore molecular mechanisms of antiproliferation of GC/GR by examining 1) the effect of GC/GR on the expression of SIRPA and its possible role in the antiproliferative effect of GC/GR in RAW264.7 cells and mouse peritoneal macrophage; 2) changes in the expression of CDK 2, cyclinD1, and cyclinB1 and the activity of MAPK1 and p38 in RAW-GR(−) cells.

Materials and methods

Materials

Dex, mouse monoclonal antibodies against β -actin, and 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemicals. Rabbit polyclonal antibodies against SIRPA and cyclinD1 were obtained from StressGene and Neo Marker companies respectively. Rabbit polyclonal antibodies against GR, cyclin B1, ERK1/2, and phosphorylated ERK1/2 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against p38 and phosphorylated p38 were purchased from Cell Signaling Technology (Beverly, MA, USA). Goat anti-rabbit-AP and goat anti-mouse-AP conjugate were from Bio-Rad Laboratories. RPMI 1640 medium was obtained from Life Technologies Inc. BCA protein assay kit was purchased from Pierce (Rockford, IL, USA). SYBR Green was from OPEN company. The SIRPA expression vector (pcDNA3-SIRPA) was generously provided by Prof Wang (Yan *et al.* 2004).

Cell lines and isolation of mouse peritoneal macrophages

Murine macrophage cell line RAW264.7 was obtained from American Type Culture Collection (Rockville, MD, USA). RAW-GR(−) and RAW-control cells were established by stable transfection of RAW264.7 cells with GR-siRNA expression vector and nonspecific-siRNA vector respectively (Zhu *et al.* 2004). Mouse peritoneal exudates macrophages were harvested by peritoneal lavage from kunming mouse by i.p. injection of 1 ml sterile ice-cold physiological saline five times. The cells were resuspended in RPMI 1640 and seeded in 96- or 24-well plate for 1–5 h, the supernatant was then discarded and the cells adhered to the plate were mouse peritoneal macrophages (An *et al.* 2007).

Cell culture

RAW264.7, RAW-GR(−), and RAW-control cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in a humidified incubator of 5% CO₂. To examine the effect of Dex on the expression of SIRPA at the mRNA and protein levels, these cells were cultured in RPMI 1640 medium containing 10% charcoal-dextran-treated FBS (CD-FBS) for 24 h, and then treated with ethanol or different concentrations of Dex for the indicated times. Cortisol in CD-FBS was not detectable by RIA. Mouse peritoneal macrophages were cultured in RPMI 1640 medium containing 10% CD-FBS and treated with ethanol or 10^{−7} M Dex with or without 10^{−6} M RU486 for 2 days.

Quantitative real-time PCR

Total RNA was extracted with TRIzol reagent and 2 µg total RNA was subjected to synthesizing first-strand cDNA by reverse transcription system (Promega) according to the manufacturer's instructions.

PCR primers used in this study are as follows: SIRPA, sense 5'-CTT TGC TCG TAG TCC TGC-3' and antisense 5'-CTG CGT ATG TGA TGT CGT-3' (177 bp); β -actin, sense 5'-CTG TAT GCC TCT GGT CGT AC-3' and antisense 5'-TGA TGT CAC GCA CGA TTT CC-3' (214 bp). SYBR Green was used as the fluorescence reagent. cDNA template (100 and 50 ng for amplifying SIRPA and β -actin respectively) was added to the mixture of 2 µl 10×buffer, 1.6 µl 25 mM MgCl₂, 0.5 µl 20 mM dNTP, 0.3 µl 20 mM primers, 1.5 U Taq enzyme, and 1.0 µl of 20×SYBR Green to a final volume of 20 µl. Amplification of SIRPA and β -actin was performed in different tubes, with the same process: 36 cycles of 15 s denaturation at 94 °C, 20 s annealing at 57 °C, 15 s elongation at 72 °C, and a final extension for 10 min at 72 °C. The mRNA ratio of *Sirpa* and actin was calculated as follows: we first drew the standard curves of SIRPA and β -actin by measuring the threshold cycles (CT) of standards of different concentrations, which had strong positive relationship with the start copy number (i.e. cDNA amounts) of a gene in linear amplification period. Then the cDNA amounts of SIRPA and β -actin of each testing sample were calculated according to their standard curves, and cDNA amount of SIRPA was normalized by the corresponding β -actin.

