UNIVERSIDADE DE LISBOA
FACULDADE DE FARMÁCIA

ROLE OF APOPTOSIS AND ITS MODULATION IN ALZHEIMER' DISEASE:
INSIGHTS FROM *IN VITRO* AND *IN VIVO* STUDIES

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DOUTORAMENTO EM FARMÁCIA
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O PAPEL DA APOPTOSE E A SUA MODULAÇÃO NA DOENÇA DE ALZHEIMER:

CONTRIBUIÇÃO DE ESTUDOS *IN VITRO* E *IN VIVO*

Dissertação apresentada à Faculdade de Farmácia da Universidade de Lisboa para obtenção do grau de Doutor em Farmácia (Bioquímica)

Rita Cruz Coelho de Mira Ramalho

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The studies presented in this thesis were performed at the Centro de Patogénese Molecular, Faculdade de Farmácia da Universidade de Lisboa under the supervision of Professor Cecília M. P. Rodrigues, at the Department of Medicine, University of Minnesota Medical School, Minneapolis, MN, USA, in collaboration with Professor Clifford J. Steer, and at the Department of Neurosurgery, University of Minnesota Medical School, Minneapolis, MN, USA, in collaboration with Professor Walter C. Low.

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Ao Cláudio

À minha família

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Preface

November 26th, 1901. Dr. Alzheimer was informed about a patient showing unusual clinical symptoms. Auguste D., 51 years old, who had never been ill prior to that time, began to suffer from delusions, having trouble remembering things, and making serious mistakes in her daily activities a few months before. Dr. Alzheimer analyzed the mental condition of Auguste D., who maintained confusing and illogical conversations. The patient showed alterations in memory, language, thought, and behavior. All symptoms worsened progressively, month by month, until 8th April, 1906, when she finally died. Her illness had lasted just over five years and Alzheimer was convinced that this was an extraordinary case. Shortly after her death, the brain was analyzed with silver impregnation staining techniques and the findings were truly surprising. Alzheimer and his co-workers, Perusini and Bonfiglio, observed lesions similar to those found in the brains of patients 70 and 80 years old suffering from dementia, but much more marked. All three firmly believed that this was an unusual case, which had never been described. Dr. Alzheimer would probably never understand the impact of his discovery. However, since then, and especially in the last decades, Alzheimer's disease (AD) has been the focus of intensive research, to establish the abnormal molecular mechanisms that lead to the onset of the disease and to develop novel therapeutic strategies. Although considered the major cause of dementia, with prevalence increasing every year, AD is still not completely understood.

It is now established that AD can be triggered by toxic extra and intracellular aggregates formed from amyloid β and tau, respectively, but the nature of these peptides was not definitively discovered until mid 1980's. It is becoming clear that these aggregates accumulate in selectively vulnerable regions of the brain, compromising the function and viability of neurons and glia. In the absence of the proper conditions to survive, neurons massively die, compromising the cognitive function of a brain affected by AD. A specific type of cell death, apoptosis, has brought much attention in the last few years.

The first observations of dying neurons was made in the 19th-century by a German naturalist, Carl Vogt, when studying the nervous system of toad embryos. However, it was not until 1951 when Ernst and Glucksmann discovered that cell death was an integral part of normal embryonic development. During the 1960s, much was learned about cell death at the ultrastructural level using electron microscopy. Finally, in 1972, John Kerry and co-authors described for the first time a specific process of cell death, when observing characteristic features of hepatocyte development. The process was termed apoptosis, from the Greek word αποπτοσισ, whose prefix "apo" (απο) generally means "separation", and the suffix "ptosis" (πτοσισ) the "act of falling off". The complete word can be translated as the falling of leaves from trees in the autumn and refers to the fragmentation of dying cells into characteristic small bodies. Decades of investigation have shown that apoptosis is an intrinsic suicide program that determines the fate of a cell. It is a common process in many types of cells and tissues. Apoptosis is not only an important event in embryonic development, but also in tissue homeostasis during adult life. In addition, its deregulation can also account for several pathological conditions, ranging from cancer to neurodegenerative disorders. In fact, recent studies suggest a critical role for apoptosis and cell death mediators in AD, even before the reduction in neuronal number.

It has become clear in recent years that prevention of cell death in disorders associated with abnormally increased levels of apoptosis may positively affect the patient outcome. Interestingly, an endogenous bile acid, ursodeoxycholic acid (UDCA) has been described as an inhibitor of apoptosis, not only in liver diseases, but also in other pathological conditions, including neurological disorders. UDCA is a major constituent of black bear bile and has been used for centuries in traditional Chinese medicine for the treatment of liver diseases. However, the mechanisms of action of the bile acid have been characterized only recently. UDCA administration can induce the protection of cholangiocytes against cytotoxicity of hydrophobic bile acids and stimulate the hepatobiliary secretion. Importantly, in 1998, Rodrigues and co-authors showed that UDCA can also have beneficial effects by inhibiting mitochondrial membrane perturbations associated with bile acid-induced apoptosis. UDCA has blossomed as a potent modulator of apoptosis, acting in a tissue-independent manner. Its effects have been tested in many pathological conditions, underscoring its potential and promising therapeutic use.

When I started the Ph.D. program in the laboratory of Professor Cecília M. P. Rodrigues, I focused on investigating the apoptotic mechanisms triggered by neurons exposed to toxic stimuli, in the specific context of AD. My first questions as a student who has just entered a new and exciting area of research, gave rise to additional new questions, which have made my last four years challenging, but rewarding. The present work provides insight into the modulation of apoptosis associated with AD, and more importantly uncovers intriguing connections and links that warrant further investigations.

The purpose of my work was to identify and characterize molecular targets for the use of taurourosodeoxycholic acid (TUDCA) as a modulator of apoptosis in AD, using *in vitro* and *in vivo* models. As a conjugated form of UDCA with

taurine, already in use for the treatment of primary biliary cirrhosis, TUDCA proved to be a potent tool in preventing apoptosis in non-hepatic diseases, such as Huntington's and Parkinson's disease. The use of TUDCA in AD came as a natural extension of this work. Chapter 1 provides a general, up-to-date review on the process of apoptosis. In addition, the role of bile acids as modulators of apoptosis is discussed. We also focus on describing AD and the role of apoptosis in this neurodegenerative disorder. In Chapter 2, we characterize the mechanisms of neuronal protection by TUDCA in *in vitro* AD. The role of cell cycle and apoptosis-related proteins in the effects of TUDCA is also presented and discussed. In Chapter 3, the role of p53, a cell cycle-related protein, in TUDCA neuroprotection is further examined, using an *in vitro* model of familial AD. In Chapter 4, we investigate the role of apoptosis in neurodegeneration using a transgenic mouse model of tauopathy. Apoptosis is presented as an early mechanism that contributes to increased toxicity, and eventually leads to characteristic neurological deficits of AD. Further, we confirmed the existence of a link between amyloid β and tau, via activation of apoptosis-related proteins, and its inhibition by TUDCA. Finally, Chapter 5 integrates our overall findings and discusses specific future perspectives.

The exact mechanism(s) that triggers AD is still obscure. Although more than 100 years have passed since its first description, and despite the efforts of a growing scientific community, an effective treatment is still not available. Nevertheless, in recent years, many mysteries of the disease have been unveiled, including the role of apoptosis as an important event in AD. With this thesis, we hope to contribute to a better understanding of the mechanisms of apoptosis in AD and provide evidences for the neuroprotective role of TUDCA. Ultimately, an increased knowledge of the disease and its potential modulation by bile acids may result in development of more efficient therapeutic interventions.

Summary

Ursodeoxycholic (UDCA) and its taurine-conjugated form, tauroursodeoxycholic acid (TUDCA), are endogenous bile acids used in the treatment of cholestatic liver disorders. Their cytoprotective effects result, in part, from their ability to modulate hepatocyte apoptosis. Interestingly, UDCA and TUDCA play a unique role in modulating the apoptotic threshold in other cell types, including neuronal cells, by interfering with classic mitochondrial pathways. In these studies, we investigated the role of apoptosis using *in vitro* and *in vivo* models of Alzheimer's disease (AD) and determined its potential modulation by TUDCA. A hallmark pathologic feature of AD is the formation of amyloid plaques composed by aggregated amyloid β (Aβ). Our results showed that TUDCA reduced Aβ-induced apoptosis in PC12 neuronal cells, through modulation of apoptosis- and cell cycle-related proteins. In fact, TUDCA treatment resulted in inhibition of E2F-1 induction, p53 stabilization and Bax expression. Further, TUDCA protected PC12 cells against p53- and Bax-dependent apoptosis induced by E2F-1 and p53 overexpression, respectively. The role of p53 in TUDCA effects was further confirmed using an *in vitro* model of familial AD. In neuroblastoma cells expressing the amyloid precursor protein (APP) with the Swedish mutation (APPswe), or double-mutated human APP and PS1 (APPswe/ΔE9), TUDCA modulated p53 activity, and Bcl-2 family changes. Moreover, overexpression of p53 was sufficient to induce apoptosis, which in turn

was reduced by TUDCA. Another pathologic feature of AD is the intracellular aggregation of tau into neurofibrillary tangles. Using the rTg4510 transgenic mouse model of tauopathy, expressing a mutated form of human tau, we confirmed the role of apoptosis in neurodegeneration. Increased levels of DNA fragmentation and caspase-3 activation were observed in the hippocampus and frontal cortex of young mice. These changes were associated with cleavage of tau into smaller intermediate fragments, which were often colocalized with active caspase-3. *In vitro*, fibrillar Aβ resulted in nuclear fragmentation, caspase activation, and caspase-3-induced cleavage of tau. Notably, incubation with TUDCA abrogated apoptosis-mediated cleavage of tau in rat cortical neurons. The results suggest that caspase-3-cleaved intermediate tau species precede cell loss in rTg54510 brains and Aβ-exposed cultured neurons. In conclusion, the work presented here underscores the role of apoptosis in neurodegeneration of AD and expands the antiapoptotic function of TUDCA. Furthermore, the results demonstrate that TUDCA regulates specific transcriptional and posttranscriptional events that impact on mitochondrial function of neurons.

