Chapter A24

APOPTOTIC CELL DEATH IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Apoptosis of effector cells as a safe mechanism in the termination of an autoimmune inflammatory attack

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Abstract: Particularly in the vulnerable CNS with a low capacity for regeneration specialized mechanisms must be active for the fast and gentle elimination of dysregulated autoaggressive immune cells.   In EAE, local apoptosis of autoimmune T-cells has been identified as a safe means for the removal of these unwanted cells. T-cell apoptosis *in situ* followed by phagocytic clearance of apoptotic remnants by glia assures a minimum of detrimental bystander damage to the local parenchyma and down-regulates the local inflammatory reaction. The pharmacological augmentation of local apoptosis of inflammatory effector cells might gain therapeutic importance also in human neuroimmunological diseases such as multiple sclerosis.

Key words: T-cell, phagocytosis, Experimental autoimmune neuritis, astrocyte, microglia, glucocorticosteroids, multiple sclerosis

1.   INTRODUCTION

Multicellular organisms face the task of safely disposing unwanted cells under physiological and pathological conditions. The active and tightly regulated cell death program during apoptosis is considered to play a major role in the physiological control of cell turnover while at the other end dysregulation of apoptosis has been shown to contribute to the pathogenesis of different autoimmune diseases.

During the last decade the role of apoptosis also in neuroimmunological diseases has been more clearly defined. In EAE and experimental autoimmune neuritis (EAN), especially apoptosis of the pathogenic effector cells has been thoroughly investigated. This review will briefly introduce definitions and detection methods for apoptosis. We will focus on apoptotic cell death of effector T-cells in EAE, proposed mechanisms of T-cell apoptosis, functional consequences and finally its potential therapeutic implications.

1.1   Apoptosis: definition and detection

Apoptosis is a specialized, morphologically and biochemically distinct form of eukaryotic cell death. Introduced in 1972 by Kerr and colleagues, the term apoptosis served to describe a set of morphological features commonly observed during cell death in various tissues and cell types which were different to those observed during necrotic cell death (1). The stereotypic series of cellular changes comprise blebbing of the plasma membrane and condensation of chromatin at the periphery of the nucleus, followed by disintegration of the cell into multiple membrane-enclosed vesicles. In contrast to the lytic processes observed during necrosis, cell death by apoptosis is not associated with secondary inflammatory tissue responses, e.g. towards potentially harmful intracellular contents of the dying cells (2). Thus, apoptosis is considered to provide a safe and gentle cell death mechanism. *In vivo*, apoptosis also appears to be a fast and efficacious mechanism for the elimination of unwanted cells, with completion of the cell death program within 4-5 hrs at least in certain tissues and under defined conditions (3).

As the traditional definitions of apoptosis were mainly based on morphological criteria, electron microscopy served as the gold standard in the detection of apoptosis. Meanwhile, the better understanding of underlying biochemical processes during apoptosis has led to the introduction of an array of detection methods at the molecular level (4,5). For example, intravital internucleosomal DNA-fragmentation which generates fragments of 180 bp and multiples thereof can reliably be detected using TUNEL or *in situ* nick translation techniques. The identification of a set of aspartate-directed cysteine proteases (Caspases) whose activation underly many of the observed morphological changes during apoptosis has further broadened the spectrum of detection methods. Thus, either the activated caspases or their proteolytic cleavage products (e.g. cytokeratin 18, Poly(ADP-Ribose)-Polymerase PARP, APP, actin cleavage products) can be identified. Also specific alterations of the cell membrane (e.g. loss of

phospholipid asymmetry detected with annexin-V) or mitochondrial changes (e.g. cytochrome c release, "permeability transition") serve as the basis for commercially available detection methods for apoptosis. However, many of these detection methods have to be interpreted cautiously (4). For example DNA-fragmentation does not only occur during apoptosis, but also during necrosis. Since different intracellular pathways lead to typical apoptotic features, a combination of different molecular detection methods is feasible. Thus, typical morphological features of apoptosis can also occur in the absence of oligonucleosomal DNA-fragmentation as well as in enucleated cells (6,7).

