RAPID COMMUNICATION

Differential expression of GAS5 in rapamycin-induced reversion of glucocorticoid resistance

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SUMMARY

This study evaluates the association between the long noncoding RNA GAS5 levels and the anti-proliferative effect of the glucocorticoid (GC) methylprednisolone (MP) alone and in combination with rapamycin in peripheral blood mononuclear cells (PBMCs) obtained from healthy donors.

The effect of MP, rapamycin, and MP plus rapamycin was determined in 17 healthy donors by labelling metabolically active cells with [methyl-3H] thymidine and the expression levels of GAS5 gene were evaluated by real-time RT-PCR TaqMan analysis. We confirmed a role for GAS5 in modulating GC response: poor responders presented higher levels of GAS5 in comparison with good responders. Interestingly, when PBMCs were treated with the combination of rapamycin plus MP, the high levels of GAS5 observed for each drug in the MP poor responders group decreased in comparison with rapamycin (*P* value = 0.0134) or MP alone (*P* value = 0.0193). GAS5 is involved in GC resistance and co-treatment of rapamycin with GCs restores GC effectiveness in poor responders through the downregulation of the long noncoding RNA. GAS5 could be considered a

biomarker to personalize therapy and a novel therapeutic target useful for the development of new pharmacological approaches to restore GC sensitivity.

Key words: Long noncoding RNA, GAS5, glucocorticoids, methylprednisolone, rapamycin.

INTRODUCTION

Glucocorticoids (GCs) are commonly used as therapeutic agents for inflammatory and autoimmune diseases, in the treatment of leukaemias and lymphomas, and in the prevention of rejection in transplant patients.[1–3] These agents exert their biological effects through binding to the GC receptor (GR), which translocates from the cytoplasm into the nucleus and binds, through its DNA-binding domain (DBD), the glucocorticoid responsive elements (GREs) in the regulatory regions of GC responsive genes.[4] However, considerable inter-individual differences in their efficacy and side effects have been reported. The molecular mechanisms involved in this variability are scarcely understood and there is presently no means to predict the response in advance.[5, 6]

Recent results obtained in the authors' laboratory suggest a role for the long noncoding RNA (lncRNA) growth arrest-specific 5 (GAS5) in modulating GC response in peripheral blood mononuclear cells (PBMCs). A previous *in vitro* study demonstrated that PBMCs resistant to GCs express higher levels of GAS5 in comparison with good responders, and hypothesized that upregulation of GAS5 could interfere with GR activity, leading to the resistance phenotype observed.[7] In addition, others have recently demonstrated that decreasing GAS5 levels can enhance GC action in airway epithelial cells.[8]

GAS5 is a lncRNA (~650 bases in humans) that interacts with the DBD of the ligand-activated GR and suppresses GR-induced transcriptional activity of GC responsive genes by inhibiting binding of the GR to target genes GREs.[9] GAS5 is a member of the 5' terminal oligopyrimidine (5'TOP) class of RNAs, whose transcript levels are controlled by the mammalian target of rapamycin (mTOR) pathway.[10, 11]

An increasing number of reports indicate that other immunosuppressive agents, among which rapamycin, the inhibitor of mTOR, can reverse GC resistance in different human cell lines,[12–16] suggesting that the poor response to GCs may derive from an impaired cross-talk between the GC and mTOR signalling pathways. However, the molecular mechanism involved in the synergistic effect of these agents has not yet been clarified. An increase in the levels of GAS5 was observed when mouse embryo NIH3T3 cells were treated with the mTOR-specific inhibitor rapamycin.[10]

Furthermore, the regulation of GAS5 transcript levels by mTOR has been confirmed in leukaemic and primary human T cells: downregulation of GAS5 using RNA interference protected both cell types from the inhibition of proliferation produced by mTOR antagonists.[11]

These findings are in contrast with our recent observation, showing an upregulation of GAS5 in GC resistant PBMCs. On these bases, this study investigates the role of GAS5 in the phenomenon of GC sensitivity restored by rapamycin in resistant PBMCs. The association between the anti-proliferative effects of methyl-prednisolone (MP) alone or in combination with rapamycin, and GAS5 gene expression in PBMCs obtained from healthy subjects was examined. The preliminary data presented here suggest that rapamycin, in combination with GCs, reverts GC resistance in PBMCs and this effect is associated with a reduction in the expression of GAS5. The study confirms that mTOR pathway is connected with GR signalling and that GAS5 could play a key role in the synergistic effect of rapamycin and GCs.