Transient transfection of cells

Cells were seeded in a 24-well plate (4×10^4 cells/well) or a 6-well plate (2×10^5 cells/well) for 24 h and then transiently transfected with 1 µg (for the 24-well plate) or 4 µg (for the 6-well plate) of the control vector (pcDNA3) or SIRPA expression plasmid using LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer's instructions.

Western blot analysis

Cells were lysed in ice-cold lysis buffer (Stamatakis *et al.* 2002; 10 mM Tris (pH 7.5), 0.1 mM EDTA, 0.1 mM EGTA, 0.5% SDS, 0.1 mM mercaptoethanol, 2 µg/ml of each of the protease inhibitors leupeptin, aprotinin, and pepstatin) for 10 min. The lysate was briefly sonicated on ice, and spun at 12 893g for 10 min to remove the insoluble material. Protein concentrations of the supernatant were measured with a BCA protein assay kit. Twenty to fifty micrograms of extracts were loaded on 10% SDS-PAGE gel. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, which were blocked with 5% nonfat milk,

and probed overnight with antibodies against SIRPA (1:200), GR (1:1000), cyclinB1, cyclinD1, CDK2 (1:1000), MAPK1, p38 (1:500), or β -actin (1:10 000). The membranes were then washed, exposed to alkaline phosphatase-conjugated secondary antibodies for 2 h, and finally colored with phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium (BCIP-NBT).

The blots were quantified with Tanon software (Shanghai, China). In each experiment, we first normalized the levels of proteins interested in treated samples and control. We then compared the normalized numbers from three independent experiments. The average level from these experiments was calculated and the significance test performed.

Cell proliferation assay

Cell proliferation of RAW264.7, RAW-GR(−), and RAW-control cells was measured by MTT assay (Carmichael *et al.* 1987) or cytometry. Briefly, cells were seeded in duplicate at 3×10^4 cells per well in a 24-well plate for MTT assay or 2×10^5 cells per well in a 6-well plate for cytometry. After incubation for 2 days, cells in each well were collected and counted with a hemacytometer or examined by MTT assay. To each well, 200 µl RPMI 1640 medium containing 5 mg/ml MTT (Sigma-Aldrich Chemicals) solution was added. Cells were incubated for 4 h at 37 °C, followed by solubilization with 200 µl dimethyl sulfoxide (DMSO) solutions. Then the O.D. value was measured using a Bio-Rad (model 550) microplate reader at 550 nm with DMSO as blank. To ensure the reliability of MTT results, the linear response range of RAW264.7 cells between the results of MTT assay and the number of cells was explored. There was good linear correlation when RAW264.7 cells were seeded at the densities from 1×10^4 to 4×10^5 cells per well in a 24-well plate and cultured in medium for 4 days (data not shown).

The proliferation of mouse peritoneal macrophage was estimated by CCK-8 assay according to the manufacturer's guidelines. This assay is based on the cleavage of the tetrazolium salt WST-8 by mitochondrial dehydrogenase in viable cells. Briefly, mouse peritoneal macrophages (2×10^5 cells/well) were incubated with 100 µl culture medium in 96-multiwell plates. After incubation with 10^{-7} M GC with or without 10^{-6} M RU486 for 2 days, the media were removed and 100 µl DMEM containing CCK-8 (10 µl) was added to each well. After a further 1-h incubation at 37 °C, the absorbance at 450 nm of each well was measured with a microplate reader. Each experiment was repeated thrice, and the data represent the mean of all measurements.

Figure 1 Dex inhibits growth of RAW264.7 cells in a dose-dependent manner. RAW264.7 cells were seeded at 1×10^4 cells per well in a 24-well plate in duplicate and cultured in medium containing ethanol, 10^{-7} M or 10^{-6} M Dex for different periods of time. Proliferation of the cells was monitored by MTT assay. Values plotted are mean $\pm$ s.d. of three independent experiments. * $P < 0.05$ between 10^{-7} M and 10^{-6} M Dex treatment, ** $P < 0.01$ versus control.