1.2   T-cell apoptosis in EAE and CNS inflammation

T-cells autoreactive to CNS-antigens such as MBP can also occur in healthy individuals (8,9). Albeit more often in MS-patients, surges of increased frequencies of circulating myelin-reactive T-cells can also be observed in healthy subjects, possibly driven by cross-reactive environmental antigens (10). These activated autoreactive T-cells are capable of entering the CNS-parenchyma and thus have the potential to induce a local immune response when they encounter their specific antigen in the context of appropriate restriction molecules (11,12). Therefore, specialized anatomic barriers such as the blood brain barrier or the absence of lymphatic drainage are not sufficient to prevent immune-mediated damage in the CNS, arguing for other mechanisms in the physiological control of autoreactive inflammatory cells *in situ*. Local apoptosis of pathogenic T-cells has been identified as a major immunological defense mechanism in the "immune-privileged" CNS, leading to an inhibition of inflammation or, once it has encroached, to rapid and non-destructive elimination of the inflammatory infiltrate (review in (13,14).

First reports on cell death by apoptosis in inflammatory brain lesions of Lewis rat EAE were from Pender and colleagues (15). Using morphological criteria the majority of the dying cells appeared to be lymphocytes and oligodendrocytes. Apoptosis of α/β-T-cells was subsequently confirmed by the same group using pre-embedding immunolabelling techniques (16). By combined T-cell immunohistochemistry, molecular labeling techniques and ultrastructural criteria, Schmied and coworkers analyzed T-cell apoptosis in the spinal cord quantitatively during the time course of different rat EAE models (17). Of all apoptotic cells, 64% were identified as T-lymphocytes, mostly expressing the α/β-T cell receptor. While another 9% of the apoptotic cells were classified as oligodendrocytes, apoptosis of macrophages was only

rarely observed. However, a considerable proportion of apoptotic cells could not be identified immunocytochemically, due to the advanced degeneration of the cells.
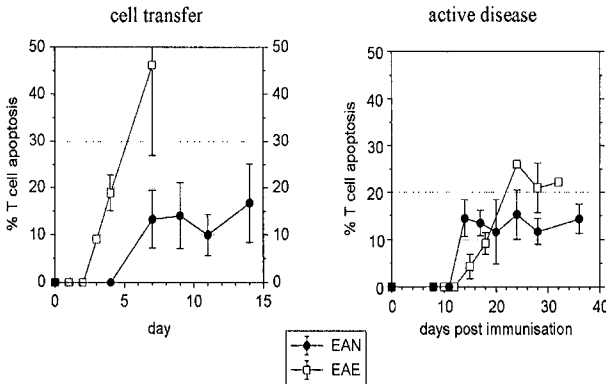

Figure 1. Time course of T-cell apoptosis *in situ.* Apoptosis during the natural course of experimental autoimmune encephalomyelitis (EAE) or neuritis (EAN), passively induced by cell transfer or by active immunization with MBP (EAE) or peripheral nerve myelin (EAN). Ordinate: percentage of apoptotic T-cells (mean +/- SD). Modified after (18).

During Lewis rat cell transfer (AT)-EAE using MBP-specific T-cells the degree of T-cell apoptosis was minimal at early stages on day 4, but peaked at the time of recovery from disease at day 7 with apoptosis rates of up to 49% (figure 1). Also in the active disease model prevalence of T-cell apoptosis *in situ* was highest when animals had recovered from the disease.

Apoptosis of inflammatory cells *in situ* also occurs in chronic relapsing EAE models, especially during clinical relapses (15,19,20,21). In the CNS of adoptively transferred chronic relapsing EAE in SJL/J mice, apoptosis of CD4+ T-cells and microglia/brain macrophages could readily be observed, while oligodendrocytes and astrocytes did not exhibit TUNEL positivity (19).

