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The role of tyrosine kinases and phosphatases in galectin-1 induced apoptosis in T cells

Summary of Ph.D. thesis

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Szeged, 2002

INTRODUCTION

Galectin-1 is prominent member of the S-type mammalian lectins, the galectin family. These proteins are characterized by high affinity for β-galactosides on complex glycoconjugates and highly conserved carbohydrate recognition domain (CDR).

Galectin-1 is expressed in tissues of different origin and differentiation stage, executing various functions. Although galectin-1 lacks the signal sequence and shows a cytoplasmic and nuclear distribution within the cells, it is also externalized by a non-classical secretory pathway. Its intracellular function is poorly understood. Recently published data indicate that galectin-1 is a component of the nuclear matrix, and participates in pre-mRNA splicing. Its expression and localization in the cytoplasm and further externalization into the extracellular matrix is tightly regulated during cell differentiation. Several studies have shown that changes in the expression of galectin-1 correlates with pathological conditions. In tumor cells galectin-1 expression is associated with malignant transformation and metastasis and is correlated with undifferentiated phenotypes. According to these findings galectin-1 expression has been proposed as a tool for cancer diagnosis and also as a marker for tumor progression. When secreted, galectin binds to N-acetyllactosamines on the cell surface glycoconjugates and the extracellular matrix proteins. It has been suggested that the secreted galectin-1 has immunomodulatory activities, since it suppresses autoimmune and inflammatory responses. Galectin-1 is present at the sites of apoptosis during normal T-cell development and maturation secreted by thymic epithelium cells. In the thymus, the immature thymocyte subsets are susceptible to galectin-1 mediated apoptosis, suggesting that this lectin may play a role in the thymocyte selection. According to this, galectin-1 induces apoptosis

of immature thymocytes, activated T-cells, and several T leukemia cell lines *in vitro*. The mechanism of galectin-1 induced apoptosis in T cells is still not clear although recent studies indicate that it is distinct from that of initiated *via* Fas/FasL, CD3 or glucocorticoids, the well-known triggers of T cell death.

Galectin-1 treatment induces partial T cell receptor (TCR) ζ chain phosphorylation, generating pp21ζ and limited receptor clustering at the TCR contact site, suggesting that galectin-1 antagonizes TCR responses. On the other hand, galectin-1 induces tyrosine phosphorylation in T cells and synergizes with anti-CD3 signals in dramatically up-regulating ERK activity and inducing apoptosis. Recently, different T cell surface receptors have been identified as galectin-1 binding proteins, including CD45, CD43, CD2, CD3, CD4 and CD7. The first candidate of mediators regulating galectin-1 induced apoptosis in lymphocytes was the receptor tyrosine phosphatase, CD45. CD45 transmits signals upon galectin-1 binding, since the binding of galectin-1 to CD45 reduces its tyrosine phosphatase activity, and consequently the dephosphorylation of Lyn kinase in a Burkitt's lymphoma B cell line. Moreover, in T cells galectin-1 induces the redistribution and segregation of CD45 and CD43, suggesting that this may be important for sending the apoptotic signal. The role of CD45 in apoptotic processes has been suggested in other studies, as well. However, a recent report has not strongly supported the importance of the CD45 in galectin-1 induced apoptosis. Currently, CD7 has been reported to be a necessary component of the galectin-1 triggered death pathway, since a CD7-deficient human T cell line (HUT78) was resistant to galectin-1 induced apoptosis.

AIMS

Galectin-1 has been shown to be apoptotic on lymphocytes, but there are no data regarding other haematopoietic cells. It is not proved, which of the identified galectin-1 binding proteins mediate its apoptotic effect. Moreover, the elements of the galectin-1 induced signal transduction pathway are not known.

The questions leading our work were:

1. Does other bone marrow derived cells than T cells bound galectin-1? What is the fate of the galectin-1 after binding to the cells?
2. Does galectin-1 influence the viability of other haematopoietic cells?
3. What signaling processes and molecules are involved in the galectin-1 induced apoptosis? Which signaling molecules regulate this process?
4. Does CD45 mediate the biological effect of galectin-1?

