Review

Evolving perspective of the pathogenesis of globoid cell leukodystrophy (Krabbe disease)

By Kunihiko SUZUKI

Neuroscience Center,*) Departments of Neurology and Psychiatry**)
University of North Carolina School of Medicine
Chapel Hill, NC 27599, U. S. A.

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Abstract: Clinical, pathological and biochemical phenotype of globoid cell leukodystrophy (Krabbe disease) has several unique characteristics that are sometimes contrary to the conventional concept of genetic lysosomal disease. It was demonstrated early that galactosylceramide has unusual capacity to elicit a globoid cell-like reaction when implanted into the brain. Then, thirty years ago a hypothesis was introduced to explain the pathogenetic mechanism underlying the rapid and complete loss of myelin and myelinating cells. It postulated that galactosylsphingosine (psychosine), which is highly cytotoxic and also cannot be degraded due to the underlying genetic defect, is responsible for the very rapid loss of the oligodendrocytes and the consequent paradoxical analytical finding – lack of accumulation of the primary substrate, galactosylceramide, in patients' brain. It took nearly ten years before the actual accumulation of psychosine was demonstrated in human Krabbe patients and also in the brain of mouse and dog models of the disease. During the intervening years, the psychosine hypothesis has been generally accepted as a critical pathogenetic mechanism in classical infantile globoid cell leukodystrophy. However, a more recent experimental mouse model due to genetic defect in saposin A, an *in vivo* galactosylceramidase activator protein, introduced new elements in our understanding of the disease process. Not only has it established the second gene, genetic defect of which can cause globoid cell leukodystrophy but it has indicated potential decoupling of the two previously postulated pathogenetic mechanisms, galactosylceramide for the globoid cell reaction and psychosine for loss of myelinating cells. Pathogenetic significance of participation of the major histocompatibility complexes and other immune mechanisms, inflammatory processes as suggested by activation of many cytokines, and possible interactions with sex hormones remain to be further explored.

Key words: Krabbe disease; globoid cell leukodystrophy; pathogenesis; psychosine hypothesis; galactosylceramide; saposin A.

Historical overview. Globoid cell leukodystrophy (GLD, Krabbe disease) is one of the classical genetic leukodystrophies first described by Krabbe in 1916.[1] Rapid and complete loss of the oligodendrocytes and myelin, reactive astrocytic gliosis and infiltration of the unique "globoid cells" are the pathological hallmark of the disease.[2] In the mid-sixties, Lehfeldt *et al.* demonstrated that galactosylceramide could uniquely elicit a globoid cell-like reaction when implanted into the brain.[3] These experimentally generated globoid cells have light and electron microscopic characteristics identical to those in patients' brain.[4),5] In 1970, my laboratory identified genetic deficiency of galactosylceramidase as the underlying cause of human as well as canine globoid cell leukodystrophy (Krabbe disease).[6),7] We also demonstrated that the myelin sheath in GLD was compositionally normal although quantitatively minuscule and that

*) Director Emeritus.
**) Professor Emeritus.
Correspondence to: K. Suzuki, M. D., 4929 Boulder Run Road, Hillsborough, NC 27278, U. S. A.

there was no overt accumulation of galactosylceramide in the brain of Krabbe disease patients.[8] This was in contrast to an equivalent disease, metachromatic leukodystrophy (MLD), in which abnormal sulfatide accumulation had been well established since the first report by Jatzkewitz[9] and in which Norton, and also O'Brien and Sampson had reported an abnormally high sulfatide content in the myelin sheath.[10),11] There were other observations in GLD that provided a puzzling contrast against those on MLD, even though the two diseases are metabolically closely related. A substantial oligodendroglial population remains in MLD but not in GLD, and there is an abnormal accumulation of sulfatide in MLD brain but no accumulation of galactosylceramide in GLD brain. Observations on MLD are more consistent with the concept of the inborn lysosomal disorder of Hers,[12] a storage disorder due to a genetic deficiency of a lysosomal hydrolase. There must be something atypical and unique about Krabbe disease. The psychosine hypothesis was introduced in order to explain the unique characteristics of Krabbe disease not shared even by such a closely related and conceptually equivalent disease, metachromatic leukodystrophy.[13] Although received with skepticism initially, the hypothesis has survived the intervening 30 years and is generally accepted as a critical pathogenetic mechanism underlying GLD.[14]

