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Human galectin-1 triggers apoptosis via ceramide mediated mitochondrial pathway

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Contents

Preface

According to an old definition,"Lectins are multivalent carbohydrate-binding proteins or glycoproteins except for enzymes and antibodies." Such a narrow definition, however, seems no longer relevant, because a significant number of exceptions are evident now. For today, a more flexible interpretation would be accepted, e.g., "lectins are simply defined as proteins which specifically bind (or crosslink) carbohydrates." As exceptions, ricin, the oldest lectin, is actually the enzyme RNA-N-glycosidase, Charcot-Leyden crystal protein (galectin-10) is known as lysophospholipase, and I-type lectins such as sialoadhesin are members of the immunoglobulin superfamily. Multivalency may not be an absolute requirement, even though it is still an important factor for most lectins. Since lectins generally have no apparent catalytic activity like enzymes, their physiological functions remain unclear. Unfortunately, for this reason, the term "lectin" has sometimes been used as a convenient taxon to "group out" carbohydrate-binding proteins, the functions of which were unknown. At present, however, probably no one will oppose the idea that lectins are "deciphers of glycocode".

In addition to the `classic' families of animal lectins such as the galectins, C-, I- and P-type lectins, there are many more animal proteins or protein domains whose functions involve oligosaccharide recognition and which could be classified under the definition of lectin. Certain cytokines such as tumor necrosis factor (TNF), IL-1, IL-2, IL-6, IL-12 have documented lectin activities.

Regarding their function has been well established that various lectin families play critical roles in the immune response. Almost all of the mammalian lectins that are involved in immunity are membrane proteins. Interesting exceptions are galectins. During the past decade, attempts to identify the functional role of galectin-1 suggested participation in the regulation of the immune response. Only in the last few years has the molecular mechanism involved in these properties been clearly elucidated, revealing a critical role for galectin-1 as an alternative signal in the generation of T cell death.

1. Introduction

1.1 Galectin family

Galectins are members of an evolutionarily highly conserved family of animal lectins widely distributed in nature from lower invertebrates to mammals[1, 2]. They share sequence similarities in the carbohydrate recognition domain (CRD) with specificity for N-acetyllactosamine-enriched glycoconjugates[1, 2]. The typical carbohydrate recognition domain consists of 135 amino acids tightly folded into a sandwich structure of 5-6 stranded β-sheets and recognizes the basic structure of LacNAc; (Gal β1-4GlcNAc)[3].

Fourteen mammalian galectins have been identified to date in a wide variety of tissues from different species[4, 5]. According to their structure, they have been classified by Hirabayashi and Kasai into prototype galectins (galectins-1, -2, -5, -7, -10, -11, -13 and B14)[6], existing as monomers or noncovalently bound homodimers, consisting of two identical CRD, chimera-type (galectin-3), containing a nonlectin domain linked to a CRD, and tandem-repeat-type (galectins-4, -6, -8, -9, and B12) composed of two distinct CRDs in a single polypeptide chain (Figure 1.1).

Studies of multiple animal lectins by X-ray crystallography have provided evidence for their oligomeric structures[7, 8]. By virtue of their multivalency, these carbohydrate binding proteins are able to cross-link specific glycoproteins and glycolipid receptors, leading to activation of different signal transduction pathways, which converge in multiple biological responses[9]. Most galectins have been proposed to exert discrete biologic effects, according to different subcellular compartmentalization, cell-activation and developmentally regulated expression[6]. Although galectins lack a signal peptide, they are secreted by a nonclassical and novel apocrine mechanism, in which the synthesized protein becomes concentrated at the level of the plasma membrane, and are externalized further to form galectin-enriched extracellular vesicles[10]. This unusual secretory route might prevent the premature binding of galectins to oligosaccharides on nascent glycoproteins. After release into the extracellular medium, galectins can crosslink β-galactoside-containing cell-surface glycoconjugates, resulting in the modulation of cell signaling, adhesion and cell survival[11].

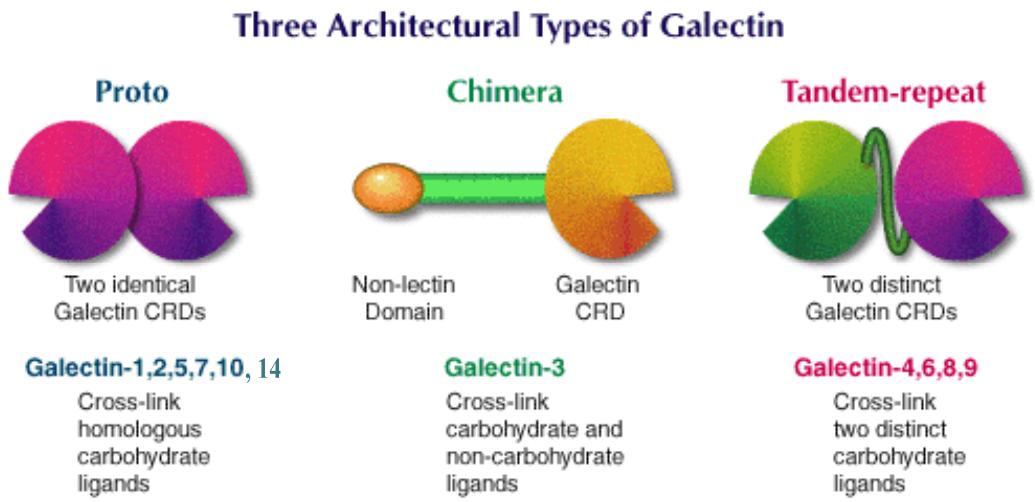

Figure 1.1 **Three structural types of galectins.**
(*taken from http:/www.glycoforum.gr.jp/science/word/lectin/LEA01E.htlm*)

1.2 Galectin-1 structure and biological functions

Galectin-1 was identified in the mid-1970 as a β-galactoside-binding protein with hemagglutinating activity in the electric organ tissue of electric eels[12], calf heart and lung[13] and chick embryos[14, 15, 16]. It is a monomeric or homodimeric protein composed of subunits of 14.5 kDa[17]. Each subunit folds as one compact globular domain[2] (Figure 1.2). Dimerization involves self-association of the monomer subunits via the hydrophobic surfaces opposite to the sugar-binding pocket[18]. Galectin-1 binds to lactose, Galβ1-4Glc and N-acetyllactosamine Galβ1-4GlcNAc with relatively low affinity (K_d in the range of 90-100 μM), and to glyco-proteins containing polylactosamine sequences $[3Gal\beta1\text{-}4GalNAc\beta1]_n$ with high affinity (K_d.1 μM). The lectin does not require terminal non-reducing galactose residues in polylactosamines for recognition[19].

Galectin-1 is expressed in adult muscles, including skeletal and smooth muscle, thymus, lymph node, prostate, spleen, liver, placenta, endothelial cells, skin, testis, olfactory neurons, developing brain, retina, macrophages, B cells, T cells[17, 20]. The expression of galectin-1 is changed during the embryonic development. It is initially synthesized in the trophectoderm of expanded blastocysts immediately prior to implantation.

Figure 1.2 **Structure of galectin-1 dimer.** Bound sugar and residues participating in the binding are shown. (*taken from ref. 28*).

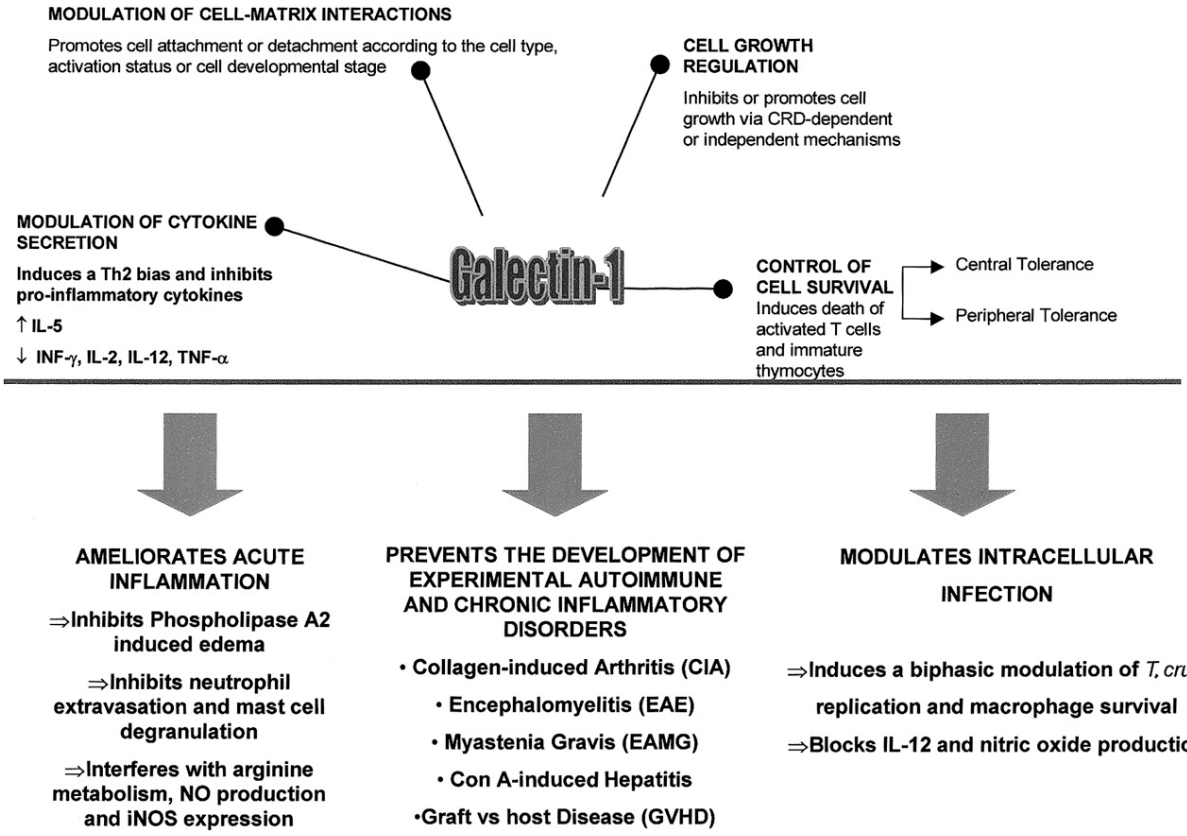

Figure 1.3 **Participation of galectin-1 in different physiological processes** (upper panel) **and pathological condition** (lower panel). (*taken from ref. 17*)

In the postimplantation embryo, the lectin is abundantly expressed in the myotomes of the somites. These changes of expression during the development are regulated by DNA methylation of the galectin-1 gene[89-91].

Galectin-1 has been implicated in several physiological and pathological processes such as cell adhesion[21], cell growth regulation[22], immunomodulation[23], apoptosis[24, 25], reproduction[26] and pre- mRNA splicing[27]. Many of these effects have been found to depend on the carbohydrate-binding activity of this lectin and some are mediated exclusively by protein-protein interactions[17]. An overview of the biological effects of galectin-1 is shown in Figure 1.3. A series of clinical and experimental evidence have been reported to support correlation between galectin-1 expression in tumorigenesis. Galectin-1 has been implicated in malignancies of pancreas, thyroid, prostate and bladder, kidney, nervous system[86].

1.2.1 Galectin-1 in cell growth and apoptosis

Role in cell growth

Both negative and positive effects have been demonstrated for galectin-1 on cell proliferation. Wells and Mallucci in 1991 purified a protein with cytostatic activity that was secreted from mouse embryonic fibroblasts (MEFs), and subsequently cloned the complementary DNA (cDNA) for this protein based on the amino acid sequences of the peptides after tryptic digestion. They have designated this protein mGBP for mouse galactoside-binding protein, which is known now as mouse galectin-1. Treatment of unsynchronized cells with recombinant galectin-1 caused cell cycle arrest at the G2 phase, and the addition of galectin-1 to quiescent (G0) MEF prevented serum-stimulated reentry of the cells into the cell cycle. The growth inhibitory effect was not related to the lectin properties, as it was not sensitive to the presence of lactose. A neutralizing monoclonal antibody was further used to confirm the cytostatic activity of the endogenous protein. Addition of this antibody to G0 cells accelerated reentry of the cells into the cell cycle after serum stimulation[43]. Recombinant galectin-1 inhibited antigen-induced proliferation of T cells[44] as well as the interleukin (IL)-2 independent proliferation of malignant T cells, by arresting them in the S and G2/M phases of the cell cycle[36]. Galectin-1 treatment of three independent human mammary cell lines which differed in oncogenic potential caused a cell cycle block prior to the cells= entry into G2 phase[45]. Overexpression of the lectin by transfection caused

transformation of BALB 3T3 fibroblasts[46], a finding that was also confirmed with the rat fibroblast cell line, Rat-1[47]. Another report showed the biphasic modulation of cell growth by recombinant galectin-1. While high doses of galectin-1 inhibited cell proliferation independently of its sugar-binding activity, low doses of galectin-1 induced cell proliferation in a lactose-inhibitable fashion[22]. Galectin-1 also suppressed the proliferation of freshly isolated mouse thymocytes by modulating TCR signaling and inhibiting IL-2 production[48]. Little is known about the mechanism of growth modulation by galectin-1. Its effects on cell proliferation can be either positive or negative, with or without the involvement of its lectin activity. In one case, the growth inhibitory activity of galectin-1 was partially mapped to a surface loop (residues 25-30) and two internal β strands, clearly distinct from the sugar-binding site[49]. It is not likely that galectin-1 directly acts on the central cell cycle machinery, and the mediators of the effects of galectin-1 remain to be identified. Oncogenic H-Ras has been identified as an intracellular galectin-1 binding protein. The transformation activity of H-Ras has been shown to be dependent on galectin-1, which appeared to direct the anchorage of activated Ras to the cell membrane, and activate its downstream effector, Raf[47]. Overexpression of galectin-1 increased the levels of membrane-associated Ras, Ras-GTP and extracellular signal-related kinase (ERK) activity, resulting in cell transformation that was blocked by dominant-negative Ras[47].

Role in apoptosis

Exogenously added galectin-1 was first reported to induce apoptosis in activated T cells and certain human leukemia T cell lines. Resting T cells also bound galectin-1, but did not undergo apoptosis. The mechanism of galectin-1 induced apoptosis appears to be distinct from that triggered by Fas as it has been shown in a Fas resistant T cell line[25] and Fas deficient *lpr* mice[44]. Baum LG et al[35] showed that galectin-1 is produced by thymic epithelial cells. The same group found that certain subsets of thymocytes are susceptible to galectin-1 induced apoptosis[50]. Exposure of thymocytes to anti-CD3 antibody enhanced galectin-1 induced apoptosis[50], suggesting that galectin-1 may be involved in thymocyte apoptosis mediated by the T cell receptor (TCR), an important process in thymic selection. Similarly, galectin-1 enhances apoptosis induced by TCR engagement in T cell hybridoma and freshly isolated thymocytes, at least in part through antagonizing IL-2 production[48]. While the binding partners

for galectin-1 are largely undefined on thymocytes, different T cell surface receptors (CD45, CD43, CD2, CD4, CD3 and CD7) have been identified as galectin-1 binding proteins[51, 53-56]. To address whether these receptors act cooperatively or independently to deliver the apoptotic signal, Pace and colleagues[55] demonstrated that galectin-1 binding to cells resulted in a dramatic redistribution of these glycoproteins into segregated membrane microdomains on the cell surface. Whereas CD45 and CD3 colocalize on apoptotic blebs, CD7 and CD43 are distributed in small patches away from the membrane blebs[55]. The relevance of CD45 in mediating apoptosis was supported by the fact that CD45 positive Jurkat T cells were more susceptible to the apoptotic effect than were CD45 negative cells[25, 51]. In contrast our recent work showed that CD45 deficient Jurkat cells exhibit susceptibility to galectin-1, which is indistinguishable from that of their wild-type counterparts[52]. The disagreement between our and others results could be explained by the difference between experimental approaches. Most of the studies were based on the inhibition of galectin-1 induced apoptosis by specific anti-bodies to CD45[25, 55]. This approach may not be adequate because immunoglobulins are glycosylated and may compete for the galectin-1 as it was observed in our experiments. However, only CD7 seems to fulfill the requirement for a transmitting receptor, as the absence of CD7 correlates with the failure of gal-1 induced apoptosis, and complementation of CD7 restores the cell death[56]. It has been speculated that the loss of CD7 in several pathologies, such as lymphoid tumors and autoimmune disease, might contribute to enhance survival and expansion of malignant and autoreactive lymphocytes[57].

There are also reports showing the involvement of caspase activation, Bcl-2 downregulation and activation of AP-1 transcription factor in galectin-1 induced apoptosis[58, 59].Treatment of alloreactive lymphocytes with a broad range caspase inhibitor (zVAD-fmk) was able to overcome cell growth inhibition triggered by galectin-1, suggesting that this protein transduces inhibitory/death signals through activation of caspases[59]. It was proved by the same group[59] that galectin-1 modulated Bcl-2 expression during the alloimmune response as galectin-1 suppressed the expression of Bcl-2 which was stimulated in T cells in a mixed lymphocyte culture. Requirement of AP-1 for galectin-1 induced apoptosis was confirmed by the dose-dependent reduction on the level of DNA fragmentation observed when cells were pretreated with curcumin before exposure to galectin-1[58].

1.2.2 Galectin-1 in the immune system

The immunomodulatory properties of galectin-1 were first identified in the context of two experimental models of autoimmune disease. Administration of recombinant galectin-1 prevented clinical and histopathological signs of experimental encephalomyelitis (EAE), a T-cell-mediated autoimmune disease in susceptible Lewis rats[23]. A homologus galectin from the fish *Elecrophorus electricus* showed prophylactic and therapeutic effects on experimental autoimmune myasthenia gravis (EAMG) in rabbits[29]. Since that, experimental data have been accumulated concerning the implication of galectin-1 in apoptosis of activated, mature T cells and particular subsets of nonselected and negatively selected $CD4^{lo}$ $CD8^{lo}$ immature thymocytes[24, 26, 30].

Another line of evidence supporting the implication of galectin-1 in the regulation of adaptive immune response comes from the striking accumulations of the protein in immune privileged sites of the body, such as placenta[17, 26] and eye[17, 32], where multiple factors operate to ensure rapid elimination of inflammatory cells. T-cell apoptosis can provide a defending mechanism to vulnerable sites from tissue damage. The expression of FasL in sites of immune privilege may have a key role in preserving this privilege by selectively killing activated T cells by apoptosis[33]. Accordingly, galectin-1 may represent an alternative regulatory signal to regulate mechanisms operating in the establishment of immune privilege. Expression of galectin-1 in the first term gestation placenta could be significant in protecting the fetus from the mother's immune system by killing infiltrating T cells[26]. Galectin-1 expression in the eye would protect this sensory organ from the devastating effects of an inflammatory response[33, 34].

Human thymic epithelial cells synthesize galectin-1, which binds to oligosaccharide ligands on the surface of thymocytes[35]. The degree of galectin-1 binding to thymocytes correlates with the maturation stage of the cells, as immature thymocytes binds more galectin-1 than mature thymocytes do. This result suggests one mechanism for the influence of thymic epithelial cells in positive selection of thymocytes bearing the appropriate T-cell receptor and in negative selection of autoaggressive clones[9]. Galectin-1 inhibits antigen-induced proliferation of naive and memory T cells, acting as an autocrine negative growth factor, hence it switches off T lymphocyte effector function, by arresting cell cycle progression at the level of the S and G2/M stages and alternatively triggering IFN-γ mediated apoptosis of activated human T lymphocytes [36].

It may be also be relevant that autoantibodies against galectin-1 have been demonstrated in sera of patients with neurological disorders, particularly those suffering from multiple sclerosis [31].

Galectin-1, inflammatory cascade and immunopathology

Lymphocytes that express self-specific clonotipic receptors will mediate auto-immune lesions if they either: **1.** escape clonal deletion in central organs; **2.** do not receive any of the multiple anergy-inducing or suppressive signals; **3.** express the appropiate combination of adhesion receptors that will allow for migration to the target tissue containing the auto-antigen; and **4.** once activated, are not counterbalanced by immunosuppresion and activation-induced apoptosis[38, 39]. The redundancy of several homeostatic systems might explain why autoimmune diseases are rarely acute, but follow a chronic progressive inflammatory course.

The inflammatory response involves the sequential release of soluble mediators and the recruitment of circulating leucocytes, which become activated at the inflammatory sites. This response is self-limiting and resolves through the release of endogenous anti-inflammatory products and the clearance of inflammatory cells.

Galectin-1 has been shown to be a powerful homeostatic signal, probably by influencing all the described mechanisms: central clonal deletion, cell adhesion, clonal suppression and peripheral T-cell death[37]. The anti-inflammatory and immunosuppressive effects of galectin-1 have been demonstrated in several experimental models of inflammation and autoimmunity (Figure 1.3).