T-cell apoptosis *in situ* has not only been identified in EAE but also in coronavirus-mediated encephalomyelitis (22). Apoptosis of T-lymphocytes also occurs in inflammatory human brain lesions, most prominently in acute disseminated leukoencephalomyelitis (ADEM) (23), an acute monophasic disease which closely mimics pathological changes of acute EAE. T-cell apoptosis can also be observed in active MS lesions albeit to a lesser extent, possibly due to the chronicity of the disease (24). The similar degree of T-cell apoptosis in the acutely autoimmune inflamed rodent (EAE) and human

CNS (ADEM) (25) with approximately 30% of all invading T-cells undergoing apoptosis highlights the importance of the local apoptotic destruction of inflammatory T-cells in the termination of CNS-inflammation.

In contrast to the high T-cell apoptosis rate observed in the CNS, the PNS and other peripheral tissues appear to have a lower capacity to induce T-cell apoptosis locally. T-cell apoptosis could also be observed in sciatic nerves of Lewis rats with active or AT-EAN (26). Whereas the time course was similar to EAE, the extent of T-cell apoptosis was markedly different: highest levels were also found during recovery, with typical T-cell rates of approximately 10% (figure 1). However, in spite of this lower apoptosis rate, also in the PNS local T-cell apoptosis can be regarded as an efficient mechanism of cell elimination. Thus, given a time frame of 4-5 hrs for the completion of apoptosis, one could assume that up to 50% of an inflammatory infiltrate would be eliminated within 24 hrs.

Negligible degrees of T-cell apoptosis were found in T-cell mediated autoimmune diseases of muscular tissue. T-cell apoptosis was less than 0.5% in CD8-T-cell dominated experimental autoimmune myositis and biopsies of different human myopathies of presumed autoimmune etiology (27,28). Thus, T-cell inflammation in muscle is not cleared by apoptosis *in situ*, which could contribute to the non-self-limiting nature of these diseases. Even in HIV-associated polymyositis and -neuropathy there is virtually no relevant T-cell apoptosis *in situ*, in spite of the pathophysiological relevance of T-cell apoptosis in HIV-infection (29).

Also T-cell infiltrates in the skin do not appear to be eliminated by local apoptosis. In Lewis rat EAE, there was negligible T-cell apoptosis in the dermal tissue adjacent to the sensitization site, despite a heavy T-cell infiltration (17). Furthermore, in skin biopsies of patients with dermatomyositis and lupus erythematosus, T-cell apoptosis was virtually absent (30)(Chan, Gold, unpublished observations).

Given the obvious disparity in T-cell apoptosis between the "immunoprivileged" CNS and other non-immunologically protected sites, tissue-specific local mechanisms must be active in the CNS, that lead to a very efficient apoptotic clearance of pathogenic T-cells.

1.3 Possible mechanisms of T-cell apoptosis in the CNS

An important question in the elucidation of possible mechanisms of T-cell apoptosis concerns the specificity of the dying cells. Thus, apoptosis-inducing mechanisms such as activation induced cell death via triggering of the T-cell receptor would lead to selective cell death only of autoantigen-specific T-cells. In contrast, apoptosis also of non-antigen specific

"bystander" T-cells, secondarily recruited into the lesion, would suggest non-selective mechanisms of apoptosis induction.

To elucidate this further, Pender and colleagues investigated T-cell apoptosis in Lewis rat EAE passively induced with a MBP-specific T-cell clone using the Vβ8.2+ T-cell receptor, which is the predominant T-cell receptor element in MBP-induced Lewis rat EAE (31,32). Apoptosis and T-cell receptor usage was then analyzed in lymphocytes isolated and enriched from spinal cord. The frequency of Vβ8.2+ T-cells was about sevenfold higher in the apoptotic cell population than in the non-apoptotic T-cells. Moreover, using MBP- and ovalbumin-specific T-cells, it appeared that MBP-specific T-cells were readily eliminated from the CNS by apoptosis, while T-cells specific for ovalbumin, an antigen not present in the Lewis rat, survived in the CNS and recirculated to peripheral lymphoid organs. However, these studies could not exclude that also ovalbumin specific T-cells underwent apoptosis in the CNS. Also, altered physicochemical properties of collapsed apoptotic cells could have precluded their quantitative recovery from spinal cord during gradient centrifugation. Moreover, other MBP-reactive T-cell receptor elements besides Vβ8.2, proven to be encephalitogenic were not analyzed in these studies (33,34).

