Cellular FLICE-inhibitory protein: an attractive therapeutic target?

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Expert Opinion on Therapeutic targets

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Abbreviations: Abeta, beta-amyloid; AD, Alzheimer's disease; AICD, activation-induced cell death; AIDS, acquired immunodeficiency syndrome; ALPS, autoimmune lymphoproliferative syndrome; CAD, caspase-activated deoxyribonuclease; CARD, caspase-recruitment domain; CNS, central nervous system; CrmA, cytokine- response modifier A; DD, death domain; DED, death effector domain; DISC, death-inducing signalling complex; DR, death receptor; EAE, experimental autoimmune encephalomyelitis; EDAR: ectodermal dysplasia receptor; FADD, Fas-associated death domain; FasL, Fas ligand; FLICE, FADD-like interleukin-1β converting enzyme; c-FLIP, FLICE/Caspase-8-inhibitory protein; gld, generalized lymphoproliferative disorder/FasL mutation; HIV, human immunodeficiency virus; IAP, Inhibitor of apoptosis; ICAD, caspase-activated deoxyribonuclease inhibitor; I-kB, inhibitor of nuclear factor kB; IL, interleukin; JNK, c-Jun N-terminal kinase; lpr, lymphoproliferative disease/Fas mutation; MHC, major histocompatibility complex; MS, multiple sclerosis; NF-kB, nuclear factor kB; NGF, nerve growth factor; NK, natural killer; OPG, osteoprotegerin; OxLDL, oxidized LDL; PKC, protein kinase C; RA, rheumatoid arthritis; RAIDD, RIP-associated ICH/CED-3-homologous protein with a death domain; RANK, receptor activator of NF-kB; RIP, receptor interacting protein; SAP, stress-activated protein kinase; SLE, systemic lupus erythematosus; TCR, T cell receptor; TNF, tumour necrosis factor; TNFR-1, tumor necrosis factor receptor 1; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL-R1 or -R2, TRAIL receptor.

Abstract:

Cellular FLIP, also known as FLICE-inhibitory protein, has been identified as an inhibitor of apoptosis triggered by engagement of Death Receptor (DR) such as Fas or TRAIL. c-FLIP is recruited to DR signalling complexes, where it prevents caspase activation. Animal models indicated that c-FLIP plays an important role in T-cell proliferation and heart development. Abnormal c-FLIP expression has been identified in various diseases such as multiple sclerosis, Alzheimer's disease, diabetes mellitus, rheumatoid arthritis and various cancers. The present review focuses on recent insights in c-FLIP dysregulation associated with human diseases, and addresses the possibilities of using c-FLIP as a therapeutic target.

1-Introduction

Tissue homoeostasis is essential for the maintenance of multicellular organisms and requires a fine tuned balance between cell proliferation and death. Elucidation of the molecular mechanisms involved in the latter process progressed exponentially over the last two decades and provided significant insights into the comprehension of pathogenesis of human diseases. Amongst the cell death mechanism characterized in mammals, namely apoptosis, necrosis and autophagy, programmed cell death or apoptosis is currently the best characterized. This a discrete and active process that was originally identified on morphological characteristics, including cell shrinkage, membrane blebbing, chromatine condensation and nuclear fragmentation [**1]**. Apoptosis has been involved in embryonic development, adult tissue homeostasis and cell response to noxious stimuli. Its triggering can proceed through an extrinsic or intrinsic pathway [**2]**.

The intrinsic pathway can be initiated by the release of proapototic molecules such as cytochrome c, from damaged mitochondria, which activate the intracellular receptor Apaf-1 (Apoptotic Protease- Activating Factor 1) forming with caspase-9 a proteotically active structure called the apoptosome [**3]**. Extrinsic activation of apoptosis, on the other hand, can occur upon stimulation of a cell membrane associated death receptor (DR). To date eight DRs have been described: TNF-R1 (CD120a) [**4, 5]**, Fas