It was shown that galectin-1 ameliorates phospholipase A2-induced edema in a selective and dose-dependent manner, when pre-injected or co-injected together with the enzyme. The lectin inhibits arachidonic acid release and prostaglandin production from lipopolysaccharide (LPS)-stimulated macrophages and blocks neutrophil extravasation, mast cell degranulation and nitric oxide synthesis[40]. Galectin-1 effectively inhibits the inflammatory and autoimmune response in collagen-induced arthritis (CIA), an experimental model of rheumatoid arthritis. Investigation of the mechanisms involved in the anti-inflammatory effects of galectin-1 revealed that this protein increases T-cell susceptibility in vivo to antigen induced cell death (AICD). This effect is antigen specific and requires signaling via the TCR. In addition to triggering apoptosis in activated T cells, immunosupression by galectin-1 can come

about by preventing synthesis and/or release of proinflammatory and Th1 cytokines. Within the arthritogenic process, galectin-1 treatment skews the overall balance toward a Th2 profile with reduction of the level of IFN-γ and a clear increase in IL-5 production[41].

An inhibitory effect of galectin-1 has also been reported on Con A-induced hepatitis, a T-cell dependent model of liver injury in mice. Galectin-1 pretreatment prevented both liver injury and T-cell liver infiltration induced by Con A. This immunosuppressive effect was also associated with inhibition of TNF-α and IFN-γ production[42].

1.3 Apoptosis in the immune system

Programmed cell death (PCD) or *apoptosis* is an essential part of the life in multicellular organisms, playing roles in development, defense, and homeostasis[92, 93]. In the vertebrates, there is one type of cell that even without transformation has the potential to cause havoc on the survival of the individual if (in principle) even a single cell functions inappropriately - **the lymphocyte**. A single lymphocyte, with specificity for an antigen expressed by our own tissues, can respond and cause crippling damage. For this reason, the regulation of the formation and function of the lymphocyte repertoire is under exquisite and extensive regulation[112].

The apoptotic process has a well established role in the physiology of the immune system. It is required to maintain immune responsiveness and to avoid the aberrations of immunodeficiency, autoimmunity and cancer[95]. During the decline of an immune response, most of the activated T cells die. Cell death is also responsible for eliminating autoreactive lymphocytes[94].

1.3.1 Hallmarks of apoptosis

Apoptosis is defined by stereotypic changes[92, 96]:

- In the nucleus chromatin condenses to compact and apparently simple geometric - globular, crescent-shaped figures
- Phosphatidylserine (PS) exposure (translocation of phosphatidylserine, which is confined to the inner leaflet of the plasma membrane in healthy cells, to the outside of

the plasma membrane where it is recognized by macrophages or neighboring cells) (Figure 1.6)

- Cytoplasmic shrinkage
- Zeiosis (dynamic plasma membrane blebbing of a dying cell, analogous to the bubbling of fermenting yeast)
- Caspases are activated
- Formation of apoptotic bodies that contain self-enclosed fragments of the nucleus surrounded by cytoplasm and a cell membrane.

A key consequence of apoptotic process is that the cellular membrane tends to remain intact, preventing the leakage of cellular contents, which in turn ensures a relatively non-inflammatory process[95].

1.3.2 Apoptotic death pathways

Diverse stimuli can initiate apoptosis, however the common biochemical and morphological alterations can be observed independent by that of the initial stimulus. This finding suggests that most apoptotic signals converge on a limited number of common effector pathways[115]. On a basic level these pathways can be distinguished by the entry site of the apoptotic stimulus, the relative timing of caspase activation and mitochondrial release of cytochrome c. In conformity with the entry site of the apoptotic stimulus, two basic pathways are described: receptor and mitochondria mediated apoptotic pathway. The receptor pathway is exemplified by the activation of death receptors and an effector caspase is activated prior to mitochondrial alterations[116]. Oligomerization of the death receptors leads to activation of caspase 8 which then initiates a caspase cascade leading to the activation of caspase 3 and cell death (Figure 1.4b). The other pathway is characterized by the release of cytochrome c from the mitochondrial intermembrane space prior to caspase activation[97]. The complex of cytochrom c, apoptosis protease-activating factor 1 (Apaf-1), dATP, and pro-caspase 9 activates caspase 9. Caspase 9 then activates caspase 3, triggering a commitment to apoptosis (Figure 1.4b)[92, 96]. While these two pathways are presented as separate, several mechanisms exist for cross-talk and positive feedback loops[92, 117]. For example, activation of caspase 8 can be followed by cleavage of Bcl family members (Bcl-2, Bcl-x_L, and Bid) leading to mitochondrial apoptosis[92, 95, 118, 119]. In addition, activated caspase 3 can activate caspase 6 [121]

which in turn activates caspase 8[120].

Death receptors

The cell surface death receptors obtained their name as their ligation can trigger apoptosis. These receptors belong to the tumor necrosis factor (TNF) receptor family. The best characterized members are Fas (CD95/APO-1) and tumor necrosis factor receptor 1 (TNF-R1)[92, 98]. Deficiency of individual members of TNF-R family (such as CD95) leads to abnormal lymphoid development and autoimmunity[95]. Death receptors are activated by cell surface bound and soluble ligands such as FasL (CD95L), tumor necrosis factor α (TNF-α) and TNF-related apoptosis inducing ligand (TRAIL)[92, 98]. The ligands are members of the TNF-α cytokine family and are homotrimeric molecules[98]. The signaling through death receptors is initiated by ligand-induced receptor trimerization[92, 98].

Activation of these receptors initiates protein-protein interactions via the death domain (DD) in their cytoplasmic tail[92, 98] (Figure 1.4a). The surface ligation is followed by the trimerization of intracellular DDs, thereby recruiting and dimerizing various DD containing adaptor molecules such as Fas- associated death domain (FADD), TNF-R1-associated protein with death domain (TRADD), and the receptor interacting protein death domain (RIP-DD)[92, 95, 98]. Further this complex binds pro-caspase 8 through the death effector domain (DED) smelting the assembly of DED present in both DD adaptors and pro-caspase 8 in death-inducing signal complex (DISC)[92, 98]. The DISC triggers the rapid self-activation of caspase 8, and initiates a caspase-first apoptotic pathway (Figure 1.4)[98]. This cascade leads to cleavage of the effector procaspase 3 and is sufficient to kill certain types of cell (‘type I cells’), including lymphocytes[95]. In ‘type I cells’ the activation of caspase 8 results in direct processing of caspase 3 and this step is independent of mitochondrial activation and cannot be blocked by overexpression of Bcl-2, an anti-apoptotic member of Bcl family[99]. In ‘type II cells’, the amount of active caspase 8 generated at the DISC is limited[99]. Apoptosis proceeds through mitochondrial perturbations and generation of active caspase 3 and 8 downstream of mitochondria and activation of both caspase 3 and 8 is blocked by overexpression of Bcl-2[99].

Mitochondrial apoptosis

In death receptor-mediated apoptosis, the apoptotic pathway is activated by the interaction of a relatively small number of structurally-related ligands with a limited number of structurally-related cell surface receptors[98]. In contrast, mitochondrial apoptosis can be induced by a variety of agents including chemo-therapeutic drugs, reactive oxygen species, kinase and phosphatase inhibitors, respiratory poisons, Ca^{2+}-ionophores, UV-and γ-irradiation, lipid mediators such as ceramide or the disialoganglioside GD3, granzyme B, and environmental stresses including growth factor withdrawal, heat, and osmolarity changes[97, 122-130].

The varied nature of these apoptotic triggers suggests that the mitochondrial apoptosis is induced by more than one mechanism[96]. After the permeabilization of the mitochondrial membrane several components are released from the mitochondrial intermembrane space. These components include cytochrome c, SMAC (also known as Diablo) and apoptosis-inducing factor (AIF).

Cytochrom c, an essential component of the mitochondrial electron transport chain, is required for mitochondrial respiration. When released into the cytoplasm, cytochrome c leads to the assembly of the apoptosome complex, containing Apaf-1, the initiator procaspase-9 and cytochrome c[127] (Figure 1.5). Apaf-1 serves as a docking protein for caspase-9 and cytochrome c. Recent evidences suggest that the apoptosome is a wheel-like particle containing seven Apaf1 monomers which, like seven spokes, radiate from a central hub[76]. At the N-terminus Apaf-1 contains a segment homologous to the caspase recruitment domain (CARD) which is located in the central hub[76] and it binds to CARD domain of procaspase-9[75, 76]. Apaf-1 contains also Walker A and B boxes and the nucleotide p-loop for nucleotide binding[75, 76] and a long carboxi-terminus domain rich in WD-40 repeats. The cytochrome c would interact with the WD40 domain, which forms the distal part of the spoke[76]. Normally Apaf1 has a compact autoinhibited shape in which the CARD domain binds the WD40 domain. Cytochrome c displaces the CARD domain from the WD40 domain taking its place and allowing the Walker A and B boxes to bind dATP/ATP and undergo a conformational change in which Apaf1 has a more extended conformation; this is required for efficient procaspase-9 binding to the exposed CARD domain and assembly of the apoptosome. This assembly leads to the cleavage of procaspase-9 into its active fragment, which initiates the downstream cascade of effector

caspases [131]. The suggestion that cytochrom c is required to carry out the cell suicide program is supported by observation that cells depleted of cytochrome c lost the ability to activate caspases. Activity was restored when purified cytochrome c was added back[75].

The release of SMAC from the mitochondria relieves the inhibition of caspase inhibitors known as inhibitor of apoptotic proteins (IAPs)[132, 133] (Figure 1.5). These inhibitors prevent caspase activation by blocking their cleavage; on release of SMAC, however, this inhibitory role is prevented, allowing downstream activation of caspase[134, 135].

The release of AIF from mitochondria has been reported to initiate an alternative pathway of cell death. The released AIF translocates to the nucleus, initiating the condensation of chromatin and subsequent fragmentation of DNA[136, 137].

1.3.3 Caspases

Caspases are specialized cysteine-proteases that are essential for apoptosis[96]. At least 14 death proteases have been identified in mammalian tissues[100] which are homologues to each other in amino acid sequence, structure, and substrate specificity[101, 102].

Caspases occur as proenzyme and are activated by proteolysis[101-104]. They are synthesized as a single polypeptide chain, and during activation, each polypeptide chain is cleaved into a large and small subunit, which then dimerize[116]. Two large and two small subunits are required for full enzymatic activity[116]. Activation occurs either by interaction with other proteins or by action of other caspases. For example, caspase-8 is activated as a result of its interaction with FADD[98], and caspase-9 is activated through an interaction with cytochrome c, dATP (or ATP), and Apaf-1[75]. Also, both active caspase-8 and caspase-9 can cleave and activate caspase-3[96]. Caspases cleave substrates at Asp-Xxx bond (after aspartic acid residues); caspase distinct substrate specificity is determined by the four residues amino-terminal to the cleavage site[105]. Based on substrate specificity they are classified into three categories. The first group (group I) contains caspases involved in inflammatory processes including caspases 1, 4, and 5. The second category (group II) of caspases contains caspases 6, 8, 9, and 10[106]. These enzymes are considered signaling caspases because they can activate other caspases, initiating a cascade[106]. The final category (group III) contains caspases 2, 3, and 7. These enzymes are known as effector caspases because they are activated by other caspases, and they cleave the cellular targets resulting in the acquisition of apoptotic morphology.

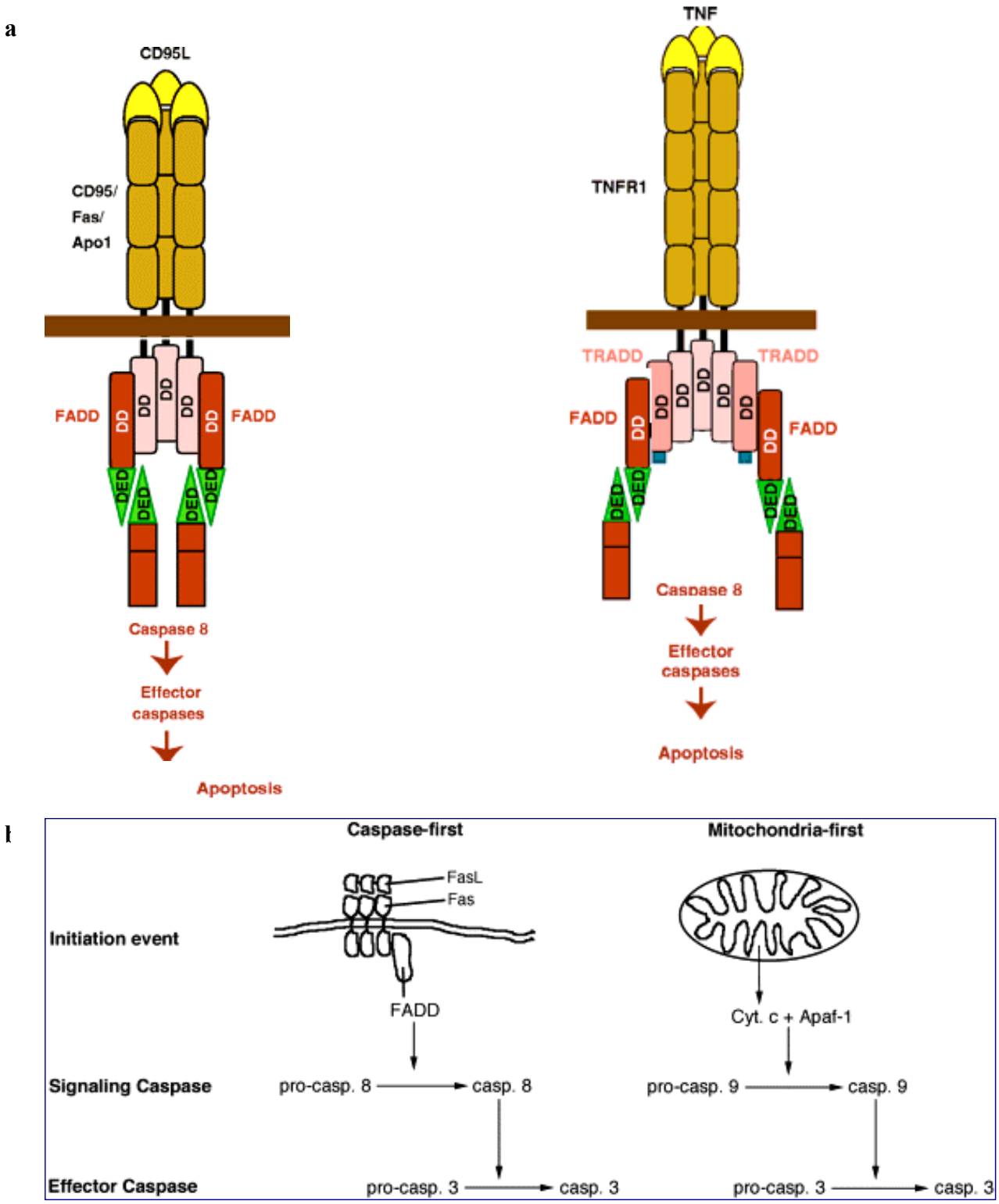

Figure 1.4 **Apoptotic pathways. a.** Apoptosis signaling by CD95 and TNF-R1 (*Figure adapted from ref 98*). **b.** The main apoptotic pathways (*Figure taken from ref 96*).

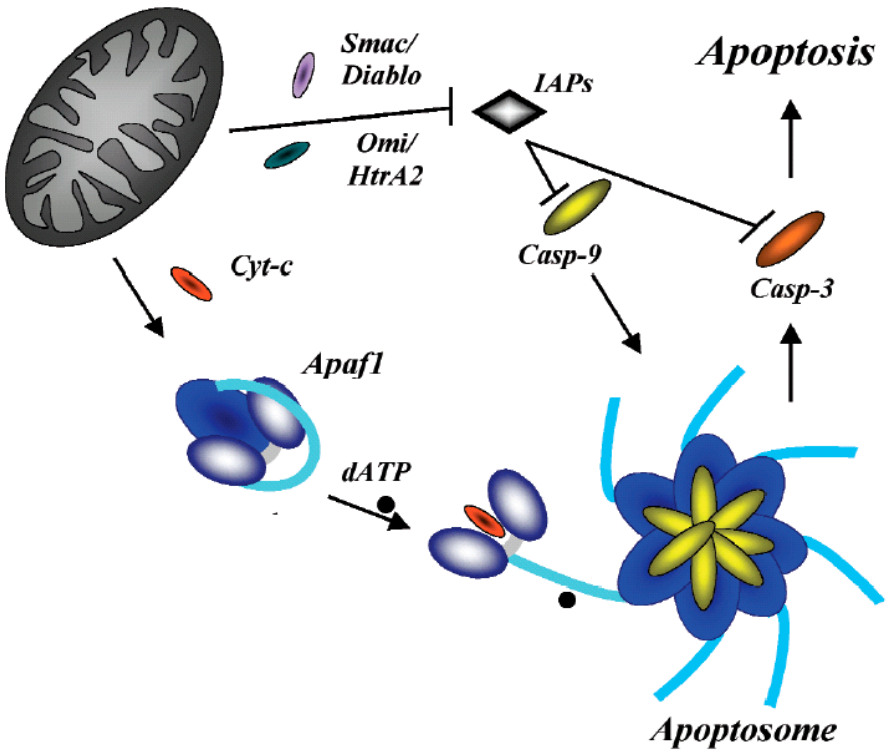

Figure 1.5. **The activation of the apoptosome.** (*Figure taken from ref 77*).

Activation of these caspases generally results in an irreversible commitment to cell death[100]. The most recently identified caspases, 12–14, have not yet been sufficiently characterized to place them in one of these three categories.

Cellular targets of the caspase activity belong to different categories of proteins, including structural elements, nuclear proteins, and signaling proteins (table 1). Some substrates are cleaved and inactivated such as cytoskeletal proteins (lamin, α-fodrin, and actin), signaling molecules (Bcl-2 family members), nuclear elements including the U1 (70 kDa) ribonuclear protein involved in RNA splicing and two enzymes involved in DNA repair, poly(ADP-ribose) polymerase (PARP) and DNA-dependent protein kinase (DNA-PK).

Other substrates are activated such as DNA fragmentation factor, an inhibitor of the caspase-activated DNAse and PKCδ that lead to DNA fragmentation[96].

Category	Target	Outcome
Signaling	Other caspases	Activation
	PKCδ	Activation, nuclear fragmentation
	p21-Activated kinase	Activation of JNK
	PISTRLE kinase	Activation
	Phospholipase A_2	Activation
	Bcl-2, Bcl-x_L	Inactivation
	Bid	Activation
Nuclear	DNA fragmentation factor	DNA fragmentation
	Inhibitor of caspase-activated DNAse (CAD)	Activation of CAD, DNA fragmentation
	Poly (ADP-ribose) polymerase	Inactivation
	DNA-dependent protein kinase	Inactivation
	U1 (70 kDa)-sn RNP	Inactivation
Structural	Lamin	Degradation
	α-Fodrin	Degradation
	Actin	Degradation

Table 1. **Caspase targets** (*taken from ref 96*)

1.3.4 Sphyngolipids in apoptosis

De novo biosynthesis of the hydrophobic core of sphingolipids, ceramide occurs in the endoplasmic reticulum (ER), as has been firmly established in both mammals[78] and yeast[79]. Ceramide serves as the precursor for all major sphingolipids in eukaryotes (such as sphingomyelin (SM) or glucosylceramide) (Figure 1.6). The breakdown of complex sphingolipids results in the formation of ceramide through the action of either sphingomyelinases (SMases) or glycosidases. The breakdown of ceramide proceeds through the action of ceramidases (CDases), and the resulting sphingoid bases serve as substrates for sphingosine kinases to form S1P or are recycled into ceramide and complex sphingolipids through the action of ceramide synthases[154].

Ceramide belongs to the class of lipid second messengers such as inositol 3 phosphate (IP_3), phosphoinoside 3 phosphate (PIP_3) and diacylglicerol (DAG)[107, 108] and it regulates diverse cellular processes including apoptosis, cell senescence, the cell cycle, and cellular differentiation[138].

Ceramide reportedly modulates the activity of several signaling pathways by interacting with a large number of proteins[146, 147]. Among these is kinase suppressor of RAS (KSR; identical to ceramide-activated protein kinase), ceramide-activated protein phosphatase (CAPP), protein kinase Cζ (PKCζ), phosphatidylinositol 3-kinase (PI3K), Jun amino-terminal

kinase (JNK), protein kinase B (PKB), stress- and mitogen-activated protein kinases[139-144]. It was shown also that ceramide can cause upregulation of FasL (CD95L), an effect that may be mediated by its activation of JNK[145].

