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Human galectin-1 triggers apoptosis via ceramide mediated mitochondrial pathway

Summary of Ph.D. thesis

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Introduction

Galectin-1 belongs to the family of β-galactoside binding animal lectins, galectins. Secreted galectin-1 plays roles in several biological processes such as immunomodulation, cell adhesion, regulation of cell growth and apoptosis. The immunoregulatory effect, at least in part, is mediated by the induction of apoptosis of activated peripheral T cells, particularly the Th1 subpopulation at inflammatory sites. The mechanism of the galectin-1 induced apoptosis is poorly defined, yet. It is still not clear which receptor transmits the apoptotic signal into T cells. It appears to be distinct from Fas/FasL pathway as it has been shown in a Fas resistant T cell line and Fas deficient *lpr* mice. The galectin-1-binding surface glycoproteins (CD2, CD3, CD7, CD43 and CD45) have been presumed as candidates. However, only CD7 seems to fulfill the requirements for a transmitting receptor, as the absence of CD7 correlates with the failure of galectin-1 induced apoptosis, and complementation of CD7 restores the cell death. The CD45, initially described as apoptotic receptor for galectin-1 has recently been shown to be dispensable for this process. The intracellular pathway involves caspase activation, Bcl-2 downregulation and activation of AP-1 transcription factor. Galectin-1 treatment induces partial TCRζ chain phosphorylation, generating pp21ζ and limited receptor clustering at the TCR contact site and hence it

antagonizes with the TCR signal transduction and promotes apoptosis. On the other hand, galectin-1 induces tyrosine phosphorylation in BI-141 T cells and synergizes with anti-CD3 signals in dramatically up-regulating ERK activity and inducing apoptosis.

The sphingolipid ceramide is frequently generated during cellular stress and apoptosis, though the exact role of the ceramide liberation is controversial in apoptotic pathways induced by various stimuli, such as TNF or FasL. It can be a consequence of the scrambling of the membrane asymmetry and the subsequent translocation and activation of a sphingomyelinase. In the absence of the increase of ceramide expression, the apoptosis (decrease of mitochondrial membrane potential and DNA degradation) may still be executed.

Aims

In spite of the well-documented fact that galectin-1 triggers apoptosis on activated T cells and T cell lines, the apoptotic pathway is not elucidated yet. In order to get new data about the galectin-1 apoptotic mechanism we investigated the following aspects:

- What is the chronology of individual apoptotic steps during

galectin-1 induced cell death?

- What signaling molecules are involved in galectin-1 triggered apoptosis? What is the significance of tyrosine phosphorylation and ceramide release in this process?
- What is the entry-site of the galectin-1 death signal?

Materials and Methods

➢ **Detection of galectin-1 binding activity.** Cells were incubated in RPMI containing 1.8 μM galectin-1 then they were stained with anti-galectin-1 followed by streptavidin-FITC and analysed on FACSCalibur cytofluorimeter.

➢ **Detection of tyrosine phosphorylation.** The cells were stimulated by adding 1.8 μM galectin-1. After lysis the cell lysate was separated on SDS polyacrylamide gel and then transferred to nitrocellulose membrane. The membranes were subsequently probed with anti-phosphotyrosine antibody and rabbit anti-mouse IgG-HRP.

➢ **Annexin V labeling.** The exposure of phosphatidyl serine on the outer leaflet of plasma membrane was analyzed in flow cytometry after staining with Annexin V.

➢ **Immunofluorescence staining of intracellular ceramide**

After treatment, the cells were settled by cyto-centrifugation, fixed, permeabilized and labeled with anti-ceramide mAb. After staining

with streptavidin-FITC the cells were analyzed on a Carl Zeiss (Axioskop 2 Mot) fluorescence microscope using a camera (AxioCam) and software (AxioVision 3.1). The contrasts of the images were adjusted using CorelDRAW 10.

Inhibition of ceramide release with BSA: For BSA extraction the cells were incubated twice with 5% BSA for 5 min on ice. Subsequently the cells were cultured in RPMI 1% FCS with or without 5% BSA.

- **Loss of mitochondrial potential** After treatment, the cells were loaded with MitoTracker Red CMX-Ros and the fluorescence intensity was measured on FACSCalibur.
- **Detection of caspases activity.** After galectin-1 treatment the Caspase-Glo^{TM} 9 or Caspase-Glo^{TM} 3 was added to the samples and caspase 9 and 3 activity were detected using LuminoScan plate-reading luminometer.
- **Detection of PARP proteolysis.** The cell lysate was separated on SDS polyacrylamide gel and then transferred to nitrocellulose membrane, probed with mouse anti-PARP and subsequently stained with rabbit anti-mouse IgG-HRP. Immunoreactive proteins were visualized by ECL plus detection system.
- **Hypodiploid, 'sub-G1' cell population.** Galectin-1 treated cells were permeabilized and stained with DNA staining buffer.

The sub-G1 (hypodiploid) population was determined with cell cycle analysis using CELLQuest software programs (Becton Dickinson) and was considered as apoptotic cells.

Results and Discussion

The intracellular steps upon galectin-1 stimulation showed the following chronology: tyrosine phosphorylation preceded PS exposure and elevation of the intracellular ceramide level that was followed by the decrease of the mitochondrial membrane potential then caspases were activated and finally the nuclear DNA was degraded.