Recent developments have forced expansion and modifications of our understanding of the pathogenesis of GLD. At the fundamental level, the newly generated saposin A-deficient mouse established that, not only galactosylceramidase deficiency, but genetic defect of saposin A, an *in vivo* galactosylceramidase activator protein, also results in a qualitatively identical disease.[15] The saposin A-deficient mouse also provided the first evidence that the two primary pathogenetic mechanisms, globoid cells due to undigested galactosylceramide and the loss of myelinating cells due to psychosine cytotoxicity, may operate independently from each other under certain circumstances, although they interact closely in the typical infantile form of the disease. Additionally, evidence has been gradually accumulating that immune, inflammatory and hormonal factors play potentially important roles in the pathogenesis of GLD.

Phenotype. *Clinical phenotype.* Since the first description of the disease by Krabbe more than 90 years ago,[1] globoid cell leukodystrophy had been recognized for its unusual clinical and pathological phenotype. Clinical phenotype of the classical infantile Krabbe disease is relatively stereotypic. Hagberg *et al.*[16] divided the steady and rapidly progressive clinical course into three stages. It is characterized by exclusive manifestations of white matter involvement and a relentless, rapidly progressive course leading to early death. Stage I is characterized by generalized hyperirritability, hyperesthesia, episodic fever of unknown origin, and some stiffness of the limbs. The child, apparently normal for the first few months after birth, becomes hypersensitive to auditory, tactile, or visual stimuli and begins to cry frequently without apparent cause. Retardation or regression of psychomotor development, vomiting with feeding difficulty, and convulsive seizures may occur as initial clinical symptoms. The cerebrospinal fluid protein level is already highly increased. In stage II, rapid and severe motor and mental deterioration develops. There is marked hypertonicity, with extended and crossed legs, flexed arms, and the backward-bent head. Tendon reflexes are hyperactive. Minor tonic or clonic seizures may occur. Optic atrophy and sluggish pupillary reactions to light are common. Stage III is the "burnt-out" stage, sometimes reached within a few weeks or months. The infant is decerebrate and blind and has no contact with the surroundings. Patients rarely survive for more than 2-3 years.

Pathology. Neuropathology of globoid cell leukodystrophy is so unique that diagnosis is rarely in doubt when brain tissue is available for examination. Pathology is, for all practical purposes, limited to the nervous system.[2] At the terminal stage, loss of myelin is nearly complete with possible exception of the subcortical intergyral arcuate fibers. Microscopically, marked paucity of myelin with some axonal degeneration is present throughout the brain. Extensive fibrillary gliosis and infiltration of numerous macrophages, often multinucleated ("globoid cells"), are the unique features. The globoid cells are abundant in the region of active demyelination and often clustered around blood vessels. Oligodendrocytes disappear rapidly. Globoid cells contain PAS-positive tubular and filamentous inclusions with polygonal cross sections that are structurally identical with chemically pure galactosylceramide.[17] The peripheral nerves are often grossly enlarged and firm with marked endoneurial fibrosis, segmental demyelination and evidence of remyelination process. Endoneurial macrophages and also Schwann cells contain tubular inclusions similar to those in the globoid cells in the cerebral white matter.

Biochemistry. The genetic cause of all so far known human patients with Krabbe disease is deficient activity of galactosylceramidase (Fig. 1).