Keywords: Amyloid β – Alzheimer's disease - Apoptosis – Bcl-2 family – Bile acids – Caspases – E2F-1 – p53 – Tau

Sumário

O ácido ursodesoxicólico (UDCA) e a sua forma conjugada com a taurina, o ácido tauro-ursodesoxicólico (TUDCA), são ácidos biliares endógenos, largamente utilizados no tratamento de doenças crónicas do fígado, como a cirrose biliar primária. No entanto, só recentemente começaram a ser conhecidos e descritos os mecanismos de acção destes ácidos biliares. Actualmente, sabe-se que o efeito citoprotector do UDCA e do TUDCA se deve, maioritariamente, à capacidade destas moléculas modularem a morte celular programada ou apoptose dos hepatocitos, fenómeno que se encontra desregulado em inúmeras patologias hepáticas. De facto, através de estudos prévios, foi possível demonstrar que o UDCA e o TUDCA desempenham este papel anti-apoptótico, em parte, através da estabilização da membrana mitocondrial, prevenindo a sua despolarização e a consequente libertação de citocromo *c* e activação de caspases, responsáveis pela execução do processo apoptótico. Porém, desconhece-se, ainda, a maior parte dos mecanismos de sinalização iniciados por estes ácidos biliares. Uma vez que os vários produtos do metabolismo lipídico, incluíndo os ácidos biliares, possuem propriedades sinalizadoras, pensa-se que a regulação da apoptose exercida pelo UDCA e TUDCA poderá passar pela modulação a nível da transcrição génica ou mesmo a nível pós-transcricional.

Curiosamente, o papel protector do UDCA parece estender-se a outros tipos celulares e em resposta a vários agentes tóxicos. Por outro lado, após a conjugação

com a taurina e quando administrado sistemicamente, em sobredosagem, o UDCA pode ser distribuído por outros tecidos, incluíndo o cérebro, o que permite a sua aplicação em doenças não hepáticas, como é o caso de várias desordens neurológicas. De facto, os efeitos protectores do TUDCA foram já testados, *in vitro* e *in vivo*, para as doenças de Hungtington e Parkinson, assim como em modelos de acidente vascular cerebral, do tipo isquémico e hemorrágico. Muitas outras patologias associadas à desregulação da apoptose poderão, também, beneficiar desta estratégia terapêutica.

A doença de Alzheimer (AD) é uma doença neurodegenerativa progressiva, à qual estão associadas graves perdas de memória e um acentuado défice cognitivo. O cérebro de um doente de Alzheimer caracteriza-se pela presença de placas amilóides, cujo principal componente é a proteína β amilóide (Aβ), e de tranças neurofibrilhares (NFT), compostas por agregados intracelulares da proteína tau. Como resultado da formação destes agregados tóxicos, os neurónios sofrem profundas alterações, tornam-se disfuncionais e acabam por morrer em grande escala. A apoptose parece desempenhar um papel importante, como mecanismo essencial de morte celular associada à AD.

No presente estudo, investigou-se o envolvimento da apoptose na neurodegenerescência associada à AD e a possível regulação dos mecanismos apoptóticos pelo TUDCA. Foi também explorada a função de proteínas específicas da apoptose e do ciclo celular no papel anti-apoptótico do TUDCA.

Numa primeira parte do trabalho, os resultados obtidos demonstraram que, apesar do aumento da expressão da proteína anti-apoptótica Bcl-2, incubações com Aβ induzem níveis significativos de apoptose em células neuronais PC12, o que foi eficazmente inibido em pré-tratamentos com TUDCA. A inibição da apoptose induzida por Aβ parece ser feita através da via E2F-1/p53/Bax, mais especificamente pela inibição da indução do factor de transcrição E2F-1, da estabilização da proteína p53 e da expressão da Bax. De facto, o TUDCA foi

capaz de proteger as células da apoptose dependente da expressão de p53 e de Bax, após sobre-expressão de E2F-1 e p53, respectivamente.

De seguida, o papel das proteínas do ciclo celular, e mais especificamente da p53, na modulação, pelo TUDCA, da apoptose induzida por Aβ, foi confirmado num modelo das formas familiares da AD. Apesar de se manifestar numa pequena percentagem da população mundial, a AD na sua forma familiar, associada a mutações na proteína precursora da Aβ (APP) ou nas presenilinas 1 e 2, possui características muito semelhantes à forma esporádica, embora o início da doença ocorra geralmente em idades mais precoces. Utilizando células de neuroblastoma que expressam APP com a mutação *Swedish* (APPswe) ou duplamente mutadas na APP e na presinilina 1 (APPswe/ΔE9), observou-se um aumento dos níveis de apoptose, em consequência da produção endógena e agregação de Aβ. De facto, detectou-se fragmentação nuclear e activação das caspases -2, -6 e -8 em células APPswe e APPswe/ΔE9. Por outro lado, observou-se também um aumento da expressão de p53 e de Bax e uma diminuição da expressão de Bcl-2. Em contrapartida, a pré-incubação com o TUDCA reduziu eficazmente os níveis apoptóticos e de activação das caspases -2 e -6, restabelecendo a expressão de p53 e de proteínas da família Bcl-2. A sobre-expressão de p53 induziu, por si só, a apoptose nas células de neuroblastoma, o que, por sua vez, foi reduzido pelo TUDCA. No entanto, a inibição da via de sobrevivência fosfatidilinositol 3'-cinase reduziu a capacidade do TUDCA para proteger a apoptose induzida pela p53. Em conclusão, estes estudos demonstram que as mutações associadas às formas familiares da AD activam mecanismos apoptóticos muito semelhantes às formas esporádicas. Por outro lado, o TUDCA é capaz de reduzir a apoptose, através da inibição da p53 e da consequente modulação dos níveis de expressão das proteínas da família Bcl-2.

Por fim, numa terceira parte do trabalho, o papel da apoptose foi avaliado num modelo de tauopatia. Na AD, assim como nas tauopatias, a proteína tau deixa

de induzir a estabilização dos microtúbulos a nível do axónio, é fosforilada de uma forma anómala e agrega em NFT no corpo celular dos neurónios. Como resultado, os neurónios deixam de ser funcionais e, eventualmente, acabam por morrer. Em estudos anteriores, o modelo transgénico rTg4510, que expressa uma forma mutada de tau humana, apresentou níveis elevados de morte neuronal, em estruturas corticais e límbicas associadas à AD, atrofia cerebral e défices cognitivos. Nos nossos estudos, observou-se um aumento dos níveis de fragmentação nuclear e activação de caspase-3, especialmente em animais mais jovens, de 2,5 meses, e nas áreas do córtex frontal e do hipocampo. De facto, a apoptose aparenta ser um evento precoce nestes animais transgénicos e a activação de caspase-3 parece estar associada à clivagem de tau na sua zona C-terminal, uma vez que se observou a co-localização da caspase-3 activa e de tau clivada no cortex frontal e hipocampo de animais com 2,5 meses. A clivagem de tau pela caspase-3 foi já descrita, por alguns autores, como sendo um acontecimento essencial à sua agregação em NFT, o que vem confirmar o papel da apoptose nesta patologia. Apesar das tauopatias estarem associadas a mutações na proteína tau, não existem quaisquer mutações descritas na AD, sendo a Aβ apontada como principal responsável pelas alterações conformacionais da tau. Em estudos *in vitro*, por nós realizados, fibrilhas de $A\beta_{1-42}$ induziram a fragmentação nuclear, a activação de caspases e clivagem de tau pela caspase-3, estabelecendo uma ligação entre as duas entidades patológicas da AD. Por outro lado, a pré-incubação com TUDCA inibiu, de forma significativa, a clivagem de tau induzida pela apoptose em neurónios corticais de rato. Deste modo, os resultados sugerem que formas intermediárias de tau, clivadas pela caspase-3, precedem a morte neuronal nos cérebros dos ratos rTg4510 e em neurónios expostos a $A\beta_{1-42}$ fibrilhar.

Em suma, o presente trabalho demonstra a importância da apoptose na AD, não só como mecanismo de morte neuronal, mas também como mediadora de efeitos tóxicos. Por outro lado, estes estudos revelam o potencial papel protector

do TUDCA em modelos de AD, actuando a montante dos eventos mitocondriais, nomeadamente através da regulação transcricional da expressão de proteínas do eixo apoptótico E2F-1/p53/Bax. Ilustrou-se, ainda, a capacidade do TUDCA para inibir mecanismos tóxicos a jusante da mitocôndria, que culminam na clivagem de tau em fragmentos indutores da sua agregação.

A caracterização do ácido biliar TUDCA como modulador transcricional e pós-transcricional da apoptose na AD, consolida o papel desta molécula como uma opção terapêutica no tratamento de doenças neurodegenerativas, expandindo o seu papel protector para além das doenças hepáticas.

Palavras chave: Ácidos Biliares – Apoptose – β amilóide – Caspases – Doença de Alzheimer – E2F-1 – Família Bcl-2 – p53 – Tau

Abbreviations

Aβ	amyloid β peptide
AD	Alzheimer's disease
AIF	apoptosis-inducing factor
ANT	adenine nucleotide translocator
Apaf-1	apoptosis protease-activating factor 1
APOE ε4	apolipoprotein ε4 allele
APP	amyloid precursor protein
APPwt	APP wild-type
APPswe	APP with the Swedish mutation
APPswe/ΔE9	APP double-mutated human APP and PS1
BH	Bcl-2 homology domain
CAT	chloramphenicol acetyltransferase
CDK	cyclin dependent kinase
DIABLO	direct IAP binding protein with low pI
DISC	death-inducing signaling complex
DTT	dithiothereitol
ER	endoplasmic reticulum
FAD	familial form of AD
FADD	Fas-associated death domain
FC	frontal cortex

FTDP-17	frontotemporal dementia with parkinsonism linked to chromosome 17
GR	glucocorticoid receptor
GSK3β	glycogen synthase kinase 3 β
HD	Hungtington's disease
IAP	inhibitor of apoptosis protein
IM	mitochondrial inner membrane
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MMP	mitochondrial membrane permeabilization
MPT	mitochondrial permeability transition
MSN	medial septal nucleus
3-NP	3-nitropropionic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NF-κB	nuclear factor κB
NFT	neurofibrillary tangles
OM	mitochondrial outer membrane
PD	Parkinson's disease
PBS	phosphate-buffered saline
PI3K	phosphatidylinositide 3'-OH kinase
pNA	*p*-nitroanilide
pRb	retinoblastoma protein
PS1	presenilin 1
PS2	presenilin 2
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
SGZ	subgranular zone

Smac	second mitochondria-derived activator of caspases
TNF	tumor necrosis factor
TNF-α	tumor necrosis factor α
TNF-R1	TNF type receptor 1
TUDCA	tauroursodeoxycholic acid
TUNEL	transferase mediated dUTP-digoxigenin nick-end labeling
UDCA	ursodeoxycholic acid
VDCA	voltage-dependent anion channel

1

General Introduction

1. Apoptosis

Apoptosis (from the Greek "falling off") was originally described in 1972 by Kerr *et al*. as a common type of programmed cell death, repeatedly observed in various tissues and cell types (Kerr et al. 1972). Apoptosis is one of the most frequent phenomena occurring in multicellular organisms and is fundamental to their health. In fact, as a physiological mechanism, apoptosis has an important role in embryogenesis, synaptogenesis, immune response and tissue homeostasis. Surprisingly, per day, the human body destroys ~ 60 x 10^9 cells through an apoptotic process, in response to physiological, pathogenic, or cytotoxic stimuli, underscoring the relevance of this orchestrated form of cellular suicide (Reed 2002).