(APO-1 or CD95) [**6, 7]**, TRAMP (DR3, Apo3, LARD or Wsl-1)[**8-12]**, TRAIL-R1 (DR4) [**13-16]**, TRAIL-R2 (DR5) [**14, 16]**, DR6 [**17]**, NGFR (p75NTR) [**18]** and EDA-R (ectodermal dysplasia receptor) [**19]**. The best characterized are TRAIL-R1/R2 and Fas, which have been shown to play a crucial role in the immune system, in both immune-cell mediated cytotoxicity and downregulation of immune responses. Upon binding with their cognate ligands, these receptors form aggregates that enable the recruitment of the adapter molecule FADD (MORT1) [**20]** and the initiator protease caspase-8 (FLICE, MACH, MCH5) [**21-23]**, hence forming the so-called Death-Inducing Signalling Complex (DISC) in which caspase-8 is processed and activated [**24]**. Both intrinsic and extrinsic systems transmit signals through protein–protein interactions that are mediated by homologous and evolutionarily related protein-interaction motifs, including the death domain (DD), the death effector domain (DED) or the caspase-recruitment domain (CARD). Execution of the apoptotic process in all instances is dependent on a family of intracellular cysteine proteases, called caspases [**7, 25-27]**. These interactions induce caspase activation by cleavage in a close proximity [**28]** that enables the activation of the caspase cascade culminating in the cleavage of various substrates, such as lamins, fodrin, gelsolin, actin or the Inhibitor of Caspase-Activated DNase (ICAD), leading to cell dismantle (Figure 1).

Dysregulation of apoptosis, however, can lead to various pathologies. Defective apoptosis generates cell accumulation that characterizes autoimmune disease and cancer, whereas excessive apoptosis plays a role in neurodegenerative diseases, heart failure and AIDS [**29]**. It appears thus essential to understand more thoroughly how this process is regulated at the cellular level, but also with respect to human diseases, in order to design new therapeutic strategies that would circumvent apoptotic dysregulation and prove beneficial for human health. To avoid uncontrolled cell or tissue damage, apoptosis is tightly controlled by a collection of cellular inhibitors, such as the Proteins of the Inhibitor of Apoptosis (IAP) family which regulate apoptosis at the post-mitochondrial level by binding to and inhibiting caspase-3, -6, -7 and -9, the Bcl-2 family members which regulate apoptosis at the mitochondrial level [**2]**, and c-FLIP, that negatively interferes with DR-induced cell death, upstream of the mitochondrial events [**25]**. This review focuses on c-FLIP whose dysregulation has been identified in various diseases

including multiple sclerosis, Alzheimer's disease, diabetes mellitus, rheumatoid arthritis, cardiovascular disorder and various cancers.

2-FLICE-Inhibitory Protein or c-FLIP.

Viral FLICE-inhibitory proteins (v-FLIPs), were initially discovered by bioinformatic search for novel virus-encoded apoptosis-regulatory molecules containing a DED. Mammalian homologues of v-FLIPs, also designed c-FLIP, CASH, Casper, CLARP, FLAME, I-FLICE, MRIT and usurpin [**30-37]**, share structural and sequence homologies with caspase-8 and -10, upstream caspases involved in the initiation of DR-induced apoptosis, such as Fas/Apo1, TRAIL or TNFR-1. c-FLIP contains two serial amino-terminal DEDs followed by a carboxy-terminal extension comprising a caspase-homologous domain similar to caspase-8 and caspase-10. However, owing to the substitution of several amino acids conserved in caspases, such as the cysteine residue within the QACXG-motif and the histidine residue within the HG-motif, c-FLIP is devoid of proteolytic activity [**25, 38]**. Consistently, a protective role for c-FLIP in DR-induced apoptosis was found in fibroblast cell lines derived from c-FLIP-knockout mice [**39]**. The DEDs of c-FLIP bind to the DED of the adaptor protein FADD, acting as dominant-negative inhibitors of the processing and release of active caspase-8 or –10 to the cytosol.

Although cellular FLIPs exists as multiple splice variants at the mRNA level, it appears that only two variants are expressed as proteins *in vivo*: short FLIP (c-FLIP (S)) of 26 kDa and long FLIP (c-FLIP (L)) of 55 kDa. Compared to c-FLIP (L), described above, c-FLIP (S) has a shorter carboxy-terminal extension of approximately 20 amino acids [**25, 40]**. Both c-FLIP (S) and c-FLIP (L) inhibit apoptosis induced by Fas, TRAIL-R1, TRAIL-R2, TRAMP and TNF-R1 [**32-34, 36, 41, 42]**. At comparable expression levels, however, c-FLIP (L) is a more potent inhibitor than c-FLIP (S) [**36]**. In the canonical Fas and TRAIL-induced caspase activation pathway, both caspase-8 and c-FLIP (L) are partially processed at the DISC level [**43, 44]**. c-FLIP (L) contains a conserved aspartic-acid cleavage site (Asp-341) between the p20- and p10-like domains [**32]**, which can be cleaved *in vitro*. In c-FLIP (L) overexpressing cells, procaspase-8 is