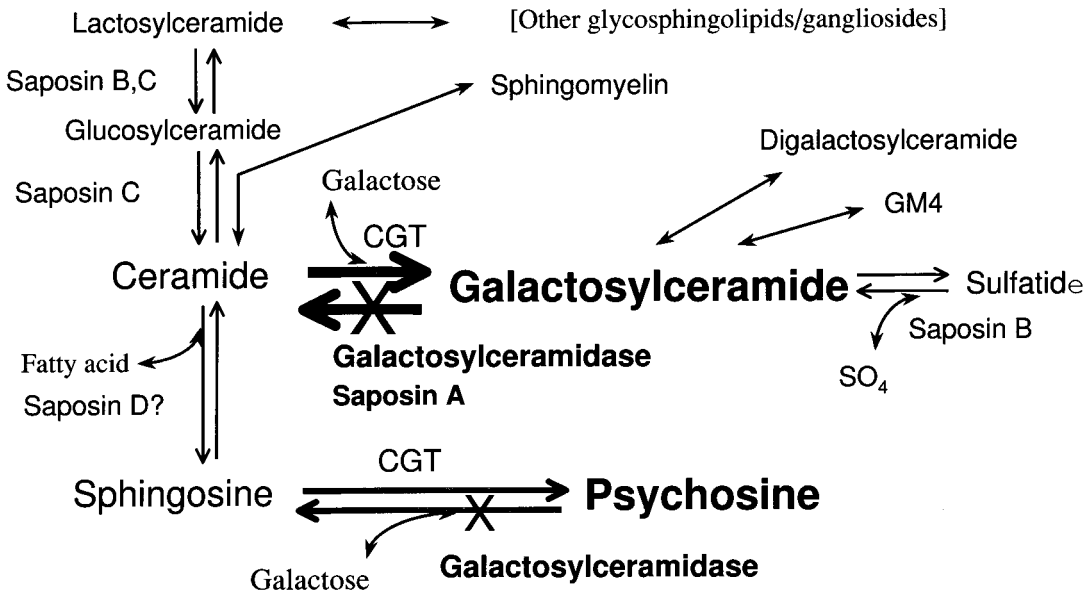

Fig. 1. Metabolic pathways pertinent to galactosylceramide and related compounds. In the synthetic pathway, sphingosine is first acylated to ceramide, which in turn is galactosylated by UDP-galactose: ceramide galactosyltransferase (CGT) to form galactosylceramide. The same enzyme can galactosylate sphingosine directly to generate psychosine. Both galactosylceramide and psychosine are degraded by galactosylceramidase, which is genetically deficient in Krabbe disease. *In vivo* degradation of galactosylceramide requires, in addition to the enzyme, a sphingolipid activator protein, saposin A. Galactosylceramide is further sulfated to form sulfatide. Both galactosylceramide and sulfatide are characteristic myelin glycolipids.

Galactosylceramidase is a hydrolytic enzyme with an acid pH optimum localized in the lysosome. Thus, the disease conceptually belongs to the category of the lysosomal disease as originally defined by Hers.[12] Essentially all lysosomal diseases are "storage diseases", in which substrates of the genetically defective enzymes accumulate to abnormally high levels. The enzyme is specific for certain glycolipids with a terminal galactose moiety in β anomeric configuration. The major natural substrate is galactosylceramide, which is almost exclusively localized in the myelin sheath. Other known natural substrates are psychosine (galactosylsphingosine), monogalactosyldiglyceride, and the precursor of seminolipid (1-alkyl, 2-acyl-, 3-galactosyl glycerol). *In vivo* degradation of these substrates requires an activator protein, saposin A, in addition to the enzyme, galactosylceramidase. The unique biochemical characteristic of Krabbe disease is lack of abnormal accumulation of galactosylceramide in the brain, contrary to what is expected from the enzymatic defect.[18),19)] This paradoxical phenomenon can be explained phenomenologically by the exclusive localization of galactosylceramide in the myelin sheath and the very rapid and early disappearance of the myelinating cells during the course of the disease. Since the very rapid disappearance of the myelinating cells eliminates the source of galactosylceramide synthesis, it does not accumulate beyond the level attained at the early stage of myelination. However, a related toxic metabolite, psychosine (galactosylsphingosine) does accumulate abnormally and is considered the key compound in the pathogenesis of the disease.[13),20)] Galactosylceramide has unique capacity to elicit infiltration of globoid cells when it is implanted into the brain[3)] and such experimentally induced globoid cells appear morphologically identical to those seen in patients with Krabbe disease.[4),5)]