RESULTS

Peripheral blood mononuclear cells obtained from healthy donors were isolated for proliferation and gene expression analyses. Subjects were divided into two groups on the basis of individual MP (250 ng/mL) response: MP good responders (MP_GR) had a percentage of inhibition ≥54% (8 subjects MP_GR, median inhibition 61%); and MP poor responders (MP_PR) had a percentage of inhibition <54% (9 subjects MP_PR, median inhibition 36%) (Fig. 1, $P = 0.0004$).

When incubated with rapamycin (100 nmol/L) alone, the MP_GR and MP_PR groups showed a significantly different growth inhibitory effect: the MP_GR subjects were significantly more sensitive to rapamycin (RAPA: MP_GR median inhibition 75%) compared with MP_PR group (MP_PR median inhibition 58%) (Fig. 1, $P = 0.0225$). The combination of rapamycin with MP in MP_PR PBMCs enhanced the growth inhibitory effect (RAPA+MP: MP_PR median inhibition 81%) compared to rapamycin or MP alone (MP_PR: RAPA+MP *vs* RAPA $P < 0.001$; RAPA+MP *vs* MP $P < 0.001$), and the same trend was observed in MP_GR (RAPA+MP: MP_GR median inhibition 91%) (MP_GR: RAPA+MP *vs* RAPA $P < 0.05$; RAPA+MP *vs* MP $P < 0.001$) (Fig. 1).

While the percentage of inhibition was significantly different between MP_GR and MP_PR both for MP and rapamycin alone, when the combination of the two drugs was used, no significant difference in growth inhibition was observed between the two groups (RAPA+MP: MP_GR *vs* MP_PR $P > 0.05$).

The expression of the GAS5 gene was analyzed in the same MP_GR and MP_PR PBMCs.

Treatment with MP alone induced a different regulation of GAS5 in the two groups, confirming recently published preliminary data from our laboratory:[7] the expression of GAS5 mRNA was significantly downregulated in MP_GR group after 72 hours of treatment with 250 ng/mL of MP in comparison with MP_PR, in which GAS5 resulted, on the contrary, upregulated (Fig. 2, $P = 0.0427$). The correlation between the percentage inhibition of PBMC proliferation and GAS5 expression level was further analyzed using the nonparametric correlation coefficient (Fig. 2, Spearman rho = –0,73; $P = 0.0009$).

Then, the effect of rapamycin alone and in combination with MP on the expression of GAS5 was checked (Fig. 3). When all subjects were considered, no differences in GAS5 expression were observed after 72 hours of treatment with rapamycin in comparison with the untreated cells (data not shown; $P = 0.1019$), as already published for MP alone.[7] When GAS5 expression was evaluated in the MP_PR group after treatment with RAPA, it was observed that the levels of GAS5 were significantly increased compared to the MP_GR group (Fig. 3; $P = 0.0302$).

Interestingly when PBMCs were treated with the combination of rapamycin plus MP, the high levels of GAS5 observed for each drug in MP_PR, decreased in comparison with rapamycin ($P = 0.0134$) or MP alone ($P = 0.0193$); it is important to point out that no significant difference between MP_PR and MP_GR groups was observed (Fig. 3; $P = 0.9502$).

DISCUSSION

Glucocorticoid resistance is a major driver of therapeutic failure in cancer, allogeneic transplantation, and immune-mediated diseases and there is presently no means to predict this phenomenon in advance.

