HSC70 blockade by the therapeutic peptide P140 affects autophagic processes and endogenous MHCII presentation in murine lupus

Nicolas Page, Frédéric Gros, Nicolas Schall, Marion Décossas, Dominique Bagnard, Jean-Paul Briand, Sylviane Muller

ABSTRACT

Background The P140 phosphopeptide issued from the spliceosomal U1-70K small nuclear ribonucleoprotein displays protective properties in MRL/lpr lupus-prone mice. It binds both major histocompatibility class II (MHCII) and HSC70/Hsp73 molecules. P140 peptide increases MRL/lpr peripheral blood lymphocyte apoptosis and decreases autoepitope recognition by T cells.

Objective To explore further the mode of action of P140 peptide on HSC70 + antigen-presenting cells.

Methods P140 biodistribution was monitored in real time using an imaging system and by fluorescence and electron microscopy. Fluorescence activated cell sorting and Western blotting experiments were used to evaluate the P140 effects on autophagic flux markers.

Results P140 fluorescence accumulated especially in the lungs and spleen. P140 peptide reduced the number of peripheral and splenic T and B cells without affecting these cells in normal mice. Remaining MRL/lpr B cells responded normally to mitogens. P140 peptide decreased the expression levels of HSC70/Hsp73 chaperone and stable MHCI dimers, which are both increased in MRL/lpr splenic B cells. It impaired refolding properties of chaperone HSC70. In MRL/lpr B cells, it increased the accumulation of the autophagy markers p62/SQSTM1 and LC3-II, consistent with a downregulated lysosomal degradation during autophagic flux.

Conclusion The study results suggest that after P140 peptide binding to HSC70, the endogenous (auto) antigen processing might be greatly affected in MRL/lpr antigen-presenting B cells, leading to the observed decrease of autoreactive T-cell priming and signalling via a mechanism involving a lysosomal degradation pathway. This unexpected mechanism might explain the beneficial effect of P140 peptide in treated MRL/lpr mice.

INTRODUCTION

Systemic lupus erythematosus is a multifactorial (multigenic) and highly polymorphic systemic autoimmune disorder. It affects multiple organs, including skin, muscle, joints and vital internal organs such as kidneys and heart. Current pharmaco- logical treatments are largely palliative and result in non-specific immunosuppression. The goal today is to design more specific and effective treatments.

P140 peptide is a 21-mer fragment of the spliceosomal U1-70K small nuclear ribonucleoprotein protein that significantly ameliorates clinical and biological manifestations in autoimmune patients with systemic lupus erythematosus and enhances survival in lupus-prone mice. This peptide contains a phosphoserine residue at position 140, a modification that specifically occurs at an early stage of apoptosis, before the cleavage of the C-terminal part of the protein by caspase-3 and the dephosphorylation of other serine residues by a PPI1 phosphatase-mediated mechanism. Originally thought to act solely via the modulation of intracellular signalling induced by αβ T-cell receptor (TCR) engagement, P140 was unexpectedly found to interact selectively with the constitutive heat-shock HSC70/Hsp73 protein. By a granzyme-B and caspase-dependent mechanism, it induces in Fas-deficient MRL/lpr lupus mice apoptosis of B cells and CD4, CD8 or double negative (DN) αβ TCR+CD4/CD8/B220+ T lymphocytes through a regulatory circuit involving γδ T cells. Intravenous P140 peptide administration into young MRL/lpr mice thus causes T- and B-cell egress from peripheral blood but does not affect T-cell priming, and does not interfere with the ability of P140-treated mice to resist to an infectious viral challenge. P140 also downregulates the expression of programmed death 1 (PD-1/CDC279) receptor, a molecule of the CD28 family, which is overexpressed at the surface of MRL/lpr CD4 T cells. P140 peptide thus displays various effects on the immune system that probably interfere with several, yet unidentified, molecular pathways leading finally to a decreased T-cell autoreactivity and to significantly lower levels of autoantibodies to native DNA.