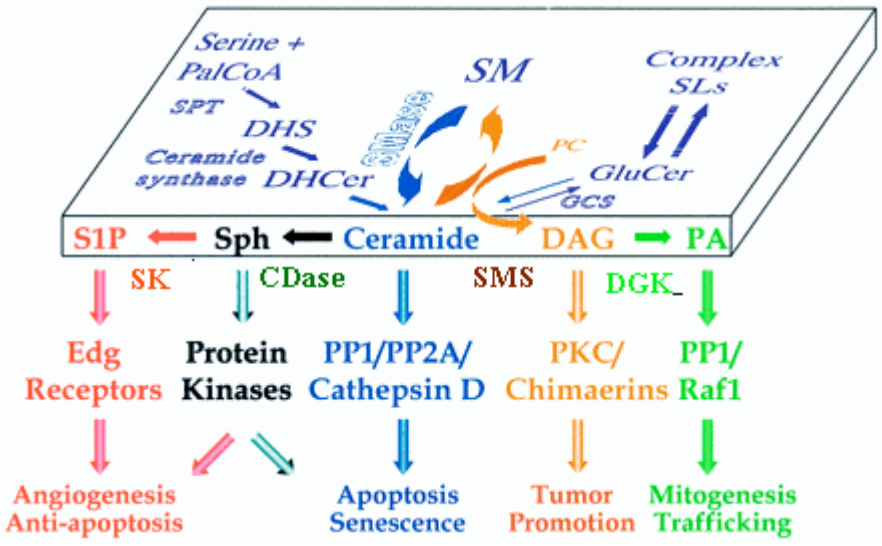

Figure 1.6 **Basic pathways of ceramide metabolism and interrelationship of regulatory pathways mediated by bioactive lipids.** Ceramide can be formed *de novo* or from hydrolysis of SM or complex glycolipids (horizontal plane in diagram). In turn, ceramide may be converted to sphingosine (*Sph*) or serve as a substrate for SM synthesis (generating DAG) or glycolipids. Each of the major bioactive lipids is then capable of interacting with specific targets leading to specific responses (vertical plane). *PalCoA*, palmitoyl-CoA; *DHS*, dihydrosphingosine; *DHCer*, dihydroceramide; *SK*, sphingosine kinase; *PC*, phosphatidylcholine; *PA*, phosphatidic acid; *SMS*, SM synthase; *DGK*, DAG kinase; *SL*, sphingolipid. (*taken from ref. 154*)

Evidence supporting ceramide as a message for apoptosis induction is based on data from many cell systems and comes from several kinds of studies. Ceramide generation can be triggered by the ligation of cell surface receptors (TNF-R[73, 80], CD95[80]), the action of chemotherapeutic drugs, reactive oxygen species, UV- and γ-radiation[96, 70, 80-82]. While most ceramide formation during apoptosis appears to involve the hydrolysis of SM by various sphingomyelinases (SMases), there are some cases in which de novo synthesis of ceramide by a ceramide synthase is activated[96, 108]. Cermide formation and PS exposure, two lipid events in the apoptotic execution phase, are functionally linked[109] (Figure 1.7). PS exposure is the direct consequence of the loss of phospholipid (PL) asymmetry in the plasma membrane. The asymmetric PL distribution (i.e., cholinephospholipids - SM and phosphatidylcholine (PC) in the outer leaflet and the aminophospholipids - PS and phosphatidylethanolamine (PE) in the inner leaflet of the lipid bilayer) is maintained by an ATP-dependent aminophospholipid translocase that continuously transports PS and PE to the inner leaflet. Bidirectional PL scrambling (flip-flop) activity, causing the loss of lipid bilayer asymmetry is thought to be mediated by a distinct enzyme, PL scramblase[85]. By using scrambling-competent Jurkat T and SKW6.4 B cells and

scrambling-deficient Raji B cells and analogues of SM and PC Tepper et al[109] showed that, as a consequence of the loss of PL asymmetry, SM moves from the outer to the inner leaflet of the plasma membrane, where it serves as a substrate for an intracellular SMase. Thus, transbilayer movement of SM during apoptosis determines substrate availability and thereby controls ceramide formation. The death receptors, Fas and TNF-R, activation have been shown to trigger the acid SMases (ASMases) and release ceramide (Figure 1.8) within seconds to minutes upon stimulation. A later release of ceramide is likely mediated by neutral sphingomyelinases. The activation of the ASMases and the release of ceramide are important for the initiation of apoptosis by Fas and TNF, since ASMases-deficient B-lymphocytes, hepatocytes and peripheral blood lymphocytes were resistant to apoptosis. Addition of C_{16}-ceramide restored Fas-induced apoptosis, indicating that it is the lack of ceramide that concludes resistance to Fas[80]. Fas activation of Jurkat cells and primary cultures of hepatocytes is followed by the translocation of ASMase into membrane rafts. The consequent hydrolysis of SM to ceramide, which self-associates, initiates coalescence of ceramide-enriched domains into larger patches and platforms. The functional significance of this reorganization of membrane structure would appear to allow for protein oligomerization. Preformed Fas trimers localize to these domains and appear to form higher-order structures, allowing for the oligomerization of the downstream adaptors FADD and caspase-8. This key step, which, at least in some systems, is necessary for transmembrane transmission of the Fas apoptotic signal, is suppressed under basal conditions because only the ligated Fas that activates ASMase appears capable of entering the membrane platforms[83]. A large number of data established that ceramide acts as a second messenger in mediating radiation-induced apoptosis in specific systems *in vitro* and *in vivo*[81, 82]. Haimovitz-Friedman et al[70] proved that irradiation of bovine aortic endothelial cells (BAEC) induced sphingomyelin conversion to ceramide within seconds and apoptosis. Analogs of ceramide, but not other potential second messengers, mimicked the effect of radiation to signal apoptosis. Additional data on the molecular ordering of ceramide signaling in response to radiation was provided by Tepper et al[150]. In Jurkat cells, radiation, etoposide or CD95-induced ceramide generation and apoptosis, while a clone resistant to CD95 resulted in cross-resistance to ionizing radiation and etoposide[150]. The apoptotic effect of ceramide is a consequence of the modulation of mitochondrial function, for instance by inhibiting the mitochondrial respiratory complex III [148, 149].

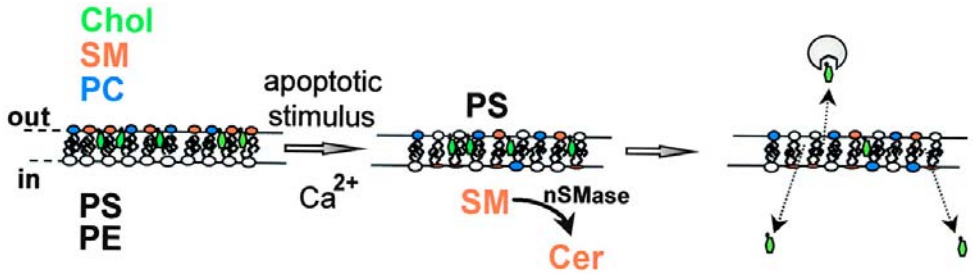

Figure 1.7 **Schematic representation of ceramide formation.** In viable cells, SM (red) and PC (blue) localize to the exoplasmic leaflet of the plasma membrane, while PS and PE (white) are sequestered in the inner leaflet. Cholesterol (Chol; green) partitions between both leaflets, but only the preferential clustering with outer leaflet SM is indicated. An apoptotic stimulus or elevated calcium induces loss of the asymmetric phospholipids distribution, and SM appears in the inner leaflet, where it is immediately hydrolyzed to ceramide by an intracellular neutral sphingomyelinase (nSMase). Reduced SM content alters biophysical properties of the lipid bilayer, allowing morphological changes such as membrane blebbing and vesicle shedding. (*taken from ref. 109*)

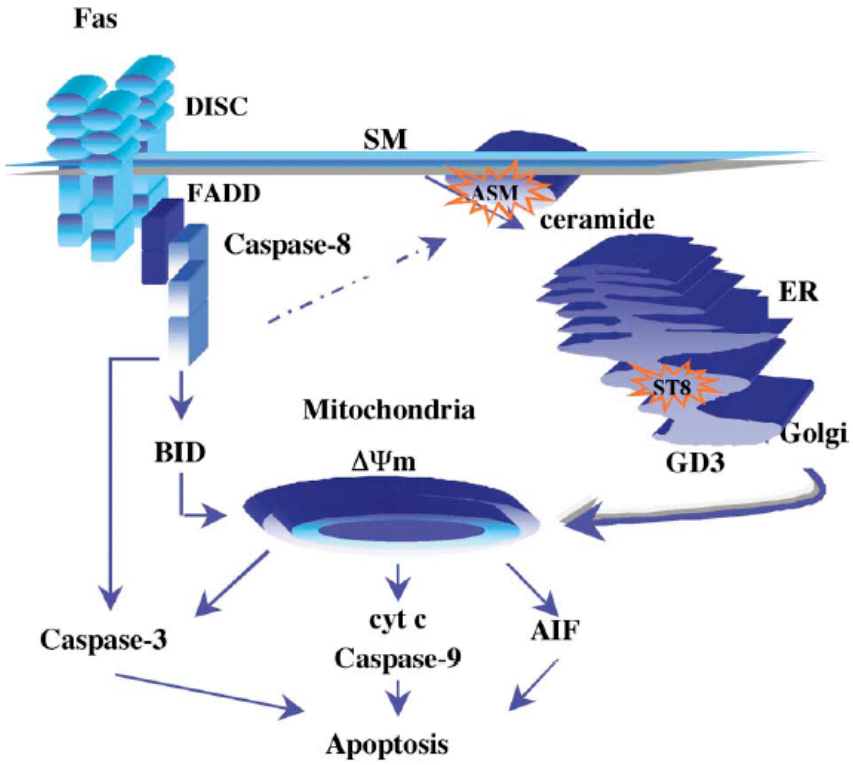

Figure 1.8 **A model for Fas-induced ceramid formation.** Two main death domain dependent pathways are activated upon crosslinking of Fas: one involves the proteolitic cascade mediated by the caspases while the other one leads to ceramide accumulation and to the synthesis of complex glycosphingolipids. The latter pathway involves the activation of an ASMase, which leads to the transient accumulation of apoptogenic ceramid. The addition of sugars and sialic acid converts ceramide into GD3 ganglioside. (*taken from ref 84*)

2. Aims of the study

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?

3. Materials and Methods

Cell lines

Jurkat cells, I 9.2 (Jurkat lacking caspase 8) (ATCC), Raji, BL-41 and Daudi were maintained in RPMI-1640 containing 5% fetal calf serum (FCS) (*appendix* **A1**) in an incubator with 5% CO_2 at 37°C. JCaM1.6 ($p56^{lck}$ deficient), JCaM1/Lck (JCaM1.6 re-transfected with $p56^{lck}$) [153], P116 (ZAP70 deficient)[60] and P116WT (P116 re-transfected with ZAP70)[60] Jurkat variants were cultured in RPMI 1640 with 10% FCS under the same conditions. The cell lines I 9.2 was a kind gift from Dr. V. Chitu (Albert Einstein College of Medicine of Yeshiva University, New York), P116 and P116WT donated by Dr. R.T. Abraham (Mayo Clinic, Rochester) and JCaM/Lck was generously provided by Dr. A. Weiss (Howard Hughes Medical Institute, San Francisco). During the apoptosis experiments the cells were cultured in RPMI containing 1% FCS with or without 1.8μM galectin-1.

Reagents

Antibodies:

mouse anti-PARP monoclonal antibody (mAb) (Serotec)

anti-ceramide mAb, MID 15B4 (Alexis Biochemicals)

anti-galectin-1 mAb, 2C1/6 (developed in our laboratory)

rabbit anti-mouse IgG conjugated to horseradish peroxidase (HRP) (DAKO)

biotinylated rabbit anti-mouse IgM (DAKO)

Inhibitors (table II):

Inhibitor	Activity	Manufacturer	Comments
caspase inhibitor I (zVAD-fmk)	Broad spectrum casapase inhibitor	Calbiochem	Stock 10 mM in DMSO. Store at -20^{0}C.
caspase 8 inhibitor I (Ac-IETD-CHO)	Caspase 8 inhibitor	Calbiochem	Stock 10 mM in DMSO. Store at -20^{0}C.
bongkrekic acid	Inhibitor of mitochondrial megachannel	Calbiochem	Stock 2 mM in 2 N NH_4OH. Store at -20^{0}C.
fumonisin B1	Inhibitor of sphingolipid biosynthesis	Sigma	Stock 5 mM in DMSO. Store at 4^{0}C.
genistein	Proteine kinase inhibitor	Sigma	Stock 20 mM in DMSO. Store at -20^{0}C.

Other reagents:

streptavidin-FITC (DAKO), AnnexinV-FITC (Pharmingen), MitoTracker Red CMX-Ros (Molecular Probes), prestained molecular weight marker (GIBCO-BRL), nitrocellulose membrane (Schleicher & Schuell), Enhance Chemiluminescence (ECL) plus detection system (Amersham Bioscience), X-ray film (Medifort SFB).

Tumor necrosis factor α (TNFα) was kindly provided by Dr. Erno Duda (Biological Research Center, Szeged Hungary).

Recombinant galectin-1 was cloned and purified by lactose affinity chromatography as previously described[61]. The empty vector was cloned and the bacterial lysate was processed on the same way as for galectin-1 purification and used as control in all experiments.

For buffers and solutions see appendix.

Detection of galectin-1 binding activity

Cells were incubated in RPMI containing 1.8 μM galectin-1 for one hour on ice. After washing two times with cold phosphate-buffered saline (PBS) (*appendix* **A3**) the cells were stained with anti-galectin-1 (10 μg/ml in immunofluorescence buffer (IFB, *appendix* **A9**)) for 45 min on ice. Afterward the samples were washed and stained with streptavidin-FITC (1:100 dilution in PBS) for 30 min. Finally, the cells were washed three times with PBS and analyzed on FACSCalibur using CELLQuest software programs (Becton Dickinson). Before data acquisition propidium iodide (*appendix* **A4**) was added to the cells to the final concentration of 1μg/ml and the propidium iodide positive cells were gated out during analysis.

Detection of tyrosine phosphorylation

The cells were stimulated at a concentration of $5x10^7$cells/ml in RPMI without FCS by adding 1.8 μM of galectin-1 for 10 min at 37^0C. Activation was stopped by addition of equal volume of 2x concentrated ice-cold lysis buffer (*appendix* **A14**). The cells were lysed for 30 min on ice and cleared off the nuclear/cytoskeletal components by centrifugation at 12,000xg for 15 min. The proteins were separated on a 7.5%-15% gradient of SDS polyacrylamide gel (*appendix* **A16**) and transferred to nitrocellulose membrane in transfer buffer (*appendix* **A20**). The membranes were blocked using Tris-buffered saline Tween 20 (TBST, *appendix* **A22**) containing 3% cold fish gelatin and subsequently probed with anti-phosphotyrosine mAb,

4G10 and rabbit anti-mouse IgG-HRP. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL plus) detection system.

Annexin-V labeling

To detect PS exposure on the outer cell membrane, the Jurkat cells were treated as indicated, then washed twice with PBS and resuspended in binding buffer (*appendix* **A8**). Annexin V-FITC and propidium iodide (10µg/ml) were added to the cells for 15 min in dark, at room temperature. After washing, the cells were analyzed on FACSCalibur cytofluorimeter (Becton and Dickinson).

Immunofluorescence staining of intracellular ceramide

After treatment, the cells were settled by cyto-centrifugation (Cytospin from Shandon Southern Products Ltd.) at 120xg for 1min, fixed in PBS containing 1% paraformaldehyde (*appendix* **A10**) for 15 min. Then the cells were washed with washing buffer (*appendix* **A12**) and permeabilized (*appendix* **A11)** for 10 min. The samples were labeled with anti-ceramide mAb (1:10 dilution in IFB) for 45 min, washed three times and followed by biotin-conjugated anti-mouse-IgM (1:250 dilution in IFB) for 30 min. Afterward it was washed and the cells were stained with streptavidin-FITC (1:100 dilution in PBS) for 30 min. Finally, the cells were washed three times with PBS and the slides where mounted with glycerol. All steps were done at room temperature. The stained 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 (*appendix* **A7**) 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 (100 ng/ml in PBS) for 15 min at 37^0C. After incubation, the cells were washed twice with PBS and the fluorescence intensity was measured on FACSCalibur.

Detection of caspases activity

The cells were cultured in 96-well plate and after 16 hours gal-1 treatment the plates were equilibrated for 15 min at room temperature. Caspase-Glo™ 9 or Caspase-Glo™ 3 was added to the samples and caspase 9 and 3 activity were detected according with manufacturer's instructions. After 1 hour incubation the luminescence was measured using LuminoScan plate-reading luminometer.

Detection of PARP proteolysis

The samples (10^7cells/ml) were lysed in reducing SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer 1x concentrated (*appendix* **A13**) and boiled for 5 min. The lysate was then centrifuged with 12,000xg for 15 min at room temperature. Twenty µl of the cell equivalent was loaded onto a 6-12% SDS polyacrylamide gel and then transferred onto nitrocellulose membrane. The membrane was blocked using TBST containing 5% nonfat milk and subsequently probed with mouse anti-PARP (1:500 dilution) overnight at 4^0C, washed and then stained with rabbit anti-mouse IgG-HRP (1:7500 dilution) for 1 hour at 4^0C. Immunoreactive proteins were visualized by ECL plus detection system. Images of immunoblots were captured with Hewlett Packard scanner, exported to Adobe Photoshop 7.0, and then Tiff images were placed for final presentation in CorelDRAW 10.

Hypodiploid, 'sub-G1' cell population

Cells were treated as indicated and subjected to DNA content analysis[114]. Briefly, the cells were harvested and washed two times with 1x sample buffer (*appendix* **A5**) then permeabilized and stained with DNA staining buffer (*appendix* **A6**). After incubation in dark for 30 min at room temperature the cells were analyzed on FACSCalibur. The sub-G1 (hypodiploid) population was determined with cell cycle analysis using CELLQuest software programs (Becton Dickinson) and was considered as apoptotic cells.

4. Results

4.1 Apoptosis induced by galectin-1 is accompanied by the release of ceramide and the reduction of the mitochondrial membrane potential

We previously showed that galectin-1 induced tyrosine phosphorylation in Jurkat cells[52] with a maximum of 10-20 minutes then reverted to the baseline level (Figure 4.1)

The loss of the membrane asymmetry and hence the exposure of the PS on the outer surface of the cell membrane is a major consequence of apoptotic trigger[62]. Jurkat cells treated with galectin-1 exposed PS as it was detected with FITC labeled AnnexinV (Ann V) (Figure 4.2). The number of $AnnV^+$/propidium $iodide^-$ (PI) cells increased up to 10 hours of galectin-1 treatment, remained on this level till 16 hours, then declined (Figure 4.2 LR on dot plots and on diagram). These cells did not allow PI to enter, indicating an intact cell membrane. The $AnnV^+/PI^+$ cell population, representing the late apoptotic cells, increased continuously up to 24 hours (Figure 4.2 UR on dot plots).

Parallel with the change of the PS orientation, ceramide, a frequent component of the apoptotic response, was released as we detected with immunostaining of the cells with monoclonal anti-ceramide antibody. The ceramide expression elevated up to 12 hours then sharply decreased (Figure 4.3). The maximum level of ceramide at 12 hours of stimulation coincided with the highest level of the PS on the outer cell membrane (Figure 4.2).

The decrease or loss of the mitochondrial membrane potential ($\Delta\psi_m$) is a central step during apoptosis triggered with certain drugs, oxidative stress or UV radiation. It was sharply reduced from 6 to 16 hours after induction with galectin-1 and then stayed steady (Figure 4.4).

The time course of the caspase activation was followed by the cleavage of a caspase substrate, the nuclear repair enzyme, poly-ADP ribosyl polymerase (PARP). This late response was first detected at 10 hours after galectin-1 treatment and elevated till 24 hours (Figure 4.5).

The final step, the degradation of the nuclear DNA, was analyzed by the formation of the ‘sub-G1’, hypodiploid cell population (Figure 4.6). This process started at 10 hours after stimulation and increased up to 24 hours.

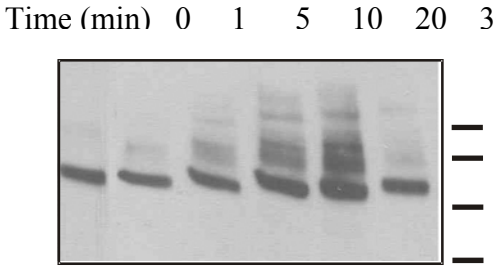

Figure 4.1 **Galectin-1 induces tyrosine phosphorylation in a time dependent manner.** Jurkat cells were treated with 1.8 μM galectin-1 for the indicated time points at 37^0C and lysed. The proteins in the postnuclear supernatants were separated by 7.5-15% gradient SDS-PAGE, and then transferred to nitrocellulose membrane. The membranes were blotted with anti-phosphotyrosine monoclonal antibody followed by anti-mouse IgG-HRP and developed with ECL system.

