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Letter to the Editor

Role of p56^{lck} and ZAP70-mediated tyrosine phosphorylation in galectin-1-induced cell death

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Dear Editor,

Galectins belong to the family of evolutionary conserved β-galactoside binding animal lectins. One of the members of the galectin family, galectin-1 (gal-1), plays roles in several biological processes such as immunomodulation, cell adhesion, regulation of cell growth and apoptosis.[1] The immunoregulatory effect, at least in part, is mediated by the induction of apoptosis of activated peripheral T cells.[2] The intracellular pathway of T-cell death involves the hyperpolarization of the mitochondria,[3] caspase activation, Bcl-2 downregulation and activation of AP-1 transcription factor.[4,5] Gal-1 treatment induces partial T-cell receptor ζ (TCRζ) chain phosphorylation, generating pp21ζ and limited receptor clustering at the TCR contact site,[6] and hence it antagonizes with the TCR signal transduction and promotes apoptosis.

Recent studies showed that T cells respond with tyrosine phosphorylation to gal-1 treatment;[6,7] however, the kinase(s) mediating this tyrosine phosphorylation and the biological significance of this early event has not been identified. Since the role of p56^{lck} has been demonstrated in ceramide[8] and mitochondrial[9] pathways of apoptosis and these pathways were indicated in gal-1-induced cell death,[3] we analyzed the involvement of Lck and its immediate target ZAP70 in gal-1 cytotoxic effect.

We previously showed that gal-1 induced tyrosine phosphorylation in Jurkat cells[7] with a maximum of 10–20 min, and then reverted to the baseline level (data not shown). The tyrosine kinase inhibitor genistein blocked the gal-1-stimulated protein tyrosine kinase activity (Figure 1a), since in the presence of the inhibitor gal-1 did not cause phosphorylation over the nonstimulated control. The phosphorylation step was significant in the process of cell death, as genistein or another tyrosine kinase inhibitor herbimycin A did not only inhibit the tyrosine phosphorylation but also the formation of the apoptotic 'sub-G1' cells (Figure 1b).

Whether or not Lck and one of its main immediate targets, ZAP70, were the responsible kinases in gal-1-induced cell death, the Lck-deficient Jurkat cells, JCaM1.6 and ZAP70 mutant, P116 were treated with gal-1. The treatment of these mutant cells did not result in tyrosine phosphorylation over the untreated control (Figure 1c). Examination of the kinase activity demonstrated that Lck and ZAP70 activities were increased upon gal-1 stimulation (Figure 1d). Phosphatidyl serine (PS) failed to be externalized (Figure 1e) in Lck- and ZAP70-deficient cells. Re-expression of the Lck (JCaM/LCK) and ZAP70 (P116WT) in JCaM1.6 and P116, respectively, restored PS exposure (Figure 1e). Unresponsiveness of Lck- and ZAP70-deficient Jurkat cells for gal-1 stimulation was not due to a general inability to respond to apoptotic signals since these cells died readily upon TNF treatment (Figure 1f), a pathway independent of Lck[8] and ZAP70.[10]