Using T-cells stably expressing specific genomic markers, Lassmann and colleagues could demonstrate that both MBP-specific and ovalbumin-specific T-cells undergo apoptosis in the CNS of EAE-animals (35). In addition, the occurence of T-cell apoptosis during EAE in bone marrow chimeras with different MHC-haplotypes of the resident glial cells and the passively transferred T-cells indicates that T-cell apoptosis is not dependent on antigen-specific mechanisms (35). Thus, both encephalitogenic, antigen-specific T-cells as well as secondarily recruited bystander T-lymphocytes appear to undergo apoptosis *in situ*.

The molecular mechanisms leading to T-cell apoptosis in the CNS *in situ* are as yet incompletely understood (14). *In vivo*, the TNF-receptor-1 (TNFR1)-mediated death pathway appears to play a major role in the induction of T-cell apoptosis in the CNS (36). Thus, in EAE of TNFR1-or TNF/lymphotoxin-deficient mice, local T-cell apoptosis was decreased. TNF-α has been demonstrated to be a potent inducer of T-cell apoptosis (37). Following this line, administration of anti-TNF-α antibody decreases local T-cell apoptosis in EAE of Lewis rats undergoing high-dose antigen-therapy with MBP, where the release of high amounts of TNF-α can be observed *in situ* (38).

T-cell apoptosis in the CNS may also be mediated via the Fas/Fas Ligand (CD95/CD95L)-pathway (review in (14,39)). Thus, one proposed mechanism is the ligation of T-cell CD95 by CD95L expressed by host cells

resident in or secondarily recruited into the CNS (40). In addition, intrathecal infusion of FasL suppresses Lewis rat EAE and augments apoptosis of inflammatory cells *in situ* (41).

Since T-cell apoptosis appears to be tissue specific, resident glial cells might sensitize T-cells towards cell death directly or through soluble factors. Antigen presentation to MBP-specific T-cells by rat microglia results in T-cell apoptosis *in vitro*, which can be prevented by exogenous IL-2 (42).

Also astrocytes, which act as non-professional antigen presenting cells (APC), render T-cells susceptible to apoptosis induced by glucocorticosteroids *in vitro* (43). Glucocorticosteroids markedly increased T-cell apoptosis when added to T-cell astrocyte co-cultures during late T-cell activation stages whereas there was no effect when thymus cells were used as antigen presenters. These results could argue for a scenario, where an infiltrating T-cell is primed for an apoptotic stimulus by a resident non-professional APC, and subsequently undergoes apoptosis under hormonal influences of the microenvironment or systemic changes. However, T-cell apoptosis during recovery from EAE also occurs in the CNS of adrenalectomized animals, arguing for additional mechanisms (44).

Among others, further proposed mechanisms of local CNS T-cell apoptosis comprise the action of reactive oxygen intermediates and nitric oxide, or cell death due to deprivation of growth factors such as IL-2 (45,46). Yet, the precise role of the different putative mechanisms as well as other to date unknown CNS-specific factors in the induction of T-cell apoptosis remain to be elucidated.

1.4   Phagocytosis of apoptotic cells in the central nervous system

The high degree of apoptosis of inflammatory cells in the autoimmune inflamed human and rodent CNS raises the need for an efficient and tightly controlled removal mechanism for these unwanted cells. A key event in the resolution of an inflammatory infiltrate is the nonphlogistic and thus safe phagocytic clearance of dying, yet intact leukocytes undergoing apoptosis (review in (47,48). Apoptosis labels unwanted cells with signals that direct the recognition, uptake and subsequent degradation by tissue-specific phagocytes, thus preventing the spilling of potentially harmful contents and inhibiting secondary immune responses directed towards the dying cells. In addition to the mere clearing of cell remnants, the ingestion of apoptotic cells

also actively elicits phagocyte responses that modulate immune reactions and inflammation.