Pathogenesis of classical infantile GLD. Three of the most characteristic pathological features of Krabbe disease are (a) the infiltration of macrophages that are often multinucleated and contain strongly PAS-positive materials ("globoid cells"), (b) the rapid and almost complete disappearance of the oligodendrocytes and (c) lack of abnormal tissue accumulation of the primary substrate of the defective enzyme, galactosylceramide, contrary to what is expected in a "storage disease" due to genetic defect in degradative enzymes. These phenotypic characteristics must be explained as consequences of the underlying genetic defect. Defective degradation of two substrates, galactosylceramide and psychosine (galactosylsphingosine),

appears to play critical roles in the pathogenesis. While these mechanisms are fundamentally distinct from each other, they are closely intertwined to result in the unique phenotype of the disease.

Globoid cells. Impaired degradation of galactosylceramide is clearly a major factor for the unique pathological feature of the disease, the globoid cells. It has long been known that free galactosylceramide has a specific capacity to elicit infiltration of macrophages into the brain.[3),21)] No other agent is known to have a similar capacity *in vivo*. Once in the brain, they phagocytize undegradable galactosylceramide and are transformed to multinucleated globoid cells. The characteristic inclusions in the globoid cells have morphological appearance identical to galactosylceramide itself.[17)] The globoid cell reaction can be reconstructed in the following way. Once the active period of myelination begins, turnover of already formed myelin also begins. In patients' brain, however, galactosylceramide cannot be degraded due to the underlying galactosylceramidase deficiency. Free galactosylceramide thus generated elicits infiltration of hematogenous macrophages, which become the characteristic PAS-positive, often multinucleated globoid cells.

Psychosine hypothesis. The devastating early destruction of the myelin-forming cells is difficult to explain on the basis of undegradable galactosylceramide because there is no experimental evidence that galactosylceramide is a metabolic toxin. Galactosylceramide implanted in the brain does not exhibit any functionally detrimental capacity other than eliciting the globoid cell reaction. On the other hand, a closely related metabolite, psychosine (galactosylsphingosine), is highly cytotoxic[22)] and causes rapidly fatal hemorrhagic infarct when implanted into the brain.[13)] At least in mammalian tissues, psychosine can be generated only by galactosylation of sphingosine by galactosylceramide synthase, UDP-galactose: ceramide galactosyltransferase (CGT), but not by de-acylation of galactosylceramide. Since CGT is nearly exclusively localized in the myelin-forming cells, synthesis of psychosine should also occur only in the oligodendrocytes and Schwann cells. Psychosine is detectable in normal brain with highly sensitive analytical methods but its concentration is minuscule (less than 10 picomoles/mg protein). It appears to be a metabolic dead-end product, which is normally degraded immediately by galactosylceramidase. Therefore, patients with Krabbe disease cannot degrade psychosine. A hypothesis, known as the psychosine hypothesis, was first proposed on the basis of this enzymological consideration[13)] and then its abnormal accumulation was analytically demonstrated in the brain of patients[19),20)] and in canine and murine models.[23)] The psychosine hypothesis postulates that, in globoid cell leukodystrophy, not only the primary substrate of the defective enzyme, galactosylceramide, but also the toxic metabolite, galactosylsphingosine (psychosine), cannot be degraded and that the consequent abnormal accumulation of psychosine causes the uniquely rapid destruction of the myelin-forming cells. The hypothesis initially met considerable skepticism but has survived the intervening 30 years.[14)] In fact, the basic premise of the hypothesis has been extended to other sphingolipidoses.[24)] For varieties of reasons, however, its plausibility for other disorders is not as firm as it is for GLD, with possible exceptions of neuronopathic form of Gaucher disease and Niemann-Pick type A disease.