In contrast to necrosis, apoptosis is an active energy-dependent process, defined by a series of biochemical and morphological modifications, including condensation of chromatin, shrinking of cytoplasm and nuclear compartments, degradation of DNA into oligonucleosome-length fragments and compartmentalization of nuclear material into vesicular apoptotic bodies (Kerr et al. 1972). These are rapidly eliminated by resident phagocytic and neighboring cells, preventing the release of cellular components into the extracellular space, and consequent inflammatory response. Defects in physiological pathways of apoptosis contribute to the development of numerous medical illnesses for which adequate therapy or prevention is lacking. In fact, insufficient levels of apoptosis are implicated in cancer, autoimmune diseases and persistent infections, while excessive apoptosis can lead to neurodegenerative disorders and hepatocellular degeneration.

The highly conserved molecular basis of apoptosis was originally described in the nematode *Caernorhabditis elegans (C. elegans)* (Ellis and Horvitz 1986). Interestingly, 113 of the 1090 embryonic somatic cell undergo apoptosis during the

development of *C. elegans*. In a coordinated process, apoptosis is regulated by three important genes: *ced-3*, *ced-4*, that induce cell death, and *ced-9*, which has an antiapoptotic role (Metzstein et al. 1998).

Apoptosis may occur by several molecular pathways. The best characterized and most prominent, however, are the extrinsic death receptor and intrinsic mitochondrial pathways (Fig.1).

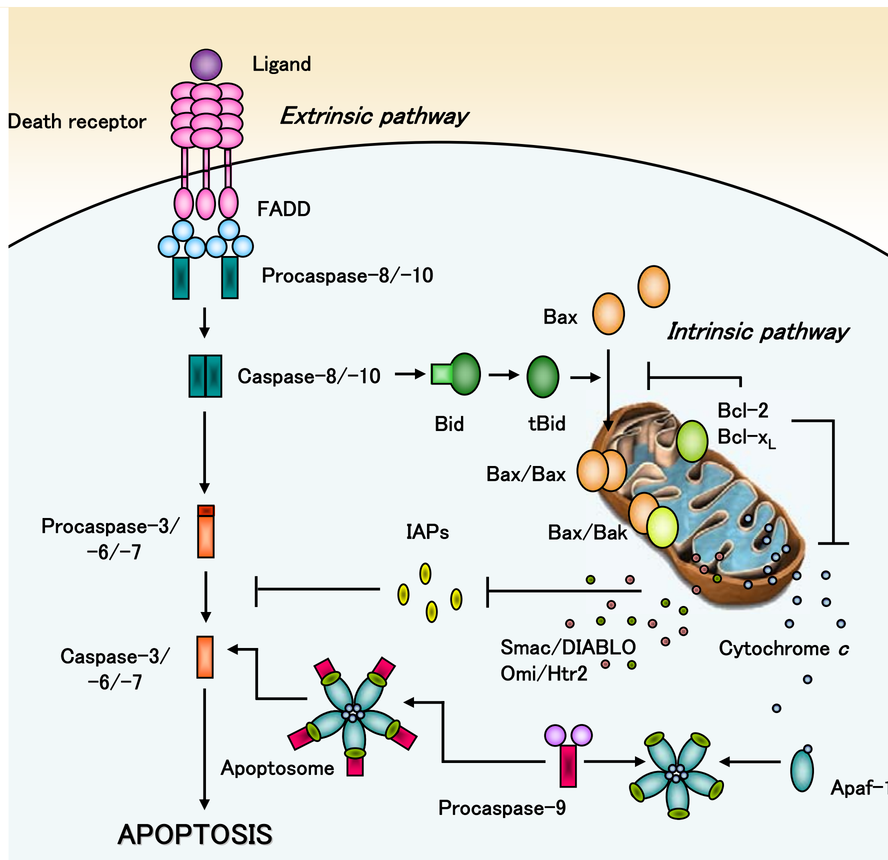

Fig. 1. Schematic overview of extrinsic and intrinsic apoptotic pathways. In the death receptor pathway, after interacting with their ligands, the death receptors recruit adaptor

proteins such as FADD and activate caspases-8 and -10. These initiator caspases then cleave effector caspases-3, -6, and -7, which activate key downstream targets and execute the apoptotic process. In the mitochondrial pathway, death stimuli target mitochondria either directly or through transduction by proapoptotic Bax and Bak. Mitochondria release cytochrome *c*, Smac/DIABLO, Omi/Htr2 and other apoptogenic factors. Cytochrome *c* induces oligomerization of Apaf-1 that recruits and activates procaspase-9. Caspase-9 then activates effector caspases. The crosstalk between both pathways is mediated by Bid, which is truncated and activated by caspases-8. See text for more complete description.

Both pathways are characterized by an initiation phase, when a signal triggers the apoptotic process; integration/decision phase, which involves the activation of several apoptotic mechanisms; and final execution/degradation phase that culminates in cell death. Although apparently independent, the two apoptotic pathways often interact in many cell types to accomplish cell death signaling.

1.1. Mitochondrial pathway

As the primary generators of energy and important regulators of intracellular calcium, mitochondria are essential organelles for cell survival. By coupling electron transport to the generation of proton gradients for oxidative phosphorylation, mitochondria produce ATP that is used in the metabolic activities of the cell. Thus, highly metabolic tissues such as the brain are particularly dependent on mitochondria. In the past decades, mitochondria have also emerged as critical players in cell death. In fact, all the energy that is used for maintaining life in healthy cells is completely redirected to serve a mortal purpose, under pathological conditions. After an apoptotic stimulus, such as oxidative stress, DNA damage, or protein misfolding, the levels of calcium are increased, the mitochondrial membrane is permeabilized, releasing apoptogenic factors from the

intermembrane space to the cytoplasm and disrupting the mitochondrial membrane potential, which culminates in cell death (Ricci et al. 2003).

1.1.1. Structural modifications of mitochondria during apoptosis

Under physiological conditions, mitochondrial inner membrane (IM) is nearly impermeable to all ions including protons. This results in an electrochemical gradient, the inner mitochondrial transmembrane potential, that is essential for cellular bioenergetics (Mitchell and Moyle 1965a, b). The permeability of the mitochondrial outer membrane (OM) is also well regulated, mainly by the presence of voltage-dependent anion channels (VDAC) (De Pinto and Palmieri 1992). After an apoptotic stimulus, mitochondria undergo several modifications leading to mitochondrial membrane permeabilization (MMP), often considered as the "point of no return" (Green and Kroemer 2004). The permeability of the OM increases, allowing the release of soluble proteins that are usually retained in the intermembrane space. The IM looses its selectivity and becomes permeabilized, which results in permanent dissipation of the transmembrane potential (Marchetti et al. 1996). Far from an accidental process, the MMP is a tightly regulated phenomenon, with the involvement of dynamic pore structures and interaction of different proteins. One of the first events is the opening of the high conductance mitochondrial permeability transition (MPT) pore. In fact, excessive calcium accumulation by mitochondria during apoptosis leads to opening of the MPT pore and massive mitochondrial swelling (Bernardi 1999). Despite the uncertainty about the molecular composition, it has been suggested that the MPT pore spans the IM and the OM and is composed of proteins from membranes and matrix. The adenine nucleotide translocator (ANT), located in the IM (Brustovetsky and Klingenberg 1996), the VDAC (De Pinto and Palmieri 1992), and the cyclophilin D, from the matrix (Crompton et al. 1998), were proposed to be part of the MPT pore complex. In addition, proapoptotic proteins from the Bcl-2 family, namely

Bax and Bak, can also engage in a close molecular cooperation with some components of the MPT complex, such as the VDAC and/or the ANT (Tsujimoto and Shimizu 2002), or form themselves pores, further enhancing mitochondrial permeabilization (Wolter et al. 1997; Kuwana et al. 2002).

1.1.2. Release of apoptogenic factors from mitochondria

As a consequence of the MMP, several apoptogenic factors are released into the cytosol, activating a family of death-inducing cysteine proteases, the caspases. Cytochrome *c*, a peripheral protein of the IM that functions as an electron shuttle between complexes III and IV of the respiratory chain, is a crucial factor in mediating mitochondria-dependent apoptosis (Li et al. 1997). Recent studies have identified an additional mitochondrial compartment, the intracristae space, that is formed by lamellar and tubular structures resulting from the convoluted folds of the IM cristae (Frey and Mannella 2000). Most of the cytochrome *c* yield (~ 85%) is contained in this compartment, which suggests that an additional step for cytochrome *c* release may occur. In fact, during apoptosis, cristae are remodeled, which results in the widening of junctions that delineate the intercristae space. This phenomenon facilitates the diffusion of cytochrome *c* to the intermembrane space and, consequently, to the cytosol through pores formed in the OM (Scorrano et al. 2002). Although not completely understood, the reorganization of cristae may require several proteins involved in mitochondrial fusion and fission processes, such as the Drp1 (Germain et al. 2005).

Once in the cytosol, cytochrome *c* binds and induces conformational changes to the apoptotic protease-activating factor-1 (Apaf-1), a CED-4 homolog, in the presence of ATP/dATP, recruiting procaspase-9, and forming the apoptosome. Caspase-9 then acquires the ability to trigger processing and activation of downstream caspases, finalizing the apoptotic process (Zou et al. 1999).

Similar to its murine homolog direct inhibitor of apoptosis proteins (IAP) binding protein with low pI (DIABLO), the second mitochondria-derived activator of caspases (Smac), is another apoptogenic factor released during MMP. Smac/DIABLO is a mitochondrial protein encoded by the nuclear genome, and is proteolytically processed within the intermembrane space to yield a mature polypeptide of 23 kDa with an IAP biding motif (Du et al. 2000). Following MMP, Smac/DIABLO is released into the cytosol, neutralizing IAPs and thus promoting caspase activation. Similar to Smac/DIABLO, the Omi/Htr2 protein is also processed in the intermembrane space into a mature form of 37 kDa (Martins 2002). Once in the cytosol, it promotes cell death either by antagonizing IAPs (caspase-dependent pathway), or via its proteolytic activity (caspase-independent pathway).

The apoptosis inducing factor (AIF) also plays an important role in the apoptotic process. In healthy cells, AIF is required for optimal detoxification of reactive oxygen species (ROS), and for the assembly or maintenance of the respiratory chain complex I (Vahsen et al. 2004). However, once released from the intermembrane space, after OM permeabilization and proteolytic maturation, AIF translocates from the cytosol to the nucleus, promoting chromatin condensation and large-scale DNA fragmentation (Susin et al. 1999). A similar role is played by endonuclease G (EndoG), a mitochondria-specific enzyme that also translocates to the nucleus during apoptosis. However, the mechanism by which EndoG cleaves DNA into nucleosomal fragments (Li et al. 2001) is not entirely known.