found in the DISC partially processed as a p44/41 fragment [**43, 45]**, corresponding to the first cleavage of procaspase-8 generally observed upon Fas or TRAIL stimulations. In contrast, c-FLIP (S) forms a caspase-8/c-FLIP (S) heterocomplex, in which no processing of caspase-8 occurs [**43, 45]**. Thus, although able to inhibit DR-induced cell death, both isoforms proceed differently, suggesting that a) these isoforms are not redundant and b) their non apoptosis-inhibitory functions, yet to be determined, could differ importantly.

When overexpressed, c-FLIP (L), but not c-FLIP (S), has been reported to be pro-apoptotic [**33]**. This cytocidal effect was suggested to result from the non-physiological aggregation of its pro-apoptotic interaction partners FADD and caspase-8 under conditions of overexpression. We have recently provided molecular evidences that the heterocomplex c-FLIP (L)/caspase-8, contrarily to c-FLIP (S)/caspase-8, could exhibit a proteolytic activity, at the DISC level [**45]**. While physiologically restricted to the plasma membrane, this proteolytic activity could account for the pro-apoptotic activity of c-FLIP (L) under non-physiological conditions.

In Fas-stimulated cells, c-FLIP (L) was suggested to be involved in the regulation of gene expression by the extracellular signal-regulated kinase (ERK)-mediated gene expression and by NF-kB, suggesting that c-FLIP (L) could play a role in proliferation and/or differentiation [**46, 47]**. Interestingly, c-FLIP (L) has been shown to interact with additional signalling molecules, such as RIP and Raf-1 [**46, 48]**, TRAF-1 and -2 [**30, 46, 48]** or caspase-10 [**32-34]**. Therefore c-FLIP could act as an adapter-like molecule for the recruitment of proteins involved in cell proliferation signals in the Fas pathway [**46, 47]**. The first indications of a non pro-apoptotic function for the Fas pathway came from the observation that FADD-deficient mice or mice expressing a dominant-negative version of FADD exhibited impaired T cell proliferation [**49-51]**. Thymocytes derived from transgenic mice expressing a dominantly interfering mutant of FADD lacking the caspase-dimerizing death effector domain, and those from mice overexpressing the poxvirus serpin, CrmA, an inhibitor of caspases downstream of FADD, are completely protected from Fas-dependent cytotoxicity. Neither transgene afforded protection from apoptosis induced during thymocyte selection and neither led to the lymphoproliferative disorders associated with deficiencies in Fas. Nevertheless, in FADD dominant negative mice, early thymocyte development was retarded and peripheral lymphocyte pools were

devoid of normal populations of T cells, suggesting that FADD is probably involved in T cell development and activation [**49-51]**. This is concordant with earlier observations indicating that Fas-engagement could induce co-stimulation of TCR-induced lymphocyte proliferation in resting human and murine memory T cells [**52-54]**. Accordingly, Fas engagement was shown to induce neurite growth through ERK [**55]**, and c-FLIP (L) was recently shown to enhance TCR-triggered proliferation in transgenic mice [**47]**.

Gene-targeted inactivation of either FADD [**49]**, caspase-8 [**56]** or c-FLIP [**39]**, in mice, provided important information on the contribution of DRs in human embryonic development and human diseases. Although carrying opposite regulatory functions, caspase-8 and c-FLIP deficient mice, exhibit a similar embryonic phenotype as FADD-/- mice. These mice die from day 10.5 to 12.5 of embryogenesis, with apparent heart defects. However, neither FADD-deficient nor c-FLIP-deficient embryos demonstrate changes in cell death rate, suggesting that the developmental defects in these mutant mice may be independent of apoptosis. However, unlike FADD-/- and caspase-8-/- cells, FLIP-/- embryonic fibroblasts are highly sensitive to apoptotic stimuli that involve death receptors. This result is consistent with the predicted role of c-FLIP to counteract FADD and caspase-8 in the regulation of DR-induced apoptosis.