In Lewis rat EAE, phagocytosis of apoptotic lymphocytes by macrophages/microglia, oligodendrocytes and astrocytes *in situ* has first been described by electron microscopy (49). Using an *in vitro* phagocytosis model, we have further investigated the phagocytosis of apoptotic T-cells by different glial cell elements and its functional consequences (50). Lewis rat microglia efficiently phagocytose specifically apoptotic, encephalitogenic MBP-specific T-cells. This process is differentially regulated by Th1-/Th2-type cytokines (51). The phagocytosis of apoptotic T-cells by Lewis rat microglia is more efficient than by other glial cell elements such as astrocytes. Moreover, phagocytosis of apoptotic T-cells leads to a profound downregulation of microglial immune functions with a suppression of pro-inflammatory cytokines and microglial T-cell activation, thus silencing the microglial phagocyte in the inflammatory context (52). Figure 2 gives a hypothetical model of the phagocytosis of apoptotic T-cells in the CNS.
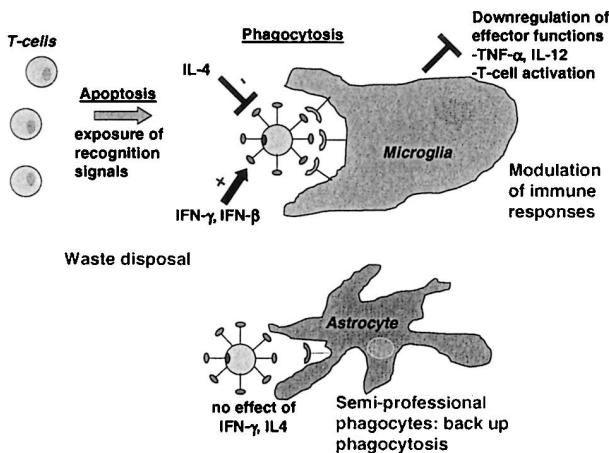

Figure 2. T-cells undergoing apoptosis exhibit specific "eat me" recognition signals for phagocytes. Phagocytosis by microglia is differentially regulated by cytokines ("waste disposal"). The uptake of apoptotic cells leads to a down-regulation of different microglial immune-functions ("modulation of immune responses"). Astrocytes have a lower capacity to phagocytose apoptotic cells and may provide a "backup" mechanism. Modified from (50).

In vitro, rat microglia/brain macrophages have also been demonstrated to phagocytose apoptotic neurons via lectin-, integrin-, and phosphatidylserine-dependent mechanisms (53).

1.5 Induction of T-cell apoptosis by immunotherapy

Current therapeutic approaches in presumably immune-mediated, demyelinating diseases of the CNS such as MS aim at the rapid termination of the inflammatory process, thereby hastening clinical recovery and potentially also preventing subsequent demyelinative and axonal tissue damage (54). Glucocorticosteroids (GS) are potent anti-inflammatory drugs, whose therapeutic efficacy in neuroimmunological diseases has especially been established in immune neuropathies, MS, lupus erythematosus and cerebral vasculitis. Intravenously administered high-dose GS serve as the current mainstay in the therapy of acute MS-relapses(55). GS can mediate their pleiotropic anti-inflammatory effects via different mechanisms, e.g. modulation of cell activation, cytokine expression, secretion of inflammatory mediators, leucocyte migration and the reduction of local edema (56). These effects are mediated via the cytosolic GS-receptor (GSR) and can be blocked by the GSR antagonist RU 468 at low GS concentrations. At higher concentrations, GS appear to induce cell death directly, possibly via non-genomic, physicochemically mediated effects on the plasma membrane (56).