In addition to the above-described, many other factors are released from mitochondria during MMP. However, their precise role, if any in cell death has not yet been elucidated. Thus, further investigation is needed for the complete characterization of the mitochondrial pathway of apoptosis.

1.2. Death receptor pathway

Many toxic and pathological situations are associated with changes in expression and/or functioning of death receptors and their ligands, leading to caspase activation and apoptosis (Fig. 1). The tumor necrosis factor (TNF) death receptor superfamily is a group of cytokines with important functions not only in apoptosis, but also in immunity and inflammation, control of cell proliferation and differentiation (Baud and Karin 2001). Nineteen different proteins have been identified within this family, including the TNF-α and the Fas/Apo-1/CD95 receptors that are activated by binding of TNF-α and Fas ligand (FasL), respectively (Brunner et al. 1995). The members of TNF receptor family are structurally similar membrane proteins of type I. They consist of extracellular (N-terminal), transmembrane, and intracellular (C-terminal) components. Ligand-binding domains of the extracellular compenent of death receptors are characterized by the presence of 2 to 6 repeats of about 40 amino acids enriched in cysteine (Banner et al. 1993). After ligand binding, adaptor proteins such as the Fas-associated death domain (FADD) are recruited (Blagosklonny 2000), forming the death-inducing signaling complex (DISC) (Kischkel et al. 1995). Once activated, death receptors induce the cleavage and activation of procaspase-8, and -10. In fact, FADD was shown to contain two death effector domains (DEDs) capable of recruiting caspase-8, and -10 (Krueger et al. 2001). Depending on cell type, caspase-8, and -10 can directly activate downstream caspases, such as caspase-3 or -7 (Peter and Krammer 2003), or transmit the death signal to mitochondria, in a cross-talk between both apoptotic pathways (Li et al. 2002) (Fig. 1). In this case, caspase-8 cleaves the inactive cytoplasmic Bid, a proapoptotic protein of the Bcl-2 family, exposing an active truncated fragment (tBid) (Scaffidi et al. 1998). Once activated, tBid translocates to mitochondria, inducing conformational changes in proapoptotic proteins, such as Bax and Bak

and, consequently, the MMP (Eskes et al. 2000). Moreover, tBid can also inhibit antiapoptotic proteins, such as Bcl-2, (Kim et al. 2000), or even directly permeabilize the mitochondrial OM (Goonesinghe et al. 2005), ultimately inducing caspase-3 activation and perpetuating the apoptotic process.

Finally, in the presence of ROS, TNF-R1 can also induce apoptosis via mitogen-activated protein kinase (MAPK) signaling, activating the c-Jun NH_2-terminal kinase (JNK) pathway (Shen and Pervaiz 2006). Interestingly, TNF-R1 is capable of triggering survival signals, including the activation nuclear factor κB (NF-κB) (Barnhart and Peter 2003), underscoring the relevance and complexity of death receptors.

1.3. Other molecular intervenients in the apoptotic process

To execute apoptosis, many proteins, enzymes, and different factors are involved. In fact, to integrate death signals and perform the multiple reactions that culminate in cell death, several components, interactions, and biochemical processes are necessary, in a complex organization that defines the efficiency of apoptosis.

1.3.1. Bcl-2 family

Modulation of apoptosis is performed by the Bcl-2 family, a group of proteins that work in regulated protein-protein interactions. This family gives its name to the first identified member, over 20 years ago, at the chromosomal breakpoint of t(14;18)(q32;q21) lymphomas, B-cell lymphoma-2 (Bcl-2). Bcl-2 is an homolog of CED-9 (Tsujimoto et al. 1985). Curiously, Bcl-2 was found to inhibit cell death, rather than promote proliferation, and since then many relatives of this family have been described. In mammals, the Bcl-2 family consists of at least 20 members, including proteins that promote apoptosis, or proapoptotic proteins, and others than inhibit it, forming a complex balancing network that determines cell fate.

Bcl-2 members can be subdivided in three groups, according to their structure and function. The antiapoptotic proteins, such as Bcl-2, Bcl-x_L and Mcl-1, among others, share 3 to 4 conserved Bcl-2 homology domains (BH1-4), forming group I. Bcl-2 and its homologues potently inhibit apoptosis in response to many cytotoxic insults. The proapoptotic proteins, such as Bax and Bak, share 3 conserved domains (BH1-3), forming group II. Group III contains a subgroup of proapoptotic proteins that only have the BH3, such as Bid, Bad, Noxa and Puma, among others (Cory et al. 2003). Both types of proapoptotic proteins are required to initiate apoptosis; the BH3-only proteins usually act as damage sensors and direct antagonists of the antiapoptotic proteins, while proteins of group II act further downstream, mainly in mitochondria disruption.

Under physiological conditions, Bcl-2 binds to the cytoplasmic face of mitochondrial OM, endoplasmic reticulum (ER) and nuclear envelope, promoting the integrity of membranes, by interacting and neutralizing proapoptotic proteins (Gottlieb 2001). Bcl-2 overexpression decreases the amount of calcium mobilized from the ER to the mitochondria, inhibiting the opening of the PT pore (Baffy et al. 1993). In addition, some studies indicate that Bcl-2 and Bcl-X_L can also interact with ANT and VDAC, inhibiting the formation of the MPT pore (Marzo et al. 1998; Shimizu et al. 2000).

Proapoptotic Bax, under normal circumstances, resides as a monomer in the cytoplasm (Hsu et al. 1997), while Bak is attached to the mitochondrial OM as an integral membrane protein (Griffiths et al. 1999). Following induction, an increased expression of proapoptotic proteins occurs, changing the balance between anti- and proapoptotic factors. Consequently, Bax undergoes conformational changes, translocates to the mitochondria, oligomerizes and inserts into the OM (Wolter et al. 1997). Once attached to mitochondria, it is thought that Bax, alone or associated with Bak, can provoke or contribute to permeabilization of the OM, allowing the release of cytochrome *c* (Kuwana et al. 2002). The

mechanism by which proapoptotic proteins permeabilize the OM, however, is still controversial and not entirely understood. Some studies suggest that Bax and Bak can themselves form hydrophobic pores in the membrane (Wolter et al. 1997; Kuwana et al. 2002), or simply destabilize membrane lipid bilayers (Basanez et al. 2002). Alternatively, Bax might interact with proteins of the MPT pore, such as VDAC and ANT, inducing the MMP (Tsujimoto and Shimizu 2002). Further, Bax and Bak can enhance the loading of the ER calcium store, boosting the calcium load to mitochondria (Scorrano et al. 2003).

Finally, BH3-only proteins act as sentinels that when activated trigger apoptosis in response to developmental cues or intracellular damage. These proapoptotic proteins exert their action by two different mechanisms. In fact, they can interact with antiapoptotic proteins, dissociating them from other BH-3-only or from BH1-3 proteins, and promoting MMP (e.g. Bad), or they can directly activate the BH1-3 proteins to initiate MMP, either by stimulating the translocation of Bax to mitochondrial OM or by local effects on Bak (e.g. tBid) (Letai et al. 2002).

1.3.2. Caspases

Caspases are a family of cysteine proteases that play an important role in the execution of apoptosis, cleaving a restricted set of target substrates after an aspartate residue in their primary sequence (Thornberry and Lazebnik 1998). In 1993, the gene *ced-3* was discovered in *C. elegans*, showing great similarities with caspase-3; a connection between caspases and apoptosis was established for the first time (Yuan et al. 1993). Since then, many other caspases have been described in mammalian and non-mammalian species.

Caspases share similarities in amino acid sequence, structure, and substrate specificity. In healthy cells, they are present in the cytosol as inactive precursors, called zymogens. Caspases contain three domains, including a N-terminal prodomain, a large subunit with the active cysteine, and a C-terminal small unit

(Thornberry and Lazebnik 1998). After an apoptotic signal, the zymogen is exposed to two cleavage events. The first proteolytic cleavage divides the chain into large and small caspase subunits, and a second cleavage removes the N-terminal prodomain (Wolf and Green 1999). The cleavage of the zymogen is not always an obligatory requirement for caspase activation. However, all activated caspases can be detected as cleaved fragments in apoptotic cells (Degterev et al. 2003).

According to their function, caspases are grouped as upstream proteases termed initiator caspases (caspases-2, -8, -9 and -10), and their downstream targets known as effector or executioner caspases (caspase-3, -6 and -7) (Thornberry and Lazebnik 1998). The initiator caspases function as signal integrators for apoptotic or proinflammatory stimuli. They contain larger prodomains and specific sequence motifs, such as the caspase recruitment domain (CARD), for caspase-2 and -9, or a pair of DEDs, for caspase-8 and -10 (Hofmann et al. 1997; Ashkenazi and Dixit 1998). These domains mediate the recruitment of zymogen to death signaling complexes, leading to its auto-catalytic activation. Moreover, the homodimerization of zymogens appears to be a crucial step for the activation of initiator caspases in contrast to executioner caspases.

Caspase-9 is a key component of the mitochondrial pathway and its activation occurs after cytochrome *c* release and formation of the apopotosome. The zymogen is recruited to the complex through CARD-CARD interactions, and rapidly processed into active caspase-9, which in turn is responsible for the activation of downstream caspases, such as caspase-3 (Thornberry and Lazebnik 1998). On the other hand, caspase-8 has a key role in the death receptor pathway and is activated in the presence of external death signals. In response to the activation of receptors of the TNF family, the zymogen is recruited to the DISC via binding to FADD, resulting in caspase-8 activation and subsequent activation of downstream caspases (Boldin et al. 1996; Varfolomeev et al. 1998).

Caspase-10 is structurally very similar to caspase-8, but its function in apoptosis is not entirely known (Degterev et al. 2003). In fact, some studies suggest that caspase-10 may have a function that overlaps with caspase-8 in Fas ligand-mediated apoptosis. Caspase-10 might be recruited to the Fas DISC, cleave Bid, and activate the mitochondrial pathway.

Finally, caspase-2 was one of the first caspases discovered, but its physiological function and activation remain obscure. Recent results suggest that caspase-2 can be activated by dimerization (Butt et al. 1998) or by recruitment of a large complex similar to the apoptosome, named the PIDDosome (Tinel and Tschopp 2004). It is often localized in the cytosol, the nucleus (Colussi et al. 1998), and the Golgi (Mancini et al. 2000), although its protein targets in these compartments remain largely unclear. Nevertheless, it was demonstrated that in some cells caspase-2 is responsible for the mitochondrial OM permeabilization and the release of apoptogenic factors in response to DNA damage (Zhivotovsky and Orrenius 2005). It can also associate with the Fas DISC, but apparently it is not required for Fas-induced cell death (Lavrik et al. 2006). Interestingly, caspase-2 can also function independently of its protease activity, such as by activation of MAPK and NF-κB signaling pathways (Lamkanfi et al. 2005).