c-FLIP (L) is expressed in many tissues, but most abundantly in the heart, skeletal muscle, lymphoid tissues and kidney. In lymphatic tissues, an additional 25 kDa c-FLIP species is detectable, which corresponds to the predicted size of c-FLIP (S) [**35]**. Although expressed constitutively in many cell types, c-FLIP is a very short-lived protein [**42, 57, 58]**, whose expression can be regulated by a variety of stimuli. Protein synthesis inhibitors [**42, 57]**, oxidized low-density lipoproteins [**59]**, chemotherapeutic agents [**60-62]**, p53 [**63]**, synthetic PPAR ligands [**64]**, sodium butyrate [**65]**, interferon β [**66]** and E1A [**67]** were shown to downregulate c-FLIP expression, through mechanisms that could involve the ubiquitin-proteasome pathway. Degradation of c-FLIP is dramatically elevated in TRAF2-deficient mice embryonic fibroblasts [**68]**. More recently, hemin-mediated erythroid differenciation was shown to downregulate both splicing variants of c-FLIP in K562 cells [**69]**.

In contrast, the PI3K/Akt [**70-72]**, and MAPK (Mitogen-Activated Protein Kinase) [**73, 74]** pathways, stem cell factor [**75]** and the Calcium/Calmodulin-dependent protein kinase II [**76]**, increased c-FLIP expression at the transcriptional level. Amongst the stimuli implicated so far in the regulation of c-FLIP expression, the discovery that c-FLIP expression was up-regulated by NF-kB signals, provided for the first time a molecular explanation of the well-known TNF-induced NF-kB-dependent survival pathway [**42, 57, 77]**.

Moreover, c-FLIP appears to be post-translationally regulated. Phosphorylation of c-FLIP, in hepatocytes treated with bile acids, was shown to affect c-FLIP binding to FADD, hence sensitizing cells to TRAIL-induced cell death without altering c-FLIP steady state levels [**78]**. In contrast, β_1 integrin-mediated adhesion of human U937 histiocytic lymphoma cells to fibronectin, increased c-FLIP (L) cytosolic solubility and availability for FADD binding by redistributing c-FLIP (L) from a preexisting membrane-associated fraction [**79]**.

3-FLIP and human diseases

3.1-Lymphoproliferative syndrome with autoimmunity

Autoimmune lymphoproliferative or Canale and Smith syndrome (ALPS) is a human disorder that is characterized by defective lymphocyte apoptosis, lymphadenopathy, splenomegaly and autoimmunity [**80]**. Its pathogenesis has been attributed to dysregulated lymphocyte homeostasis, a process involving negative selection and activation-induced cell death (AICD) [**80]**. Genetically, ALPS was first described in MRL *lpr* and in MRL *gld* mice, two animal models of human lupus, which exhibit mutations in Fas receptor and Fas ligand respectively [**81**]. In humans, ALPS is defined by functional analysis of lymphocyte sensitivity to Fas-induced apoptosis *in vitro* in 3 categories ranging from a complete (APLS 0) or partial Fas deficiency (ALPS Ia and II) to an absence of defect in the Fas pathway (ALPS Ib and III)[**81**]. So far, heterozygous mutations in Fas, Fas ligand or caspase-10 underlie most cases of human inherited genetic deficiency with deregulated lymphocyte proliferation. Recently, inherited caspase-8 deficiency has been described in human patients [**82**]. Individuals with

homologous mutations in caspase-8 manifest defective lymphocyte apoptosis and homeostasis but, unlike individuals affected with ALPS, also have defects in T lymphocytes, B lymphocytes and natural killer cells activation, which leads to immunodeficiency. Surprisingly, caspase-8 deficiency in humans is compatible with normal development, in contrast to mice for which caspase-8 gene inactivation is lethal [**56, 82**]. The observation that c-FLIP (L) expression is modulated in activated T cells in an IL-2-dependent manner suggests that c-FLIP (L) could play a role in the control of T-cell activation [**36, 83-85**]. This idea is consistent with a recent report providing evidence that c-FLIP (L), rather than preventing AICD, could enhance TCR-triggered proliferation [**47]**. Since c-FLIP can in the one hand inhibit cell death and in the other hand contribute to TCR-triggered lymphocyte proliferation, further experiments will be required before considering its therapeutic modulation in the context of lymphoproliferative diseases.