Recent reports have shown that the lncRNA GAS5 could act as a riborepressor of the GR. In particular, GAS5 exon 12-derived sequence has been shown to structurally mimic the GREs, preventing the binding of the activated GR complex to its target DNA sequences.[9] Moreover, GAS5 is a member of the 5’ terminal oligopyrimidine (5’TOP) class of RNAs, whose transcript levels are controlled by the mTOR pathway,[10] a potential target to restore GC effectiveness.[14, 15]

This study provides new insights on the phenomenon of GC sensitivity restored by rapamycin in which GAS5 could be directly involved. In particular, the results demonstrate for the first time the synergistic effect of MP and rapamycin in MP_PR PBMCs, confirming previous studies in tumor cell lines.[12–15] Indeed, it has been recently shown that rapamycin sensitizes GC resistant haematological malignant cells to Dexamethasone-induced apoptosis; this is demonstrated by an increased inhibition of cell growth, cell cycle arrest at G1 and apoptosis.[14, 15, 17] In particular,

the combination of rapamycin and GCs was able to both inhibit the mTOR signalling pathway, as confirmed by downregulation of p-p70S6k, and increase in the phosphorylation status of the GR.[15]

Previous studies conducted in our laboratory have shown that endogenous GAS5 is upregulated in resistant PBMCs after *in vitro* treatment with MP, possibly through the binding to the DBD site, and consequent interference with the GR transcriptional activity,[7] however, no data are available on the effect of rapamycin reversion of GC resistance on GAS5 expression levels in PBMCs. Our data demonstrate that mTOR inhibition increases GAS5 transcript levels in MP_PR PBMCs compared to the MP_GR group. This upregulation observed in MP_PR PBMCs is probably ascribable to the fact that GAS5 is a 5'TOP RNA, whose stability is controlled by the mTOR pathway.[10] An accumulation of GAS5 transcript in NIH3T3 cells treated with rapamycin has been described already by Smith and Steitz.[10] Furthermore, the regulation of GAS5 transcript levels by mTOR has been studied indirectly in leukaemic and primary human T cells: downregulation of GAS5 using RNA interference protected both cell lines from the inhibition of proliferation produced by rapamycin.[11] The different regulation of GAS5 expression in the MP_GR and MP_PR PBMCs observed in the current study is unexpected and requires further investigation. Currently studies are in progress on the involvement of the lncRNA GAS5 antisense RNA 1 (GAS5-AS1) gene: the partial overlap between the two lncRNAs could be important to regulate GAS5 RNA stability.

When PBMCs were treated with the combination of rapamycin plus MP, the high levels of GAS5 observed for each drug in MP_PR group decreased in comparison with rapamycin or MP alone. It was very interesting to find that the combination of rapamycin and MP could reverse GC resistance in PBMCs, not only through the inhibition of mTOR but also decreasing GAS5 levels. Again, the downregulation of this lncRNA is associated with a good response to GCs.[7] This downregulation seems not to be related to the direct effect of the single drug but rather to a possible cross-talk between their pathways, however, further investigation is needed to shed light on the interacting mechanism.

Taken together, the results provide evidence that high GAS5 levels in PBMCs of healthy donors after GC treatment *in vitro* could represent a molecular index of GC resistance. Therefore, if these data could be confirmed in patients in therapy with GCs, GAS5 levels could represent a specific molecular target to be evaluated. The development of an assay based on GAS5 screening in patients' PBMCs treated with GCs *in vitro* could predict the *in vivo* response and help clinicians in the adjustment of the current protocols. In patients with high levels of GAS5, a co-treatment with rapamycin could eventually be proposed. In conclusion, GAS5 could be considered a biomarker to

personalize therapy and a novel therapeutic target useful for the development of new pharmacological approaches to restore GC sensitivity.

METHODS

Subjects

Samples from 17 blood donors were collected between January 2014 and October 2014 from the Transfusion Centre, Azienda Ospedaliera Universitaria, Trieste, Italy. Blood samples were obtained by venipuncture between 0800 and 1000 hours to minimize the variability due to circadian rhythm, and immediately processed. All donors signed an individual review-board-approved consent for blood sampling and use for research purposes. Blood samples were delivered to the University of Trieste with no individually identifiable information. A total of 9 mL of each buffy coat was used for the isolation of PBMCs.