MATERIALS AND METHODS

- Analysis of galectin-1 binding to cell surface by flow cytometry: the cells were incubated either with FITC or biotin conjugated galectin-1 and then analyzed on FACSCalibur cytofluorimeter.
- Identification of galectin-1 binding proteins: the cells were cell surface biotinylated, then lysed. The galectin-1 binding proteins were purified from the whole cell lysate by galectin-1 coupled Sepharose 4B. The proteins were then analyzed in Western blot using streptavidin-horseradish peroxidase.
- The intra- or extracellular localization of galectin-1 was followed by flow cytometry: FITC or biotin conjugated galectin-1 was added to the cells, then incubated for 3 hours either at 4°C to inhibit or at 37°C to promote the

internalization process. The galectin-1-biotin treated cells were further incubated with streptavidin-FITC to show the cell surface level of galectin-1. In the case of galectin-1-FITC treated samples lactose was added to remove the cell surface fluorescence signal. For the simultaneous detection of the cell surface level of galectin-1 and CD2, CD3, CD43 or CD45, the cells were incubated with biotinylated galectin-1 for 3 hours either at 4°C or at 37°C. The cells were then incubated with the monoclonal antibodies against the cell surface markers, followed by goat-anti-mouse-FITC and streptavidin- Quantum Red. The cells were then analyzed on FACSCalibur cytofluorimeter.

- For the detection of apoptotic cell we used flow cytometry: during the early apoptotic processes the membrane phosphatidyl serine is translocated from the inner to the outer leaflet of the plasma membrane and is detected by binding of Annexin V - FITC. In the late apoptotic cells the DNA lost was measured by DNA content analysis.
- The galectin-1 induced early signal transduction steps were analysed in Western blot: the cells were stimulated by adding different amounts of galectin-1, then lysed. 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 conjugated to horseradish peroxidase.
- The components of galectin-1 induced signal transduction pathway were analysed either with tyrosine kinase and phosphatase inhibitors (genistein, sodium-vanadate, phenylarsine oxide), or with kinase ($p56^{lck}$ and ZAP70) and phosphatase (CD45) mutant Jurkat cells.

RESULTS AND DISCUSSION

- All the cell lines tested, e.g. the T leukemia cell line, Jurkat, a T lymphoblastic cell line ,MOLT-4, with $CD4^+/CD8^+$ immature phenotype, a glucocorticoid-sensitive T cell line, CEM, Burkitt's lymphoma B cell lines, BL-41, Raji, Daudi and non-lymphoid cell lines, such as the myelo-monocytic U937 and the erythro-leukemic K562 bound galectin-1. The binding was sugar dependent, since the galectin-1 bound to the cells was almost completely removed by excess of β-galactosides (lactose). Affinity precipitation form Jurkat cells using Sepharose coupled galectin-1 beads resulted in the detection of a series of cell surface proteins including proteins with molecular weight about 200 kDa. Patterns of galectin-1 binding proteins isolated from CD45 deficient Jurkat variant, J45.01 and wild type Jurkat cells were similar, except that the proteins with 200 kDa were missing from J45.01. The latter proteins turned out to be CD45 glycoprotein, when the galectin-1 binding proteins were hybridized with antibodies recognizing CD45. However, it is noteworthy that CD45 was not the major galectin-1-binding protein since several proteins with molecular mass of 160, 134, 95 and 58 kDa were bound with similar strength.

- We explored the post-binding behavior of galectin-1 by monitoring its extra- and intracellular localization at 37°C. The flow cytometry experiments showed the internalization of galectin-1 within 3 hours in all the cell lines listed above. The internalization did not occur at 4°C indicating that internalization was mediated via cell surface receptor(s). Searching for the possible mediator of endocytosis we analyzed whether the cell surface quantity of known galectin-1 binding glycoproteins, such as CD45, CD43, CD3 and CD2 decreased together with the surface bound galectin-1. Our results showed that the internalization of galectin-1 was not accompanied with the down-regulation of the cell surface

level of these glycoproteins, indicating that these receptors were not involved. Other identified galectin-1 binding proteins (CD4, CD7) or a not yet identified receptor(s) could mediate the down-regulation of the surface bound galectin-1. It is also possible that galectin-1 is internalized by another mechanism and not by receptor mediated endocytosis.