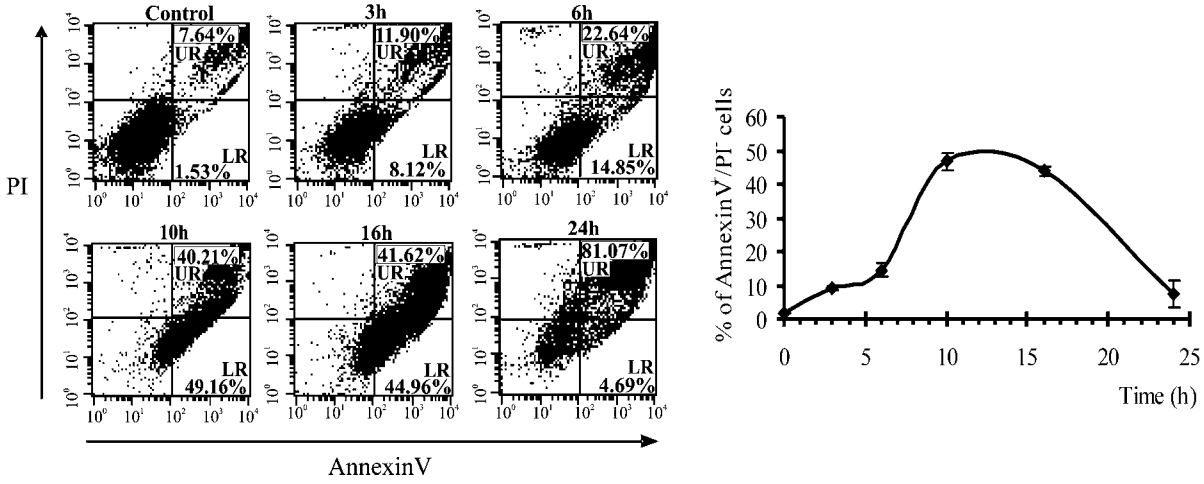

Figure 4.2 **Phosphatidyl serine is exposed after galectin-1 treatment.** Jurkat cells ($5x10^5$/ml) were incubated with 1.8 μM galectin-1 for 3, 6, 10, 16 and 24 hours or left unstimulated (for control see Materials and methods), then stained with AnnexinV-FITC and propidium iodide and analyzed with cytofluorimetry. The right panel shows the dot plots of the cytofluorimetry. The percentage of AnnexinV positive and propidium iodide negative cells is presented graphically on the left (mean± SD of duplicates). (UR: upper right; LR: low right)

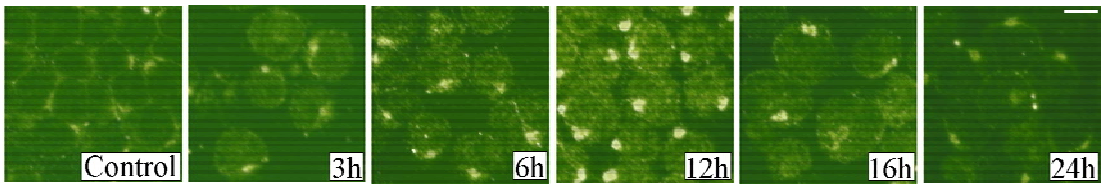

Figure 4.3 **Galectin-1 induces ceramide release.** The cells treated with 1.8 μM galectin-1 for the indicated times were cytospined, fixed, permeabilized and stained with anti-ceramide antibody followed with biotinylated anti-mouse IgM and streptavidin-FITC and investigated by microscopy. Bar 20 μm.

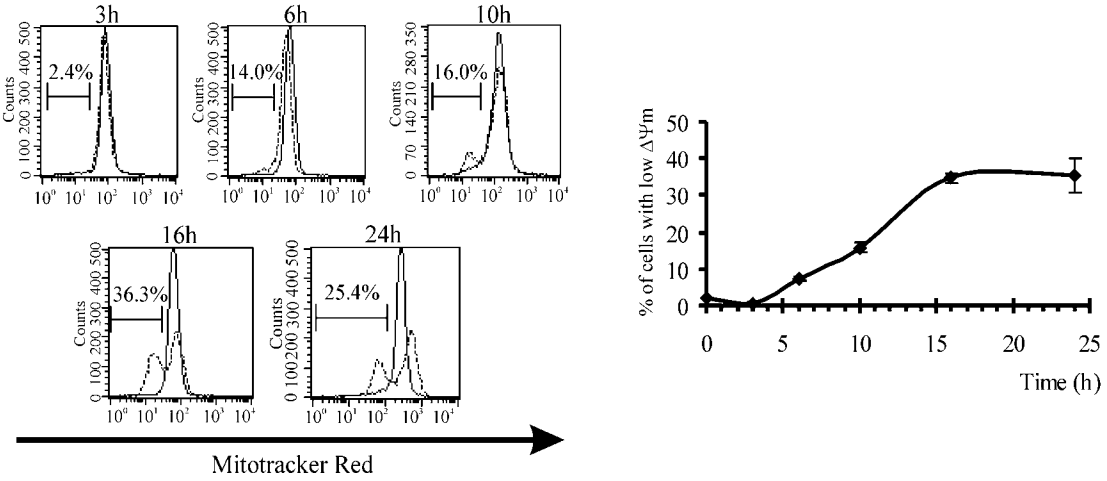

Figure 4.4 **Loss of mitochondrial potential occurs in galectin-1 treated cells.** Jurkat cells were treated with galectin-1 as described at Figure 4.2 (dashed line) or left untreated (continuous line) for the indicated times. After treatment the cells were loaded with MitoTracker Red CMX-Ros and Δψm was analyzed with cytofluorimetry. The histograms obtained for each time points are shown on the right. The mean values of the loss of Δψm of two samples/time point (±SD) are presented by the time graphically (left).

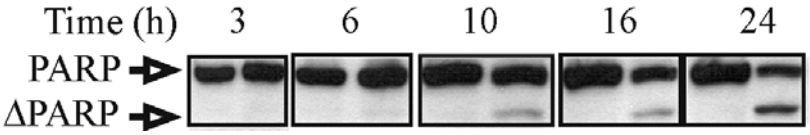

Figure 4.5 **PARP is cleaved in galectin-1 treated cells.** Lysates prepared from cells treated with galectin-1 for different times were subjected to SDS-PAGE and transferred to nitrocellulose membrane. Cleavage of PARP was detected in Western blotting using mouse anti-PARP followed by anti-mouse IgG-HRP and ECL detection.

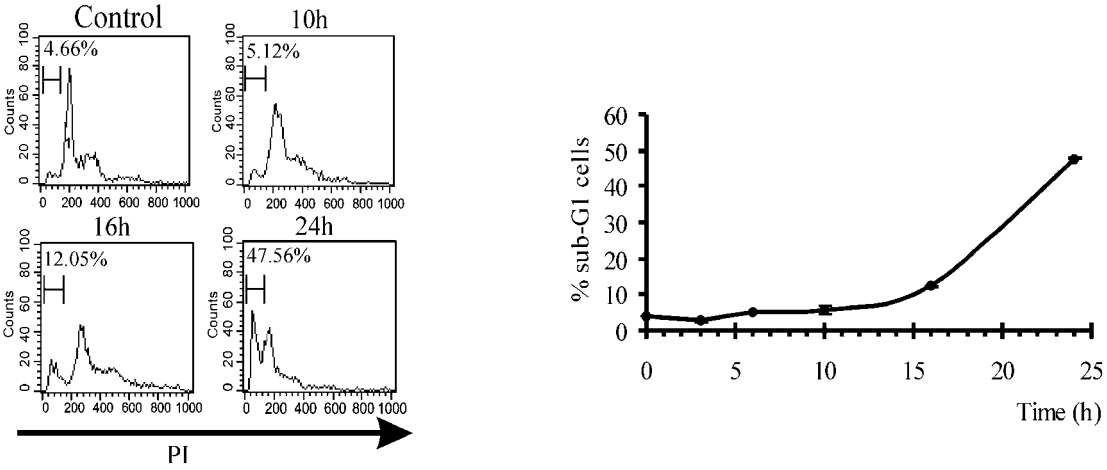

Figure 4.6 **DNA is fragmentated upon galectin-1 treatment.** The time course of formation of 'sub-G1' cell population was determined by cytofluorimetry using propidium iodide labeling of permeabilized cells. The histograms obtained for each time points are shown on the right. The mean values of the 'sub-G1' population of two samples/time point (±SD) are presented by the time graphically (left).

Galectin-1 induced apoptosis depends on p56^{lck} and ZAP70 mediated tyrosine phosphorylation

The tyrosine kinase inhibitor, genistein blocked the galectin-1 stimulated protein tyrosine kinase (PTK) activity (Figure 4.7), since in the presence of the inhibitor galectin-1 did not cause phosphorylation over the non-stimulated control. The phosphorylation step was significant in the process of cell death, as genistein did not only inhibit the tyrosine phosphorylation, but also the increase of the ceramide level (Figure 4.8) and the formation of the apoptotic 'sub-G-1' cells (Figure 4.9). The essential role of the tyrosine kinase, p56^{lck} in the ceramide[111] and mitochondrion mediated[110] apoptotic pathways in Jurkat cells has recently been well documented. Whether or not Lck and one of its main immediate target, ZAP70 were the responsible kinases in galectin-1 induced cell death, the Lck deficient Jurkat cells, JCaM1.6 and ZAP70 mutant, P116 were treated with galectin-1. The treatment did not cause tyrosine phosphorylation over the untreated control (Figure 4.10) and the PARP cleavage was not detectable (Figure 4.11). Accordingly, the intracellular ceramide levels in stimulated and non-stimulated cells were similar (Figure 4.12) and the PS exposure was severely damaged (Figure 4.13) in Lck and ZAP70 deficient cells. The loss of $\Delta\psi_m$ was dramatically inhibited (Figure 4.14) in JCaM1.6 and P116. Re-expression of the Lck (JCaM/LCK) and ZAP70 (ZAP70WT) in JCaM1.6 and P116, respectively restored the ceramide release (Figure 4.12), PS exposure (Figure 4.13) and decrease of $\Delta\psi_m$ (Figure 4.14) upon gal-1 treatment.

4.3 Ceramide is indispensable component in galectin-1 triggered cell death signaling

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 [111]. It can be a consequence of the scrambling of the membrane asymmetry and the subsequent translocation and activation of a sphingomyelinase or it can be produced via the *de novo* synthesis of sphingolipids. In the absence of the increase of the ceramide expression, the apoptosis (decrease of mitochondrial membrane potential and DNA degradation) is still executed [109]. To understand the role of the ceramide release in galectin-1 triggered cell death (Figure 4.3), the ceramide expression was modulated by several

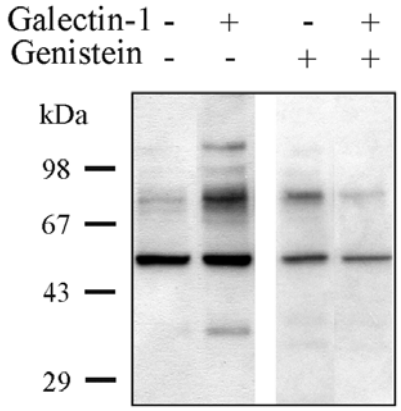

Figure 4.7 **Galectin-1 induced tyrosine phosphorylation in T cells.** Jurkat cells were stimulated with or without 1.8 µM galectin-1 in the presence or absence of 250 µM genistein. For analysis of tyrosine phosphorylation, the cells were treated for 10 minutes at 37°C and lysed. Tyrosine phosphorylation was then analyzed as described under Figure 4.1.

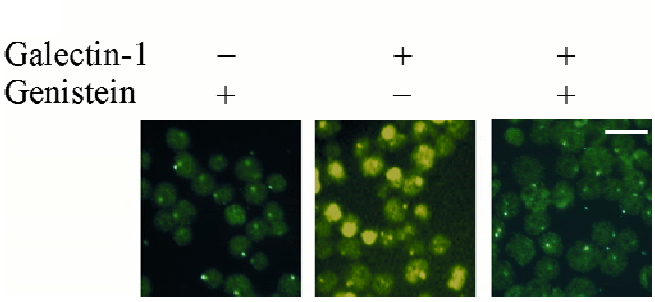

Figure 4.8 **Tyrosine phosphorylation is a regulator of the ceramide release.** Jurkat cells were stimulated with or without 1.8 µM galectin-1 in the presence or absence 75 µM genistein. The ceramide release was detected after 12 hours of stimulation with galectin-1. The cytocentrifuged cells were prepared and stained as described at Figure 4.3. Bar 20µm.

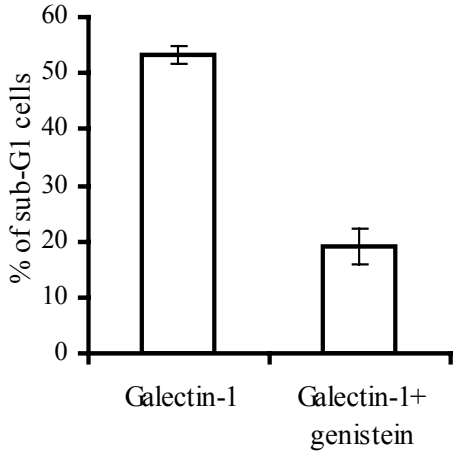

Figure 4.9 **Genistein blocked DNA fragmentation.** The cells were treated as described at Figure 4.8. 'Sub-G1' cell population was determined in cell cultures after 24 hours treatment with galectin-1, as described at Figure 4.6. The results are shown as mean values of three samples ±SD. The appropriate control values were subtracted from the sample values.

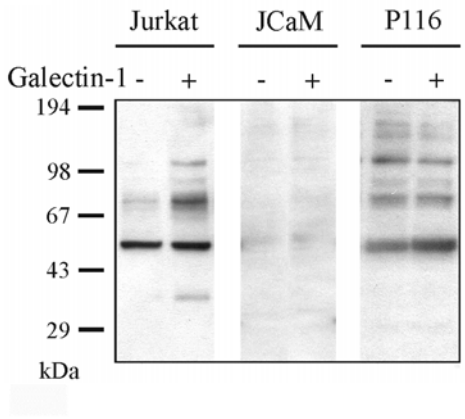

Figure 4.10 **Absence of p56^{lck} and ZAP70 blocked tyrosine phosphorylation induced by galectin-1.** Jurkat cells and the p56^{lck} and ZAP70 deficient Jurkat variants (JCaM1.6 and P116, respectively) were stimulated with galectin-1 for 10 minutes at 37°C or left unstimulated. Tyrosine phosphorylation was then analyzed as described under Figure 4.1.

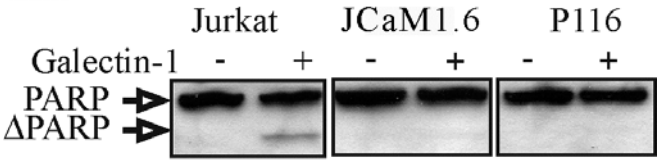

Figure 4.11 **Cleavage of PARP is not detectable in kinase deficient cell lines.** Jurkat, JCaM1.6 and P116 cells were treated with galectin-1 for 24 hours or left untreated. Cleavage of PARP was detected as described under Figure 4.5.

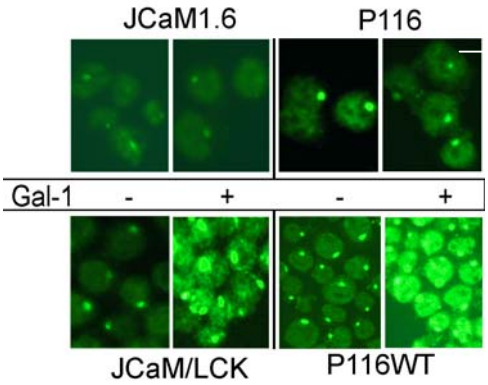

Figure 4.12 **The ceramide release induced by galectin-1 requires the presence of p56^{lck} and ZAP70.** Deficient Jurkat cells, JCaM1.6, P116 and back retransfected variants (JCaM/LCK and P116WT) were incubated with galectin-1 for 12 hours then the release of ceramide was analyzed with immunocytochemistry as described under Figure 4.3. Bar 20µm.

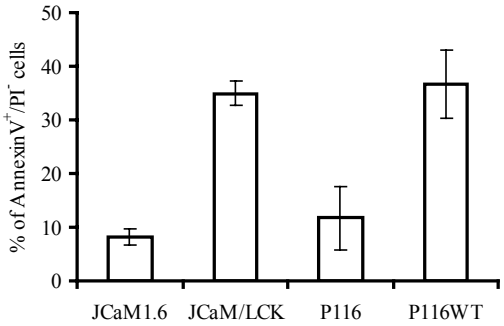

Figure 4.13 **Absence of p56^{lck} and ZAP70 damaged the exposure of PS.** The cells were treated or untreated with galectin-1 and the PS exposure was analyzed by staining the cells with Annexin-V-FITC/propidium iodide as described under Figure 4.2. The assay was done in triplicates and results are shown as the mean ±SD. The appropriate control values were subtracted from the sample values.

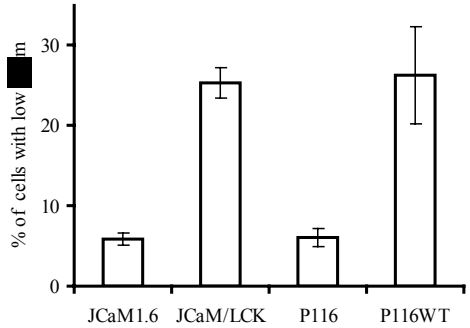

Figure 4.14 **Absence of p56^{lck} and ZAP70 impairs the decrease of ΔΨm.** The loss of the mitochondrial potential was measured after treating the cells with galectin-1 for 16 hours and then was analyzed as described under Figure 4.4. The assay was done in triplicates and results are shown as the mean ±SD. The appropriate control values were subtracted from the sample values.

means. The extraction of the outer membrane lipids with bovine serum albumin (BSA)[109] resulted in a reduction of the apoptotic response of Jurkat cells (Figure 4.15-4.18). Jurkat cells failed to expose PS on the outer surface of the plasma membrane (Figure 4.15) and release ceramide (Figure 4.16) in the presence of 5% BSA in the culture medium following BSA extraction. As a consequence, the $\Delta\psi_m$ and formation of 'sub-G1' cells were completely inhibited (Figure 4.17 and 4.18). The inhibition of the apoptosis was not cause by the change in the binding of galectin-1 to its ligands since the binding capacity remained unaltered in the presence of 5% BSA (Figure 4.19). The protein tyrosine kinase (PTK) pathway was also implicated in ceramide release since the inhibition of PTKs with genistein stopped the elevation of ceramide (Figure 4.8). To provide further evidence that ceramide played role as a second messenger in galectin-1 induced apoptosis, we used Raji, a membrane scrambling-deficient Burkitt lymphoma cell line. It was published by Tepper et al. [109] that Raji cells failed to expose PS and hence to produce ceramide upon apoptotic stimuli but it still died upon Fas ligation. When treated with galectin-1, Raji cells failed to respond with apoptosis (Figure 4.20), although other B cell lines of Burkitt lymphoma origin, Daudi and BL41 died upon the same treatment (table III). To gain a direct evidence that ceramide is not generated through sphingolipid pathway we used fumonisin B1 a specific inhibitor of ceramide synthesis. This drug did not inhibit the effect of galectin-1 indicating that the production of ceramide upon galectin-1 stimulation did not occur through the synthetic route (Figure 4.21).

The anti-apoptotic metabolic product of ceramide, sphingosine-1 phosphate (S1P) that counteracts with the apoptotic effect of ceramide[63] reduced the galectin-1 caused $\Delta\psi_m$ (Figure 4.22), PARP cleavage (Figure 4.23) and the size of the 'sub-G1' cell population (Figure 4.24). As it was expected, the presence of S1P also blocked the cytotoxic effect of the exogenously added ceramide (C_6-Cer) (Figure 4.24).

In vivo the S1P is produced by phosphorylation of sphingosine by sphingosine kinase (SPHK), an enzyme which is activated by protein kinase C (PKC)[64]. When the PKC/SPHK pathway was stimulated by a phorbol-ester, PDBu, it diminished all measured steps of the apoptosis ($\Delta\psi_m$, PARP breakdown, elevation of 'sub-G1' cell number, (Figure 4.22, 4.23 and 4.24, respectively), as it did for C_6-Cer induced cell death (Figure 4.24).

These results did not only suggest that ceramide was an indispensable messenger of

galectin-1 induced apoptosis, but also that ceramide release occurred upstream to the mitochondrial changes, caspase activation and breakdown of the nuclear DNA as downregulation ceramide expression inhibited the other apoptotic steps.

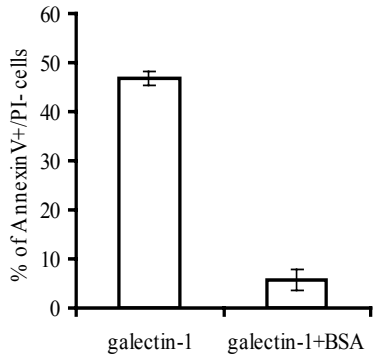

Figure 4.15 **Jurkat cells failed to expose PS in the presence of BSA**. The cells were preincubated twice with 5% BSA for 5 minutes on ice (BSA extraction). Subsequently, the cells were treated with galectin-1 in the presence or absence of 5% BSA for 12 hours. Then the PS exposure was analyzed as described under Figure 4.2. The experiments have been carried out in triplicates; the results are shown as the mean ±SD. The appropriate control values were subtracted from the sample values.