In the postulated P140 mode of action, binding to the chaperone HSC70 protein seems to be central. The implication of or any defect of the HSC70 pathway in regulating immune functions in lupus is not documented. It is not known either if HSC70-chaperoned molecules, such as other Hsps and major histocompatibility complex (MHC) molecules, are affected by P140–HSC70 binding. To address these major questions, we evaluated the effect of P140 administration on the expression of HSC70 and MHCII molecules, and studied the possible influence of P140 on autophagosomal pathways implicated in the endogenous MHCII loading. Since HSC70 is involved in certain phases of these pathways, we expected that binding of P140 to HSC70 might alter the delivery of intracellular material into the endosomal/lysosomal pathway and therefore thwart the final presentation of self-antigens to autoreactive T cells.

METHODS

Synthetic peptides and biochemical assays
Peptides were assembled and purified as described. The P140 peptide was also synthesised...
with a cysteine residue added at its N-terminus. The luciferase renaturation assay was as described previously. The antibodies were anti-HSC70 antibody labelled with FITC (clone 2G9; BD Biosciences (Le Pont de Claix, France)). Fluorescence activated cell sorting analysis was as described previously. For co-localisation experiments, spleen sections were permeabilised and incubated with either fluorescein isothiocyanate (FITC)-labelled anti-CD3e antibody or biotin-labelled anti-CD4-allophycocyanin, anti-CD8-allophycocyanin, anti-B220-PerCP, anti-B220-allophycocyanin, anti-CD11b-PerCP and anti-Gr-1-FITC. HSC70 and MHCII levels were expressed as the ratio of relative mean fluorescence intensity for specific antibody/mean fluorescence intensity for isotype-matched control antibody.

RESULTS

In vivo P140 peptide effects and biodistribution

We reported previously that intravenous administration of P140 to MRL/lpr mice significantly improves their clinical and biological manifestations and prolongs their survival. We show here that P140 significantly decreases vasculitis with fewer perivascular inflammatory infiltrates in kidneys (figure 1A; \(p<0.0079\)), slows down glomerulonephritis (figure 1B; \(p<0.0001\)) and reduces the appearance of dermatitis (figure 1C).

P140 biodistribution after intravenous administration to MRL/lpr mice was monitored in real time by bioimaging in living mice. Alexa Fluor488-labelled P140 accumulated particularly in the lungs and spleen, a distribution that was not observed with an unrelated peptide (figure 1D; online supplementary figure S1). Similar results were obtained in 6- and 12-week-old MRL/lpr mice. Fluorescence in the spleen was detectable 10–15 min after P140 injection and remained for at least 90–120 min. Similar results were observed in MRL/lpr mice that received rhodamine-labelled P140 intravenously (supplementary figure S2A). In the spleen, fluorescence was mainly localised in the lymphoid white pulp, mostly in the mantle and marginal zones (supplementary figure S2B). It co-localised essentially with splenic B cells and resident macrophages (figure 1E) as well as with CD11b- and F4/80-positive cells. The macrophage F4/80 antigen is absent from macrophages located in T-cell areas of the spleen and lymph nodes (LNs). Thus, P140 preferentially homes in organs enriched in activated B cells and, more generally, in antigen-presenting cells (APCs).

P140 administration significantly decreased peripheral hypercellularity in MRL/lpr mice (supplementary figure S3; no effect was seen with the ScP140 scrambled analogue). B cells, T cells (including \(\gamma\delta\) T cells and DN T cells) and granulocytes/monocytes were affected. In CBA/J control mice, however, even an overdosage of P140 did not produce any observable effect while, in contrast, the glucocorticoid prednisolone, which is active in MRL/lpr mice, and ciclosporin A induced a decrease of PBL counts in both CBA/J and MRL/lpr mice (figure 1F). Peripheral B and T cells from non-treated MRL/lpr mice and remaining peripheral B and T cells from P140-treated mice responded equally well ex vivo to B-cell mitogen and slightly less well (\(p=0.024\)) to T-cell mitogen (figure 1G). No effect of P140 was observed in CBA/J mice. This was not the case for ciclosporin A, which significantly affected the abilities of peripheral B and T cells from MRL/lpr and CBA/J mice to respond to mitogens, and prednisolone, which affected the abilities of CBA/J B and T cells to respond to mitogens. In the spleen, the proportion of T cells (including activated T cells) was significantly decreased after P140 treatment (\(p=0.0022\); figure 1H). The percentage of B cells remained unchanged.