Figure 2 Inhibition of GR expression in RAW264.7 cells by stably or transiently transfected with GR RNA interference vectors promotes proliferation of the cells. Proliferation of RAW264.7 cells was assessed by MTT assay after stably transfected with (A) GR RNA interference vector pSilencer2.1-U6-GR (RAW-GR(-)) or (B) 4 days following transiently transfected with pSilencer2.1-U6-GR. (mean $\pm$ s.d., $n = 3$), * $P < 0.05$ versus RAW-control cells (stably transfected with pSilencer2.1-U6-control), ** $P < 0.01$ versus RAW264.7 cells transiently transfected with pSilencer2.1-U6-control. GR expression was detected by western blot.

RNAi of SIRPA

Small interfering RNA (siRNA) of SIRPA was used to inhibit the expression of SIRPA in RAW264.7 cells by transient transfection with SIRPA siRNA oligonucleotide. Two SIRPA siRNA oligonucleotides designed by Genechem Co., (Shanghai, China) matched nonconserved 19 nucleotide sequences within the mouse *Sirpa* mRNA. The sequences of oligonucleotides used to create SIRPA RNAi were: 5'-CCC TGA CTA TCT GGT CTT A-3'; 5'-GCC TGA CACAGA AATACA A-3'. They expressed a hairpin siRNA with limited homology to any known sequences in human, mouse, and rat genomes. The oligonucleotides were annealed before transient transfection of RAW264.7 cells. The siRNA control was provided by the same company.

Figure 3 Inhibition of GR expression in RAW264.7 cells by RNA interference decreases SIRPA expression at both mRNA and protein levels. (A) *Sirpa* mRNA expression in RAW-GR(-) and RAW-control cells was detected by real-time PCR (mean $\pm$ s.d., $n = 3$), ** $P < 0.01$ versus RAW-control. (B) Protein levels of SIRPA in RAW-GR(-) and RAW-control cells were examined by western blot analysis. Similar results were obtained from three independent experiments. Using Tanon software (Shanghai, China), the blots of SIRPA in these two cells were quantified and normalized against β -actin. Then the normalized numbers from three independent experiments were compared. The values are expressed as percent of the level of RAW-control, as shown in the histogram of (C). (mean $\pm$ s.d., $n = 3$), ** $P < 0.01$ versus RAW-control.

Statistical analysis

Data are expressed as mean $\pm$ s.d. of the separate experiments. Statistical significance was determined by Student's *t*-test with a value of $P < 0.05$ considered as statistically significant.

Results

GC/GR negatively regulates the proliferation of RAW264.7 cells

The effect of Dex on RAW264.7 cell growth was examined by MTT assay. Figure 1 shows that the growth of RAW264.7 cells was inhibited by Dex in a concentration-dependent manner. About 27 and 34% inhibition of cell proliferation was observed at day 4 after treatment with 10^{-7} and 10^{-6} M Dex respectively, and no further increase of inhibition rate was observed at day 6. Furthermore, when RAW264.7 cells were stably

transfected with GR siRNA expression vector, the expression of GR was significantly decreased, accompanied by an increase in cell proliferation (Fig. 2A). The number of RAW-GR(–) cells was almost twofold of that of the RAW-control cells after 4-day culture. The proliferation promotion by GR knockdown was further confirmed in transiently transfection assay (Fig. 2B). These results indicate that GC/GR negatively regulates the growth of RAW264.7 cell.