- Galectin-1 induced apoptosis is different T and B cell lines, but not in non-lymphoid cell lines. The T cell lines, Jurkat, MOLT-4 and CEM representing different stages of T cell differentiation showed similar level of apoptotic death after galectin-1 treatment. In addition to T cells, the different B cell lines, BL-41, Raji and Daudi were also killed by galectin-1. The sensitivity of the cells did not correlate with the amount of the bound galectin-1, since the B cell lines bound similar amounts of galectin-1 but Daudi was the most sensitive and Raji was the least responsive to galectin-1 induced apoptosis. In contrast to T and B cells, the non-lymphoid cell lines, U937 and K562 did not undergo apoptosis. The apoptotic effect of galectin-1 is cell specific and is restricted to T and B lymphocytes.

- Galectin-1 induced a time and concentration dependent protein tyrosine phosphorylation in T and B cell lines. The galectin-1 induced tyrosine phosphorylation was essential for the subsequent apoptosis, since tyrosine kinase inhibitor, genistein blocked the apoptosis when added together with galectin-1 to the cells. In contrast to the wild type Jurkat cells, galectin-1 treatment did not result in elevated tyrosine phosphorylation of intracellular proteins either in ZAP70 or Lck deficient cells compared to the non-stimulated deficient cells. Consistently, the kinase mutant cells showed about 50% less apoptotic cells after 24 hours of galectin-1 treatment as Jurkat cells did

indicating that the specific kinases, Lck and ZAP70 played a role in galectin-1 induced T cell apoptosis.

- Galectin-1 treatment resulted in a definite tyrosine phosphorylation in CD45 deficient Jurkat cells, similar to that of wild type Jurkat. Jurkat and the CD45 deficient J45.01 responded with a similar degree of apoptosis when co-cultured with galectin-1 under identical conditions. In contrast to recent studies our results proved that galectin-1 induced tyrosine phosphorylation and apoptosis was not mediated by the CD45. However, it could not be excluded that other galectin-1 signals were transmitted via the phosphatase. Since tyrosine phosphatase inhibitors abolished the galectin-1 induced induced tyrosine phosphorylation and apoptosis, one could hypothesize that phosphatases other than CD45 were participating in the biological effect of galectin-1.

SUMMARY

1. Galectin-1 binds to and is internalized in cell lines of lymphoid and non-lymphoid origin. The internalization of galectin-1 is not mediated by CD2, CD3, CD43, CD45, the identified galectin-1 binding proteins.
2. Galectin-1 induces cell death only in T and B cell lines, but not in the non-lymphoid (erythroid and granulocytic) cells.
3. Galectin-1 induces tyrosine phosphorylation in T and B cell lines. This early cell response is important in the apoptotic process induced by the lectin. The tyrosine kinases, $p56^{lck}$ and ZAP70 are necessary in the signaling pathway triggered by galectin-1. These kinases are specifically but not solely involved in apoptosis induced by galectin-1 in T cells.

4. CD45 tyrosine phosphatase does not play a role in galectin-1 induced tyrosine phosphorylation and apoptosis.

ACKNOWLEDGEMENTS

I wish to thank Éva Monostori and István Raskó for providing the opportunity to work in the Institute of Genetics, Biological Research Center of Hungarian Academy of Sciences.

I thank my supervisor, Éva Monostori for the advices and support in solving the problems. I am grateful to all my colleges who contributed to this work: Gabriela Ion, Ádám Légrádi, Dmytro Demydenko and Marianna Szemes. I also acknowledge to Kinga Székely Szűcs, Andrea Gercsó and Edit Kotogány for the excellent technical assistance, Mária Tóth for the preparation of photographs and figures, and András Frankó for careful reading of the manuscript.

I thank Robert T. Abraham (Mayo Clinic, Rochester), Tamás Oravecz (NIH, Bethesda) and Gabriella Sármay (Department of Immunology, Eötvös Lóránd University, Budapest) for the provided cell lines. I acknowledge to Michel Caron (Université Paris 13, Bobigny Cedex) for the useful collaboration. This work was supported by the grants OTKA T-029706 and ETT T05/016/2000, and by Biromedicina Rt.

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