In vitro proliferation assay

Using the method reported by Cuzzoni and colleagues,[18] the effect of MP, rapamycin and MP plus rapamycin on the proliferation of PBMCs was determined. The I_{250} was calculated and defined as the inhibition of proliferation achievable at 250 ng/mL concentration of MP. PBMCs were treated for 72 hours with MP 250 ng/mL, rapamycin 100 nmol/L and with the combination. Subjects were divided into two groups based on their individual response to MP and considered good or poor responders if their I_{250} values were respectively above or below the median of the whole population analyzed.

Quantitative real-time PCR (TaqMan)

The PBMCs treated as above were collected and preserved in RNAlater solution (Ambion, Austin, TX) at –80°C. RNA extraction using the purelink RNA Isolation Kit (Ambion, Life Technologies, Carlsbad, CA) was performed according to the manufacturer's instructions. The RNA concentration and purity were calculated by Nano Drop instrument (NanoDrop 2000, ThermoScientific, Wilmington, Delaware USA). Expression levels of GAS5 gene were evaluated by real-time RT-PCR TaqMan analysis using the CFX96 real-time system-C1000 Thermal Cycler (Bio-Rad Laboratories, Foster City, CA). The reverse transcription reaction was carried out with the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA) and the real-time PCR was performed in triplicate using the TaqMan Gene Expression Assay to assess GAS5 mRNA expression, according to the manufacturer's instructions. The expression levels of GAS5 were

evaluated using the comparative Ct method (2-ΔΔCt method). Ct values were corrected based on PCR efficiencies using LinRegPCR. The GAS5 expression values were normalized using the 18S as housekeeping gene.

Statistical analysis

Statistical analyses were performed using Graph-Pad Prism version 4.00 (GraphPad, La Jolla, CA). Two way ANOVA, Spearman correlation and t test were used for the analysis of inhibition of proliferation and gene expression between good and poor responders. *P* values < 0.05 were considered statistically significant.

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DISCOLSURE

The authors declare that they have no competing interests.

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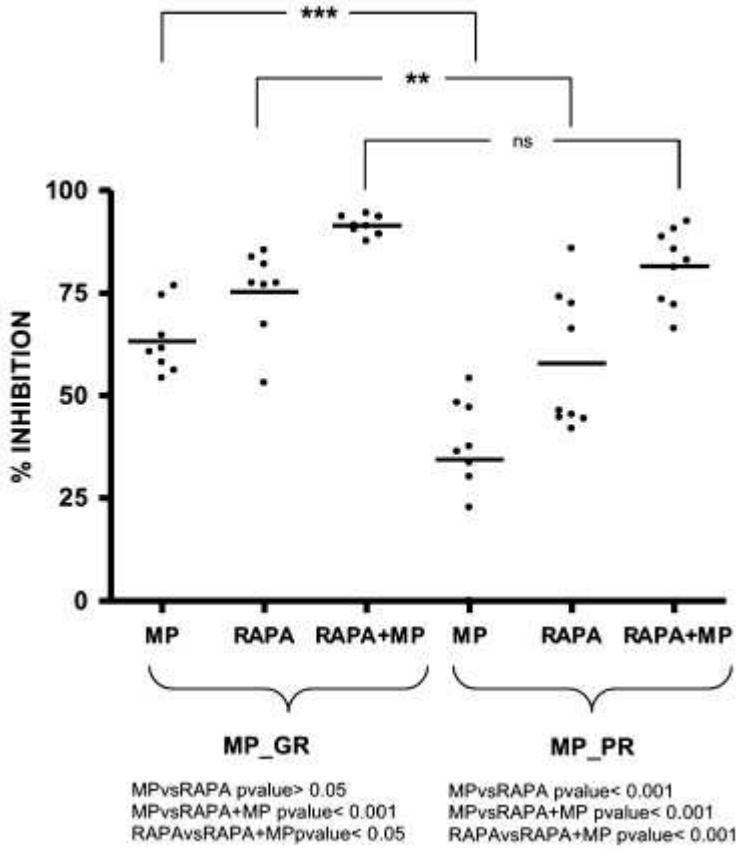