Recent new developments. *Saposin A-deficient mouse.* A recent experimental generation of an entirely new mouse model of globoid cell leukodystrophy due to genetic deficiency of saposin A, an *in vivo* galactosylceramidase activator protein, provided a new set of complications as well as insight regarding the pathogenesis of GLD.[15)] Most importantly, this model established that the defect in the galactosylceramidase gene is not the only possible genetic cause underlying globoid cell leukodystrophy. Another important insight this new model provides is that the two pathogenetic mechanisms, the globoid cell infiltration due to undegraded galactosylceramide and the loss of the oligodendrocytes due to psychosine toxicity, can operate independently from each other. A saposin A-deficient mouse line was generated experimentally by introducing an amino acid substitution (C106F) into the saposin A domain by the *Cre/lox*P system[15)] that eliminated one of the three conserved disulfide bonds considered essential for the functional properties of saposins. In humans, an equivalent mutation in the 4th cysteine to phenylalanine in saposin C causes specific saposin C deficiency, and a mutation of the 5th cysteine to serine in saposin B causes specific saposin B deficiency. Saposin A-deficient mice developed slowly progressive hind leg paralysis with the clinical onset around 2.5 months and survival up to 5 months. Tremors and shaking prominent in other myelin mutants were not conspicuous until the terminal stage. Both males and females of saposin A-deficient mice are fertile, and females are able to raise pups up to three times. In every respect, pathology – firm and thick peripheral nerves, demyelination, reactive astrogliosis, and infiltration of "globoid cells" that contain

the characteristic inclusion bodies – and analytical biochemistry – consequences of impaired degradation of all of the galactosylceramidase substrates in the brain, kidney and testis – were qualitatively identical with but generally much milder than those seen in the twitcher mouse, a naturally occurring GLD model due to galactosylceramidase deficiency.[25] Accumulation of psychosine in the brain was only twice normal compared to the 10-20-fold increase in twitcher mice. The saposin A-deficient mouse clearly established that saposin A is indispensable for galactosylceramidase activation *in vivo* in the sense that normal cellular functions cannot be maintained in its absence.

Pregnancy dramatically alleviates saposin A deficient phenotype. During routine breeding process, it was noted that affected saposin A-deficient females that were continually pregnant showed greatly improved neurological symptoms compared to affected females that do not experience pregnancy, or affected males.[26] The pathological hallmark of globoid cell leukodystrophy, demyelination with infiltration of globoid cells, largely disappeared. The immune-related gene expression (MCP-1, TNF-α) was significantly down-regulated in the brain of pregnant saposin A-deficient mice. In addition, intense expression of estrogen receptors (ER-α and ER-β) on the globoid cells, activated astrocytes and microglia in the demyelinating area of saposin A-deficient mice was observed. When saposin A-deficient mice were subcutaneously implanted with time-release 17β-estradiol (E2) pellets from 30 to 90 days, the pathology was vastly improved. These findings suggest that higher level of estrogen during pregnancy is one of the important factors in the protective effect of pregnancy.

Involvement of apoptotic process. While it is well accepted now that cytotoxicity of psychosine is likely to be responsible for the characteristic rapid disappearance of the oligodendrocytes, the exact mechanism is not well characterized. Involvement of the cellular apoptotic processes has been explored in this regard and increasing number of oligodendrocytes in twitcher mouse brains were found to be dying from apoptotic processes.[27),28] This observation is consistent with an *in vitro* study, which indicated that psychosine is as potent an inducer of apoptosis as C-6 ceramide.[29]

