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

Nicolas Page,1 Frédéric Gros,1 Nicolas Schall,1 Marion Décossas,1 Dominique Bagnard,2 Jean-Paul Briand,1 Sylviane Muller1

ABSTRACT

Background The P140 phosphopeptide issued from the spliceosomal U1-70K small nuclear ribonucleoprotein protein 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 MHCII 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 pharmacological 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.1-2 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 PP1 phosphatase-mediated mechanism.3 Originally thought to act solely via the modulation of intracellular signalling induced by αβ autoreactive T-cell receptor (TCR) engagement,2,4 P140 was unexpectedly found to interact selectively with the constitutive heat-shock HSC70/Hsp75 protein.5 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) αβTCR+CD4/CD8/B220+ T lymphocytes through a regulatory circuit involving ΔΥT cells.6 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.5,7 P140 also downregulates the expression of programmed death 1 (PD-1/CDD279) receptor, a molecule of the CD28 family, which is overexpressed at the surface of MRL/lpr CD4 T cells.8 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.1,5 The P140 peptide was also synthesised...
with a cysteine residue added at its N-terminus. The luciferase
renaturation assay was as described7 8 using either intact or
heat-denatured luciferase and rabbit reticulocyte lysate in
the presence of increasing concentrations of P140 peptide.
Luciferase activity was determined using Bright-glo reagent
(Fromega, Charbonnières, France).

In vivo experiments and cell studies
Protection experiments and clinical monitoring have been
described previously.1 All experimental protocols were carried
out with the approval of the local Institutional Animal Care
and Use Committee (CREMEAS). The renal pathology was evaluated
by measuring proteinuria levels and observing vasculitis corre-
sponding to the area of cell infiltrates. For in vivo imaging, Alexa
Fluor655-labelled P140 was injected through the retro-orbital
venous sinus of anaesthetised mice. Mice were immediately
transferred into the cabinet of the NightOwl Bioimager (Berthold
Technologies, Thoiry, France) and images were taken periodi-
cally for 3 h. Mice were then killed to collect organs for final
fluorescence acquisition and validation of biodistribution. Signal
intensity was evaluated using the Indigo software.

Histology was carried out on frozen section of organs collected
from MRL/lpr mice that received rhodamine-labelled P140 intra-
venously. For co-localisation experiments, spleen sections were
permeabilised and incubated with either fluorescein isothiocya-
nate (FITC)-labelled anti-B220 antibody or biotin-labelled anti-
CD3e antibodies, followed by Alexa Fluor670-labelled streptavidin,
or biotin-labelled anti-MOMA-1/CD169 antibodies specific for
metallophilic macrophages, followed by Alexa Fluor488-labelled
streptavidin. Other antibodies tested were: biotinlabelled anti-
CD11b and biotin-labelled anti-F4/80 antibodies.

Immunofluorescence and immunoelectron microscopy
experiments were performed on peripheral blood lymphocytes
(PBLs) and splenocytes collected from MRL/lpr mice that had
received Alexa Fluor680-labelled P140 intravenously in 1 h before
examination. Some experiments used splenocytes incubated for
1 h at 37°C with 5 μM Alexa Fluor680-labelled P140. HSC70 and
MHCII molecules were revealed with specific antibodies (clone
1B5; Stressgen Enzo Life Sciences, Villeurbanne, France) and
2G9; BD Biosciences (Le Pont de Claix, France) respectively.
Fluorescence activated cell sorting analysis was as described
previously.5 The antibodies were anti-HSC70 antibody labelled
with R-phycocerythrin (clone 1B5) and anti-1A/1E antibody
labelled with FITC (clone 2G9). For specific analysis of cellular
subtypes, antibodies used were: anti-CD3e-FITC, anti-CD3e-
allophycocyanin, anti-B220-PerCP, anti-B220-allophycocyanin,
anti-CD4-allophycocyanin, anti-CD8-allophycocyanin, anti-
CD11b-PerCP and anti-G1-1-FTTC. 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.

For measuring lymphocyte proliferation, MRL/lpr and CBA/J
mice (four to five/group) were given P140 peptide or ScP140
(100 μg/mouse) or saline only, daily for 10 consecutive days
via the intraperitoneal route. Ciclosporin A (20 mg/kg/day) and
prednisolone (feeding at 4 mg/kg/day) were tested in parallel.
Ciclosporin A (20 mg/kg/day) and (100

mice (four to five/group) were given P140 peptide or ScP140
or biotin-labelled anti-MOMA-1/CD169 antibodies specifi c for
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,9 and immunosuppressant
ciclosporin A induced a decrease of PBL counts in both CBA/J
and MRL/lpr mice (fi gure 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 mito-
gen (fi gure 1G). No effect of P140 was observed in CBA/J mice.
This was not the case for ciclosporin A, which signifi cantly
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 signifi cantly decreased after P140 treat-
ment (p=0.0022; fi gure 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 (fi gure 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-localised in the cytoplasm of freshly isolated, permeabi-
lised MRL/lpr PBLs 1 h after intravenous administration of
Alexa Fluor680-labelled P140 (fi gure 2B1). This co-localisation
was confirmed by immunofluorescence and immunoelectron
microscopy (fi gure 2B2, C), and was mainly cytoplasmic.
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 $\mu$g 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 HSC70 is present at the surface of non-permeabilised MRL/lpr and CBA/J peripheral B and T cells (notably B220+/TCR$\beta$+ activated T cells and DN T cells) as well as on splenic MRL/lpr B220–/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 $\mu$g 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).

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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-localised in permeabilised MRL/lpr splenocytes (figure 3A and supplementary figure S5). After in vitro incubation, co-localisation of P140 and MHCII molecules 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.

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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. 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. 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, the basal accumulation of LC3-II expression was increased in a P140 concentration-dependent manner.

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-locates 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,
treated individual. Autoreactive CD4 T-cell response in a beneficial pathway for the P40- and class II MHC molecules are destabilised, therefore compromising of antigens into MIIC compartments for class II presentation is altered antigens processing by lysosomal hydrolysis. (4) Subsequent delivery chaperone-mediated autophagy (CMA) leading to the alteration of (auto) (putative) and slows down the autophagic flux, and/or (3b) affects complex of co-chaperones involved in the regulation of macroautophagy in acting as a chaperone for unfolded substrate protein in association with the membrane receptor Lamp2a. In certain conditions, such as an elevation of co-chaperone BAG-3 level, HSC70 can also associate with p62. Proper turnover of p62 by autophagy is critical, notably to prevent spontaneous aggregate formation. 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 mice and the absence of lupus patient’s PBL proliferation ex vivo in the presence of P140.

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.

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REFERENCES


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