GC/GR induces the expression of SIRPA at mRNA and protein levels in RAW264.7 cells

Although GR has been proved to play an important role in inhibiting cell proliferation, only a few target genes that mediate antiproliferative effect of GR/GC on macrophages are known. Considering that SIRPA is highly expressed in macrophages and inhibits the proliferation of different cell-type-derived tumor cells, we presumed that SIRPA may be involved in the

Figure 4 Dex increases SIRPA expression in a dose- and time-dependent manner in RAW264.7 cells. (A and C) *Sirpa* mRNA expression in RAW264.7 cells after treatment with different concentrations of Dex and with 10^{-7} M Dex for different time was examined by real-time PCR, (mean $\pm$ s.d., $n=3$), $*P < 0.05$ versus control. (B and D) Protein level of SIRPA in RAW264.7 cells was examined by western blot analysis. Similar results were obtained from three independent experiments. The blots of SIRPA were quantified by Tanon software and normalized against β -actin. Then the normalized numbers from three independent experiments were compared. The values are expressed as fold of the level of control, as shown in the histograms of B and D. (mean $\pm$ s.d., $n=3$), $*P < 0.05$ versus control.

antiproliferative characteristic of GC/GR. To this end, the expression of SIRPA in RAW-GR(–) cells was then examined by quantitative real-time PCR and western blot analysis. The results showed that mRNA and protein levels of SIRPA in RAW-GR(–) cells reduced to about 16 and 24% of RAW-control cells respectively (Fig. 3).

To determine whether GC up-regulated the expression of SIRPA, we further investigated the mRNA and protein levels of SIRPA in RAW264.7 cells treated with different concentrations of Dex or with 10^{-7} M Dex for different times by quantitative real-time PCR and western blot respectively. Figure 4 shows that Dex induced the expression of SIRPA at mRNA and protein levels in dose- and time-dependent manner. The mRNA level of *Sirpa* significantly increased 1 h

after treatment with 10^{-7} M Dex, with the maximal induction effect at 2 h (2.2-fold of the control, $P < 0.05$; Fig. 4C). A similar increase in SIRPA protein expression was also observed following the incubation of cells with 10^{-7} M Dex ($P < 0.05$; Fig. 4D).

SIRPA signaling is involved in GC/GR-mediated inhibition of RAW264.7 cell proliferation

As the expression of SIRPA was regulated by GC/GR in RAW264.7 cells and increased proliferation was associated with a significant decrease in the expression of SIRPA in RAW-GR(–) cells, we hypothesized that SIRPA might be involved in the regulation of RAW264.7 cell growth by GC/GR, and therefore we examined the effect of SIRPA on cell proliferation by MTT assay and cytometry. It was found that the expression of SIRPA in RAW-GR(–) cells and in RAW264.7 cells transiently transfected with SIRPA plasmids increased by about 2.5-fold (Fig. 5A), which resulted in about 40 and 34% inhibition of proliferation in RAW-GR(–) cells and in RAW264.7 cells respectively (Fig. 5B and C). Then we further examined the role of SIRPA in GC/GR-induced proliferation inhibition of RAW264.7 cells by inhibiting the expression of SIRPA using small interference RNA. As shown in Fig. 6A, the level of

Figure 5 Transfection of RAW-GR(–) and RAW264.7 cells with SIRPA expression plasmid inhibits cell proliferation. (A) RAW-GR(–) and RAW264.7 cells were transfected with SIRPA plasmid or control vector for 24 h and the protein levels of SIRPA was detected by western blot analysis. These blots are representative of three independent experiments. After transfection with SIRPA vector or control vector, cells were seeded and cultured in duplicate for 2 days in (B) a 6-well (2×10^5 cells/well) plate for cytometry or (C) a 24-well plate (3×10^4 cells/well) for MTT assay. (mean $\pm$ s.d., $n=3$), ** $P < 0.01$ versus cells transfected with control vector, $n=3$.

Figure 6 Inhibition of SIRPA expression in RAW264.7 cells by RNA interference attenuates Dex-induced inhibition of cell proliferation. (A) RAW264.7 cells were transfected with small interference RNA (siRNA) for SIRPA or siRNA control and the protein levels of SIRPA in the cells were detected by western blot analysis. These blots are representative of three independent experiments. After transfection, RAW264.7 cells were seeded at 1×10^4 cells per well in a 24-well plate in duplicate and cultured in medium containing ethanol, 10^{-7} M or 10^{-6} M dexamethasone (Dex) for 4 days. (B) Proliferation of the cells was monitored by MTT assay. The values of A550 values were normalized against corresponding blank control to show clearer the difference of inhibition rate. Then the normalized numbers from three independent experiments were compared. The values are expressed as percent of the level of corresponding control. ** $P < 0.01$ versus control, * $P < 0.05$ versus control.