Involvement of immune and inflammatory processes. One of the issues related to the pathogenesis of GLD is the extent and nature of immune and inflammatory system involvement. With progression of demyelination, GFAP-, Mac-1- and F4/80-positive cells increased both in the twitcher CNS and PNS. Some Mac-1-positive cells also expressed the major histocompatibility complex (MHC) class II (Ia). Emergence of Ia-expressing cells was largely coincident with the onset of demyelination. Ia-immunoreactive cells gradually increased in areas of demyelination, reached a plateau between 30 to 40 days of age in the cerebrum and then rapidly decreased despite continuous demyelination. In the spinal cord, however, Ia-immunoreactive cells did not decline even at 50 days.[30] These results may indicate either a specific involvement of immunological factors in the pathogenesis of this genetic demyelinating disease or a non-specific reaction to degenerating tissue components, such as myelin. Cross-breeding the twitcher mice with a MHC class II knockout mouse line generated a twitcher mice simultaneously also deficient with MHC class II molecules.[31] In these mice, clinical symptoms and histopathology of the cerebrum and the brain stem-cerebellar region were milder than those in twitcher mice with the MHC-II positive background but there was no noticeable improvement in the pathology of the spinal cord.[32] Preliminary analysis of psychosine level also showed less accumulation than in the MHC-II-positive twitcher mice, consistent with the lesser degree of pathology in these mice. Interleukins, cytokines and their receptors have also been examined in the twitcher mice to evaluate participation of inflammatory processes in the pathogenesis of GLD.[26),33)-37] The dramatic improvement of saposin A-deficient female mice during pregnancy as mentioned above may also be related to the known anti-inflammatory capacity of estrogen.

Current understanding of pathogenesis. Fig. 2 depicts what I now understand as the basic pathogenetic mechanism of globoid cell leukodystrophy. The recent radical change in this scheme is the addition of saposin A as another causative gene. Saposin A-deficient GLD is now known only experimentally in the mouse but corresponding human patients can be anticipated. Thus, genetic defects in either galactosylceramidase or saposin A can be the underlying cause of the disease. The common consequence is impaired degradation of the substrates of galactosylceramidase. In the brain, this means galactosylceramide and psychosine (galactosylsphingosine). Beyond this point, close interactions of two pathogenetic mechanisms can explain the most important aspects of the phenotype of Krabbe disease. Since synthesis of both galactosylceramide and psychosine is limited to actively myelinating cells, the disease process does not begin until the active myelination period. This point was clearly demonstrated by a

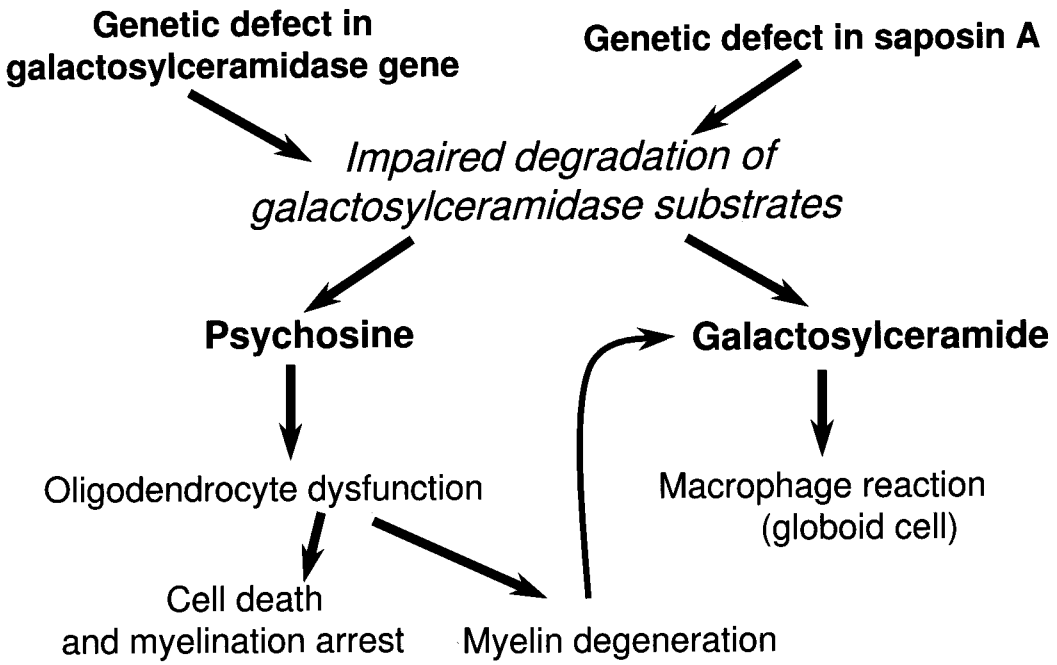

Fig. 2. Pathogenetic mechanisms operating in Krabbe disease as understood at this time. See text for explanation.