HSC70 and MHCII overexpression in MRL/lpr mice is reduced after P140 treatment

P140 binds to HSC70 protein both at 25°C and 4°C (figure 2A), suggesting that P140 recognises both intracellular and cell surface-expressed HSC70 (result obtained with splenocytes, LN cells and PBLs). Fluorescent staining of P140 and HSC70 co-localises in the cytoplasm of freshly isolated, permeabilised MRL/lpr PBLs 1 h after intravenous administration of Alexa Fluor488-labelled P140 (figure 2B). This co-localisation was confirmed by immunofluorescence and immunoelectron microscopy (figure 2B, C), and was mainly cytoplasmic.
Extended report

mice appeared to be correlated with an increased HSC70 mRNA expression in MRL/lpr splenocytes (figure 2E; not visualised in LN cells and thymocytes). Interestingly, in MRL/lpr mice that received intravenous injections of 100 μg P140 daily over 6 days, HSC70 expression in splenic B cells was decreased by 32% (figure 2F; expression of inducible Hsp70/Hsp72 protein was not affected).

Interaction of HSC70 with antigen is an important component of the MHCII antigen processing pathway. 10–14 In accordance with this, HSC70 is present at the surface of non-permeabilised MRL/lpr and CBA/J peripheral B and T cells (notably B220+/TCRβ+ activated T cells and DN T cells)2 as well as on splenic MRL/lpr B220−/CD3/CD11b+/MAC-1/Gr-1− monocytes/macrophages and B220−/CD3/CD11b+/Gr-1+ granulocytes (figure 2D1). In the spleen, surface and intracellular HSC70 expression levels were raised in MRL/lpr mice (figure 2D and supplementary figure S4; observed also in LN cells, notably at the surface). The HSC70 expression increase observed in the spleen of MRL/lpr mice appeared to be correlated with an increased HSC70 mRNA expression in MRL/lpr splenocytes (figure 2E; not visualised in LN cells and thymocytes). Interestingly, in MRL/lpr mice that received intravenous injections of 100 μg P140 daily over 6 days, HSC70 expression in splenic B cells was decreased by 32% (figure 2F; expression of inducible Hsp70/Hsp72 protein was not affected).

Interaction of HSC70 with antigen is an important component of the MHCII antigen processing pathway. 10–14 In accordance

Figure 1  In vivo effect and biodistribution of P140 peptide in MRL/lpr mice. (A, B) MRL/lpr mice received saline alone or 100 μg P140 peptide/injection/mouse on four occasions. Kidneys were collected from 25-week-old MRL/lpr mice and fixed in formalin. Haematoxylin and eosin-stained kidney paraffin sections were scored for (A) vasculitis (n=5/group; p=0.0046 between the two groups) and (B) glomerulonephritis grade (five mice/group, corresponding to 132–177 glomeruli). (C) Observation of skin lesions in both groups of 16-week-old mice. (D) In vivo biodistribution of Alexa Fluor633-labelled P140 peptide following retro-orbital injection of 100 μg peptide in NaCl (complete data in online supplementary figure S1). The yellow circle designates the position of the spleen. At the end of the experiment (180 min), organs were collected and fluorescence was measured in the isolated organs (1, thymus; 2, spleen; 3, lung; 4, salivary gland; 5, axillary lymph node). (E) Spleen sections were co-stained with antibodies to B220 and MOMA-1/CD169 to identify B cells and marginal zone metallophilic macrophages, respectively. (F) Peripheral blood lymphocyte (PBL) counts measured in 10-week-old MRL/lpr and CBA/J mice (n=3–4 mice tested individually/group) that received at daily intervals 10 intraperitoneal injections of P140 (100 μg/mouse), Scp140 (100 μg/mouse), ciclosporin A (20 mg/kg) or saline or that received prednisolone orally at a 4 mg/kg dose. (G) Effect of P140 overdosage on the ability of PBLs to respond to T- and B-cell mitogens. Cultures for 48 h in the presence of 3 μg/ml concanavalin A (ConA) or 5 μg/ml lipopolysaccharide (LPS). NT, not tested. (H) P140 peptide administration reduces the percentage of total CD3 T cells among splenocytes. Splenocytes from 6-week-old MRL/lpr mice were collected 3 days after a single intraperitoneal injection of 200 μg P140 or saline (n=6/group). Representative results of two independent experiments are shown. *p<0.05, **p<0.01, ***p<0.001. NS, not significant (Mann–Whitney U test in panels A, B, F and H; Student t test in panel G). Bars, 100 μm.
lymphoblast-like cells (figure 3C; MHCII monomer expression remained unaffected). Thus, P140 associates with both HSC70 and MHCII molecules and decreases directly or indirectly their surface expression on splenic B cells.