3.2-**Alzheimer's Disease**

Alzheimer's disease (AD) is a neurodegenerative disease characterized by elevated levels of β-Amyloid (Aβ) in the brains [**86**]. Senile plaques deposition of Aβ and dysregulated apoptosis are though to be involved in the pathogenesis of Alzheimer's disease [**29, 87**]. Aβ-induced neuronal death has been attributed to be triggered by two members of the TNF superfamily, namely t□ □ □ p75(NGFR) nerve growth factor receptor and Fas through an indirect mechanism. It has been shown that Aβ □ □ □ □ □ □ □ □ □ □ □ □ □ □ p75(NGFR) and activates neuronal cell death [**88**] via the JNK pathway **[89]**. Albeit described as a caspase-8-independent, but mitochondria-dependent mechanism [**88**], the Aβ-induced cortical neuronal cell death was also shown by others to be triggered by Fas indirectly through a JNK-dependent-Fas-ligand upregulation [**90**] or activation of the caspase-8 pathway [**91**]. Although not accounting for the total cell death induced by Aβ, Fas ligand has been shown to play a substantial role in this process [**90**], suggesting a critical role for the JNK pathway in the regulation of Aβ-induced apoptosis in AD patients and the potential involvement of inhibitory molecules located downstream of the Fas signalling pathway, such as c-FLIP. The JNK-Fas ligand pathway can also be triggered by survival factor withdrawal in neurons [**92**],

or by stress stimuli as UV-lights or gamma irradiation [**93, 94**]. Interestingly, a recent report suggested that a viral homologue of c-FLIP, the HHV8-v-FLIP could rescue growth factor withdrawal-induced apoptosis in the TF-1 human myeloid leukemia cell line [**95**]. In addition, studies evaluating the expression levels of caspases or apoptosis-related proteins, in human postmortem brain cerebellum or frontal cortex tissues of patients with AD supported the notion that dysregulation of apoptosis-induced by the Fas pathway could contribute to the pathology of AD [**96, 97**]. In these studies, c-FLIP expression was indeed shown to be decreased in AD patients as compared to controls. Therefore, therapeutic strategies aiming at increasing c-FLIP expression in neurons, should both prevent progression of the disease and stabilize cognitive functions of patients showing early signs of AD.

3.3-Multiple sclerosis

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) that is characterized pathologically by perivascular mononuclear cell infiltrates and myelin damage [**98**]. Experimental autoimmune encephalomyelitis (EAE) is an animal model of this disease that is characterized by increased permeability of the blood-brain barrier, perivascular inflammatory infiltrates comprised of T cells, B cells, macrophages, and granulocytes, and demyelination leading to an ascending paralysis of the extremities. In this model, the principal targets of the autoimmune attack are oligodendrocytes and myelin.

Although the mechanisms causing myelin disruption and damage to axons in the CNS are still unclear, this autoreactive immune-mediated disorder is thought to be initiated by activated T lymphocytes recognizing myelin components of the CNS [**99**], and autoreactive B lymphocytes directed against oligodendroglial and myelin antigens [**100, 101**]. In animal EAE models, Fas-mediated apoptosis of activated T cells and other inflammatory cells in the CNS modulates the inflammatory response [**102**]. In the early phases of the disease however the Fas pathway may be responsible for myelin destruction since administration of neutralizing antibody against Fas ligand was shown to suppress

the acute progression phase of EAE [**103**]. In patients with MS, the Fas pathway has also been suggested to be involved [**104-107**].

Overexpression of c-FLIP (L) by retroviral gene transfer in hemopoietic stem cells increases the severity of myelin oligodendrocyte glycoprotein-induced EAE [**108**]. Consistently, c-FLIP (L) and c-FLIP (S) are overexpressed in cortical-spinal- fluid lymphocytes and activated-peripheral T cells from patients with clinically active MS, which may contribute to maintaining autoreactive T cells [**109, 110**]. Interestingly, interferon β, known to reduce clinical exacerbations in MS, was recently shown to downregulate c-FLIP [**66]**. Other ligands of the TNF superfamily, such as TRAIL, were suggested to be able to contribute to the regulation of inflammatory cells in both EAE and MS, however this issue yielded controversial results [**111 , 112 , 113 , 114 , 115 , 116**].

Nevertheless, as several DR-induced apoptotic signals are regulated by c-FLIPs, these findings demonstrate that effective apoptotic elimination of inflammatory cells to achieve disease remission may be crucial. Therefore, downregulation of c-FLIPs could prove beneficial for the prevention of MS pathogenesis.