SIRPA protein in siSIRP 1 and siSIRP 2 cells reduced to 29 and 31% of that in cells treated with control siRNA respectively. The inhibition of SIRPA expression attenuated Dex-induced inhibition of cell proliferation in siSIRP 1 cells as compared with that in control cells (Fig. 6B), indicating that SIRPA negatively regulates the proliferation of RAW264.7 cells and SIRPA signaling is involved in the inhibitory effect of GC/GR on the proliferation of these cells.

SIRPA signaling is also associated with GC/GR-mediated inhibition of mouse peritoneal macrophage proliferation

Does the proliferation inhibition effect of Dex on RAW264.7 cells exist in primary mouse peritoneal macrophages? Is the proliferation inhibition effect of Dex on primary mouse peritoneal macrophages also associated with SIRPA? To this end, we examined the effect of Dex on the proliferation of mouse peritoneal macrophages by CCK-8 assay and found that cell growth was decreased obviously two days after treatment with 10^{-7} mol Dex and the inhibition of proliferation was completely reversed by GR antagonist, RU486, while RU486 itself had no effect on cell proliferation (Fig. 7A). At the same time, we detected the expression of mRNA of *Sirpa* by real-time PCR and found that the expression of SIRPA was increased remarkably by treatment with 10^{-7} M Dex and RU486 partially inhibited the enhancement of SIRPA induced by GC, while RU486 itself had no effect on the expression of SIRPA (Fig. 7B). These results indicated that GC also inhibited the growth of primary mouse peritoneal macrophage through GR mediation and the inhibitory effect of GC/GR was also associated with SIRPA.

Increased expression of CDK2, cyclinD1, and cyclinB1 but not ERK1/2 and p38 phosphorylation may be associated with the increased proliferation and cell cycle change of RAW-GR(–) cells

Previous studies demonstrated that GCs inhibited cell growth by lengthening the cell cycle, mainly through arresting cells in the G1 phase (Funakoshi *et al.* 2005, Sundberg *et al.* 2006). The decreased percentage of G1 phase cells and the increased percentage of S phase cells were found in RAW-GR(–) cells as compared with that of RAW-control cells (Fig. 8A). CDK2, cyclinD1, and cyclinB1 play pivotal roles in cell cycle, especially promoting G1/S phase transition (Kaldis & Aleem 2005, Schwartz & Shah 2005, Walker & Assoian 2005), so we detected the expression of these proteins in RAW-GR(–) cells. Figure 8B shows that the expression of CDK2, cyclinD1, and cyclinB1 proteins increased

Figure 7 Increase in *Sirpa* mRNA induced by Dex in mouse peritoneal macrophages is associated with the proliferation inhibition of Dex on the cells. Mouse peritoneal macrophages were isolated and seeded at 4×10^5 cells per well in a 96-well plate in duplicate and cultured in medium containing ethanol, 10^{-7} M Dex with or without GR antagonist 10^{-6} M RU486 for 2 days. (A) Proliferation of the cells was monitored by CCK-8 assay. Values plotted are mean $\pm$ s.d. of three independent experiments. $**P < 0.01$ versus control. Mouse peritoneal macrophages were isolated and seeded at 2×10^6 cells per well in a 24-well plate and treated with 10^{-7} M Dex with or without 10^{-6} M RU486, then *Sirpa* mRNA expression in the cells was examined by real-time PCR (B) (mean $\pm$ s.d., $n=3$), $**P < 0.01$ versus control.

significantly in RAW-GR(–) cells by about 2.1-, 1.7-, and 2.2-fold respectively, as compared with that in RAW-control cells, suggesting that these positive regulators of cell cycle may be associated with faster growth resulted from GR knocking down.

