The mechanism of T cell apoptosis caused by soluble and cell-derived Gal-1; a comparative study to determine the physiological effect of Gal-1

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Ph.D. thesis

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Introduction

Galectin-1 (Gal-1) is the first discovered, well characterized member of a family of highly conserved glycan-bindig proteins. Gal-1 has arisen as a regulator of immune cell tolerance and homeostasis. This endogenous lectin widely expressed at sites of immunoprivilege and tumor growth and has been postulated as an attractive immunosuppressive agent to restore immune cell tolerance and homeostasis in autoimmune and inflammatory situations.

Anti-inflammatory effect of Gal-1 is predominantly exerted by induction of apoptosis of Th1 and Th17 cells. In addition, Gal-1 induces immunoregulation underlying distinct, but complementary mechanisms, including: deviation of cytokine balance toward a Th2 dominant profile, controlling immunosuppressive activity and expansion of regulatory T cells and modulation of activation, adhesion and transendothelial migration of T cells.

Gal-1 is differentially expressed by various normal and pathological tissues. Thus, not only the tumor cells produce Gal-1, but mesenchymal stem cells (MSCs) derived from physiological, healthy tissues also express and secrete Gal-1 to the cell surface and may contribute to immunoregulation by these cells. MSCs have a powerful *in vitro* and *in vivo* immunosuppressive effect on all cells of the immune system, including direct suppression of allogeneic and mitogenic T cell proliferation, induction of T cell anergy or apoptosis, modulation of cytokine production, inhibition of dendritic cell maturation and antigen presentation. Moreover, MSCs regulate immune response by downregulating Th1 and CTL, as well as NK cell response. Thus, they possess a powerful anti-inflammatory effect. The mechanisms underlying these effects remain to be fully elucidated, but are suggested to be mediated by direct cell-to-cell interactions in combination with soluble factors. MSC derived soluble factors, hepatocyte growth factor, transforming growth factor-β, prostaglandin-E2, indolamine-2,3-dioxygenase, nitrogen monoxide, soluble HLA-G and IL-10, all act as anti/inflammatory mediators. There is no evidence, however, that any of these factors would be solely and sufficiently responsible for the immunosuppressive effect *in vitro* or *in vivo*.

Aims

1. Many studies have emerged analyzing Gal-1 signal transduction mechanism during T cell apoptosis, but the data remain very controversial. We have aimed to resolve this controversy by comparing cell death induced by low (1.8 μM, lowGal-1) and high (18 μM, highGal-1) concentration of soluble Gal-1.
2. Using of soluble recombinant protein in any concentration likely does not reflect the *in vivo* effect of Gal-1 because of two reasons: a) Gal-1 is not supposed to exist in soluble form as it binds to high affinity Gal-1-binding structures on the secreting or neighboring cells or extracellular matrix components; b) Soluble Gal-1 samples may differ from lab to lab due to the differences in the methods of purification, moreover Gal-1 requires relatively high concentration of reducing agent for its biological effect. To avoid artificial results originating from the mentioned conditions we used Gal-1 producing cell lines as effectors for inducing apoptosis of activated T cells (peripheral blood cells or Jurkat lymphoblasts) as targets in co-culture system. Our aim was to determine whether Gal-1 secreted from tumor or MSC cells acted as a soluble or membrane associated factor.
3. Next, we have compared the role and mechanism of distinct, normal or pathological cell-derived Gal-1 in the T cell apoptotic process.

Methods

The presence of cell produced Gal-1 on the cell membrane was detected by flow cytometry using anti-Gal-1 antibody produced in our laboratory. Measurements were evaluated by using CellQuest^{TM} software.
Apoptotic cells (Sub-G1 cell population) were subjected to DNA content analysis by flow cytometry.
Gal-1 induced T cell apoptosis was also investigated using fluorescent dye-conjugated AnnexinV labeling and analyzed by both microscope and flow cytometry. Involvement of $p56^{lck}$ and ZAP70 kinases in apoptotic process was proved by enzyme deficient T cell lines. Mitochondrial membrane depolarization was showed by using JC-1 which is

mitochondrial membrane potential-dependent dye. This molecule accumulates in the healthy mitochondria and then emits in red color, while it can not enter into the mitochondria with decreased membrane potential therefore only the green monomer form of the dye is visible in the cytoplasm. Activation of caspase 3 in the T cells during the apoptotic pathway was proved by using an antibody which recognizes only the active, cleaved caspase 3.