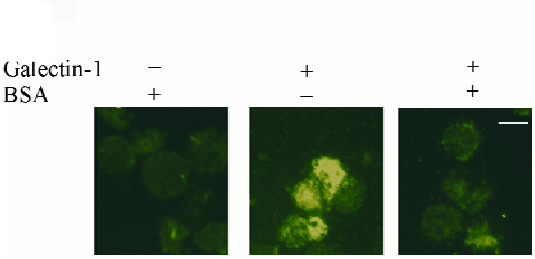

Figure 4.16 **Ceramide release is blocked by the continuous presence of BSA in the culture medium.** Jurkat cells were cultured with galectin-1 in the presence (after BSA extraction) or absence of 5% BSA for 12 hours and subjected to immunocytochemistry using anti-ceramide antibody (see Figure 4.3). Bar 20μm.

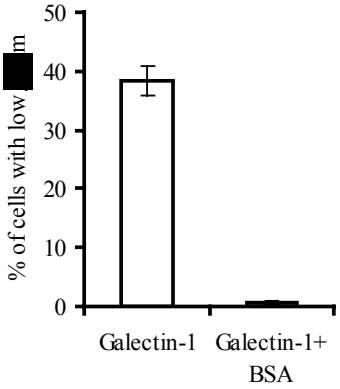

Figure 4.17 **Loss of mitochondrial potential is sharply reduced in the presence of BSA.** Jurkat cells were cultured for 16 hours with or without galectin-1 in the presence (after BSA extraction) or the absence of 5% BSA. Then cells were subjected to analysis of Δψm as under Figure 4.4. The experiment has been carried out in triplicates; the results are shown as the mean ±SD. The appropriate control values were subtracted from the sample values.

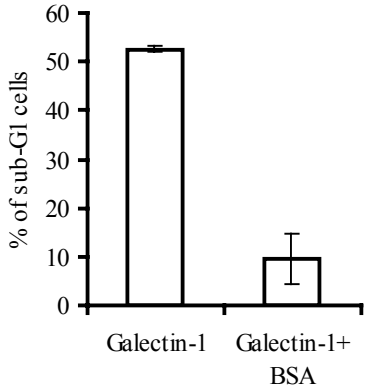

Figure 4.18 **Galectin-1 induced apoptosis does not occur in the absence of ceramide release.** After BSA extraction the cells were treated with galectin-1 in the presence of 5% BSA for 24 hours. The 'sub-G1' cell population was analyzed as described under Figure 4.6. The experiments have been carried out in triplicates; the results are shown as the mean ±SD. The appropriate control values were subtracted from the sample values.

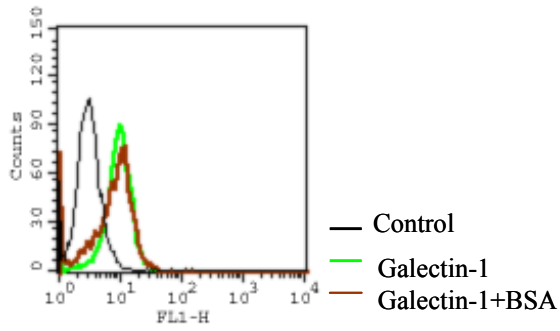

Figure 4.19 **The binding capacity of galectin-1 is not affected by the presence of BSA.** Jurkat cells were treated for one hour with or without 1.8 µM galectin-1 in the presence or the absence of 5% BSA. Then the cells were subjected to flow cytometry analysis using anti-galectin-1 antibody.

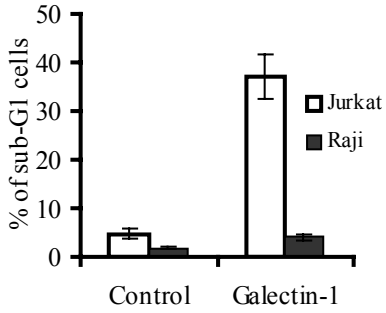

Figure 4.20 **Raji cells failed to respond with apoptosis after galectin-1 treatment.** Jurkat and Raji cell lines were treated with galectin-1 for 24 hours and then the 'sub-G1' cell population was analyzed (see Figure 4.6). The experiments have been carried out in triplicates; the results are shown as the mean ±SD.

Cells	% of 'sub-G1'cells
BL-41	30.4
Daudi	35.4

Tabel III. **DNA content analysis of B cell lines.** BL-41 and Daudi cells were treated with galectin-1 for 24 hours and then the 'sub-G1' cell population was analyzed as described under Figure 4.6.

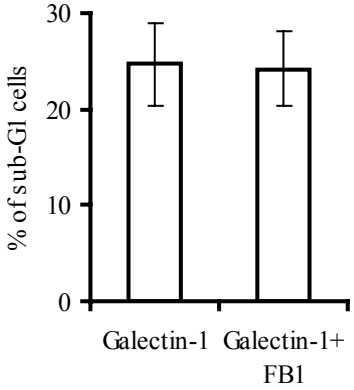

Figure 4.21 **Galectin-1 induced apoptosis does not depend on the novo synthesis of ceramide.** Jurkat cells were treated with galectin-1 in the presence or absence of 10 µM fumonisin B1 for 24 hours. The 'sub-G1' cell population was analyzed as described under Figure 4.6. The experiments have been carried out in triplicates; the results are shown as the mean ±SD. The appropriate control values were subtracted from the sample values.

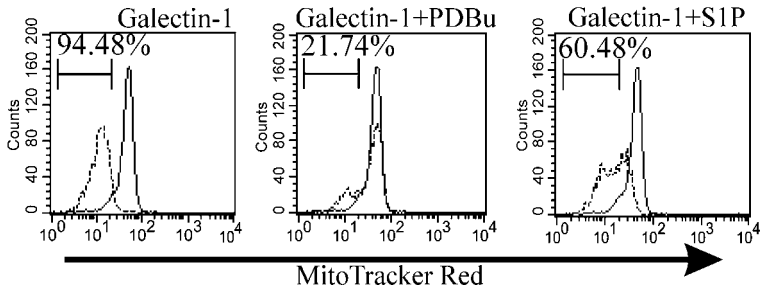

Figure 4.22 **Galectin-1 mediated changes in mitochondrial Δψ can be modulated by S1P and PDBu.** Jurkat cells ($5x10^5$/ml) were treated with (dashed line) or without (continuous line) 1.8 μM galectin-1 in the presence or absence of 5 μM S1P or 50 ng/ml PDBu. The cells were subjected to analysis of $\Delta\psi_m$ (see Figure 4.4). The percentage of cells with low $\Delta\psi_m$ is presented.

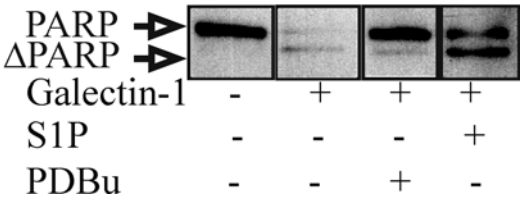

Figure 4.23 **Cleavage of PARP is repressed by PDBu and S1P.** Jurkat cells were treated as described under Figure 4.22. After 24 hours treatment the cells were lysated followed by immunoblotting detecting PARP degradation (see Figure 4.5).

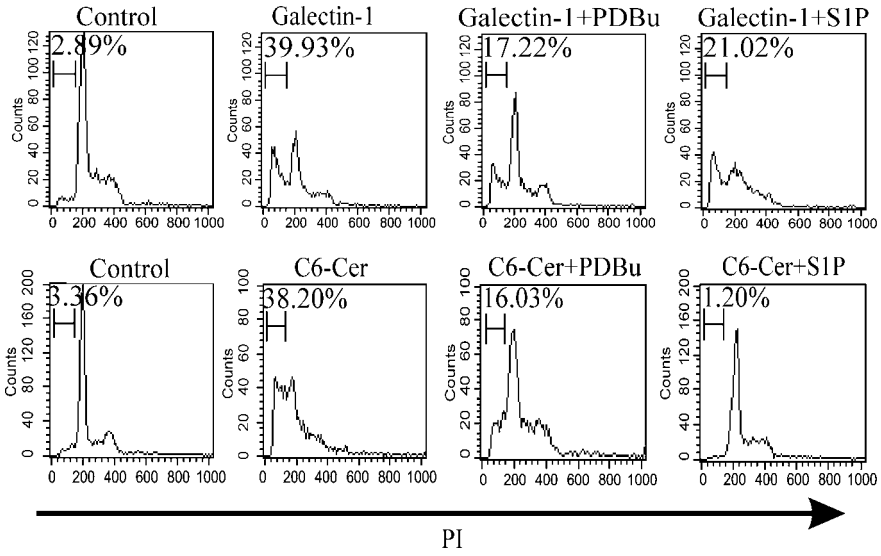

Figure 4.24 **PKC activation and ceramide metabolite, S1P inhibit DNA fragmentation induced by galectin-1.** Jurkat cells were treated with or without 1.8 μM galectin-1 or 10 μM C6-Cer in the presence or absence of 5 μM S1P or 50 ng/ml PDBu. The DNA content was investigated as described for Figure 4.6. The percentage of 'sub-G1' cells is presented.

4.4 Galectin-1 induced apoptosis belongs to the 'mitochondrion- first' type cell death

The caspase cascade can be initiated either by the death receptor mediated caspase 8 or by the mitochondrion mediated caspase 9 activation. To confirm the role of caspases in galectin-1 triggered apoptosis and to determine the initiator caspase, the caspase activities were investigated. The broad-spectrum caspase inhibitor, zVAD-fmk but not the initiator caspase 8 inhibitor, Ac-IETD impeded the activation of the caspase cascade and hence the PARP cleavage (Figure 4.25) and the formation of the 'sub-G1' cells (Figure 4.26). On the other hand, the inhibition of the caspase 8 activity blocked the apoptosis triggered by TNFα (Figure 4.26), a known pathway initiating cell death via caspase 8. To confirm that caspase 8, the initiator caspase in death receptor mediated apoptosis, was not involved, we used caspase 8 deficient Jurkat cells (I 9.2). Both Jurkat and I 9.2 cell lines were sensitive to galectin-1 but the caspase 8 mutant cells were resistant to apoptosis initiated via the TNF receptor stimulation (Figure 4.27). The initiator caspases 9 showed an enhanced substrate cleavage and accordingly, activity of caspase 3 increased as well (Figure 4.28).

To determine whether galectin-1 triggers the apoptosis on a 'caspase-first' or 'mitochondrion-first' type way (this terminology was taken from the review of N.B. Blatt and G.D. Glick[96]) the involved caspases and the order of the caspase activation in relation to the mitochondrial events were also investigated. The presence of bongkrekic acid (BA) an inhibitor of the mitochondrial events blocked the destruction of the nuclear DNA (Figure 4.29) indicating that this step was essential in the execution of the apoptosis. The loss of the $\Delta\psi_m$ was not affected by the presence of general caspase inhibitor, zVAD–fmk (Figure 4.30). Using H_2O_2, a model reagent of oxidative stress, for apoptosis induction, which acted directly on the mitochondrion[65], the cell death was also similar in the presence and absence of zVAD–fmk (Figure 4.30). These results supported that galectin-1 initiated the 'mitochondrion-first' pathway since the caspase activation occurred downstream to the mitochondrial events.

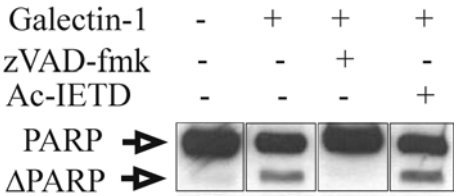

Figure 4.25 **Cleavage of PARP is not affected by caspase-8 inhibitor.** Jurkat cells were cultured with or without galectin-1 in the presence or absence of 50 μM zVAD-fmk or 50 μM Ac-IETD for 24 hours. Then the PARP degradation was analyzed with immunoblotting (see Figure 4.5).

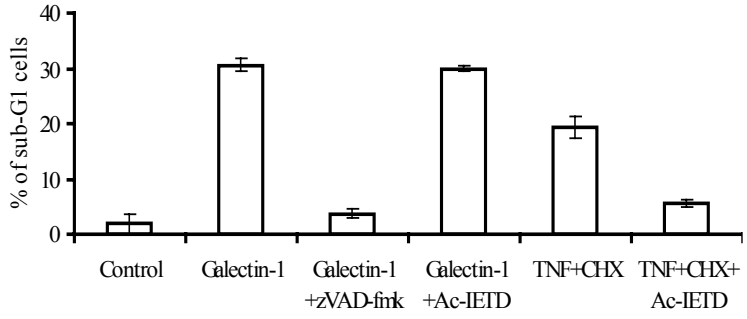

Figure 4.26 **Caspase-8 is not the initiator caspase in apoptosis induced by galectin-1.** The cells were treated with galectin-1 or 1.5 μg/ml cycloheximide (CHX) together with 50 ng/ml TNFα in the presence or absence of 50 μM zVAD-fmk or 50 μM Ac-IETD for 24 hours. Then the samples were subjected to DNA content analysis (see Figure 4.6). The results are presented as the mean ±SD of three independent experiments.

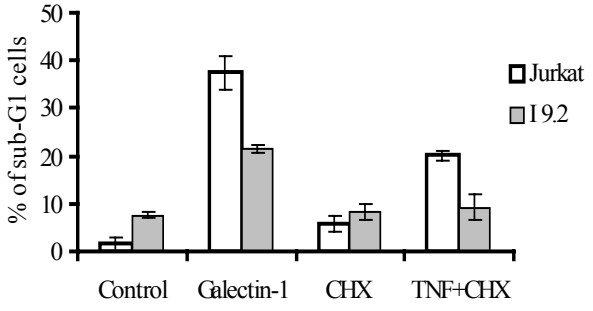

Figure 4.27 **Caspase-8 deficient cell line is sensitive to apoptosis induced by galectin-1.** Jurkat and caspase 8 deficient Jurkat (I 9.2) cells were cultured for 24 hours with or without galectin-1, 1.5 μg/ml CHX alone or 1.5 μg/ml CHX together with 50 ng/ml TNFα. Then cells were subjected to DNA content analysis. The results are presented as the mean ±SD of three independent experiments.

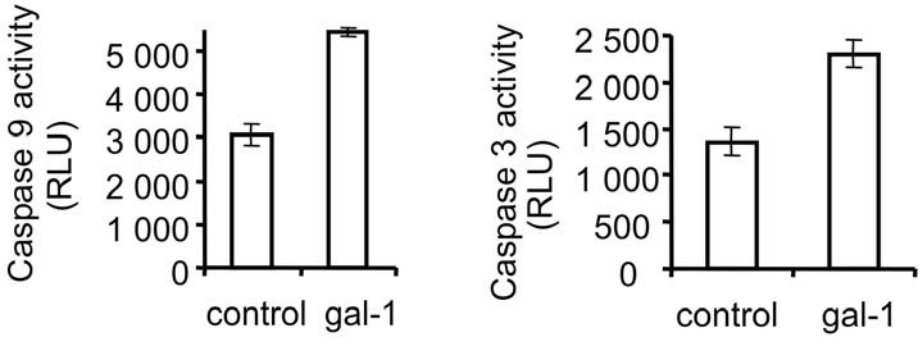

Figure 4.28 **Caspase 9 and 3 are involved in apoptosis induced by galectin-1.** Jurkat cells were treated with galectin-1 for 16 hours then they were subjected for caspase 9 (on the left) and caspase 3 (on the right) activity using Caspase-Glo™ 9 or Caspase-Glo™ 3 as substrate. The experiments were done in triplicates and they are presented as the mean ± SD.

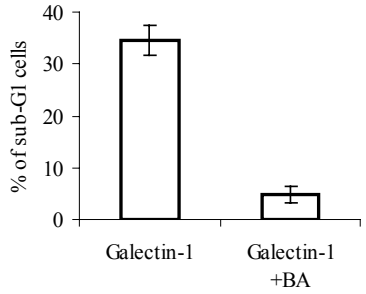

Figure 4.29 **Caspase activation occurs downstream to the mitochondrial events.** Jurkat cells were treated with or without galectin-1 in the presence or absence of 50 μM bongkrekic acid for 24 hours. Then cells were subjected for analysis of 'sub-G1'cell population as described under Figure 4.6. The appropriate control values were subtracted from the sample values. The results are presented as the mean ±SD of three independent experiments.

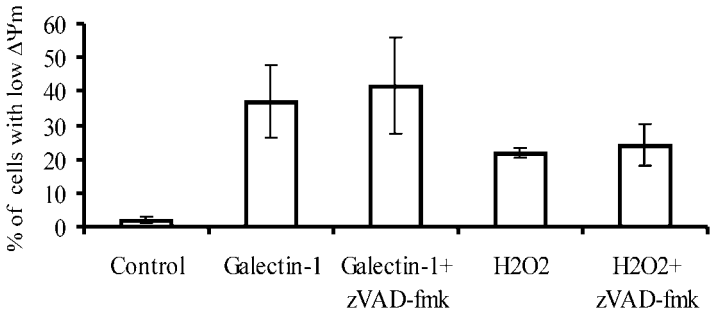

Figure 4.30 **The decrease of mitochondrial membrane potential is not affected by zVAD-fmk.** Jurkat cells were left untreated or treated with 1.8 μM galectin-1 or 20 μM H_2O_2 in the presence or absence of 50 μM zVAD-fmk for 16 hours. Then cells were subjected for analysis of $\Delta\psi_m$ (see Figure 4.4). The results are presented as the mean ±SD of three independent experiments.

5. Discussion

The present study offers an insight into the mechanism of the galectin-1 induced apoptosis. 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[25]. Accordingly, activated T cells from Fas deficient *lpr* mice also respond with apoptosis to galectin-1[44]. 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 (Figure 4.25, 4.26 and 4.27). In contrast, caspase 9 activity elevated upon gal-1 stimulation (Figure 4.28). It was previously published that caspase 3 was the effector caspase in galectin-1 induced apoptosis [58] and according to this finding we showed that caspase 3 was activated (Figure 4.28).

With the intention of determining the order of the intracellular steps and their significance in galectin-1 induced apoptosis, we analyzed their time course and sequence.

1. After galectin-1 stimulation, tyrosine kinase activity was triggered and the resulting tyrosine phosphorylation was essential for the further events as tyrosine kinase inhibitor, genistein blocked the ceramide release and the later apoptotic steps (Figure 4.7, 4.8, 4.9). Two kinases, p56^{lck} and ZAP70 played central roles since Jurkat cells deficient in these enzymes (JCaM1.6 and P116, respectively) responded with no tyrosine phosphorylation and with dramatically reduced apoptosis to galectin-1 stimulation (Figure 4.10, 4.11, 4.12 , 4.13, 4.14). The significance of the Lck/ZAP70 pathway was supported using JCaM/LCK and P116WT where the re-expression of Lck and ZAP70 in the deficient cells restored the apoptosis (Figure 4.12, 4.13, 4.14). Although the contribution of Lck to ceramide and mitochondrion mediated apoptotic processes has been proven[66, 110, 111] 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 function of the Lck/ZAP70 kinases has been supported by the recent finding that upon galectin-1 treatment the T cell receptor ζ chain is partially phosphorylated, a biochemical step that occurs during T cell apoptosis via this pathway[67]. The above kinases also regulate the activation of phospholipase C in T cells resulting in the production of diacylglycerol[68], which

in turn contributes to the ceramide release by activation of the sphingomyelinase[69].

2. During the classical apoptotic pathways the cell membrane asymmetry changes and as a result, PS turns to the outer surface of the membrane and serves as a phagocytic signal for the neighboring cells. Parallel with the PS exposure, sphingomyelin flips inside and serves as a substrate for sphingomyelinases to generate ceramide, the apoptotic second messenger[109]. Both events were detected with a similar time of maximum around 12 hours of galectin-1 stimulation (Figure 4.2 and 4.3). Although ceramide release was shown to be a general feature of apoptosis triggered by different ways, such as TNFR[70], or Fas[71] stimulation, in some cases this event was not essential for the execution of cell death[109]. To determine whether ceramide release was required for galectin-1 cytotoxicity, the ceramide pathway was modulated by several means. The inhibition of the ceramide release with BSA extraction of the outer membrane lipids[109] and the subsequent continuous presence of 5% BSA in the culture medium obstructed entirely the increase of the ceramide level and the subsequent cell death (Figure 4.15, 4.16, 4.17 and 4.18). The early tyrosine phosphorylation was critical for ceramide release and the downstream steps of the apoptosis as it was confirmed by inhibition of the tyrosine kinase activity (Figure 4.7, 4.8 and 4.9). The key role of functional Lck in ceramide mediated apoptosis was well demonstrated[111]. According to this finding the absence of the tyrosine kinase, p56^{lck} blocked the galectin-1 effect (Figure 4.10, 4.11, 4.12, 4.13, 4.14). Moreover, the presence of ZAP70 was also required for completing the apoptosis (Figure 4.10, 4.11, 4.12, 4.13, 4.14).

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 [72]. The phorbol-ester, PDBu, a potent activator of PKC, and the presence of exogenous S1P significantly blocked the galectin-1 induced apoptosis (Figure 4.22, 4.23, 4.24), strongly supporting a principal role for ceramide in this process.

The inhibition of the SMase activity 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.