P140 alters autophagic processes in MRL/lpr B cells

It has been shown earlier in B-cell lines that manipulating APC expression of HSC70 modifies the presentation of epitopes derived from intracellular proteins but not from exogenous antigens to CD4 T cells. Since HSC70 is involved in autophagy pathways, notably in chaperone-mediated autophagy, leading to endogenous MHCII loading, we explored this mechanism, hypothesising that P140 might disrupt normal functions of HSC70 in this catabolic pathway. First, using a refolding test of heat-denatured luciferase by rabbit reticulocyte lysate, we with observations showing that in B cells HSC70 associates with HLA-DRB1*0401, and that HSC70 and MHCII molecules are associated in B-cell exosomes, for example, we found that HSC70 and MHCII molecules staining co-localises in permeabilised MRL/lpr splenocytes (figure 3A and supplementary figure S5). After in vitro incubation, co-localisation of P140 and HSC70 staining was observed in 25% of cells, and co-localisation of P140, HSC70 and MHCII molecules staining was visualised in 5–10% of cells. Interestingly, we found that after daily intraperitoneal injections of P140 into 12-week-old MRL/lpr mice, overexpression of MHCII molecules in spleen B cells significantly decreased (figure 3B). We thus examined further the effect of P140 on the stability of functional MHC molecules using Raji cells. In a dose-dependent manner, P140 hampered the formation of stable HLA-DRαβ dimers in these lymphoblast-like cells (figure 3C; MHCII monomer expression remained unaffected). Thus, P140 associates with both HSC70 and MHCII molecules and decreases directly or indirectly their surface expression on splenic B cells.

Figure 2  P140 peptide interacts with HSC70 and, in vivo, diminishes HSC70 overexpression in MRL/lpr lymphocytes. (A) Pull-down experiments showing the interaction of P140 peptide with HSC70 at 25°C and 4°C (representative results of five independent experiments). (B1) Co-localisation in permeabilised MRL/lpr dissociated splenocytes of P140 peptide and HSC70 examined 1 h after retro-orbital injection of 100 μg Alexa Fluor488-labelled P140 in NaCl. (B2) Co-localisation of P140 and HSC70 on spleen sections from MRL/lpr mice that received intravenously 200 μg rhodamine-labelled P140. (C) Co-localisation of P140 and HSC70 staining shown by immunoelectron microscopy (example with a single cell). HSC70, 15 nm gold particles; P140, 6 nm gold particles. (D) Fluorescence activated cell sorting analysis showing HSC70 expression at the surface of (D1) or intracellularly (D2) in different spleen cell subsets from CBA/J and 12-week-old MRL/lpr mice (n=5): B cells (B220⁺CD3), activated T cells included double negative T cells (B220⁺CD3), naïve T cells (B220⁻CD3), granulocytes/macrophages (CD11b⁺Gr-1⁻). HSC70 levels were expressed as the mean fluorescence intensity (MFI) for specific antibody/MFI for isotype-matched control antibody (Ab) ratio. Error bars represent SD. Representative results of at least two independent experiments. (E) Levels of HSC70 mRNA detected by qRT-PCR in lymph node, splenic and thymic cells from 6- and 12-week-old MRL/lpr mice compared with CBA/J mice. Mean of two experiments in triplicate, normalised to actin-β expression. (F) Intracellular staining of HSC70 and Hsp70 in spleen B cells collected from untreated or P140-treated (six times at daily intervals; 100 μg/injection/mouse) 6-week-old female MRL/lpr mice. *p<0.05, **p<0.01, ***p<0.001 (Student t test). Bars = 100 μm, except in (C) where bars = 500 and 100 nm in the picture and inset, respectively.
Representative results of two independent experiments. DRβ control. dimers or MHCII DRαβ were analysed by Western immunoblotting for expression of MHCII -mercaptoethanol. Cell lysates P140 and then lysed with or without β in human lymphoma B cells. Raji cells were incubated for 24 h with fluorescence intensity. (C) P140 alters the stability of MHCII dimers determined using the Student t test. *p<0.05, ***p<0.001. MFI, mean fluorescence intensity. (C) P140 alters the stability of MHCII dimers in human lymphoma B cells. Raji cells were incubated for 24 h with P140 and then lysed with or without β-mercaptoethanol. Cell lysates were analysed by Western immunoblotting for expression of MHCII DRβ dimers or MHCII DRα chain, compared with actin-β control. Representative results of two independent experiments.