Once activated, initiators caspases cleave effector caspases, which lack the long prodomain and the ability to self-activate. Effector caspases are responsible for cleaving most of the cellular substrates, finalizing the apoptotic process. Caspase-3 is the main downstream effector caspase, which can be activated via the death receptor pathway, following activation by caspase-8, and through the mitochondrial pathway, by caspase-9 (Porter and Janicke 1999). It is responsible for cleavage of many substrates, including nuclear lamins and cytoskeletal proteins, such as fodrin and gelsolin that are associated with morphological changes in apoptotic cells (Kothakota et al. 1997). In addition, caspase-3 cleaves the inhibitor of caspase-activated DNAse (ICAD), promoting the activation of the

endonuclease CAD, which induces the characteristic nucleosomal DNA fragmentation (Sakahira et al. 1998). Caspase-3 is also responsible for the cleavage of poly(ADP-ribose) polymerase (PARP), inhibiting its capacity to repair DNA (Rosen and Casciola-Rosen 1997).

Caspase-7 is highly homologous to caspase-3, with similar substrate specificity and redundant functions in the majority of general apoptotic events. It can be activated by caspase-8 (Hirata et al. 1998) and caspase-9 (Li et al. 1997), and has also a specific role in the ER-stress response pathway (Rao et al. 2001). Caspase-6, although structurally similar to caspase-3 and -7 has different substrate specificities. Its function and activation are still not entirely understood. However, some caspase-6 substrates have been already described and include lamin A (Takahashi et al. 1996).

Even with apparently similar functions, effector caspase-3, -6 and -7 have different relevance to the apoptotic process. In fact, depletion of caspase-3 in a cell-free apoptotic system inhibited most of the downstream events, including DNA fragmentation and chromatin condensation, while elimination of caspase-6 and -7 did not produce the same effects (Slee et al. 2001). Thus, caspase-3 appears to be the primary effector caspase, with more specialized functions. Nevertheless, if caspase-3 is missing or not functioning, the other effector caspases can compensate the catalytic mechanisms, creating alternative and novel networks (Zheng et al. 2000).

Other caspases play important roles in the inflammation process, such as caspase-1, -5 and -11. In fact, caspase-1 is involved in proinflammatory cytokine maturation (Ghayur et al. 1997), while caspase-5 is associated with the formation of the inflammasome. This protein complex is responsible for the activation of inflammatory caspases (Martinon et al. 2002). Caspase-11 was proposed to be the murine functional orthologue of human caspase-5 (Lin et al. 2000). Finally,

caspase-12 also appears to have a distinct role in ER-stress mediated pathway, which is correlated with disruption of calcium homeostasis (Lamkanfi et al. 2004).

Overall, caspases are the key executioners of apoptosis, with different functions, integrating and terminating the mechanisms that lead to cell dysfunction and death. Their expression and activation are spatially and temporally regulated, depending on cell type and development stage, underscoring versatility and wide spread function. Giving their importance and power to destroy cells, caspases are tightly regulated in normal cell function. The IAPs are the primary inhibitors of caspase activation, whose homologues have been subsequently described in all eukaryotes, from yeast to humans (Crook et al. 1993). The p53 protein (Clem et al. 1991) and CmrA (a cytokine response modifier gene) (Ray et al. 1992) can also regulate the activation of caspases. In the last years, several peptide and non-peptide inhibitors have been developed, providing novel therapeutic tools in prevention of apoptosis associated with pathogenic situations, such as neurodegenerative and infectious diseases, and ischemia-reperfusion disorders. However, most studies did not result in less cell death, since the use of caspase inhibitors often sensitizes cells to necrosis and/or autophagy (Vandenabeele et al. 2006). Moreover, cells can also undergo apoptosis in caspase-independent pathways, involving several other proteases such as cathepsins, calpains and granzymes. This compromises the expected regulation and inhibition by therapeutic drugs, challenging science to discover and develop better solutions.

1.3.3. Cell cycle-related proteins

The balance between cell death and proliferation may be the most important phenomenon in tissues homeostasis. Typically, eukaryotic cells replicate with a complexity of events involving a large number of proteins. However, after stress stimuli or DNA-damaging events, cells undergo several modifications to induce

either cell cycle arrest and DNA repair, or apoptosis when injury compromises survival (Fig. 2).

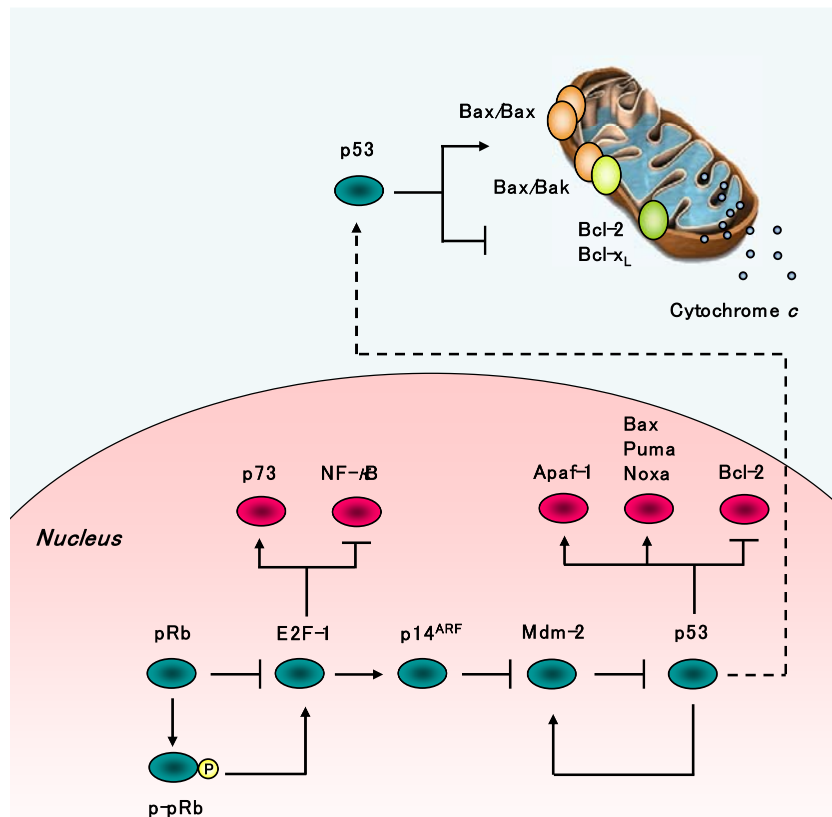

Fig. 2. Schematic representation of modulation of apoptosis by cell cycle-related proteins. Under normal conditions, the transcription factor E2F-1 and p53 are downregulated by pRb and Mdm-2, respectively. Following an apoptotic stimulus, E2F-1 is released and either activates or represses its target genes, including those encoding for p73 and p14^{ARF}, and NF-κB. Consequently, p14^{ARF} inhibits Mdm-2 and indirectly stabilizes p53. p53 regulates the expression of proapoptotic *Bax*, *Noxa, Puma*, among others. In addition, it can transcriptionally repress *Bcl-2* and induce *Apaf-1* expression, further enhancing the apoptotic response. p53 also regulates the mitochondrial death pathway, in a transcription-

independent manner, by inhibiting Bcl-2 and Bcl-x_L, and activating Bax and Bak. See text for more complete description.

A central player in protecting the integrity of the genome is the tumor suppressor p53, a transcription factor that regulates the expression of a large number of target genes. The protein p53 is present at low levels under physiological conditions but becomes rapidly stabilized and activated in response to a variety of stimuli. The p53 network is activated through at least three independent pathways. These include DNA damage via the protein kinase ataxia telangiectasia mutated (ATM) and Chk2; aberrant hyperphosphorylation and cell cycle re-entry triggered by oncogenes *Ras* or *Myc* and by $p14^{ARF}$, and by cytotoxic stimuli such as chemotherapeutic agents, in a ATM, Chk2 or $p14^{ARF}$-independent pathway (Vogelstein et al. 2000). Once activated, p53 can either cause cell cycle arrest by transactivation of p21, or induce apoptosis by both transcription-dependent and -independent mechanisms (Steele et al. 1998).

The precise mechanisms by which p53 becomes stabilized are not entirely clear, but may involve post-translational modifications of p53 and its repressor Mdm-2. Under unperturbed conditions, p53 is tightly regulated by Mdm-2, an E3 ubiquitin ligase that binds to and poly-ubiquitinates p53, targeting it for degradation (Iwakuma and Lozano 2003). Moreover, Mdm-2 is itself a transcriptional target of p53, in a negative feedback loop that maintains low physiological levels of p53 (Zauberman et al. 1993). In addition, several proteins have recently been shown to cooperate with Mdm-2 in p53 regulation, such as the homolog MdmX protein (Parant et al. 2001). In toxic conditions that lead to activation and increased levels of p53, the Mdm-2/p53 interaction is affected by conformal changes and phosphorylation of p53 in specific residues (Lakin and Jackson 1999). Once stabilized, p53 accumulates in the nucleus, regulating

expression of numerous proapoptotic genes, such as *Bax* (Miyashita and Reed 1995), *Noxa* (Oda et al. 2000), and *Puma* (Nakano and Vousden 2001). In addition, it can also transcriptionally repress Bcl-2 (Miyashita et al. 1994) and induce Apaf-1 expression (Robles et al. 2001), further enhancing the apoptotic response. Moreover, p53 is capable of transactivating genes involved in the death receptor apoptotic pathway, such as FasL and Fas (Vogelstein et al. 2000). Recent evidence also indicates that p53 regulates the mitochondrial death pathway, in a transcriptional-independent, non-nuclear mechanism. In fact, the results suggest that p53 binds and inhibits Bcl-2 and Bcl-x_L, and activates proapoptotic and multi-domain Bax and Bak, inducing permeabilization of the mitochondrial OM (Schuler and Green 2005). Thus, it is clear that p53 is more than a transcription factor, working in a varied and complex manner to promote efficient elimination of malfunctioning cells.

E2F-1 is also a transcription factor, member of the E2F family that comprises six elements with the ability to regulate many target genes involved in the control of cell proliferation. Regulation of E2F-1 is mediated primarily by interaction with unphosphorylated retinoblastoma protein (pRb) that masks and inhibits the transactivation domain of E2F-1. Following phosphorylation of pRb by cyclin-cyclin dependent (CDK) complexes, E2F-1 is released and free to mediate activation of target genes (Dyson 1998). Interestingly, E2F-1 can also regulate apoptosis by at least three different mechanisms. In fact, E2F-1 stabilizes p53 by induction of $p14^{ARF}$, a possible direct target of E2F-1, which binds to Mdm-2 and prevents p53 degradation (Kamijo et al. 1998). On the other hand, E2F-1 transcriptionally upregulates p73, a homolog of p53 that shares the ability to induce apoptosis (Irwin et al. 2000). E2F-1 can also inhibit antiapoptotic factors, such as NF-κB, thus promoting cell death (Phillips et al. 1999). It is thought that different apoptotic pathways induced by E2F-1 occur simultaneously in cooperative actions that further enhance the death signal.