Ceramides, as second lipid messengers, mediate the apoptosis induced by a variety of death stimuli[73]. The mitochondrial permeability transition and hence the breakdown of

mitochondrial membrane potential is directly regulated by ceramides in isolated mitochondria[74]. Moreover, ceramides act on enzymes participating in the transduction of death signals, for instance by activating the protein phosphatase A2 which rapidly dephosphorylates and inactivates Bcl-2[151] and on the contrary the phosphorylation of Bcl-2 is inhibited by the inactivation of PKCα which phosphorylates Bcl-2[152.] In this regard the direct target of ceramide in galectin-1 induced apoptosis has to be revealed.

3. 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 (Figure 4.29). 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 (Figure 4.30).

Apoptotic processes can be classified as two simplified pathways[96]: **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. In galectin-1 induced apoptosis it was clearly shown that the mitochondrial changes preceded the caspase activation therefore this apoptosis pathway belonged to the 'mitochondrion first' type cell death.

Based on the presented data we propose a model for the mechanism of the galectin-1 induced apoptosis (Figure 5.1). Galectin-1 binds to a not yet identified receptor on T cells. An early cell response triggered by galectin-1 is the induction of tyrosine phosphorylation, a step that requires the presence of functional $p56^{lck}$ and ZAP70. The following steps are the ceramide release and the subsequent depolarization of the mitochondria. As a result the caspase 3 is activated and after the proteolysis of those caspase substrates, the apoptosis is executed by the breakdown of the nuclear DNA.

The apoptotic mechanism for several others galectin members has been settled. The apoptotic pathway induced by galectin-9 differs from that triggered by galectin-1, as galectin-9 required Ca^{2+} influx, calpain and caspase-1 activation. Mitochondrial events may not be involved in galectin-9 induced apoptosis, since an inhibitor for caspase 9 required for mitochondria-associated apoptosis does not suppress galectin-9 mediated apoptosis[87].

Galectin-3 triggers a similar mitochondria apoptotic pathway to that of galectin-1 as it induces cytochrome c release as well as caspase 3 activation, but not caspase 8 activation[88].

The biological significance of the mechanism of the galectin-1 induced apoptosis is its role in immunosuppression. As a potent anti-inflammatory agent[41, 59] 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.

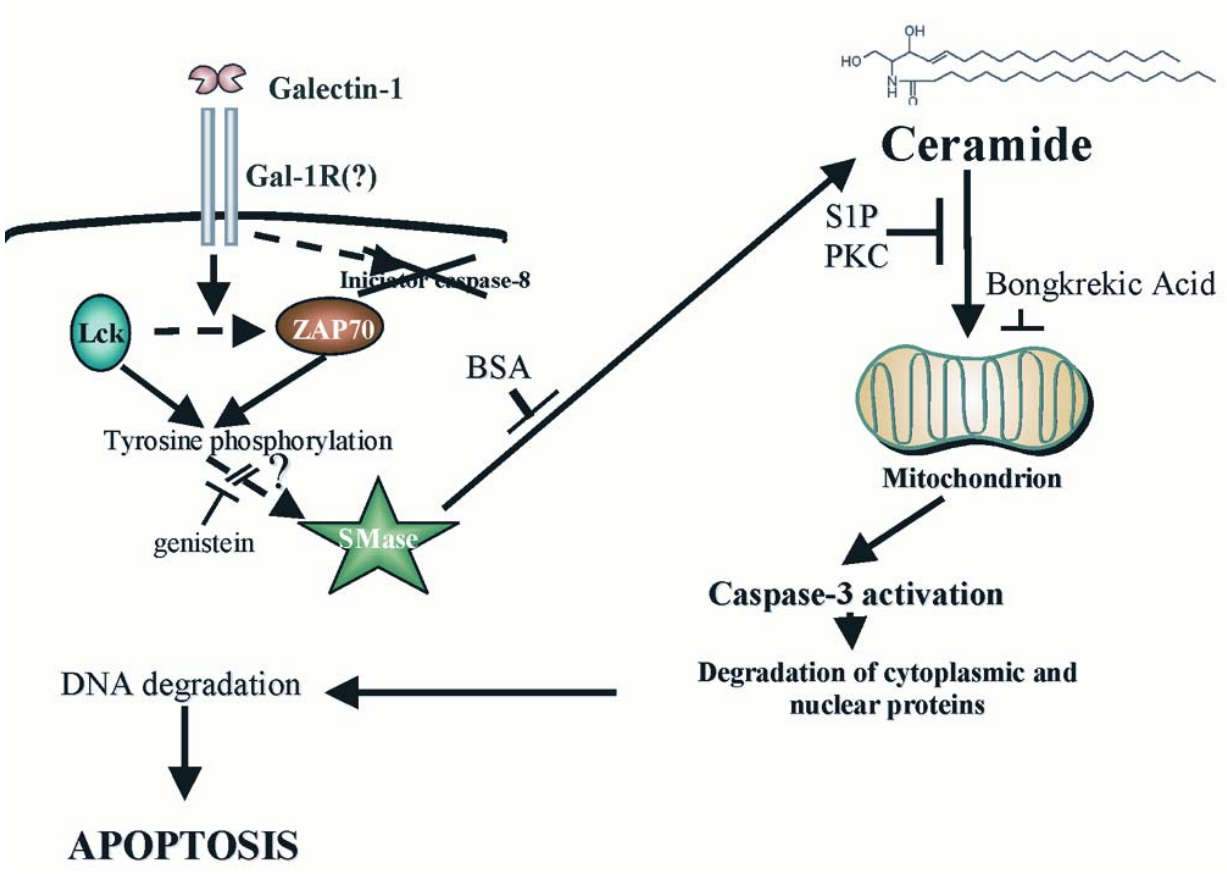

Figure 5.1 **Model of the galectin-1 mediated cell death.** Galectin-1 triggers early tyrosine phosphorylation which involves $p56^{lck}$ and ZAP70. The individual steps between the tyrosine kinase activation and the release of ceramide are not yet known (dashed arrow). The ceramide (release is blocked with the presence of BSA) acts on the mitochondria (this step is inhibited by activation of PKC and S1P). Depolarization of the mitochondrial membrane (blocked by bongkrekic acid) results in the activation of the caspase cascade, and as a consequence, the degradation of caspase substrates and the breakdown of the nuclear DNA.

6. Concluding remarks

Recent findings implicated galectin-1 in the regulation of the thymocyte differentiation and the downregulation of the peripheral T cell response[37]. Galectin-1, produced by the thymic epithelial cells, also regulates the selection of the thymocytes by promoting the death of non-selected and negatively selected $CD4^{lo}$ $CD8^{lo}$ immature thymocytes[50] and is cytotoxic to leukemia T cell lines[25,41].

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 p56lck 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.

7. References

1. Barondes SH, Castronovo V, Cooper DN, Cummings RD, Drickamer K, Feizi T, Gitt MA, Hirabayashi J, Hughes C, Kasai K, et al. (1994) Galectins: a family of animal beta-galactoside-binding lectins. *Cell* **76**:597-598

2. Barondes SH, Cooper DN, Gitt MA, Leffler H (1994) Galectins. Structure and function of a large family of animal lectins. *J Biol Chem.* **269**:20807-20810

3. Kasai K, Hirabayashi J (1996) Galectins: a family of animal lectins that decipher glycocodes. *J Biochem.* (Tokyo) **119**:1-8

4. Cooper DN, Barondes SH (1999) God must love galectins; he made so many of them. *Glycobiology* **9**:979-984.

5. Dunphy JL, Barcham GJ, Bischof RJ, Young AR, Nash A, Meeusen EN (2002) Isolation and characterization of a novel eosinophil-specific galectin released into the lungs in response to allergen challenge. *J Biol Chem.* **277**:14916-14924

6. Hirabayashi J, Kasai K (1993) The family of metazoan metal-independent beta-galactoside-binding lectins: structure, function and molecular evolution. *Glycobiology* **3**:297-304

7. Rini JM (1995) Lectin structure. *Annu Rev Biophys Biomol Struct.* **24**:551-577

8. Liao DI, Kapadia G, Ahmed H, Vasta GR, Herzberg O (1994) Structure of S-lectin, a developmentally regulated vertebrate beta-galactoside-binding protein. *Proc Natl Acad Sci.* **91**:1428-1432

9. Rabinovich GA (1999) Galectins: an evolutionarily conserved family of animal lectins with multifunctional properties; a trip from the gene to clinical therapy. *Cell Death Differ.* **6**:711-721

10. Hughes CR (1999) Secretion of the galectin family of mammalian carbohydrate-binding proteins. *Biochim Biophys Acta* **1473**:172-185

11. Rabinovich GA, Baum LG, Tinari N, Paganelli R, Natoli C, Liu FT, Iacobelli S (2002) Galectins and their ligands: amplifiers, silencers or tuners of the inflammatory response? *Trends Immunol.* **23**:313-320

12. Teichberg VI, Silman I, Beitsch DD, Resheff G (1975) A beta-D-galactoside binding protein from electric organ tissue of Electrophorus electricus. *Proc Natl Acad Sci.* **72**:1383-1387

13. de Waard A, Hickman S, Kornfeld S (1976) Isolation and properties of beta-galactoside binding lectins of calf heart and lung. *J Biol Chem.* **251**:7581-7587

14. Kobiler D, Barondes SH (1977) Lectin activity from embryonic chick brain, heart, and liver:

changes with development. *Dev Biol.* **60**:326-30

15. Nowak TP, Kobiler D, Roel LE, Barondes SH (1977) Developmentally regulated lectin from embryonic chick pectoral muscle. Purification by affinity chromatography. *J Biol Chem.* **252**:6026-6030

16. Den H, Malinzak DA (1977) Isolation and properties of beta-D-galactoside-specific lectin from chick embryo thigh muscle. *J Biol Chem.* **252**:5444-5448

17. Rabinovich GA, Rubinstein N, Fainboim L (2002) Unlocking the secrets of galectins: a challenge at the frontier of glyco-immunology. *J Leukoc Biol*. **71**:741-752

18. Cho M, Cummings RD (1996) Characterization of monomeric forms of galectin-1 generated by site-directed mutagenesis. *Biochemistry* **35**:13081-13088

19. Cho M, Cummings RD (1997) Galectin-1: oligomeric structure and interactions with polylactosamine. *Trends Glycosci Glycotechnol.* **9**:47-56

20. Perillo NL, Marcus ME, Baum LG (1998) Galectins: versatile modulators of cell adhesion, cell proliferation, and cell death. *J Mol Med.* **76**:402-412

21. Cooper DN (1997) Galectin-1: secretion and modulation of cell interactions with laminin. *Trends Glycosci Glycotechnol.* **9**:57-67

22. Adams L, Scott KG, Weinberg CS (1996) Biphasic modulation of cell growth by recombinant human galectin-1. *Biochim Biophys Acta* **1312**: 137-144

23. Offner H, Celnik B, Bringman TS, Casentini-Borocz D, Nedwin GE, Vandenbark AA (1990) Recombinant human beta-galactoside binding lectin suppresses clinical and histological signs of experimental autoimmune encephalomyelitis. *J Neuroimmunol.* **28**:177-184

24. Rabinovich GA, Iglesias MM, Modesti NM, Castagna LF, Wolfenstein-Todel C, Riera CM, Sotomayor CE (1998) Activated rat macrophages produce a galectin-1-like protein that induces apoptosis of T cells: biochemical and functional characterization. *J Immunol.* **160**:4831-4840

25. Perillo NL, Pace KE, Seilhamer JJ, Baum LG (1995) Apoptosis of T cells mediated by galectin-1. *Nature* **378**:736-739

26. Iglesias MM, Rabinovich GA, Ivanovic V, Sotomayor C, Wolfenstein-Todel C **(**1998) Galectin-1 from ovine placenta--amino-acid sequence, physicochemical properties and implications in T-cell death. *Eur J Biochem*. **252**:400-407

27. Vyakarnam A, Dagher SF, Wang JL, Patterson RJ (1997) Evidence for a role for galectin-1 in pre-mRNA splicing. *Mol Cell Biol.* **17**:4730-4737

28. Bianchet MA, Ahmed H, Vasta GR, Amzel LM (2000) Soluble beta-galactosyl-binding lectin (galectin) from toad ovary: crystallographic studies of two protein-sugar complexes. *Proteins* **40**:378-388

29. Levi G, Tarrab-Hazdai R, Teichberg VI (1983) Prevention and therapy with electrolectin of experimental autoimmune myasthenia gravis in rabbits. *Eur J Immunol.* **13**:500-507

30. Zuniga E, Rabinovich GA, Iglesias MM, Gruppi A (2001) Regulated expression of galectin-1 during B-cell activation and implications for T-cell apoptosis. *J Leukoc Biol.* **70**:73-79

31. Lutomski D, Joubert-Caron R, Lefebure C, Salama J, Belin C, Bladier D, Caron M (1997) Anti-galectin-1 autoantibodies in serum of patients with neurological diseases. *Clin Chim Acta*. **262**:131-138

32. Ogden AT, Nunes I, Ko K, Wu S, Hines CS, Wang AF, Hegde RS, Lang RA (1998) GRIFIN, a novel lens-specific protein related to the galectin family. *J Biol Chem.* **273**:28889-28896

33. Griffith TS, Ferguson TA (1997) The role of FasL-induced apoptosis in immune privilege. *Immunol Today.* **18**:240-244

34. Gold R, Hartung HP, Lassmann H (1997) T-cell apoptosis in autoimmune diseases: termination of inflammation in the nervous system and other sites with specialized immune-defense mechanisms. *Trends Neurosci.* **20**:399-404

35. Baum LG, Pang M, Perillo NL, Wu T, Delegeane A, Uittenbogaart CH, Fukuda M, Seilhamer JJ (1995) Human thymic epithelial cells express an endogenous lectin, galectin-1, which binds to core 2 O-glycans on thymocytes and T lymphoblastoid cells. *J Exp Med.* **181**:877-887

36. Allione A, Wells V, Forni G, Mallucci L, Novelli F (1998) Beta-galactoside-binding protein (beta GBP) alters the cell cycle, up-regulates expression of the alpha- and beta-chains of the IFN-gamma receptor, and triggers IFN-gamma-mediated apoptosis of activated human T lymphocytes. *J Immunol.* **161**:2114-2119

37. Rabinovich GA, Rubinstein N, Toscano MA (2002) Role of galectins in inflammatory and immunomodulatory processes. *Biochim Biophys Acta* **1572**:274-284

38. Van Parijs L, Abbas AK (1998) Homeostasis and self-tolerance in the immune system: turning lymphocytes off. *Science* **280**:243-248

39. Kruisbeek AM, Amsen D (1996) Mechanisms underlying T-cell tolerance. *Curr Opin Immunol.* **8**:233-244

40. Rabinovich GA, Sotomayor CE, Riera CM, Bianco I, Correa SG (2000) Evidence of a role for galectin-1 in acute inflammation. *Eur J Immunol.* **30**:1331-1339

41. Rabinovich GA, Daly G, Dreja H, Tailor H, Riera CM, Hirabayashi J, Chernajovsky Y (1999) Recombinant galectin-1 and its genetic delivery suppress collagen-induced arthritis via T cell apoptosis. *J Exp Med*. **190**:385-398

42. Santucci L, Fiorucci S, Cammilleri F, Servillo G, Federici B, Morelli A (2000) Galectin-1 exerts immunomodulatory and protective effects on concanavalin A-induced hepatitis in mice. *Hepatology* **31**:399-406

43. Wells V, Mallucci L (1991) Identification of an autocrine negative growth factor: mouse beta-galactoside-binding protein is a cytostatic factor and cell growth regulator. *Cell* **64**:91-97

44. Blaser C, Kaufmann M, Muller C, Zimmermann C, Wells V, Mallucci L, Pircher H (1998) Beta-galactoside-binding protein secreted by activated T cells inhibits antigen-induced proliferation of T cells. *Eur J Immunol.* **28**:2311-2319

45. Wells V, Davies D, Mallucci L (1999) Cell cycle arrest and induction of apoptosis by beta galactoside binding protein (beta GBP) in human mammary cancer cells. A potential new approach to cancer control. *Eur J Cancer* **35**:978-983

46. Yamaoka K, Ohno S, Kawasaki H, Suzuki K (1991) Overexpression of a beta-galactoside binding protein causes transformation of BALB 3T3 fibroblast cells. *Biochem Biophys Res Commun.* **179**:272-279

47. Paz A, Haklai R, Elad-Sfadia G, Ballan E, Kloog Y (2001) Galectin-1 binds oncogenic H-Ras to mediate Ras membrane anchorage and cell transformation. *Oncogene* **20**:7486-7493

48. Vespa GN, Lewis LA, Kozak KR, Moran M, Nguyen JT, Baum LG, Miceli MC (1999) Galectin-1 specifically modulates TCR signals to enhance TCR apoptosis but inhibit IL-2 production and proliferation. *J Immunol.* **162**:799-806

49. Scott K, Zhang J (2002) Partial identification by site-directed mutagenesis of a cell growth inhibitory site on the human galectin-1 molecule. *BMC Cell Biol*. **3**:3-9

50. Perillo NL, Uittenbogaart CH, Nguyen JT, Baum LG (1997) Galectin-1, an endogenous lectin produced by thymic epithelial cells, induces apoptosis of human thymocytes. *J Exp Med.* **185**:1851-1858

51. Walzel H, Schulz U, Neels P, Brock J (1999) Galectin-1, a natural ligand for the receptor-type protein tyrosine phosphatase CD45. *Immunol Lett.* **67**:193-202

52. Fajka-Boja R, Szemes M, Ion G, Legradi A, Caron M, Monostori E (2002) Receptor tyrosine phosphatase, CD45 binds galectin-1 but does not mediate its apoptotic signal in T cell lines. *Immunol Lett.* **82**:149-154

53. Walzel H, Blach M, Hirabayashi J, Kasai KI, Brock J (2000) Involvement of CD2 and CD3 in galectin-1 induced signaling in human Jurkat T-cells. *Glycobiology* **10**:131-140

54. Fouillit M, Joubert-Caron R, Poirier F, Bourin P, Monostori E, Levi-Strauss M, Raphael M, Bladier D, Caron M (2000) Regulation of CD45-induced signaling by galectin-1 in Burkitt lymphoma B cells. *Glycobiology* **10**:413-419

55. Pace KE, Lee C, Stewart PL, Baum LG **(**1999) Restricted receptor segregation into membrane microdomains occurs on human T cells during apoptosis induced by galectin-1. *J Immunol.* **163**:3801-3811

56. Pace KE, Hahn HP, Pang M, Nguyen JT, Baum LG (2000) CD7 delivers a pro-apoptotic signal during galectin-1-induced T cell death. *J Immunol.* **165**:2331-2334

57. Gabius HJ (2001) Probing the cons and pros of lectin-induced immunomodulation: case studies for the mistletoe lectin and galectin-1. *Biochimie* **83**:659-666

58. Rabinovich GA, Alonso CR, Sotomayor CE, Durand S, Bocco JL, Riera CM (2000) Molecular mechanisms implicated in galectin-1-induced apoptosis: activation of the AP-1 transcription factor and downregulation of Bcl-2. *Cell Death Differ.* **7**:747-753

59. Rabinovich GA, Ramhorst RE, Rubinstein N, Corigliano A, Daroqui MC, Kier-Joffe EB, Fainboim L (2002). Induction of allogenic T-cell hyporesponsiveness by galectin-1-mediated apoptotic and non-apoptotic mechanisms. *Cell Death Differ.* **9**:661-670

60. Williams BL, Schreiber KL, Zhang W, Wange RL, Samelson LE, Leibson PJ, AbrahamRT (1998) Genetic evidence for differential coupling of Syk family kinases to the T-cell receptor: reconstitution studies in a ZAP-70-deficient Jurkat T-cell line. *Mol Cell Biol.* **18**:1388-1399

61. Fouillit M, Levi-Strauss M, Giudicelli V, Lutomski D, BladierD, Caron M, Caron RJ (1998) Affinity purification and characterization of recombinant human galectin-1. *J Chromatogr B Biomed Sci Appl.* **706**:167-171

62. Somersan S, Bhardwaj N (2001) Tethering and tickling: a new role for the phosphatidylserine receptor. *J Cell Biol.* **155**:501-504

63. Cuvillier O, Rosenthal DS, Smulson ME, Spiegel S (1998) Sphingosine 1-phosphate inhibits activation of caspases that cleave poly(ADP-ribose) polymerase and lamins during Fas- and ceramide-mediated apoptosis in Jurkat T lymphocytes. *J Biol Chem.* **273**:2910-2916

64. Buehrer BM, Bardes ES, Bell RM (1996) Protein kinase C-dependent regulation of human erythroleukemia (HEL) cell sphingosine kinase activity. *Biochim Biophy Acta* **1303**:233-242

65. Dumont A, Hehner SP, Hofmann TG, Ueffing M, Droge W, Schmitz ML (1999) Hydrogen peroxide-induced apoptosis is CD95-independent, requires the release of mitochondria-derived reactive oxygen species and the activation of NF-kappaB. *Oncogene* **18**:747-757