3.4- Rheumatoid Arthritis (RA)

Rheumatoid arthritis (RA) is a chronic inflammation of the synovial joints characterized by infiltration of activated T cells, macrophages and plasma cells [**117, 118**]. Its etiology although poorly understood, is thought to involve both impaired clearance of activated infiltrating cells (T cells, macrophages) and increased cartilage and bone sensitivity to cell death. Human chondrocytes [**119**] and osteoblasts [**120**] have been shown to express functionnal Fas receptor, and as T cells infiltrating the RA-affected joints express FasL [**121, 122**], it is thougth that the Fas pathway plays an important role in cartilage and bone destruction. Indeed, it has recently been shown that arthritic lesions could be induced, *in vitro*, by persistent engrafted syngeneic lymphocytes overexpressing FasL [**123**]. In contrast to Fas, TRAIL was suggested to be a potent inhibitor of autoimmune arthritis and blocking endogenous TRAIL with soluble TRAIL-R1 impaired this inhibition and enhanced proliferation of autoreactive lymphocytes and synovial cells.

Furthermore, by use of deficient mice, TRAIL has recently been shown to play an important role in autoimmune diseases and in particular in the clearance of autoreactive synovium-infiltrating cells in RA [**124**]. The inhibitory effect of TRAIL on arthritis proceeds through inhibition of cell cycle progression and/or cytokine production inhibition [**116, 125**]. Interestingly, synoviocytes and particularly synovial macrophages which are thought to be the main trigger of the inflammatory response in RA, via the production of TNF and IL-1 [**126**], are naturally sensitive to anti-Fas monoclonal antibody-induced apoptosis *in vitro*, but particularly resistant to this process when isolated from arthritic mice [**127**] and RA patients [**128, 129**]. Furthermore, RA synovial macrophages express high levels of c-FLIP [**130**], and c-FLIP expression was shown to be increased in synovial biopsy specimens from patients with early RA, especially in RA synovial macrophages which express high levels of c-FLIP.

Altogether, these data point to c-FLIP as an interesting therapeutic target for the treatment of rheumatoid arthritis for several reasons. 1) c-FLIP is involved in both Fas and TRAIL-induced apoptosis inhibition. 2) c-FLIP is highly expressed in RA macrophages which account for the sustained joint inflammation through TNF and IL-1. 3) Inhibiting TNF and IL-1 macrophage production, ameliorates RA symptoms and joint destruction [**126**]. 4) c-FLIP expression is increased by TNF via NF-kB [**42**]. Therefore, downregulating c-FLIP locally in activated infiltrating synovial T cells and macrophages could sensitize these cells to Fas- or TRAIL-induced clearance and prove useful for the treatment of RA.

3.5- Diabetes mellitus

Diabetes mellitus is the most common metabolic disease worldwide. Type 1 diabetes results from autoimmune destruction of Langerhans islets pancreatic β cells causing

insulin deficiency. Type 2 or noninsulin-dependent diabetes mellitus (NIDDM) is a polygenic disease that accounts for more than 90% of cases of diabetes, in which long-term adaptation of pancreatic islets β cells mass expansion in response to glucose is impaired. Autoimmune diabetes results from β cell destruction by islet-reactive T cells, a process that involves β cell apoptosis. Apoptosis via Fas/Fas ligand (FasL) interactions has been proposed to be a major T-cell-mediated effector mechanism in autoimmune diabetes [**131**]. Nonobese diabetic (NOD) mice develop a type 1 diabetes mellitus, in which the Fas pathway has been shown to be involved in the destruction of insulin-producing cells and the development of diabetes, in a T-cell-dependent [**132**] or -independent manner [**133**]. Overexpressing c-FLIP (L) in β cells prevents from TNF-induced apoptosis [**134**], and predisposition of NOD mice to develop autoimmune disease is usually attributed to defects in peripheral tolerance mechanisms, which has recently been attributed to the up- regulation of c-FLIP (L) in activated T cells [**135**]. This suggests that the Fas pathway or death receptors of this family could also contribute to the progression of the disease or associated side effects [**124**]. Diabetic macular edema is the most prevalent cause of vision loss in diabetes, was recently shown to result from leukocyte-mediated Fas-FasL-dependent retinal endothelial cell apoptosis [**136**]. Taken together, these data indicate that increasing c-FLIP expression in specific cells by therapeutic targeting may prove beneficial for the treatment of diabetes, or associated retinopathy.