- The induction of tyrosine kinase activity was essential for the further events. The tyrosine phosphorylation was attributed to $p56^{lck}$ and ZAP70 since the deficiency in these enzymes abolished the galectin-1 induced cell death and restoration of Lck and ZAP70 expression restored apoptosis. Although the contribution of Lck to ceramide and mitochondrion mediated apoptotic processes has been recently proven, the immediate targets of Lck activation have not yet been identified. The involvement of ZAP70 suggests that it can be at least one of its targets.
- The failure of induction of ceramide release by galectin-1 in Lck, or ZAP70 deficient cells or in the presence of BSA resulted in the collapse of the apoptotic response. The inhibition of the

ceramide pathway blocked the decrease of the mitochondrial membrane potential and the DNA breakdown verifying that ceramide release preceded the mitochondrial events, caspase activation and nuclear events. The consequence of the absence of Lck was according to the finding that Lck played key role in ceramide mediated apoptosis. The activation of PKC was previously demonstrated to counteract with the ceramide effect since it phosphorylated and activated the sphingosine kinase and hence contributed to the generation of the anti-apoptotic sphingolipid, S1P. The down-modulation of the immediate ceramide effects by the activation of PKC and addition of the anti-apoptotic ceramide metabolite, S1P also indicated the contribution of ceramide to the galectin-1 induced apoptotic pathway. Ceramide could be generated in apoptotic cells by either catabolic or anabolic way through SMase or ceramide synthase activity, respectively. Although we did not directly prove the implication of SMase, the failure of inhibition of galectin-1 triggered apoptosis by blocking the *de novo* ceramide release with Fumonisin B1 strongly indicated the induction of the catabolic pathway. Ceramide plays role in cell survival and homeostasis on diverse manners. Early and transient release of ceramide essentially contributes to the formation of functional membrane microdomains as it occurs upon Fas ligation in T cell lines. Galectin-1 treatment does not result in early

elevation of ceramide, indicating that ceramide should act as an apoptotic second messenger. Late generation of intracellular ceramide, in hours following apoptosis induction, have been recently described. Despite that it was shown to be a general feature of apoptosis triggered by different ways, such as TNFR, or Fas stimulation or UV irradiation, in some cases this event was not essential for execution of apoptosis. Nevertheless, the "second messenger concept" cannot be ruled out since ceramide has several well-defined signaling targets.

- Apoptotic processes can be classified as two simplified pathways: a/ The 'caspase first' type apoptosis is initiated via the oligomerization of one of the death receptors followed by the activation of the initiator caspase 8 and the subsequent activation of the effector caspase 3.

b/. The other type is the 'mitochondrion first' type apoptosis in which the direct target is the mitochondrion and the caspase activation occurs downstream to the mitochondrial events with the involvement of caspase 9.

It has been previously shown, that cell death triggered by galectin-1 is not mediated by the interaction of Fas/FasL since MOLT-4 T cells, which are insensitive to the FasL mediated cell death, also die from galectin-1 treatment. Accordingly, activated T cells from Fas deficient *lpr* mice also respond with apoptosis to galectin-1.

Our results supported these data, since the function of the initiator caspase in death-receptor induced apoptosis, caspase 8, was not required for galectin-1 cytotoxicity, as galectin-1 caused cell death in the presence of caspase 8 inhibitor, Ac-IETD or in the absence of caspase 8 expression. In contrast, caspase 9 activity elevated upon galectin-1 stimulation. It was previously published that caspase 3 was the effector caspase in galectin-1 induced apoptosis and according to this finding we showed that caspase 3 was activated.

The contribution of the mitochondrion to the galectin-1 induced apoptosis was proved by using bongkrekic acid, a potent inhibitor of the mitochondrion mediated death pathway, which entirely blocked the apoptosis. The caspase cascade was activated downstream to the mitochondrial steps as the decrease of the mitochondrial membrane potential freely occurred in the presence of the caspase inhibitor.

The biological significance of the mechanism of the galectin-1 induced apoptosis is its role in immunosuppression. As a potent anti-inflammatory agent it has been implicated in the therapy of inflammatory and auto-immune disease. The findings presented in this thesis may contribute to the future application of galectin-1 as therapy for diverse immune diseases.

Summary

In order to establish the biochemical mechanism for the human recombinant galectin-1 mediated programmed cell death of Jurkat T lymphocytes we found that:

- **the apoptotic signaling steps occur in the following order:**
  1. the tyrosine kinases $p56^{lck}$ and ZAP70 are activated
  2. parallel with the phosphatidyl serine exposure on the extracellular side of the cell membrane, ceramide is released and acts like apoptotic second messenger
  3. the mitochondrial membrane potential decrease
  4. the caspase cascade is activated
  5. apoptosis is completed by DNA fragmentation
- **the release of ceramide is essential for galectin-1 induced cell death.** Our data strongly indicated that $\Delta\psi_m$ was regulated by the release of the intracellular apoptotic messenger, ceramide.
- **mitochondrion is the entry-site of the galectin-1 death signal.** We showed that in galectin-1 induced apoptosis the mitochondrial changes preceded the caspase activation therefore this apoptotic pathway belonged to the ’mitochondrion first’ type cell death

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