Gal-1 production in the cell lines was shown by Western blot.

Results and Discussion

1. We showed that lowGal-1 and highGal-1 triggered phosphatidylserine exposure, generation of rafts and mitochondrial membrane depolarization. In contrast, lowGal-1 but not highGal-1 induced signaling events were dependent on the presence of p56lck and ZAP70 and activation of the caspase cascade. The results allow the conclusion that the cell-death mechanism strictly depends on the used concentration of Gal-1.

2. In co-culture system Gal-1 remains as a native, functional protein without any chemical modification and the apoptosis assay also avoids addition of reducing agent. Both Jurkat and activated peripheral T cells died when co-cultured with various Gal-1 expressing cells, but HeLa, a Gal-1 non-expressing cervix carcinoma cell line did not affect T cell viability. Removing cell surface Gal-1 with lactose analogue, TDG, a competitor for the carbohydrate recognition or knocking down Gal-1 expression in Gal-1 producing tumor cells resulted in the diminution of the cytotoxic effect of these cell lines. Moreover, T cell apoptosis required intimate interaction between the effector tumor and target T cells since neither conditioned supernatant harvested from the tumor cells, nor physical separation of tumor and T cells in the same medium triggered T cell death. These results show that Gal-1, expressed by the examined tumor cells is responsible for inducing T cell apoptosis in the co-culture system and Gal-1 does not act as a soluble, but as a membrane associated factor.

3. Mechanism of apoptosis by tumor cell-bound Gal-1 was comparable to that of low concentration of soluble recombinant Gal-1. Requirement for $p56^{lck}$ and ZAP70 kinases

were indispensable to trigger T cell apoptosis induced by tumor cell-derived Gal-1. Cermide, an essential intracellular mediator in lowGal-1 induced apoptosis was also detected in apoptotic T cells in co-culture. Thus, our results show that cell-derived Gal-1 and low concentration of the soluble lectin triggers identical pathway of T cell apoptosis in contrast to high concentration soluble Gal-1 which act on a different fashion.

For further studies we have used coculture of murine MSCs (BM-MSC) and activated mouse T cells. TDG competition and BM-MSCs derived from Gal-1 knock out mice (Gal-1 KO BM-MSCs) were used in parallel to prove the necessity of Gal-1 in BM-MSCs induced T cell death. Gal-1 was removed from MSCs surface with TDG and it resulted in decreased T cell apoptosis and accordingly, Gal-1 KO BM-MSCs did not trigger T cell death compared to wild type BM-MSCs. BM-MSC-derived Gal-1 triggered T cell apoptosis via the same apoptotic pathway as low concentration of soluble or tumor cell derived Gal-1 did. T cell death progressed on mithocondrial pathway, which involved ceramide production and activation of caspase cascade. These results show that one of the major T cell cytotoxic factors in MSC is Gal-1, which acts similarly to that of soluble lowGal-1.

Thus, the low but not high concentration of soluble Gal-1 and cell-derived Gal-1 produced by either pathological (tumor) or physiological (MSC) cells trigger.similar pathway of T cell apoptosis.

Summary

- Both low and high concentrations of Gal-1 induced cell surface exposure of phosphatidylserine, raft reorganization and loss of mitochondrial membrane potential. There were differences between the function of Lck and Zap70 tyrosine kinases as well as the role of caspases. As long as high concentration of Gal-1 induced caspase independent apoptosis, which occurred without the activity of kinases, low concentration of Gal-1 required the activation of caspase cascade and tyrosine kinases.

- To evolve its cytotoxic effect, Gal-1 needs direct cell-cell interaction, because the separation of effector and target cells with microporous membrane inhibited apoptosis.
- Tumor cell derived Gal-1 and low concentration of Gal-1 acts via the same siglaling pathway: both required Lck and Zap70 tyrosine kinases for the mitochondrial depolarization and caspase dependent apoptosis.
- Apoptosis induced by murine MSCs, which express a high level of Gal-1 acted on the same pathway as those induced by low concentration of Gal-1.

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