The induction of tyrosine kinase activity is essential for the further events. The tyrosine phosphorylation is attributed to p56^{lck} and ZAP70 since the deficiency in these enzymes abolishes the gal-1-induced cell death and restoration of Lck and ZAP70 expression restores apoptosis. Although the contribution of Lck to ceramide and mitochondrion-mediated apoptotic processes has been recently confirmed,[8,9] the immediate receptor(s) mediating Lck activation and the direct target(s) of Lck activity have not yet been identified. The involvement of ZAP70 suggests that it can be at least one of its targets. The function of the Lck/ZAP70 kinases has been supported by the recent finding that upon gal-1 treatment, the TCRζ chain is partially phosphorylated, a biochemical step that occurs during T-cell apoptosis via this pathway.[6] This observation underlines a potential mechanism for ZAP70 activation and the subsequent tyrosine phosphorylation: Accordingly, gal-1 binds to a not yet identified receptor, and as a consequence, induces the coclustering of Lck and TCRζ chain. Lck phosphorylates TCRζ polypeptide, which in turn serves as a docking site for ZAP70 and activates this kinase. The participation of Lck/ZAP70 pathway in the gal-1-induced apoptosis suggests the feasible involvement of TCR as a mediator of gal-1 effect. However, our results do not confirm it since TCR-negative CEM cells (TCR^{neg}) or T-cell lines expressing different amounts of TCR such as MOLT-4 (TCR^{low}), Jurkat (TCR^{medium}) or HPB-ALL (TCR^{high}) respond equally well to gal-1 cytotoxic effect (data not shown). Moreover, the pattern and kinetics of the tyrosine phosphorylation are well distinguishable from those of TCR stimulation (data not shown). It has to be mentioned that Vespa *et al*[6] showed that Lck activity was not required for gal-1-induced apoptosis in CD4$^-$/CD8$^-$ murine T-cell hybridoma. The disagreement between our and their results may lie on the different experimental systems. Briefly, they used a T-cell line transfected with constitutively active or inactive kinase, while we used Lck-nonexpressing and Lck-retransfected Jurkat cells. Moreover, Vespa *et al* did not show whether gal-1 induced the tyrosine phosphorylation in cells expressing kinase negative enzyme. Most importantly, they used 10 times more gal-1 (20 μM) as we did (1.8 μM) and the much higher concentration of gal-1 may initiate the apoptosis through a distinct receptor(s) on a different signaling route.