examined in vitro whether P140 could hamper folding properties of the HSC70 molecule. At 40 and 80 μM, P140 inhibited luciferase refolding by 50.2% and 100%, respectively (figure 4A; ScP140 showed no effect). This effect was reversed by adding an excess of exogenous HSC70 (figure 4B).

Next, we examined P140-treated cells for altered expression of lysosomal or autophagy pathways markers. We studied the conversion in MRL/lpr cells of the soluble form of microtubule-associated protein-1 light chain 3 (LC3-I), a mammalian homologue of yeast Atg8, into the hydrophobic, mostly membrane-associated form LC3-II, which is enriched in the autophagic vacuole fraction but is rapidly degraded by lysosomal proteases when the autophagic flux is intense.19 We observed that splenic B cells purified from 8-week-old MRL/lpr mice exhibited almost undetectable levels of LC3-I and rather low LC3-II levels, which were dramatically enhanced in the presence of lysosomal protease inhibitors E64D (calpain and cathepsin B inhibitor) and pepstatin A (cathepsin D inhibitor) (figure 5A). This reflects the existence of a constitutive autophagic flux in resting MRL/lpr B cells that can be blocked by specific inhibitors before the autolysosome formation, at the fused autophagosome–lysosome step.19 In the presence of P140, the basal accumulation of LC3-II expression was increased in a P140 concentration-dependent manner, and strikingly, the autophagic flux was proportionally less intense (figure 5A). This suggests that in the presence of P140 B cells, P140 downregulates autophagic flux at the autolysosome stage. Interestingly, this stage is also affected by E64D and pepstatin A, for example, as well as by chloroquine,20 21 an antimalarial drug used in lupus treatment.

This result was confirmed by measuring the levels of p62 (SQSTM1/sequestosome-1), one of the specific substrates that are degraded through the autophagy-lysosomal pathway. p62 is selectively incorporated into autophagosomes via direct interaction with LC3.22 23 In a peptide dose-dependent manner, p62 accumulated in P140-treated MRL/lpr B cells (figure 5B; the same effect, although at a lower extent, observable in CBA/J B cells), again consistent with an effect of P140 on the steady-state autophagy in these cells.

DISCUSSION

We present evidence here that P140 seems to interfere with a signalling pathway engaged in lysosomal degradation, leading to instability of MHCII molecules supposed to present endogenous antigens to autoreactive T cells in murine lupus. After intravenous injection into MRL/lpr mice, P140 concentrates mainly in the lungs and the spleen, and in the latter it co-localises essentially with B cells and resident macrophages of the lymphoid white pulp. Its localisation (if any) is poor in LNs and thymus. This suggests that P140 might possess a certain degree of specificity for targeting some but not all tissues/ organs containing HSC70+ cells, and within these tissues and organs, again, it seems to target certain, but not all, HSC70+ cell subsets.