Thus, after a toxic stimulus and under appropriate conditions, cells have mechanisms to either arrest cell cycle and repair DNA, or trigger a complex and efficient mechanism that culminates in death.

2. Role of bile acids in apoptosis

Bile acids are produced in the liver and secreted into the intestine, where they play crucial biological roles such as the solubilization of lipids in the intestinal lumen, among many others. However, certain hydrophobic bile acids are cytotoxic molecules that can increase cell proliferation in the intestinal tract (Bayerdorffer et al. 1993) and/or induce cell death by necrosis and apoptosis (Patel and Gores 1995). In contrast, more hydrophilic species can be cytoprotective (Heuman et al. 1991).

2.1. Bile acid biosynthesis and physiology

Bile acids are the major components of bile, synthesized in the liver from neutral sterols by a complex series of chemical reactions (Russell and Setchell 1992). They are a class of acidic steroids with a cyclopentanoperhydrophenanthrene nucleus (ABCD-ring) containing 19 carbons, and most commonly a C5 side chain with a terminal carboxylic acid (Rodrigues et al. 2004). In humans and most animal species, bile acids are produced primarily from the cholesterol metabolic pathway. The complete synthesis of bile acids requires approximately seventeen enzymes. The expression of these enzymes is tightly regulated by nuclear hormone receptors and other transcription factors, which ensure a steady supply of bile acids to a highly demanding metabolic environment. Importantly, the initial and rate-limiting step for the major bile acid biosynthetic pathway is the 7α-hydroxylation of cholesterol, catalyzed by the cytochrome P450 enzyme,

cholesterol 7α-hydroxylase (CYP7A1). Different bile acid species have diverse degrees of hydrophobicity, as determined by their biochemical and physicochemical properties. The amphipathic structure allows these water-soluble compounds to interact with proteins and insert into lipid bilayers. These effects will have severe influences on cell function and structure, particularly when intracellular concentrations of bile acids exceed certain limits, as it is the case in cholestasis.

Primary bile acids are synthesized in the liver, conjugated with the amino acids glycine or taurine, and then secreted via the bile ducts and gallbladder into the lumen of small intestine (Russell and Setchell 1992). Bile acids act as detergents to emulsify dietary lipids and fat-soluble vitamins, but they can also solubilize bilirubin and other catabolites. Furthermore, the expression of genes that synthesize cholesterol, fatty acids, and bile acids are regulated by intermediates and/or end-products of the bile acid pathway itself (Repa and Mangelsdorf 1999). While emulsified nutrients are taken up by enterocytes in the proximal segments of the gut, bile acids re-enter the liver via the portal vein, and are transported back into the gallbladder for use in the next feeding cycle (Russell and Setchell 1992).

The biliary bile acid pool also includes secondary bile acids, such as deoxycholic and lithocholic acids. These bile acids are not formed in the liver, but rather result from the metabolism of primary bile acids by intestinal bacteria. Biotransformations of primary bile acids include also the formation of ursodeoxycholic acid (UDCA), by oxidation of chenodeoxycholic acid to 7-oxolithocholic acid, followed by reduction yielding the 7β-isomer.

2.2. Bile acids and apoptosis

2.2.1. Bile acid-induced apoptosis

Accumulation of toxic bile acids is a common feature of several chronic human liver diseases, resulting from interruption in bile flow. This pathological condition, known as cholestasis, can promote liver cell death, leading to cirrhosis (Hofmann 2002). It was thought that hydrophobic bile acids, such as glycochenodeoxycholic and taurochenodeoxycholic acids could induce cytotoxicity by acting as detergents on cell membranes. However, other evidence suggests that basic cellular mechanisms of hepatocyte injury might be primarily involved (Schmucker et al. 1990), ultimately causing cell death by either necrosis or apoptosis. Importantly, the presence of classic Councilman bodies and cell failure suggests that apoptosis may play a key role in cholestasis. Bile acid-induced apoptosis has been shown *in vivo* as well as in primary rat hepatocytes and human hepatoma HuH-7 cells (Rodrigues and Steer 2000). However, the predominant type of liver injury may depend upon several factors, such as the cell type, level of exposure, and metabolic status of the cell.

The mechanisms by which bile acids induce apoptosis in hepatocytes are still not entirely known. Several studies have shown that caspase activation, mitochondrial dysfunction, and cellular distribution of Bcl-2-related proteins determine the fate of hepatocytes in models of cholestasis (Maher 2004). In addition, toxic bile acids can also induce ligand-dependent and -independent death receptor pathways, via Fas- and tumor necrosis factor-related apoptosis inducing ligand (TRAIL) receptors (Faubion et al. 1999; Higuchi et al. 2003). Subsequently, FADD is recruited and activates caspase-8 and Bid, which results in downstream activation of effector caspases and cathepsin B (Roberts et al. 1999; Sokol et al. 2001). The activation of death receptors invariably signals the mitochondrial pathway of apoptosis in hepatocytes. In fact, deoxycholic acid

(DCA) was shown to induce the MPT pore formation in isolated mitochondria (Rodrigues et al. 1998a), as well as mitochondrial depolarization, increased ROS production, translocation of Bax to mitochondria and cytochrome *c* release (Rodrigues et al. 1999; Rodrigues et al. 2003a). Further, MPT was prevented by antioxidants and cyclosporine A, an inhibitor of the megapore channel (Botla et al. 1995; Rodrigues et al. 1998b; Sokol et al. 2001).

Curiously, the liver has the ability to limit apoptosis during cholestasis by triggering specific mechanisms. Although it has been shown that Bcl-2, Bcl-x_L, and Bax are expressed in the liver, only cholangiocytes and not hepatocytes normally express antiapoptotic Bcl-2. However, induction of cholestasis by bile duct ligation leads to Bcl-2 expresion in hepatocytes, which may represent an adaptative phenomenon to protect hepatocytes (Kurosawa et al. 1997). In addition, the activation of NF-κB and subsequent regulation of antiapoptotic genes (Schoemaker et al. 2003), as well as the cytoplasmic sequestration of p53 (Oh et al. 2002) are complementary mechanisms triggered by the liver to inhibit or modulate apoptosis induced by toxic bile acids.

2.2.2. Inhibition of apoptosis by ursodeoxycholic acid

In contrast to toxic hydrophobic bile acids, UDCA improves liver function in patients with hepatobiliary disorders (Lazaridis et al. 2001). It is normally present in human bile in a low concentration, representing only 3% of total bile acids. In black bears, however, UDCA is the major biliary bile acid. Bear bile has been used for centuries in traditional Chinese medicine as a remedy for liver disorders (Hagey et al. 1993). In the Western world, UDCA has been used for a few decades as a therapeutic agent for chronic cholestatic liver diseases. At the present, it is the only drug approved by the United States Food and Drug Administration for the treatment of primary biliary cirrhosis (Lazaridis et al. 2001; Paumgartner and Beuers 2004).

Both unconjugated UDCA and its amidated conjugates, tauroursodeoxycholic acid (TUDCA) and glycoursodeoxycholic acid (GUDCA) are effective modulators of toxicity induced by more hydrophobic bile acids (Rodrigues and Steer 2000). The mechanisms of action of UDCA in cholestasis may involve the protection of injured cholangiocytes, stimulation of impaired biliary secretion, detoxification of hydrophobic bile acids, and/or inhibition of hepatocyte apoptosis (Paumgartner and Beuers 2004). It is not clear which of these mechanisms play a primary role for the beneficial therapeutic effects of UDCA. Most likely, UDCA acts in a coordinated process involving several effects, depending on the type and stage of the disease.

The role of UDCA in preventing apoptosis in cholestasis has been intensely studied . In fact, toxic bile acids fed to rats induced apoptosis in the liver, while UDCA inhibited this effect *in vivo*, in part by preventing translocation of proapoptotic Bax from the cytosol to the mitochondria (Rodrigues et al. 1998b) (Fig. 3). These studies were subsequently extended to show that UDCA plays a unique role in modulating apoptosis in different cell types, in response to a variety of agents, acting through different apoptotic pathways (Rodrigues et al. 1998a). The antiapoptotic effect of UDCA appears to involve the mitochondrial membrane. In fact, UDCA and its conjugates prevent the release of cytochrome *c*, caspase activation, and PARP cleavage associated with mitochondrial depolarization and Bax channel formation induced by apoptotic stimuli (Rodrigues et al. 1999). Moreover, UDCA partially prevented apoptosis via the death receptor pathway in primary mouse hepatocytes co-cultured with fibroblasts expressing the Fas ligand, possibly by its direct effects at the mitochondrial membrane (Azzaroli et al. 2002)

Additional mechanisms of action for UDCA may also be engaged, where the bile acid interferes with alternate molecular targets (Fig. 3). In fact, DNA microarray analysis showed that UDCA can significantly modulate the expression of 96 different genes, most of them involved in apoptosis, but also in cell cycle

regulation and proliferation (Castro et al. 2005). Apaf-1 was found to be downregulated in rat hepatocytes in response to UDCA incubations. Importantly, UDCA can also interfere with molecular targets upstream of the mitochondria. UDCA inhibited TGF-β1-induced E2F-1 transcriptional activation, p53 stabilization and p53-associated Bax expression, independently of its effect on the mitochondria and/or caspases (Solá et al. 2003b). UDCA also inhibited the downregulation of Bcl-2 by TGF-β1, which is consistent with decreased p53 stabilization and/or of NF-κB degradation. Furthermore, recent evidence showed that both UDCA and TUDCA reduce transcriptional activation and expression of cyclin D1 in primary rat hepatocytes incubated with deoxycholic acid (Castro et al. 2005; Castro et al. 2007). The modulation of cyclin D1 expression appears to contribute to the antiapoptotic effects of the bile acid, in part through a p53-dependent mechanism. In fact, UDCA modulates the E2F-1/Mdm-2/p53 apoptotic pathway in hepatocytes via a nuclear steroid receptor (NSR)-dependent mechanism (Solá et al. 2004). UDCA upregulated both glucocorticoid (GR) and mineralocorticoid (MR) receptor expression in hepatocytes during TGF-β1-induced apoptosis. Moreover, it was shown that UDCA promotes GR/hsp90 dissociation, inducing subsequent NSR translocation (Solá et al. 2005). The deletion of the C-terminal region of GR inhibited the capacity of UDCA to induce GR/hsp90 dissociation, GR translocation and modulation of apoptosis, indicating that the ligand binding domain of GR is required for the antiapoptotic function of UDCA. These results strongly suggest that UDCA translocates to the nucleus, in a complex with GR, where it may modulate apoptosis-related genes. In contrast, recent evidence suggests that the modulation of induced neuronal apoptosis by TUDCA requires an interaction with MR (Solá et al. 2006).