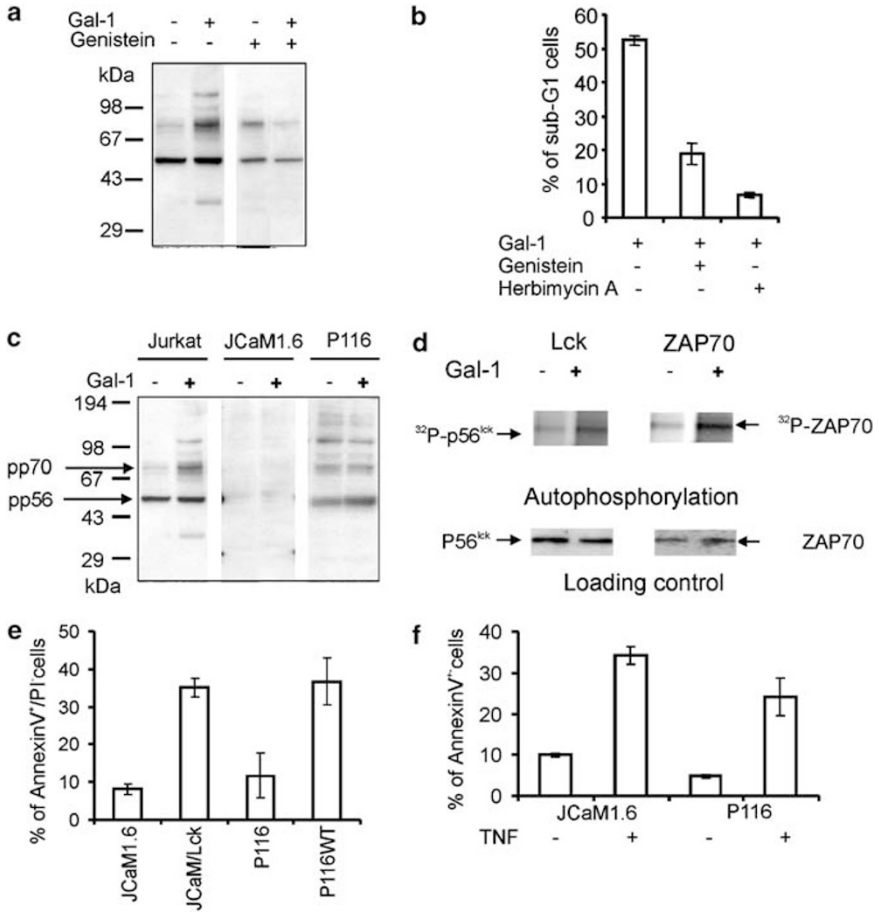

Figure 1 Jurkat (**a**–**d**), JCaM1.6 (Lck-deficient Jurkat), JcaM/Lck (Lck-retransfected JCaM1.6), P116 (ZAP70-deficient Jurkat) and P116WT (ZAP70-retransfected P116) (**c**, **e** and **f**) cells were stimulated with or without 1.8 μM recombinant gal-1 (**a**–**e**) or with 50 ng/ml TNFα and 1.5 μg/ml CHX (**f**) in the presence or absence of 250 μM (**a**) or 75 μM genistein or 1 μM herbimycin A (**b**) for 15 min (**a**, **c** and **d**), 12 h (**e** and **f**) or 24 h (**b**). For analysis of tyrosine phosphorylation, the cells were lysed in RIPA buffer (25 mM HEPES (pH 7.4), 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 125 mM NaCl, Phosphatase inhibitor cocktail (1 : 100 dilution), 10 μg/ml leupeptin and 1 mM PMSF) (**a**, **c** and **d**)). The proteins in postnuclear supernatants were separated on a 7.5–15% gradient of SDS-PAGE (**a** and **c**) and transferred to nitrocellulose membrane (Schleicher & Schuell). The membranes were blocked using Tris-buffered saline (TBS) containing 0.05% Tween-20 and 3% cold fish gelatin and probed with antiphosphotyrosine mAb, 4G10 (Upstate Biotechnology Inc.) and rabbit anti-mouse IgG-HRP (DAKO). Immunoreactive proteins were visualized by ECL plus detection system (Amersham Bioscience). (**d**) *In vitro* kinase assay was carried out by mixing the cell lysates with 10 μg rabbit anti-ZAP70 (produced in our laboratory) or 4 μg rabbit anti-Lck (Santa Cruz Biotechnology) for 16 h at 4°C. Then, protein G–Sepahrose (Pharmacia) (30 μl/sample) was added for 3 h at 4°C. The immunoprecipitates were washed with lysis buffer followed with kinase buffer without dithiotreitol (DTT) (25 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM $MnCl_2$, 100 μM Na_3VO_3, 5 mM $MgCl_2$). For the kinase assay, the beads were suspended in 30 μl kinase buffer supplemented with 5 mM DTT and 10 μCi [^{32}P]ATP (Isotpe Inst. Kft, Budapest, Hungary) and incubated for 20 min at 37°C. Autophosphorylation was stopped adding SDS sample buffer, and after boiling for 5 min, the supernatants were loaded onto 10% SDS-PAGE. After blotting, the radioactivity was analyzed with Phosphorimager 445 SI (Molecular Dinamics). Later the same filter was analyzed with anti-ZAP70 (BD Transduction Laboratory) and anti-Lck (produced in our laboratory) mAbs followed with mouse anti-IgG-HRP and visualized with ECL. (**b**) 'Sub-G1' cell population was determined by permeabilizing and staining the cells with 0.1% Triton X-100, 0.1% Na_3 citrate, 10 μg/ml RNase and 10 μg/ml propidium iodide and analyzed with cytofluorimetry (FACSCalibur, Becton and Dickinson). (**e** and **f**) To determine PS exposure, the cells were labeled with Annexin V–FITC (Pharmingen) for 15 min at room temperature and analyzed with cytofluorimetry. The results for (**b**, **e** and **f**) are shown as mean values of three samples $\pm$ S.D. Reagents not specified were purchased from Sigma

Based on the presented data, we propose that an early response triggered by gal-1 in T cells is the induction of tyrosine phosphorylation, a step that is indispensable for the execution of apoptosis. This event requires the function of p56*lck* and ZAP70 in completing tyrosine phosphorylation.

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