We found that HSC70, which is present at the surface of splenic and LN MRL/lpr and CBA/J B cells, T cells (activated,
and B and T cells proliferate normally in response to specific mitogens. We also found that the remaining B and T cells from P140-treated mice responded normally to mitogens. These data add to earlier studies showing that P140-treated and untreated MRL/lpr mice mount equivalent immune responses to a flu virus challenge.6

HSC70 protein displays numerous housekeeping chaperoning functions. It is notably involved in nascent protein folding, prevention of protein aggregation under stress conditions, modulation of the assembly/disassembly of protein complexes and stabilisation of RNA messengers. HSC70 protein acts with a series of co-chaperones including Nag-4, CHIP, hip, Bcl-2-associated athanogene BAG-1, BAG-3 and Hsp40. Studies have suggested that depending on the cytokine environment, HSC70 preferentially associates with certain of these co-chaperones leading to different effects. We found that P140 inhibits the ability of HSC70 to refold denatured protein. It might therefore disrupt certain interactions of HSC70 with cofactors and chaperones. Future studies should provide insight into this topic and determine if this is relevant to some effects described in this study and lupus phenotype.

HSC70 is central in protein translocation across membranes and lysosomal transport of proteins.24 25 It plays a pivotal role in chaperone-mediated autophagy in acting as a chaperone for unfolded substrate protein in association with the membrane receptor Lamp2a.14 16 18 26 In certain conditions, such as an elevation of co-chaperone BAG-3 level, HSC70 can also associate with p62.27 Proper turnover of p62 by autophagy is critical, notably to prevent spontaneous aggregate formation.23 26 31 This protein is regarded as a key signalling player in the cell survival/death balance. Thus, certainly the most remarkable information raised in this work, is the striking effect of P140 on lysosomal degradation and class II antigen presentation (figure 5C). P140 directly impairs the molecular chaperoning properties of HSC70 by altering its ability to promote proper folding of polypeptide. In MRL/lpr B cells, P140 increases the accumulation of the autophagy markers p62/SQSTM1 and LC3-II, consistent with a downregulation of lysosomal degradation at the autolysosome stage or with a lysosomal dysfunction. Moreover, the expression of stable MHCII molecules is dramatically affected. This might explain our earlier observations showing a decreased T-cell response to various T-cell autoepitopes in P140-treated MRL/lpr mice6 32 and the absence of lupus patient’s PBL proliferation ex vivo in the presence of P140.4

Autophagy processes have not been explored in lupus. Although much has still to be determined to fully understand the impact of this complex pathway in MHCII presentation, inflammation and central or peripheral tolerance processes, our studies show that a peptide that can modulate lysosomal degradation can be beneficial in MRL/lpr mice by reducing autoreactive T-cell and B-cell activation. Of importance, P140 peptide exerts its effects without affecting normal B and T cells, possibly thanks to the expression levels of HSC70, which seems to act as a sensor receptor on MRL/lpr B cells.

Acknowledgements The authors are indebted to Monique Duval for valuable assistance. The authors acknowledge the Imaging facility (Plateforme Inter-Unités de Microscopie et d’Imagerie Cellulaire Strasbourg-Esplanade).

Funding This research was funded by the Centre National de la Recherche Scientifique (CNRS), Région Alsace and ImmuPharma France.

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.
REFERENCES


HSC70 blockade by the therapeutic peptide P140 affects autophagic processes and endogenous MHCII presentation in murine lupus

Nicolas Page, Frédéric Gros, Nicolas Schall, Marion Décossas, Dominique Bagnard, Jean-Paul Briand and Sylviane Muller

Ann Rheum Dis 2011 70: 837-843 originally published online December 20, 2010
doi: 10.1136/ard.2010.139832

Updated information and services can be found at:
http://ard.bmj.com/content/70/5/837

These include:

Supplementary Material
Supplementary material can be found at:
http://ard.bmj.com/content/suppl/2010/11/15/ard.2010.139832.DC1

References
This article cites 32 articles, 10 of which you can access for free at:
http://ard.bmj.com/content/70/5/837#BIBL

Open Access
This paper is freely available online under the BMJ Journals unlocked scheme, see http://ard.bmj.com/info/unlocked.dtl

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections
Articles on similar topics can be found in the following collections

Open access (605)
Immunology (including allergy) (5144)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/