mouse line doubly deficient in galactosylceramide/psychosine synthesis and galactosylceramidase.[38] Once myelination begins, its metabolic turnover also starts. This generates free galactosylceramide in the brain of patients because of the inability to degrade galactosylceramide, which in turn elicits the characteristic globoid cell reaction. Galactosylceramide synthase also synthesizes psychosine within the actively myelinating cells. Normally, it is immediately degraded and never reaches beyond barely detectable levels. In Krabbe disease, however, an abnormal accumulation of psychosine reaches the level toxic to cellular metabolism. This causes the other characteristic feature of the disease, a rapid and almost complete disappearance of the oligodendrocytes. Psychosine is as potent an apoptosis inducer as C6 ceramide.[29] The cellular death results in further destruction of already formed myelin, which contributes more undegradable galactosylceramide that in turn further elicits the globoid cell infiltration. On the other hand, myelination ceases at a very early stage due to the near-complete loss of the oligodendrocytes. This explains the paradoxical characteristics of the disease that the primary substrate of the defective enzyme, galactosylceramide, does not accumulate abnormally.

The saposin A-deficient mouse demonstrated that, although the two pathogenetic mechanisms interact closely, some degree of decoupling of the two mechanisms could occur under certain circumstances. Abnormal psychosine accumulation in saposin A-deficient mouse is only twice normal. This is in contrast to human infantile patients, the twitcher mouse and other animal models due to galactosylceramidase deficiency where psychosine accumulation can reach 20-30-fold. From our tissue culture studies, it is unlikely that the twice normal level of psychosine is sufficiently high to exert cytotoxic effects on the myelinating cells to cause their apoptotic death.[29] Thus, in the saposin A-deficient mouse, the disease process may be primarily due to the globoid cell infiltration and associated immune/inflammatory phenomena rather than psychosine cytotoxicity. Then, the oligodendrocytes survive much longer than in the more severe twitcher mouse model and destruction of already synthesized myelin will be slower. This also slows down globoid cell infiltration. It seems to be a plausible extrapolation that a similar decoupling of the two mechanisms might occur in the late-onset, slowly progressive form of human GLD.

Questions for the future. While I believe that the basic pathogenetic mechanisms of GLD are getting clearer, there remain many points of uncertainty. Clearly, immune and inflammatory processes play important roles in the pathogenesis but exact mechanism of their participation is yet to be clarified. More intriguing

are the recent observations that both galactosyl- and glucosyl-psychosine can generate multinuclear cells resembling globoid cells in tissue culture[39] and possible existence of psychosine receptor.[40] These findings may further extend our understanding of the disease processes operating in GLD. On the other hand, there are a few apparent discrepancies between these findings and known facts about GLD. Both galactosyl- and glucosylpsychosine were equally capable of generating multinuclear cells, but neither generates such cells *in vivo* when implanted into the brain. They are extremely cytotoxic and kill experimental animals with massive hemorrhagic infarct. Only galactosylceramide is known to cause globoid cell-like reactions when implanted into the brain. It is known that glucosyl-psychosine accumulates in the brain of patients with neuronopathic form of Gaucher disease but there are no globoid cells in Gaucher brain. The reported psychosine receptor had similar and high binding constants toward galactosyl- and glucosyl-psychosine. The high binding constant of the receptor may indicate that the natural ligand(s) for this receptor may not be psychosines. Despite these cautionary notes, these recent findings should be followed up further since they may well have important bearing to our understanding of the pathogenetic mechanisms of globoid cell leukodystrophy. This subject is still constantly evolving and we can look forward to more exciting discoveries in the future.

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