66. Hur YG, Yun Y, Won J (2004) Rosmarinic acid induces p56lck-dependent apoptosis in Jurkat and peripheral T cells via mitochondrial pathway independent from Fas/Fas ligand interaction. *J Immunol.* **172**:79-87

67. Chung CD, Patel VP, Moran M, Lewis LA, Miceli MC (2000) Galectin-1 induces partial TCR zeta-chain phosphorylation and antagonizes processive TCR signal transduction. *J Immunol.* **165**:3722-3729

68. Chmura SJ, Nodzenski E, Beckett MA, Kufe DW, Quintans J, Weichselbaum RR (1997) Loss of ceramide production confers resistance to radiation-induced apoptosis. *Cancer Res.* **57**:1270-1275

69. Cifone MG, Roncaioli P, De Maria R, Camarda G, Santoni A, Ruberti G, Testi R (1995) Multiple

pathways originate at the Fas/APO-1 (CD95) receptor: sequential involvement of phosphatidylcholine-specific phospholipase C and acidic sphingomyelinase in the propagation of the apoptotic signal. *EMBO J.* **14**:5859-5868

70. Haimovitz-Friedman A, Kan CC, Ehleiter D, Persaud RS, McLoughlin M, Fuks Z,.Kolesnick RN (1994) Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. *J Exp Med.* **180**:525-535

71. Tepper CG, Jayadev S, Liu B, Bielawska A, Wolff R, Yonehara S, Hannun YA, Seldin MF (1995) Role for ceramide as an endogenous mediator of Fas-induced cytotoxicity. *Proc Natl Acad Sci.* **92**:8443-8447

72. Johnson KR, Johnson KY, Becker KP, Bielawski J, Mao C, Obeid LM (2003) Role of human sphingosine-1-phosphate phosphatase 1 in the regulation of intra- and extracellular sphingosine-1-phosphate levels and cell viability. *J Biol Chem.* **278**:34541-34547

73. Pettus BJ, Chalfant CE, Hannun YA (2002) Ceramide in apoptosis: an overview and current perspectives. *Biochim Biophys Acta* **1585**:114-125

74. Pastorino JG, Tafani M, Rothman RJ, Marcinkeviciute A, Hoek JB, Farber JL, Marcineviciute A (1999) Functional consequences of the sustained or transient activation by Bax of the mitochondrial permeability transition pore. *J Biol Chem.* **274**:31734-31739

75. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997) Cytochrome c and dATP-Dependent Formation of Apaf-1/Caspase-9 Complex Initiates an Apoptotic Protease Cascade. *Cell* **91**:479-489

76. Acehan D, Jiang X, Morgan DG, Heuser JE, Wang X, Akey CW (2002) Three-dimensional structure of the apoptosome. Implications for assembly, procaspase-9 binding, and activation. *Mol Cell*. **9**: 423-432

77. Ferraro E, Corvaro M, Cecconi F (2003) Physiological and pathological roles of Apaf1 and the apoptosome. *J Cell Mol Med.* **7**:21-34

78. Van Echten G, Sandhoff K (1993) Ganglioside metabolism. Enzymology, topology and regulation. *J Biol Chem.* **268:** 5341–5344

79. Dickson RC, Lester R L (2002) Sphingolipid functions in *Saccharomyces cerevisiae*. *Biochim Biophys Acta.* **1583:** 13–25

80. Gulbins E, Grassme H (2002) Ceramide and cell death receptor clustering. *Biochim Biophys Acta.* **1585:** 139-145

81. Pena LA, Fuks Z, Kolesnick R (1997) Stress-induced apoptosis and the sphingomyelin pathway. *Biochem Pharmacol.* **53**:615-621

82. Mathias S, Pena LA, Kolesnick RN (1998) Signal transduction of stress via ceramide. Biochem J. **335**:465–480

83. Kolesnick R (2002) The therapeutic potential of modulating the ceramide/sphingomyelin pathway *J Clin Invest.* **110**:3-8

84. Tomassini B, Testi R (2002) Mitochondria as sensors of sphingolipids. *Biochimie* **84**:123-129

85. Zwaal RFA, Schroit AJ (1997) Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood* **89**:1121-1132

86. Danguy A, Camby I, Kiss R (2002) Galectins and cancer. *Biochim Biophys Acta.* **1572**:285-293

87. Kashio Y, Nakamura K, Abedin MJ, Seki M, Nishi N, Yoshida N, Nakamura T, Hirashima M (2003) Galectin-9 Induces apoptosis through the calcium-calpain-caspase-1 pathway. *J Immunol.* **170**:3631-3636

88. Fukumori T, Takenaka Y, Yoshii T, Kim HRC, Hogan V, Raz A (2003) CD29 and CD7 mediate galectin-3-induced type II Y-cell apoptosis. *Cancer Res.* **63**:8302-8311

89. Poirier F, Timmons PM, Chan CTJ, Guenet JL, Rigby PWJ (1992) Expression of the L14 lectin during mouse embryogenesis suggests multiple roles during pre- and post-implantation development. *Development* **115**:143-155

90. Benvenuto G, Carpentieri ML, Salvatore P, Cindolo L, Bruni CB, Chiariotti L (1996) Cell specific transcriptional regulation and reactivation of galectin-1 gene expression are controlled by DNA methylation of the promoter region. *Mol Cell Biol.* **16**:2736-2743

91. Salvatore P, Benvenuto G, Caporaso M, Bruni CB, Chiariotti L (1998) High resolution methylation analysis of the galectin-1 gene promoter region in expressing and nonexpressing tissues. *FEBS Lett.* **421**:152-158

92. Leist M, Jaattela M (2001) Four deaths and a funeral: from caspases to alternative mechanisms. *Nat Rev.* **2**:589-598

93. Douglas RG (2003) Overview: apoptotic signaling pathways in the immune system. *Imm Rev.* **193**:5-9

94. Jaattela M, Tschopp J (2003) Caspase-independent cell death in T lymphocytes. *Nat Imm.* **4**:416-423

95. Opferman JT, Korsmeyer ST (2003) Apoptosis in the development and maintenance of the immune system. *Nat Imm.* **4**:410-415

96. Blatt NB, Glick GD (2001) Signaling pathways and effector mechanisms pre-programmed cell death. *Bioorg & Med Chem.* **9**:1371-1384

97. Kroemer G, Zamzami N, Susin SA (1997) Mitochondrial control of apoptosis. *Immunol Today* **18**:

44-51

98. Ashkenazi A, Dixit VM (1998) Death receptors: signaling and modulation. *Science* **281**:1305-1308

99. Scaffidi C, Fulda S, Li F, Friesen C, Srinivasana A, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME (1998) (Apo-1/Fas) signaling pathways. *EMBO J.* **17**:1675-1687

100. Nunez G, Benedict MA, Hu Y, Inohara N (1998) Caspases: the proteases of the apoptotic pathway. *Oncogene* **17**:3237-3245

101. Hengartner MO (2000) The biochemistry of apoptosis. *Nature* **407**: 770-776

102. Thornberry NA, Lazebnik Y (1998) Caspasaes: enemies within. *Science* **281**:1312-1316

103. Wang KKW (2000) Calpain and caspases: can you tell the difference? *Trends Neurosci.* **23**:20-26

104. Grutter MK (2000) Caspases: key players in programmed cell death. *Curr Op in Struct Biol.* **10**:649-655

105. Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia-Calvo M, Houtzager VM, Nordstrom PA, Roy S, Vaillancourt JP, Chapman KT, Nicholson DW (1997) A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J Biol Chem.* **272**: 17907-17911

106. Garcia-Calvo M, Peterson EP, Leiting B, Ruel R, Nicholson DW, Thornberry NA (1998) Inhibition of human caspases by peptide-based and macromolecular inhibitors. *J Biol Chem.* **273**:32608-32613

107. Divecha N, Irvine RF (1995) Phospholipid signaling. *Cell* **80**:269-278

108. van Blitterswijk WJ, van der Luit AH, Veldman RJ, Verheij M, Borst J (2003) Ceramide:second messenger or modulator of membrane structure and dynamics? *Biochem J.* **369**:199-211

109. Tepper AD, Ruurs P, Wiedmer T, Sims PJ, Borst J, van Blitterswijk WJ (2000) Sphingomyelin hydrolisis to ceramide during the execution phase of apoptosis results from phospholipids scrambling and alters cell-surface morphology. *J Cell Biol.* **150**:155-164

110. Belka C, Gruber C, Jendrossek V, Wesselborg S, Budach W (2003) The tyrosine kinase Lck is involved in regulation of mitochondrial apoptosis pathways. *Oncogene* **22**:176-185

111. Manna SK, Sah NK, Aggarwal BB (2000) Protein tyrosine kinase p56lck is required for ceramide-induced but not tumor necrosis factor-induced activation of NF-kappa B, AP-1, JNK, and apoptosis. *J Biol Chem.* **275**:13297-13306

112. Green DR, Droin N, Pinkoski M (2003) Activation-induced cell death in T cells. *Imm Rev.* **193**:70-81

113. Fadok VA, Bratton DL, Frasch SC, Warner ML, Henson PM (1998) The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. *Cell Death Differ.* **5**:551-562

114. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C (1991) A rapid and simple method

for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* **139**:271-279

115. Huang DC, Hahne M, Schroeter M, Frei K, Fontana A, Villunger A, Newton K, Tschopp J, Strasser A (1999) Activation of Fas by FasL induces apoptosis by a mechanism that cannot be blocked by Bcl-2 or Bcl-x(L). *Proc Natl Acad Sci.* **96**:14871-14876

116. Krammer PH (1999) CD95(APO-1/Fas)-mediated apoptosis: live and let die. *Adv Immunol.* **71**:163-210

117. Strasser A, O'Connor L, Dixit VM (2000) Apoptosis signaling. *Annu Rev Biochem.* **69**:217-245

118. Fujita N, Nagahasi A, Nagashima K, Rokudai S, Tsuruo T (1998) Acceleration of apoptotic cell death after the cleavage of Bcl-XL protein by caspase-3-like proteases. *Oncogene* **17**:1295-1304

119. Gross A, Yin XM, Wang K, Wei MC, Jockel J, Milliman C, Erdjument-Bromage H, Tempst P, Korsmeyer SJ (1999) Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. *J Biol Chem.* **274**:1156-1163

120. Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, Wang HG, Reed JC, Nicholson DW, Alnemri ES, Green DR, Martin SJ (1999) Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J Biol Chem.* **144**:281-292

121. Slee EA, Adrain C, Martin SJ (2001) Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *J Biol Chem.* **276**:7320-7326

122. Hirsch T, Marchetti P, Susin SA, Dallaporta B, Zamzami N, Marzo I, Geuskens M, Kroemer G (1997) The apoptosis-necrosis paradox. Apoptogenic proteases activated after mitochondrial permeability transition determine the mode of cell death. *Oncogene* **15**:1573-1581

123. Nicotera P, Leist M, Manzo L (1999) Neuronal cell death: a demise with different shapes. *Trends Pharmacol Sci.* **20**:46-51

124. Amarante-Mendes GP, Finucane DM, Martin SJ, Cotter TG, Salvesen GS, Green DR (1998) Anti-apoptotic oncogenes prevent caspase-dependent and independent commitment for cell death. *Cell Death Differ.* **5**:298-306

125. Bossy-Wetzel E, Newmeyer DD, Green DR (1998) Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *EMBO J*. **17**:37-49

126. Heibein JA, Barry M, Motyka B, Bleackley RC (1999) Granzyme B-induced loss of mitochondrial inner membrane potential (Delta Psi m) and cytochrome c release are caspase independent. *J Immunol.* **163**:4683-4693

127. Kluck RM, Bossy-Wetzel E, Green DR**,** Newmeyer DD (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* **275**:1132-1136

128. Lilly M, Sandholm J, Cooper JJ, Koskinen PJ, Kraft A (1999) The PIM-1 serine kinase prolongs survival and inhibits apoptosis-related mitochondrial dysfunction in part through a bcl-2-dependent pathway. *Oncogene* **18**:4022-4031

129. Wadia JS, Chalmers-Redman RM, Ju WJ, Carlile GW, Phillips JL, Fraser AD, Tatton WG (1998) Mitochondrial membrane potential and nuclear changes in apoptosis caused by serum and nerve growth factor withdrawal: time course and modification by (-)-deprenyl. *J Neurosci.* **18**:932-947

130. Bojes HK, Feng Z, Kehrer JP, Cohen GM (1999) Apoptosis in hematopoietic cells (FL5.12) caused by interleukin-3 withdrawal: relationship to caspase activity and the loss of glutathione. *Cell Death Differ.* **6**:61-70

131. Liu X, Kim CN, Yang J, Jemmerson R, Wang X (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* **86**:147-157

132. Du C, Fang M, Li Y, Li L, Wang X (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspases activation by eliminating IAP inhibition. *Cell* **102**:33-42

133. Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* **102**:43-53

134. Duckett CS, Nava VE, Gedrich RW, Clem RJ, Van Dongen JL, Gilfillan MC, Shiels H, Hardwick JM, Thompson CB (1996) A conserved family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. *EMBO J.* **15**:2685-2694

135. Uren AG, Pakusch M, Hawkins CJ, Puls KL, Vaux DL (1996) Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumor necrosis factor receptor-addociated factors. *Proc Natl Acad Sci.* **93**:4974-4978

136. Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* **397**:441-446

137. Joza N, Susin SA, Daugas E, Stanford WL, Cho SK, Li CY, Sasaki T, Elia AJ, Cheng HY, Ravagnan L, Ferri KF, Zamzami N, Wakeham A, Hakem R, Yoshida H, Kong YY, Mak TW, Zuniga-Pflucker JC, Kroemer G, Penninger JM (2001) Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature* **410**:549-554

138. Ruvolo PP (2003) Intracellular signal transduction pathways activated by ceramide and its metabolites. *Pharm Res.* **47**:383-392

139. Westwick JK, Bielawaska AE, Dbaibo G, Hannun YA, Brenner DA (1995) Ceramide activates the stress-activated protein kinases. *J Biol Chem.* **270**:22689-22692

140. Basu S, Kolesnick R (1998) Stress signals for apoptosis:ceramide and c-Jun kinase. *Oncogene* **17**:3277-3285

141. Jarvis WD, Fornari FA, Auer KL, Freemerman AJ, Szabo E, Birrer MJ, Johnson CR, Barbour SE, Dent P, Grant S (1997) Coordinate regulation of stress- and mitogen-activated protein kinases in the apoptotic actions of ceramide and sphingosine. *Mol Pharmacol.* **52**:935-947

142. Basu S, Bayoumy S, Zhang Y, Lozano J, Kolesnick R (1998) BAD enables ceramide to signal apoptosis via Ras and Raf-1. *J Biol Chem.* **273**:30419-30426

143. Bourbon NA, Yun J, Kester M (2000) Ceramide directly activates protein kinase C ζ to regulate a stress-activated protein kinase signaling complex. *J Biol Chem.* **275**:35617-35623

144. Schubert KM, Scheid MP, Duronio V (2000) Ceramide inhibits protein kinase B/Akt by promoting dephosphorylation of serine 473. *J Biol Chem.* **275**:13330-13335

145. Herr I, Wilhekm D, Bohler T, Angel P, Debatin KM (1997) Activation of CD95 (APO-1/Fas) signaling by ceramide mediates cancer therapy-induced apoptosis. *EMBO J.* **16**:6200-6208

146. Heinrich M, Wickel M, Schneider-Brachert W, Sandberg C, Gahr J, Schwandner R, Weber T, Saftig P, Peters C, Brunner J et al. (1999) Cathepsin D targeted by acid sphingomyelinase-derived ceramides. *EMBO J.* **18**:5252-5263

147. Venkataraman K and Futerman A H (2000) Ceramide as a second messenger: sticky solutions to sticky problems. *Trends Cell Biol* **10**:408-412

148. Garcia-Ruiz C, Colell A, Mari M, Morales A, Fernandez-Checa JC (1997) Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of reactive oxygen species. Role of mitochondrial glutathione. *J Biol Chem.* **272**:11369 -11377

149. Quillet-Mary A, Jaffrezou JP, Mansat V, Bordier C, Naval J, Laurent G (1997) Implication of mitochondrial hydrogen peroxide generation in ceramide-induced apoptosis. *J Biol Chem.* **272**:21388-21395

150. Tepper AD, de Vries E, van Blitterswijk WJ, Borst J (1999) Ordering of ceramide formation, caspase activation, and mitochondrial changes during CD95- and DNA damage-induced apoptosis. *J Clin Invest.* **103**:971–978

151. Ruvolo PP, Deng X, Ito T, Carr BK, May WS (1999) Ceramide induces Bcl2 dephosphorylation via a mechanism involving mitochondrial PP2A. *J Biol Chem.* **274**:20296-20300

152. Lee JY, Hannun YA, Obeid LM (1996) Ceramide inactivates cellular protein kinase Calpha. *J Bio Chem.* **271**:13169-13174

153. Straus DB and Weiss A (1992) Genetic evidence for the involvement of the lck tyrosine kinase in

signal transduction through the T cell antigen receptor. *Cell* **70**: 585-593

154. Hannun YA and Obeid LM. (2002) The Ceramide-centric Universe of Lipid-mediated Cell Regulation: Stress Encounters of the Lipid Kind. *J Biol Chem***. 277:** 25847-25850

8. Abbreviations

Ac-IETD: Ac-Ile-Glu-Thr-Asp-CHO
Ann V: AnnexinV
Apaf-1: apoptosis protease-activating factor 1
BA: bongkrekic acid
BSA: bovine serum albumine
CARD: caspase recruitment domain
CHX: cycloheximide
DD: death domain
DED: death effector domain
DISC: death-inducing signal complex
FADD: Fas-associated death domain
FB1: fumonisin B1
FCS: fetal calf serum
IFB: immunofluorescence buffer
PAGE: polyacrylamide gel electrophoresis
PARP: poly (ADP-ribose) polymerase
C6-Cer: C6-ceramide
PDBu: phorbol dibutyrate
PBS: phosphate-buffered saline
PS: phosphatydil serine
PTK: protein tyrosine kinase
SM: sphingomielin
SMases: sphingomyelinases
S1P: sphingosine 1 phosphate
TBS: Tris-buffered saline
TBST: Tris-buffered saline Tween 20
TNF: tumor necrosis factor
TNF-R1: tumor necrosis factor receptor 1
TRADD: TNF-R1 associated protein with death domain
zVAD-fmk: N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone

9. Appendix

Buffers and Solutions

A1. RPMI cell culture medium

1.04% (w/v) RPMI 1640 powder (Gibco BRL)

0.2% (w/v) $NaHCO_3$ (Reanal)

10 NE/ml penicillin (Biogal)

0.1 mg/ml streptomycin (EGIS)

after sterilization 0.63mg/ml L-glutamine, 5% (v/v) or 10% (v/v) heat inactivated foetal calf serum (FCS, Protein GMK or Plaz Med Kft.) were added.

A2. Trypan Blue solution for cell counting

0.16% (w/v) trypan blue (Reanal)

0.9% (w/v) NaCl (Merck, Reanal)

A3. Phosphate-buffered saline (PBS) 10X concentrate pH 7.4

43 mM Na_2HPO_4 x $2H_2O$ (Reanal)

14 mM KH_2PO_4 (Reanal)

1.37 M NaCl (Merck, Reanal)

27 mM KCl (Reanal, Sigma)

For a working solution (1X), dilute 1 part PBS to 9 parts distilled H_2O.

A4. Propidium iodide stock solution

1 mg/ml propidium iodide (Sigma, Fluka),

Store at 4^0C.

A5. Sample buffer stock

1% glucose (Reanal)

10X PBS

Sterile filtered, store at 4^0C.

A6. DNA staining buffer (freshly prepared)

PBS supplemented with:

0.1% (v/v) Triton X-100, stock 10%, (Sigma)

0.1% (w/v) 3Na-citrate, stock 1%, (Reanal)

10 µg/ml RNase, stock 10mg/ml, (Sigma)

10 µg/ml propidium-iodide, stock 1mg/ml, (Sigma, Fluka)

A7. 5% Bovine serum albumine (BSA)

5g BSA (Sigma)

100 ml PBS

Sterile filtered, store at 4^0C.

A8. Annexin V binding buffer, 10X concentrate

0.1 M HEPES pH 7.4, stock 1M, (Sigma)

1,4 M NaCl, stock 5M, (Merck, Reanal)

25 mM $CaCl_2$, stock 1M, (Reanal)

For a working solution (1X), dilute 1 part binding buffer to 9 parts distilled H_2O. Store both the 10X concentrate and working solution at 4^0C.

A9. Immunofluorescence buffer (IFB) (PBS, 1%BSA, 0.1%NaN_3)

5 g BSA (Sigma)

5 ml NaN_3, stock 10%, (Sigma)

500 ml PBS

Store at 4^0C.

A10. Fixation solution (2% paraformaldehyde) stock

1 g paraformaldehyde (Sigma)

50 ml PBS

Dissolve paraformaldehyde at 50-70^0C, 1h. Cool to RT and adjust pH to 7.2. Store protected from light 1 month at 4^0C.

A11. Permeabilization solution (0.1% Triton-X)

50 µl Triton-X (Sigma)

50 ml PBS

Store 1 month in amber container at 4^0C. Warm up to RT before use.