In recent years, evidence has emerged that GR plays important roles in regulating cell proliferation by modulating the activity of ERK1/2 and p38 (Miller *et al.* 2005, Qin *et al.* 2005), so we examined the activated status of these kinases in RAW-GR(–) and RAW-control cells by western blot. We failed to find significant difference in phosphorylated-ERK1/2 or -p38 level between RAW-GR(–) and RAW-control cells (Fig. 9).

Discussion

GC effectively suppresses immune and inflammatory responses and inhibits the growth of several types of cells, but the role of GC and its receptor on macrophage proliferation is unclear. In our previous work, we found that RAW-GR(–) cells stably

Figure 8 Increase in CDK2, cyclinD1, and cyclinB1 in RAW-GR(–) cells may be associated with the increased proliferation and cell cycle change of RAW-GR(–). (A) Change of cell cycle in RAW-GR(–) cells as compared with RAW-control cells. The RAW-GR(–) and RAW-control cells were cultured for 12 h with 10% fetal bovine serum followed by 24 h starving with serum-free medium. The cells were collected to determine the cell percentage of G1, G2, and S phases by FACSCalibur flow cytometer. Values shown are expressed as proportion of each phase and are the mean $\pm$ s.d. of three independent experiments, as shown in the histogram. * $P < 0.05$ versus RAW-control. (B) The protein levels of CDK2, cyclinD1, and cyclinB1 in RAW-GR(–) and RAW-control cells were examined by western blot analysis. These blots are representative of three independent experiments. The blots of CDK2, cyclinD1, and cyclinB1 in these two cells were quantified by Tanon software (Shanghai, China) and normalized against β -actin. Then the normalized numbers from three independent experiments were compared. The values of expression levels are expressed as fold of the level of RAW-control, as shown in the histogram. (mean $\pm$ s.d., $n = 3$), ** $P < 0.01$ versus RAW-control.

transfected RAW264.7 cells with GR-siRNA expression vector grew faster by about twofold (Zhu *et al.* 2004). A similar result was obtained by transient transfection with GR RNAi plasmid in this study. While treatment with 10^{-7} M Dex for 4 days resulted in only 27% proliferation inhibition of RAW264.7 cells. More important, the proliferation of RAW-GR(–) cells shows no significant difference between cultured in RPMI 1640 medium containing 10% heat-inactivated FBS and 10% charcoal-stripped serum. The inhibition effect of GC/GR on cell proliferation was also found in primary mouse peritoneal macrophages. These results indicated that GR negatively regulated the growth of RAW264.7 cells and mouse peritoneal macrophages, and the inhibitory effect of GR may work mainly in a GC-independent manner. Several studies on hormone-sensitive carcinomas *in vitro* and *in vivo* in nude mice also showed similar results in that loss of expression of functional steroid receptors was often associated with the development of less differentiated tumors with a consequently poor prognosis (Baldi *et al.* 2003, Nightingale *et al.* 2003, Litvinov *et al.* 2004). For example, the growth of prostate cancer cell lines lacking androgen

receptor (e.g. PC-3 cells) was faster than that of cells expressing androgen receptors (e.g. LNCap cells) (Chlenski *et al.* 2001), and expressing transfected androgen receptor in PC-3 cells decreased the proliferation rate and cloning efficiency and induced a more differentiated phenotype (Yuan *et al.* 1993). Similar increased proliferation also found in some breast cancer cell lines that lack estrogen receptors (Platet *et al.* 2004). These phenomena found in our laboratory and by other researchers indicate that expression of functional GR and other steroid receptors is very important for cells to become a more differentiated and less proliferative phenotype.