3.6- **Cardiovascular diseases**

The cardiovascular system is continuously subjected to stressfull haemodynamic forces due to blood pressure and flow. Although essential for the maintenance of organic structure and function, this mechanical stress can eventually induce apoptosis and lead to cardiovascular disorders such as heart failure, hypertension or atherosclerosis [**137**]. Some members of the TNF family could be involved in these diseases.

TNF was shown to be elevated in the cardiomyocytes and in the circulation of heart failure patients [**138, 139**], and chronic overexpression of TNF results in the development of a dilated cardiomyopathy also leading to heart failure [**140**]. DNA

microarray analysis of a collection of human left ventricular myocardium samples obtained from explanted cardiomyopathic hearts from patients with end-stage heart failure undergoing heart transplantation, showed a clear reduction in expression of anti-apoptotic genes such as TNFR-1,c-FLIP and A20, together with an increase in expression of TRAIL [**141**].c-FLIP is highly expressed in the adult human and murine healthy heart, but its expression is severely reduced in cardiomyocytes originating from cardiomyopathic hearts that have undergone apoptosis [**35, 142**].

Normal vascular endothelial cells express both Fas and FasL but are resistant to Fas-mediated apoptosis, however overexpression of FasL was shown to promotes atherosclerosis in a rabbit experimental model [**143**]. Interestignly, vascular endothelial cells express c-FLIP [**59]**, but downregulation of its expression by oxidized lipids sensitize endothelial cells to DR-induced apoptosis, suggesting that c-FLIP could play an important role in controlling vascular tissue destruction [**59, 144**] and that dysregulated expression of c-FLIP could be involved in the etiology and pathogenesis of atherosclerosis [**39]**.

Although different, the pathological mechanisms underlying heart failure, hypertension or atherosclerosis are believed to be related to sustained mechanical overload or stress, leading to proliferation, differentiation or to cell death [**137**]. With respect to apoptosis, c-FLIP has been suggested to could play a major role in heart development as well as in pathogenesis of heart failure and atherosclerosis [**39]**. Interestingly, FADD-, caspase-8- or FLIP-, but not Fas- nor tumor-necrosis-factor receptor-1 (TNFR-1)-deficient mice, show symptoms of impaired heart development [**39, 49, 56, 145, 146**]. Furthermore, the observation that the Fas-deficient mice phenotype differ from that of FLIP-deficient mice, suggests that other receptors of the TNF superfamily, as TRAIL receptors, may play a major role in cardiovascular diseases. Therefore, therapeutic up-regulation of c-FLIP-expression levels might turn out to be useful for the treatment of cardiovascular diseases such as heart failure or atherosclerosis.

3.7- Cancer

Cancer is a malignant disease generally arising from impaired cellular homeostasis and resulting in the excessive accumulation of unwanted cells in the body, which eventually lead to a loss of organic function and death. Dysregulation of cellular homeostasis can be inherited or acquired. For example, neuroblastoma tumour cells show complex combinations of acquired genetic aberrations [**147**] while colon carcinomas result from multiple genetic alterations, some of which may be inherited, while others reflect somatic mutations. The latter may themselves be the indirect result of environmental factors such as diet [**148, 149**]. The etiology and the progression of cancers is the addition of cumulative genetic changes, combining both the activation of oncogenes with the inactivation of tumour suppressor genes.

In recent years it became evident that TRAIL receptors are key regulators of immune surveillance against tumours [**150-154**]. Trail appears to be the most promising new anti-tumour therapeutic tool since this cytokine is capable of inducing apoptosis in tumour cells, but is devoid of severe toxicity towards normal cells, both *in vitro* and *in vivo* [**155**]. However, not all tumour cells are sensitive to TRAIL, and c-FLIP, which inhibits TRAIL-induced cell death, is often over-expressed in tumours and was suggested to be a tumour-progression factor [**156-158**]. Accordingly, expression of c-FLIP has been shown to correlate with resistance to Fas-induced apoptosis *in vitro* in certain tumour cell lines derived from B-cell lymphomas [**159-161**], and with tumour escape from T-cell immunity and enhanced tumour progression *in vivo* [**157, 162**]. Moreover, abnormal overexpression of c-FLIP (L) is a frequent event in colon adenocarcinomas [**163**]. In Fas-resistant melanoma cell lines and in melanoma tissue, c-FLIP was shown to be highly expressed [**164, 165**], compared to surrounding normal melanocytes. However, other studies could not correlate c-FLIP expression levels with resistance of melanoma cell lines towards Fas or TRAIL-induced apoptosis [**164, 166-168**]. The reasons for the discrepancies in the above-mentioned studies on melanomas are unclear, however other anti-apoptotic factors could control the DR resistance of tumour cells, as the alternative mitochondrial pathway for instance [**2, 169**]. Recently, it has been demonstrated that c-FLIP (L) could protect MHC class I-deficient tumours from rejection mediated by NK cells, in the absence of perforine [**170, 171**]. These data, which are in good agreement with the anti-apoptotic function of c-FLIP (L) towards TRAIL-induced cell death and the