TUDCA was also shown to regulate the ER stress-mediated pathway in HuH-7 cells, reducing the calcium efflux and the activation of caspases-12 (Xie et al. 2002) (Fig. 3). Moreover, treatment of obese and diabetic mice with TUDCA

resulted in normalization of ER-induced hyperglycemia and restored systemic insulin response (Ozcan et al. 2006). TUDCA was also responsible for resolution of fatty liver disease and enhancement of insulin action in liver, muscle, and adipose tissues, expanding its beneficial role to type 2 diabetes.

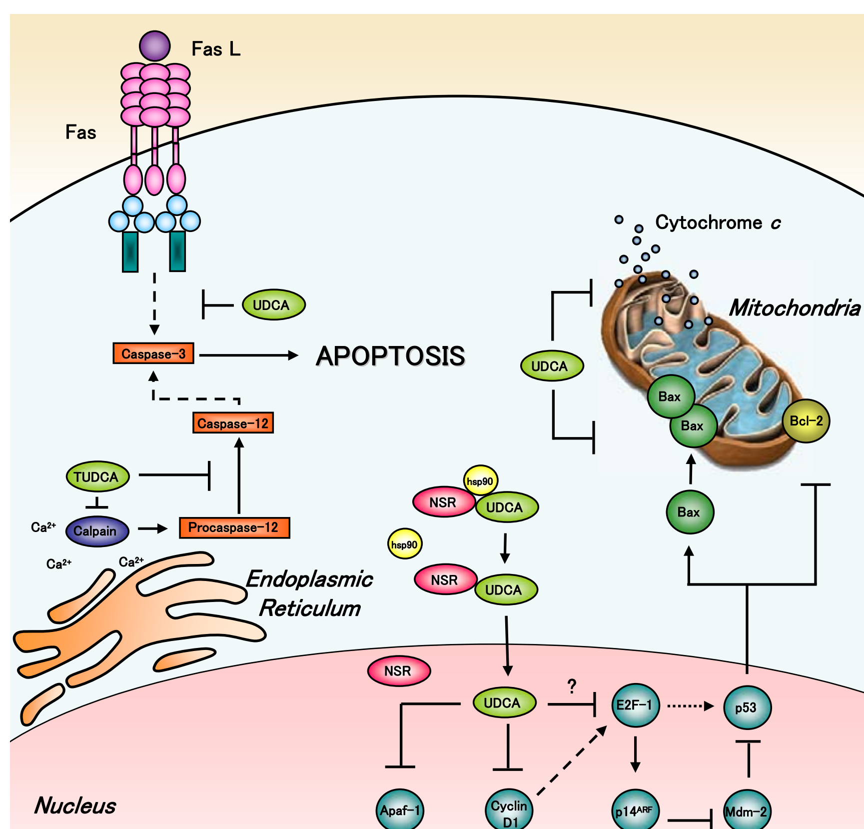

Fig. 3. Proposed mechanisms for the antiapoptotic actions of UDCA and TUDCA. UDCA negatively modulates the mitochondrial pathway by inhibiting Bax translocation, ROS formation, cytochrome *c* release and caspases-3 activation. UDCA can also interfere with the death receptor pathway, inhibiting caspase-3 activation. Moreover, TUDCA inhibits apoptosis associated with ER stress, by modulating intracellular calcium levels, and inhibiting calpain and caspase-12 activation. UDCA interacts with NSR, leading to NSR/hsp90 dissociation and nuclear translocation of NSR. The nuclear trafficking of

UDCA allows it to modulate the E2F-1/p53/Bax pathway, preventing apoptosis. Finally, UDCA downregulates cyclin D1 and Apaf-1, further inhibiting the mitochondrial apoptotic cascade. See text for more complete description.

Finally, it is becoming increasingly evident that activation of survival pathways may represent an important additional mechanism by which UDCA inhibits apoptosis. One possible survival pathway involves the activation of NF-κB by several molecules, including the inflammatory cytokines TNF-α and interleukin-1β (Bradham et al. 1998). In addition, TUDCA was shown to protect mitochondria-controlled apoptosis in primary rat hepatocytes by activating the phosphatidylinositol 3-kinase (PI3K) and MAPK pathways (Schoemaker et al. 2004).

2.3. Bile acids for the treatment of neurodegenerative disorders

The therapeutic role of UDCA has been established in the treatment of certain liver diseases. Importantly, UDCA has the ability to modulate apoptosis at several levels, suggesting a common mechanism of cell survival regulation that is independent of cell type. Thus, the use of UDCA to non-liver diseases in which increased levels of apoptosis contribute to their pathogenesis is now a major consideration.

Mitochondrial dysfunction and subsequent oxidative damage have been implicated in several neurobiological disorders, such as acute stroke and chronic neurodegenerative diseases. Moreover, increasing evidence suggests that apoptosis plays a crucial role in the pathogenesis of such disorders (Mattson 2006). Interestingly, after conjugation with taurine, UDCA administrated in high doses can be delivered to other tissues, including the brain (Keene et al. 2001). *In vitro*,

TUDCA inhibits apoptosis induced by several stimuli in neuronal cells (Rodrigues et al. 2000; Solá et al. 2003a). Furthermore, the protective role of TUDCA has been extended to several models of neurological disorders, including Hungtington's disease (HD), Parkinson's disease (PD), and acute ischemic and hemorrhagic stroke.

HD is a genetically dominant neurological disorder caused by abnormal expansion of the trinucleotide (CAG) repeat sequence in exon 1 for the gene *Htt* encoding the huntingtin protein (Walker 2007). In HD, mitochondrial function is compromised, which is demonstrated by abnormal energy metabolite levels, impaired striatal mitochondrial respiratory chain complex II/III activity and increased stress-induced mitochondrial depolarization, ROS production, and associated oxidative damage. A consequence of mitochondrial dysfunction may be the activation of certain apoptotic pathways, confirmed by activation of caspases and DNA fragmentation in HD brains. Chronic administration of 3-nitropropionic acid (3-NP), an irreversible inhibitor of succinate dehydrogenase provides a relatively accurate animal model of HD, closely resembling the pathology and symptoms of the disease. The systemic administration of TUDCA in the 3-NP rat model of HD was shown to reduce the associated morphologic striatal lesions (Keene et al. 2001). Moreover, behavioral studies correlated with histopathological findings, since the significant neuroprotection resulted in almost complete prevention of hyperactive behavior associated with 3-NP administration, while maintaining neophobia characteristic of cognitively intact animals. TUDCA also markedly reduced the mitochondrial perturbations associated with apoptosis induction in cultured neuronal cells incubated with 3-NP (Rodrigues et al. 2000). These data suggested that TUDCA could possibly act as a therapeutic agent for regulating cell survival in HD. Thus, initial studies were extended to the R6/2 transgenic mouse model of HD, which express exon 1 of the human *huntingtin* gene with ~ 150 CAG repeats and the human *huntingtin* promoter. Administration

of TUDCA induced a marked reduction in striatal cell apoptosis and degeneration of R6/2 transgenics (Keene et al. 2002). In addition, TUDCA prevented striatal atrophy, reduced the average size and number of individual characteristic intracellular inclusions of huntingtin and ubiquitin, and, consequently, improved the locomotor and sensorimotor abilities of R6/2 transgenic mice.

Transplantation of human embryonic dopamine neurons in patients with PD is currently being evaluated in clinical trials. Although transplantation of nigral tissue ameliorates deficiencies characteristic of PD, a major hurdle for successful neural grafting is the poor survival of dopaminergic neurons implanted into the brain of recipient patients. There is strong evidence showing that the majority of cell death occurring in neuronal grafts results from apoptosis. Thus, based on its antiapoptotic properties, TUDCA was added to cell suspensions prior to transplantation to improve cell survival of nigral grafts in a rat model of PD (Duan et al. 2002). The results showed that TUDCA reduced the levels of cultured cells undergoing apoptosis, increasing the number tyrosine-hydroxylase-positive neurons. In transplantation studies, where TUDCA treated-cell suspensions were injected into the striatum of 6-hydroxydopamine lesioned rats, there was a significant improvement of amphetamine-induced rotation scores, resulting from increased survival of dopamine positive cells. More recently, TUDCA was shown to partially rescue a PD model of *C. elegans* from mitochondrial dysfunction (Ved et al. 2005).

Prompted by these results, the antiapoptotic role of TUDCA was extended to acute conditions, particularly to a rat model of transient focal cerebral ischemia (Rodrigues et al. 2002). Neuronal cell death resulting from acute stroke is a complex process and appears to involve a variety of different pathways. Although originally thought to be mainly necrotic, cell death can also occur via apoptosis in both ischemic and hemorrhagic stroke (Friedlander et al. 1997; Matsushita et al. 2000). Intravenous administration of TUDCA resulted in improved neurologic

function and reduced infarct volumes by ~ 50%. In addition, TUDCA significantly reduced the number of apoptotic cells, prevented mitochondrial swelling and membrane disruption, and partially inhibited downstream caspases activation and endogenous substrate cleavage associated with apoptosis. TUDCA also reduced the levels of apoptosis, caspase-3-like activation and histological damage of the peri-hematoma region in a collagenase-induced hemorrhagic model of stroke (Rodrigues et al. 2003b). In addition, TUDCA inhibited NF-κB activation, maintained elevated the levels of Bcl-2, and activated the PI3K survival pathway, consequently improving neurological functions.

Cell death induced by glutamate may be involved in neuronal injury associated with chronic neurodegenerative disorders, including HD, and with acute conditions, such as hypoxia-reperfusion. The mechanisms by which neurons die when exposed to glutamate can either be necrotic or apoptotic, depending on the severity of the insult (Bonfoco et al. 1995). Interestingly, pretreatment with TUDCA also significantly reduced glutamate-induced apoptosis of rat cortical neurons (Castro et al. 2004). TUDCA inhibited the release of cytochrome *c* from mitochondria as well as the activation of caspases-3. In addition, TUDCA induced marked phosphorylation and translocation of Bad to cytosol, which was prevented after inhibition of the PI3K survival pathway. These results suggested that TUDCA modulates glutamate-induced apoptosis in part by activating a PI3K-dependent Bad signaling pathway.

Taken together, these studies provide evidence for the antiapoptotic role of UDCA and TUDCA. As a hydrophilic acid, TUDCA is readily water-soluble, can be administrated orally and intravenously, and is associated with minimal toxicity. These characteristics make TUDCA a potent therapeutic tool in several disorders associated with higher susceptibility to apoptotic cell death. Thus, it would be relevant and interesting to further extend its clinical applications to other neurodegenerative diseases, such as Alzheimer's disease (AD).

3. Alzheimer's disease

In 1906, the physician Alois Alzheimer gave a lecture at a congress of psychiatry in Tübingen, Germany. For the first time, he described a disease that Kraepelin some years later named AD. Over several years, Alzheimer had been observing a patient at the Frankfurt Asylum named Auguste D., who presented strange behavioral symptoms, including a decline in short-term memory, confusion and disorientation. After her death, staining techniques revealed the presence of amyloid plaques and neurofibrillary tangles (NFT) in the brain, which are today recognized as hallmarks of the disease. AD is the most common form of dementia. However, after 100 years of its discovery, the triggering mechanisms of AD are not entirely understood, and effective diagnosis and treatment are still lacking.