As mentioned in introduction, SIRPA is highly expressed in macrophages, and plays important roles in immune regulation and inhibits the proliferation of several types of cells. So we wondered whether the expression of SIRPA was regulated by GC/GR and whether there was a correlation between SIRPA expression and cell proliferation in RAW264.7 cells and mouse peritoneal macrophages. The expression of SIRPA was induced by Dex time and dose dependently in RAW264.7 cells, and decreased markedly in RAW-

Figure 9 No change of protein levels of phosphorylated p38 and ERK1/2 is found in RAW-GR(–) cells compared with RAW-control cells. (A) RAW-GR(–) and RAW-control cells were starved with serum-free medium for 24 h, and then recovered to be cultured with 10% fetal bovine serum for 5, 20, or 60 min. The cells were collected to determine the activated status of p38 and ERK1/2 at the indicated above time with the antibody of anti-phosphorylated p38 and ERK1/2 by western blot analysis. The total p38 and ERK1/2 were used as the quantitative standard. (B) The ratio of p-p38 and total p38 was expressed as fold of that of RAW-control cells at 0 min. There was no obvious difference between RAW-GR(–) and RAW-control cells at three time points. Values shown are the mean $\pm$ s.d. of three independent experiments. (C) The ratio of p-MAPK1/total MAPK1 was expressed as fold of the level of RAW-control at 0 min, and values shown are the mean $\pm$ s.d. of three independent experiments.

GR(–) cells. Overexpression of SIRPA in RAW-GR(–) and RAW264.7 cells transiently transfected with SIRPA expression vector obviously inhibited the proliferation of these cells, while knockdown of SIRPA expression by siRNA attenuated Dex-induced inhibition of cell proliferation. In addition, the proliferation inhibition of GC/GR on mouse peritoneal macrophages was also associated with Dex-induced enhancement of SIRPA. These results support our hypothesis that SIRPA is a negative regulator of the proliferation in RAW264.7 cells and mouse peritoneal macrophages, and SIRPA signaling is involved in antiproliferative effect of GC/GR on these cells.

It is well known that GR up-regulates gene transcription by direct binding with GRE located in the promoter region of target genes (Dondi *et al.* 2001, Kudawara *et al.* 2001, Frankfurt & Rosen 2004). We analyzed the sequence at the promoter region of *Sirpa* gene, and found no consensus GRE, but just several potential GRE half-sites (data not shown). It is not known whether these GRE half-sites could mediate the

effect of Dex on SIRPA transcription. In addition to binding with GREs, GR can indirectly regulate gene expression by interacting with other classes of transcription factors, such as NF- κ B, AP-1 (Fos/Jun), TRP53 to exert its antiproliferative role (Crochemore *et al.* 2002, Sengupta & Waslyk 2004, Bladh *et al.* 2005). Whether these molecules are involved in GR-mediated SIRPA regulation remains to be investigated.

GC/GR has been known to play an antiproliferative role through inducing cell cycle arrest and regulating the expression of regulatory proteins of cell cycle in several types of cells, including osteosarcoma cells, hepatoma cells, and embryonic neural stem cells (Kudawara *et al.* 2001, Yamamoto *et al.* 2002, Frankfurt & Rosen 2004, Sundberg *et al.* 2006). We had examined the protein levels of the cyclin-dependent kinase inhibitors (CDKi) CDKN1A and CDKN1B, and found that the expression of CDKN1B, but not CDKN1A was reduced in RAW-GR(–) cells (Zhu *et al.* 2004). In this study, we further demonstrated that increased proliferation of RAW-GR(–) cells was associated with

significant increase in positive regulatory proteins of cell cycle (CDK2, cyclinD1, and cyclinB1). These results indicate that GR plays a crucial role in cell cycle arrest through regulating the expression of regulatory proteins of cell cycle in macrophage cells.

ERK1/2 and p38 are also involved in the control of cell proliferation. Whether ERK1/2 and p38 are involved in GR knockdown-induced pro-proliferation process remains unknown. Our results showed no change in phosphorylated ERK1/2 and p38 expression, suggesting that ERK1/2 and p38 pathways did not participate in the regulation of GR-knockdown-sensitized proliferation in RAW264.7 cells.

In summary, in this study, we first demonstrated that GC/GR inhibited the proliferation of both established RAW264.7 cell line and mouse peritoneal macrophage. The novel evidence is provided that SIRPA, a negative modulator of cell growth induced by GC/GR, is involved in the antiproliferative effect of GC/GR on macrophages. Furthermore, the increase in CDK2, cyclinD1, and cyclinB1 may be also related to the increased proliferation of RAW-GR(−) cells.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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