NK-mediated immune-surveillance function of TRAIL [**151, 152, 157, 172**], comfort the notion that c-FLIP can be regarded as a tumour progression factor.

Efficacy of chemotherapeutic drugs is however hampered by the occurrence of intrinsic and acquired drug resistance. A variety of studies have suggested that DRs and chemotherapeutic agents, which share common apoptotic pathways, could show cross-resistancy to apoptosis [**46, 173-175**]. Moreover, tumour cells can become resistant to chemotherapeutic agents, but the combination of DNA-damaging agents or metabolic inhibitors, such as the 5FU, together with TRAIL can circumvent this resistance in various tumour cells *in vitro* [**61, 173, 176-189**] and *in vivo* [**183, 190, 191**]. Hence, sensitization of tumour cells to Fas- or TRAIL-induced cell death can be achieved by a variety of stimuli inducing the downregulation of c-FLIP, such as antisense cDNA constructs [**192, 193**], short interfering RNAs [**194**], proteasome inhibitors [**63, 64]**, protein or RNA synthesis inhibitors [**58, 177**] or chemotherapeutic agents [**60-62, 195, 196**]. In addition, overexpression of viral analogues of c-FLIP or c-FLIP (L) itself was shown to impair chemotherapy-induced apoptosis [**197, 198**]. Likewise, resistance to DR- or anti-tumour drug-induced apoptosis is often associated with a loss of function of caspase-8, the major target of c-FLIPs [**199-202**]. Loss of caspase-8 expression has been shown to occur in a variety of tumour cells including the common childhood primitive neuroectodermal brain tumours/medulloblastomas, highly malignant human neuroblastomas, Ewing tumours, and melanomas [**173, 202-205**]. Reexpression of caspase-8 through promoter demethylation or gene transfer restored tumour cell sensitivity to DR- or chemotherapeutic drug-induced cell death [**173, 202**].

Taken together, since c-FLIP expression can be regulated by certain anti-tumour agents, monitoring c-FLIP-expression levels, might therefore, turn out to be of diagnostic value for certain tumours, and could improve cancer therapy at the clinical level.

4- Conclusions

The understanding of the molecular basis of apoptosis has dramatically progressed in the last decade and has led to the identification of many target genes whose dysregulated expression contributes substantially to human diseases. Amongst these target genes, c-FLIP, which plays a central role in the inhibition of death receptors of the TNF superfamilly, has been shown to be dysregulated in multiple sclerosis, Alzheimer's disease, diabetes mellitus, rheumatoid arthritis and cancer, as documented in this review. Therefore, developing analytical tools to evaluate c-FLIP expression in biological samples originating from these diseases would likely be beneficial to therapeutic strategies targeting immunologic or neurologic disorders associated with c-FLIP dysregulation.

Targeting c-FLIP may notably have beneficial impact in oncology. For the past decades, anti-tumour drug design strategies have mainly focused on inhibiting cellular proliferation. However, it is becoming increasingly clear that cell death dysregulation can also lead to neoplastic transformation and development. Therefore, strategies aiming at the restoration of the apoptotic machinery in tumour cells, may prove to be useful for the treatment of cancer patients in the future.

The growing number of c-FLIP expression regulators (Table 1) extends considerably the possibilities of developing novel therapeutic protocols, which may prove useful in improving patient's care. Yet the molecular mechanisms of c-FLIP regulation still remains poorly understood and further studies are required, especially for the understanding of c-FLIP isoforms. Since c-FLIP is a major regulator of DR-induced apoptosis, and since dysregulated c-FLIP expression is associated with a growing number of pathologies, monitoring c-FLIP levels might be of diagnostic value, and drugs that specifically alter c-FLIP-expression levels will certainly turn out to be of therapeutic benefit.

I apologize to researchers whose work could not be cited in this review due to space limitation.

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