Fig. 1 Percent inhibition of proliferation in methylprednisolone (MP) good responder (MP_GR) and poor responder (MP_PR) groups after treatment for 72 hours with MP (250 ng/mL), rapamycin (RAPA, 100 nmol/L) and the combination (RAPA+MP). Two way ANOVA ***$P < 0.001$; ** $P < 0.01$.

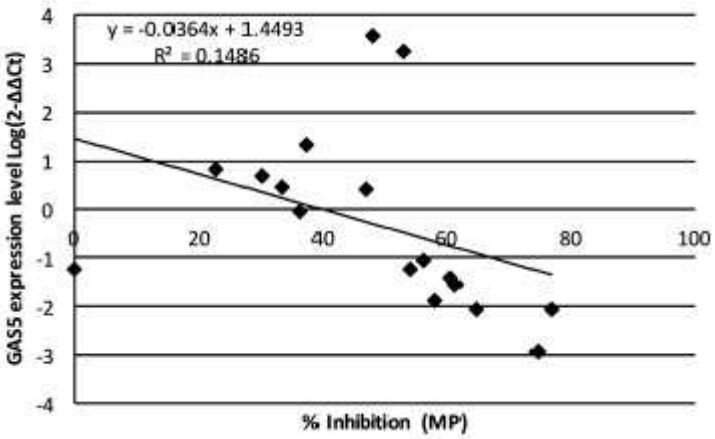

Fig. 2 Correlation between the percentage inhibition and the GAS5 expression level. Spearman rho = –0,73; $P = 0.0009$.

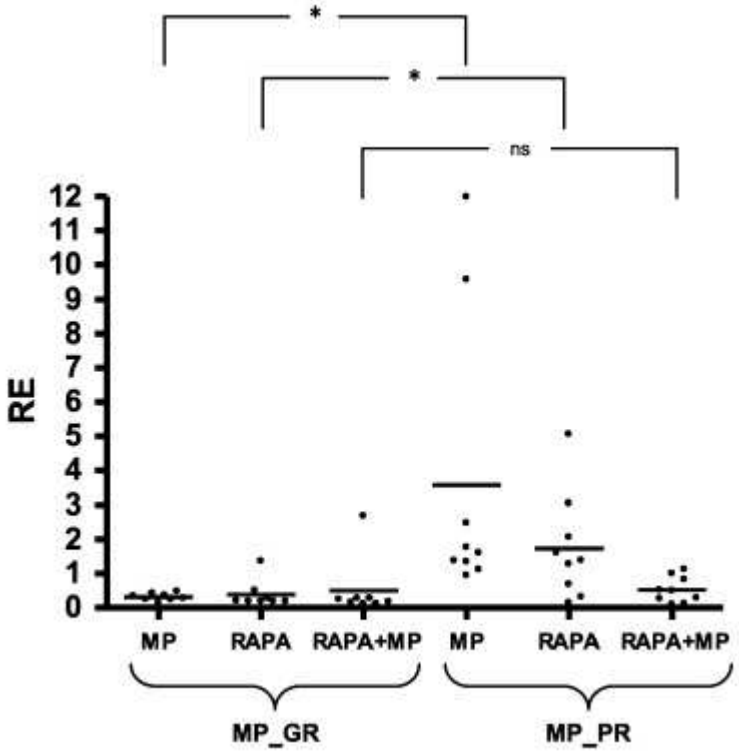

Fig. 3 Relative expression (RE) of GAS5 in methylprednisolone (MP) good responder (MP_GR) and poor responder (MP_PR) groups after treatment with MP (250 ng/mL), rapamycin (RAPA; 100 nmol/L) and in combination for 72 hours. t test $*P < 0.05$.