In Lewis rat EAE, iv. methylprednisolone (MP) therapy administered at the clinical disease maximum increased T-cell apoptosis in the spinal cord *in situ* and decreased T-cell infiltration (57). This effect was clearly dose-dependent with a dosage of at least 10 mg/kg body weight MP required to achieve an increase of T-cell apoptosis and a marginal decrease in T-cell infiltration. Higher dosages of 50 mg/kg body weight MP were superior in both respects, and were also effective in mild EAE, in contrast to the lower dosage of MP. The strong apoptosis-promoting effect of GS has also been demonstrated on peripheral blood leukocytes (PBL) of MS-patients (58). After intravenous high-dose corticosteroid treatment, apoptosis of PBLs was markedly augmented in different MS-subgroups, predominantly affecting CD4-positive T cells.

Also in the peripheral nervous system, high-dose GS augment T-cell apoptosis (59). In Lewis rat EAN, a 4-5-fold increase of T-cell apoptosis could be observed in the sciatic nerve after therapeutic administration of GS (10 mg/kg body weight prednisolone). Similar results could be observed in Lewis rat experimental autoimmune myositis (EAM), a model for human idiopathic myosites of presumed autoimmune-inflammatory etiology (27).

Here, a clear increase of apoptotic endomysial T-cells could be demonstrated, with up to 50% of these apoptotic T-cells being CD8-positive. This indicated that glucocorticosteroids also induce CD8-T-cell apoptosis, even in organs where normally T-cell apoptosis does not occur.

The type 1 interferon (IFN) IFN-β, is one of the current mainstays in the immunomodulatory therapy of MS (60). IFN-β appears to affect particularly inflammatory aspects of MS, with a reduction of the relapse rate and positive effects on inflammatory changes observed in MRI scans. In the experimental models, IFN-β has been shown to inhibit progression of relapsing-remitting EAE (61), while early discontinuation of IFN-β led to exacerbation of active EAE (62). While the exact mechanism of action of IFN-β is still unknown, numerous putative mechanisms have been proposed (review in (63)). Whether the therapeutic benefit of IFN-β is also based on the induction of apoptosis in inflammatory cells is still controversially discussed (64,65). In Lewis rat AT-EAE, no major increase of T-cell apoptosis *in situ* could be observed after treatment with IFN-β (66). However, IFN-β clearly increases the phagocytosis of apoptotic, encephalitogenic, MBP-specific T-cells by microglia *in vitro* (Chan, A., Seguin, R., submitted). Thus, theoretically IFN-β could help to engulf and eliminate T-cells driven into apoptosis by other therapeutically used agents, e.g. corticosteroids.

Antigen-specific therapy with CNS or PNS-autoantigens has been shown to be effective in EAE and EAN (67,68). The underlying mechanism appears to involve T-cell receptor reengagement at an appropriate stage of the cell cycle which leads to T-cell apoptosis (69). In Lewis rat cell transfer (AT)-EAE, high dose antigen (guinea pig MBP) therapy not only led to apoptotic cell death of invading T-cells *in situ* but also of resident oligodendrocytes (38). This was accompanied by a profound modulation of the cytokine network with a rapid induction of the Th1-type cytokines TNF-α and IFN-γ as well as inducible nitic oxide synthase. Neutralization of TNF-α *in vivo* led to a decrease in T-cell and oligodendrocyte apoptosis but did not change the beneficial clinical effect of high-dose antigen therapy. This argues for a central role of TNF-α as a mediator of local apoptosis which may have different functional effects. Similar results could also be observed in antigen-specific therapy of Lewis rat AT-EAN using P2-protein (70,71).

Conclusions

Apoptosis of inflammatory effector cells constitutes an effective protective mechanism in the autoimmune-inflamed CNS. Thus, the selective

induction of apoptosis in pathogenic cells is an attractive therapeutic target in neuroimmunological diseases. However, apoptosis of resident CNS cells represents also a major mechanism in the pathogenesis of different neurodegenerative disorders, where selective blockade of apoptotic pathways may be benificial. Therefore, the identification of the molecular, tissue- and cell-specific factors that lead to local apoptosis remain to be elucidated in order to take advantage of this phylogenetically old and important defense mechanism.

Acknowledgements

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