A12. Washing buffer (0.05% Tween 20, 1% BSA)

25 µl Tween 20 (Sigma)

0.5 g BSA (Sigma)

50 ml PBS

Store at 4^0C.

A13. Reducing SDS-PAGE sample buffer, 2X concentrate

80 mM Tris pH 6.8, stock 1M, (Reanal)

10% (v/v) glycerol (Reanal)

1% (w/v) Sodium Dodecyl Sulfate (SDS), stock 10%, (Sigma)

1% (v/v) β-mercaptoethanol (Merck)

bromfenolblue (Bio-Rad).

Store at -20^0C.

A14. Lysis buffer, 2X concentrate

50 mM HEPES pH 7.4, stock 1M, (Sigma)

1% Triton X-100, stock 10%, (Sigma)

150 mM NaCl, stock 5M, (Merck, Reanal)

20 mM NaF, stock 500mM, (Sigma)

200 µM Na_3VO_4, stock 200mM, (Merck)

2 mM EDTA, stock 200mM, (Reanal)

1 mM phenylmethyl-sulfonylfluoride, stock 100mM, (Sigma)

10µg/ml leupeptin (Sigma)

A15. Stacking gel

5% (w/v) acrylamide / 0.13% (w/v) bis-acrylamide (Bio-Rad)

125 mM Tris/HCl pH 6.8 (Reanal)

0.1% (w/v) SDS (Sigma)

0.033% (w/v) ammonium-peroxydisulfate (APS) (Sigma)

0.07% (v/v) N, N, N, N'- tetrametil-ethylene-diamine (TEMED) (Bio-Rad)

A16. Running gel

7-15% (w/v) acrylamide 0.182-0.39% (w/v) bis-acrylamide (Bio-Rad)

125 mM Tris/HCl pH 8.8 (Reanal)

0.1% (w/v) SDS (Sigma)

0,033% (w/v) APS (Sigma)

0.07% (v/v) TEMED (Bio-Rad)

A17. Running buffer

25 mM Tris/HCl pH 8.3 (Reanal)

194 mM glycin (Molar Chemicals Kft.)

0.1% (w/v) SDS (Sigma)

A18. Coomassie Brilliant Blue G-250 for staining of protein gels

0.1% (w/v) Coomassie Brillant Blue G-250 (Reanal)

50% (v/v) methanol (Reanal)

10% (v/v) acetic acid (Reanal)

A19. Protein gel destainer

10% (v/v) acetic acid (Reanal)

A20. Transfer buffer

25 mM Tris/HCl pH 8.3(Reanal)

192 mM glycin (Molar Chemicals Kft.)

20% (v/v) methanol (Reanal)

A21. Tris-buffered saline (TBS)

10 mM Tris/HCl pH 7.5 (Reanal)

150 mM NaCl (Merck, Reanal)

A22. Tris-buffered saline Tween 20 (TBST)

10 mM Tris/HCl pH 7.5 (Reanal)

150 mM NaCl (Merck, Reanal)

0.05% (v/v) Tween 20 (Sigma)

10. List of publications

1. **Gabriela Ion**, Roberta Fajka-Boja, Imre Gombos, János Matkó, Gábor K. Tóth, Michel Caron, Éva Monostori. Galectin-1 induced cell death triggers a sequence of signaling events including $p56^{lck}$, ZAP70 mediated tyrosine phosphorylation, ceramide release and the mitochondrial pathway of apoptosis. *Cell Death and Differ* 2005 (accepted for publication). IF 7.008

2. Virág Vas, Roberta Fajka-Boja, **Gabriela Ion**, Valèria Dudics, Ėva Monostori, and Ferenc Uher. Biphasic effect of recombinant galectin-1 on the growth and death of early hematopoietic cells. *Stem Cells* 2005; 23:279-87. IF 5.802

3. E. Monostori, **G. Ion**, R. Fajka-Boja, A. Legradi. Human galectin-1 induces T cell apoptosis via ceramide mediated mitochondrial pathway. *Tissue Antigens* 2004. 64: 423 IF 1.737(citable abstract)

4. Fajka-Boja R, Szemes M, **Ion G**, Legradi A, Caron M and Monostori É. Receptor tyrosine phosphatase, CD45 binds Galectin-1 but does not mediate its apoptotic signal in T cell lines. *Immunol. Lett.* 2002; 82(1-2):149-154 IF 1.874

5. Fajka-Boja R, Hidvegi M, Shoenfeld Y, **Ion G**, Demydenko D, Tomoskozi-Farkas R, Vizler C, Telekes A, Resetar A, Monostori E. Fermented wheat germ extract induces apoptosis and downregulation of major histocompatibility complex class I proteins in tumor T and B cell lines. *Int J Oncol* 2002 Mar; 20(3):563-70. IF 2.931

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12. Summary in English

Human galectin-1 is an endogenous immunomodulatory protein with remarkable immunosuppressive effect. Recent findings implicated galectin-1 in the regulation of the thymocyte differentiation and the downregulation of the peripheral T cell response. Secreted galectin-1 plays role 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. Galectin-1, produced by the thymic epithelial cells, also regulates the selection of the thymocytes by promoting the death of non-selected and negatively selected $CD4^{lo}$ $CD8^{lo}$ immature thymocytes and is cytotoxic to leukemia T cell lines. 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. Here we show that an early cell response is the induction of tyrosine phosphorylation, a step that requires the presence of functional $p56^{lck}$ and ZAP70. The phosphorylation is necessary for the downstream steps: ceramide release and the subsequent breakdown of the mitochondrial membrane potential. The activation of the caspase cascade and hence the proteolysis of the caspase substrates follows the mitochondrial response, and finally the apoptosis is executed by the degradation of the nuclear DNA.

We analyzed the time course and sequence of the morphological and biochemical changes occurred during galectin-1 induced apoptosis on Jurkat, a T leukemia cell line:

1. Galectin-1 triggered tyrosine kinase activity and the resulting tyrosine phosphorylation was critical for the further apoptotic events as tyrosine kinase inhibitor, genistein blocked the ceramide release and the DNA fragmentation. Two kinases, $p56^{lck}$ and ZAP70 played essential roles since Jurkat cells deficient in these enzymes (JCaM1.6 and P116, respectively) responded with no tyrosine phosphorylation and with noticeably reduced apoptosis to galectin-1 stimulation. The significance of the Lck/ZAP70 pathway was supported using JCaM/LCK and P116WT cells where the re-expression of Lck and ZAP70 in deficient cells restored the apoptotic response to galectin-1.

2. The exposure of phosphatidyl serine (PS) and the generation of ceramide from sphingomyeline were detected with a similar time of maximum around 12 hours of galectin-1 stimulation. PS exposure was assessed by binding of Annexin-V-FITC; ceramide release was investigated by immunofluorescence staining using anti-ceramide mAb. To establish whether ceramide release was essential for galectin-1 cytotoxicity, the ceramide expression was modulated by several means. The extraction of the outer membrane lipids with bovine serum albumin (BSA) resulted in the failure of the ceramide release and as a consequence the subsequent cell death was inhibited. The different apoptotic events were investigated by flow cytometry (the loss of the mitochondrial membrane potential ($\Delta\psi_m$) and the formation of 'sub-G1', hypodiploid cell population) or by Western blotting (the cleavage of nuclear repair enzyme, poly-ADP ribosyl polymerase (PARP)). The inhibition of apoptosis was not caused by the change in the binding of galectin-1 to its ligands since the binding capacity remained unaltered in the presence of 5% BSA. To provide further evidence that ceramide played role as a second messenger in galectin-1 induced apoptosis, we used Raji, a membrane scrambling-deficient Burkitt lymphoma cell line. According with the literature data Raji cells failed to respond with apoptosis after galectin-1 treatment although other B cell lines of Burkitt lymphoma origin, Daudi and BL41 died upon the same treatment. The activation of proteine kinase C (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, sphingosine 1 phosphate (S1P). The phorbol-ester, PDBu, a potent activator of PKC, and the presence of exogenous S1P significantly blocked the galectin-1 induced decrease of $\Delta\psi_m$, PARP cleavage and the formation of the 'sub-G1' cell population. This strongly supported a principal role for ceramide since the same results were obtained for C_6-Cer induced cell death. To gain a direct evidence that ceramide is not generated through sphingolipid pathway we used fumonisin B1 a specific inhibitor of ceramide synthesis. This drug did not inhibit the effect of galectin-1 indicating that the production of ceramide upon galectin-1 stimulation did not occur through the synthetic route.

These results did not only suggest that ceramide was an essential messenger of galectin-1 induced apoptosis, but also that ceramide release occurred upstream to the mitochondrial changes, caspase activation and nuclear DNA fragmentation as downregulation of ceramide expression inhibited the other apoptotic steps.

3. The role of the mitochondrion to the galectin-1 induced apoptosis was confirmed by using bongkrekic acid (BA), a potent inhibitor of the mitochondrion mediated death pathway. BA blocked the destruction of the nuclear DNA indicating that this step was essential in the execution of the apoptosis. The involved caspases and the order of the caspase activation in relation to the mitochondrial events were also investigated. The loss of the $\Delta\psi_m$ was not affected by the presence of general caspase inhibitor, zVAD–fmk. Using H_2O_2, a model reagent of oxidative stress, for apoptosis induction, which acted directly on mitochondrion, the cell death was also similar in the presence and absence of zVAD–fmk. These results showed that caspase cascade was activated downstream to the mitochondrial steps since the decrease of the mitochondrial membrane potential freely occurred in the presence of the caspase inhibitor. Our results demonstrated that the initiator caspase in death-receptor induced apoptosis, caspase 8, is not required for galectin-1 cytotoxicity, as galectin-1 induced PARP cleavage and the formation of the 'sub-G1' cells in the presence of caspase 8 inhibitor, Ac-IETD. On the other hand, the inhibition of the caspase 8 activity blocked the apoptosis triggered by TNFα, a known pathway initiating cell death via caspase 8. To confirm that caspase 8, the initiator caspase in death receptor mediated apoptosis, was not involved in galectin-1 induced cell death, we used caspase 8 deficient Jurkat cells (I 9.2). Both Jurkat and I 9.2 cell lines were sensitive to galectin-1 but the caspase 8 mutant cells were resistant to apoptosis to the TNF receptor stimulation.

According to our results we can conclude that ceramide release and exposure of phosphatidyl serine on the outer membrane of the cells requires the presence of functional $p56^{lck}$ and ZAP70. Downstream steps are the breakdown of the mitochondrial membrane potential followed by the activation of the caspase cascade, proteolysis of the caspase substrates and the final execution of the apoptosis by the degradation of nuclear DNA.

13. Summary in Hungarian
Összegzés

A humán galekin-1 egy endogén immunmoduláló fehérje, jelentős immunszupresszív hatással. A legújabb eredmények kimutatták a galektin-1 szabályozó szerepét a T sejt differenciálódási folyamatokban, és a perifériás T sejtes immunválasz lecsendesítésében. A szekretált galektin-1 szerepet játszik számos fontos biológiai folyamatban, mint például az immunhomeosztázis fenntartásában, a sejtadhézióban, a sejtnövekedés és az apoptózis szabályozásában. A galektin-1 immunreguláló hatása, legalább is részben, az apoptózis indukción keresztül valósul meg, melyet a periférián lévő aktivált, elsősorban a gyulladási zónákban lévő Th1 szubpopulációt alkotó T sejteken fejt ki. A tímusz epiteliális sejtjei által termelt galektin-1 részt vesz a T sejtek szelekciójában, azáltal hogy, apoptózist indukál a nem szelektált és a negatívan szelektált $CD4^{lo}$ $CD8^{lo}$ sejtfelszíni markereket hordozó T limfocitákon. A galektin-1 szintén citotoxikus hatást fejt ki a leukémiás T sejtvonalakon. Annak ellenére, hogy az irodalomban számos helyen leírták a galektin-1 apoptózist indukáló hatását, az hogy milyen útvonalon keresztül indukál apoptózist még nem ismert. Jelen munkában kimutattuk, hogy a galektin-1 által kiváltott apoptotikus folyamatok első lépése a tirozin foszforiláció, amihez szükséges a működőképes $p56^{lck}$ és ZAP70 kinázok jelenléte. A foszforiláció szükséges az apoptotikus folyamatok további lépéseihez is: a ceramid felszabaduláshoz majd az azt követő mitokondriális membránpotenciál összeomláshoz. A mitokondriális membránpotenciál összeomlását a kaszpáz kaszkád aktiválódása, majd a kaszpázok szubsztrátjainak proteolízise követi, majd végül a nukleáris DNS fragmentálódásával zárulnak az apoptotikus folyamatok. Ebben a munkában a galektin-1 által kiváltott apotózis folyamán bekövetkező morfológiai és biokémiai folyamatok időbeli lefolyását és sorrendiségét vizsgáltuk Jurkat leukémiás T sejtvonalon.

1. A galektin-1 által kiváltott tirozin kináz aktiváció majd a kinázok által elvégzett tirozin foszforiláció krititikus a további apoptotikus események szempontjából, mivel a genistein tirozin kináz inhibitor gátolta az apoptózis folyamán bekövetkező ceramid felszabadulást, és a nukleáris DNS fragmentálódását. A galektin-1 által kiváltott apoptotikus folyamatokban

különösen fontos szerepe van a $p56^{lck}$ és a ZAP-70 kináznak, mivel azon Jurkat sejtekben amelyek hiányosak a fenn említett két kinázra (a $p56^{lck}$ kinázra hiányos JCaM1.6, és a ZAP-70 kinázra mutáns P116), galektin-1 stimuláció hatására nem volt kimutatható a tirozin foszforiláció és a vad típusú Jurkat sejtekhez képest jóval kisebb mértékű apoptózis volt megfigyelhető. Az Lck/ZAP-70 foszforilációs útvonal fontosságát JCaM/LCK és P116 WT sejtek vizsgálatával is alátámasztottuk, amelyek jellemzője, hogy egy transzfekciónak köszönhetően újra expresszálják a Lck és ZAP-70 kinázt és ennek köszönhetően a galektin-1 kezelésre adott apoptotikus válaszképessége is visszatért a sejteknek.

2. Mind a foszfatidil szerin külső membránon történő megjelenése, mind a szfingomielinen keresztül történő ceramid felszabadulás azonos időben körübelül 12 órával a galektin-1 stimulációt követően volt detektálható. A foszfatidil-szerin megjelenését a külső membránban FITC –cel konjugált Annexin V segítségével mutattuk ki, míg a ceramid felszabadulást monoklonális anti ceramid ellenanyaggal vizsgáltuk. Annak kiderítésére, hogy a ceramid felszabadulás szükséges-e a galektin-1 citotoxikus hatásához, a ceramid felszabadulást különböző módokon próbáltuk befolyásolni. A külső membrán lipid molekuláit BSA (marha sérum albumin) felhasználásával eltávolítottuk, így megakadályoztuk a ceramid molekulák megjelenését a külső membránban, aminek következtében a sejtek nem adtak apoptotikus választ a galektin-1 kezelésre. Számos egyéb az apoptózis során végbemenő változást vizsgáltunk áramlási citometriával (mitokondriális membránpotenciál csökkenése ($\Delta\psi_m$), és az un. sub G1, hipodiploid fázisba tartozó sejtek mennyiségének mérése), és Western-blot segítségével (egy sejtmagi javító enzim a poli-ADP ripozil polimeráz (PARP) apoptózis során bekövetkező hasadása). A BSA kezelés hatásásra bekövetkező apoptózis gátlás nem annak volt a következménye, hogy a galektin-1 kötődése megváltozott az eddig még nem ismert ligandjához, mivel a sejtek galektin-1 kötőképességét az 5%BSA jelenléte nem befolyásolta. Annak bizonyításásra, hogy a ceramid másodlagos hírvivő szerepet tölt be a galektin-1 által kiváltott apoptikus folyamatokban, membrán rendezetlenséget előidéző enzimre hiányos Raji nevezetű Burkit limfóma eredetű sejtvonalat használtunk Az irodalmi adatoknak megfelelően a Raji sejtek nem adtak apoptotikus választ a galektin-1 kezelésre, szemben más Burkit limfóma eredetű B sejtekkel (Daudi, Bl-41). Ismert, hogy a protein kináz c (PKC) részt vesz a ceramid nevével fémjelzett apoptotikus útvonalban, mivel foszforilálja, és ezáltal aktiválja a szfingozin kinázt, amely működése eredményeként egy anti-apoptotikus szfingolipidet a szfingozin-1

foszfátot hoz létre. A PKC ismert aktivátora a PDBu nevezetű forbol-észter, csakúgy, mint a sejtekhez kívülről adott S1P, gátolta a galektin-1 által kiváltott mitokondriális membránpotenciál csökkenést, PARP hasítást és sub-G1 sejtpopuláció létrejöttét. Hasonló jelenséget tapasztaltunk, ha az apoptózist a sejtekhez kívülről hozzáadott C_6-ceramiddel idéztük elő, mindez a ceramid galektin-1 által kiváltott apoptotikus folyamatokban betöltött alapvető fontosságára utal. Annak bizonyítására, hogy a ceramid nem szintetikus úton az un. szfingolipid útvonalon keresztül jön létre, a ceramid szintézis egyik specifikus inhibitorát a fuminozin B1 nevezetű molekulát használtuk. A fuminozin B1 kezelés nem gátolta a galektin-1 által kiváltott apoptotikus folyamatokat, mindez azt jelzi, hogy a ceramid nem a szintetikus úton keresztül keletkezik.

A fennt említett eredmények nemcsak a ceramid alapvető fontosságú hírvivő szerepét támasztják alá a galektin-1 által kiváltott apoptotikus folyamatokban, hanem azt is bizonyítják, hogy a ceramid a galektin-1 által kiváltott apoptotikus útvonalon a mitokondriális membránpotenciál csökkenés a kaszpáz aktiváció és a nukleáris DNS fragmentáció fölött helyezkedik el, mivel az összes fenn említett folyamatot gátolta a ceramid felszabadulás gátlása.

3. A mitokondrium szerepét a galektin-1 által kiváltott apoptotikus folyamatokban bongkrekic acid (BA) segítségével vizsgáltuk, amely molekula a mitokondriumon keresztül ható apoptotikus folyamatok ismert gátlószere. A BA molekula jelenléte gátolta a nukleáris DNS fragmentálódását, ami esszenciális lépése az apoptózisnak. A galektin-1 által kiváltott apoptózisban résztvevő kaszpázokat, aktivációjuk sorrendjét, valamint a kaszpáz aktiváció és a mitokondriális események kapcsolatát szintén vizsgáltuk. A mitokondriális memebránpotenciál ($\Delta\psi_m$) csökkenése nem volt gátolható a zVAD–fmk nevezetű általános kaszpáz inhibitorral. H_2O_2 használatával, ami az oxidatív stressz segítségével indukál apoptózist, és közvetlenül a mitokondriumon keresztül hat, az apoptózis mértéke, csakúgy, mint a galektin-1 esetében, közel azonos volt a kaszpáz inhibitor (zVAD–fmk) jelenlétében, illetve hiányában. A fennt említett erdmények azt jelzik, hogy a mitokondriális folyamatok még a kaszpázok aktivációja előtt végbemennek, mivel a mitokondriális membrán potenciál csökkenése a kaszpáz inhibitorok jelenlétében is mérhető volt. Eredményeink azt igazolják, hogy a “halál-receptoron” keresztül végbemenő folyamatokban kulcsszerepet játszó kaszpáz, a kaszpáz -8 jelenléte nem szükséges a galaktin-1 által kiváltott apoptotikus folyamatokhoz,

mivel a galektin-1 által kiváltott PARP hasítás és a szub-G1 sejtpopuláció növekedés Ac-IETD molekula jelenlétében is végbemegy, ami a kaszpáz-8 egyik ismert inhibitora. A kaszpáz -8 gátlása megakadályozta a TNFα által kiváltott apoptózist, ami bizonyítottan a kaszpáz -8 részvételével zajlik. Annak bizonyítására, hogy a halál receptoron keresztüli útvonal iniciátor kaszpáza, a kaszpáz-8 nem vesz részt a galektin-1 által kiváltott apoptotikus folyamatokban kaszpáz-8 mutáns Jurkat (I 9.2) sejtvonalat használtunk. Mind a Jurkat, mind az I 9.2 kaszpáz-8 mutáns sejtvonal érzékeny a galektin-1 által indukált apoptózisra, de az I 9.2 sejtvonal sejtjei a TNFα által indukált apoptózisra nem voltak érzékenyek.

Eredményeink alapján elmondhatjuk, hogy a galektin-1 által indukált apoptózis során bekövetkező ceramid felszabaduláshoz és a sejt külső membránján történő foszfatidil-szerin megjelenéshez szükséges a müködőképes $p56^{lck}$ és ZAP70 kinázok megléte. A ceramid felszabadulását kővetően a mitokondriális membrán potenciál összeomlik, aktiválódik a kaszpáz kaszkád, lebomlanak a kaszpázok szubsztrátjai és az apoptózis utolsó lépéseként a sejtmagi DNS fragmentálódik.