3.1. Epidemiology and risk factors

AD is a severe and common cause of dementia, accounting for 50-60% of all cases (Ferri et al. 2005). This neurodegenerative disease targets specific brain regions early in its course, especially the cholinergic basal forebrain and medial temporal lobe structures, including the hippocampus, amygdale and entorhinal cortex. With disease progression, the pathogenesis spreads to other regions, including the posterior cingulate, temporal and parietal isocortical regions (Braak and Braak 1991). AD is characterized by progressive memory loss, anomia, constructional apraxia, anasognosia, and variable degrees of personality changes (Katzman 1992). The prevalence of AD is less than 1% in individuals aged 60-64 years, but the probability of developing the disorder increases with age. In Western countries, the prevalence reaches 24-33% in people aged 85 years or older. In 2001, more than 24 million people in the world had dementia. With life expectancy increasing, the numbers are predicted to double every 20 years, reaching up to 81 million in

2040 (Ferri et al. 2005). In Portugal, there are ~ 60,000 patients with AD, although the numbers might be under estimated, given the difficulty of diagnosis.

In the vast majority of cases, the cause of AD remains unclear. Like most other chronic conditions, AD probably develops as a result of multiple factors, rather than a single cause. Undoubtedly, the greatest risk factor is advancing age, but epidemiological studies have suggested several possible associations. Decreased reserve capacity of brain, including reduced brain size, low levels of education and occupation, low mental ability in early life, and reduced mental and physical activities during late life, can increase the susceptibility to develop AD (Mayeux 2003). In addition, it has been suggested that head injury can also be linked to AD (Jellinger 2004). Other risk factors are hypo- and hyperthyroidism, hypercholesterolemia, hyperthension, atherosclerosis, heart disease, smoking, obesity, and diabetes (Mayeux 2003).

Although environmental influences might increase the susceptibility to sporadic AD, genetic factors have a significant impact in the development of the disease. The genetic factor that accounts for more cases of late-onset sporadic AD is the apolipoprotein ε4 allele (*APOE*) located on chromosome 19. *APOE* ε4 increases the risk of developing AD 3-fold in heterozygotes and by 15-fold in homozygotes, and also influences the age of disease onset (Farrer et al. 1997; Meyer et al. 1998). The mechanisms by which *APOE* ε4 triggers AD are not entirely understood, but may include enhanced rate of amyloid β (Aβ) deposition, as well as reduced protection against oxidative stress and efficacy of neuronal repair (Horsburgh et al. 2000). The search for additional susceptibility genes for sporadic AD is still ongoing, but none has been verified with certainty.

A small percentage of AD patients have a familial form of the disease, related to a specific hereditary mutation. Familial AD is rare, with a prevalence below 0.1 % (Harvey et al. 2003). It is an autosomal-dominant disorder with early onset, striking patients in their mid-thirties to mid-fifties. To date, more than 100

mutations of 3 genes have been identified to cause this specific form of AD. The first mutation related to familial AD was identified in the amyloid precursor protein (*APP*) gene on chromosome 21 (Goate et al. 1991). Since then, several additional mutations have been described. The largest number of mutations, however, is located in the presenilin 1 (*PS1*) gene, responsible for most cases of familial AD (Sherrington et al. 1995). Finally, a small number of mutations have also been identified in the presenilin 2 (*PS2*) gene in chromosome 1 (Levy-Lahad et al. 1995). All mutations result in elevated Aβ levels. Although related to hereditary factors, familial AD patients develop the same histopathological features and cognitive deficits observed in sporadic cases.

3.2. Pathogenesis

Neurodegeneration in AD is estimated to begin 20-30 years before clinical onset (Davies et al. 1988). During this preclinical phase and after diagnosis, several pathologic mechanisms occur in specific areas of the brain. At the microscopic level, Aβ and tau deposit and aggregate into characteristic external amyloid plaques and internal NFT, respectively. At the same time and/or consequently, other cellular abnormalities occur, such as neuronal dysfunction, cell cycle deregulation, ROS production, inflammation and mitochondrial failure. Ultimately, all these pathological mechanisms culminate in neuronal death and cognitive decline.

3.2.1. Amyloid plaques

The main constituent of amyloid plaques found in AD brains is the Aβ peptide (Glenner and Wong 1984). Initially, Aβ was thought to be an abnormal protein.

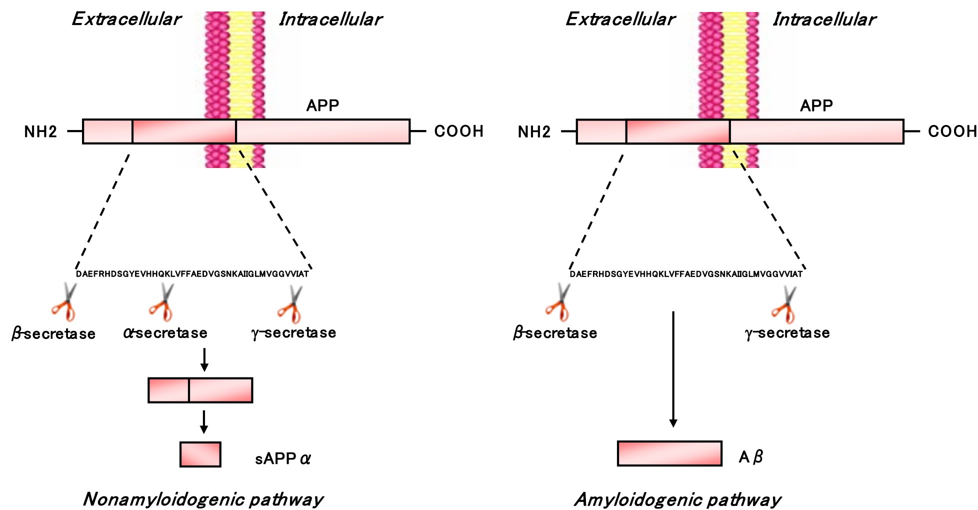

Fig. 4. Diagram of APP processing. In the nonamyloidogenic pathway, APP is cleaved by β-, γ- and α-secretases, generating a soluble sAPPα fragment. In contrast, the cleavage of APP by β- and γ-secretases produces Aβ, with high capacity to aggregate into amyloid plaques. See text for more details.

However, it is also constitutively produced during normal cell metabolism (Haass et al. 1992). Aβ is generated by proteolytic cleavage of APP, mediated by the transmembrane proteins β- and γ-secretases (Vassar et al. 1999) (Fig. 4). The β-site APP cleaving enzyme (BACE1), a member of the pepsin family of aspartyl proteases, cleaves the ectodomain of APP to generate a membrane bound C-terminal fragment, which is subsequently, cleavage by γ-secretase. Importantly, although not entirely understood, the metabolism of APP appears to be modulated both directly and indirectly by phosphorylation and phosphorylation-dependent events (da Cruz e Silva and da Cruz e Silva 2003). Depending on the exact cleavage site, peptides with 38 to 43 amino acids may be generated. Nevertheless,

the most common and toxic forms of Aβ contain 40 and 42 amino acids in length, and are termed $A\beta_{1-40}$ and $A\beta_{1-42}$, respectively. These fragments of ~ 4 kDa account for the majority of Aβ found in AD brains. Finally, APP can also be cleaved by a third protease, the α-secretase (Allinson et al. 2003). α-secretase cleavage is nonamyloidogenic and precludes subsequent BACE1 cleavage, generating a soluble sAPPα fragment that appears to be neuroprotective (Meziane et al. 1998). Under normal conditions, the brain also has the capacity to degrade Aβ by the peptidases insulin-degrading enzyme, neprilysin, and by endothelin-converting enzyme (Carson and Turner 2002). Moreover, Aβ is cleared from the brain in a process balanced by a system of efflux and influx that allows the peptide to cross the blood-brain barrier (Tanzi et al. 2004).

The amyloid cascade hypothesis is considered the major cause of AD (Hardy and Selkoe 2002). According to this, an imbalance between production and clearance of Aβ in the brain triggers neuronal degeneration and dementia, as the initial step of the process. Support for this hypothesis includes the finding of mutations in APP, and in PS1 and PS2, implicated in familial AD. Moreover, the discovery of a third *APP* gene in people with Down's syndrome, who develop AD early in life, suggested that life-long APP overexpression triggers Aβ deposition. Following cleavage, soluble Aβ undergoes conformational changes in its β-sheet content. Next, the peptide self-aggregates by a complex process starting with self-dimerization, followed by oligomerization, protofibril formation, and eventual aggregation into large insoluble fibrillar structures (Urbanc et al. 2004). Importantly, increasing evidence suggest that the ratio $A\beta_{1-42}/A\beta_{1-40}$ is important in AD pathogenesis, rather than the total amount of Aβ produced. In fact, due to its hydrophobic nature, $A\beta_{1-42}$ has a higher tendency to aggregate and is believed to initiate amyloid plaques formation, by triggering the misfolding of other Aβ species (Jarrett et al. 1993; Iwatsubo et al. 1994).

Initially, only Aβ plaques were assumed to be neurotoxic. However, there are several phenomena that can not be explained by fibrillar deposits alone. Indeed, affected neurons do not necessarily colocalize with amyloid plaques in the brain (Li et al. 1994). Similarly, amyloid plaques do not correlate with cognitive decline, as it would be expected if plaques were the main toxic entity in AD (Naslund et al. 2000). Thus, it has been suggested that intermediate soluble oligomers of Aβ may be toxic entities (McLean et al. 1999). These protofibrils are present in AD brain, and represent a distinct form of low molecular weight Aβ, containing 9 to 50 molecules of the peptide. Interestingly, exposure of neuronal cultures and mouse brains to soluble oligomers of Aβ resulted in higher levels of toxicity and neuronal dysfunction, compared to fibrillar species, but the results are still controversial (Dahlgren et al. 2002; Kirkitadze et al. 2002). Moreover, the role of tau in AD pathogenesis can not be neglected and it possibly accounts for other dysfunctions not directly correlated with Aβ aggregation.

3.2.2. Neurofibrillary tangles

Neurobrillary tangles, in addition to amyloid plaques, are another pathological feature of AD, whose major component is the protein tau. Tau is a family of microtubule associated proteins, abundant in the central nervous system and predominantly expressed in axons (Binder et al. 1985). Through its microtubule-binding domains, tau promotes microtubule assembly and stability. In addition, it can also play a role in vesicular transport and axonal polarity. In adult human brain, tau contains six different isoforms, ranging from 45 to 65 kDa, resulting from alternative splicing. The isoforms differ by the presence of either three- (3R) or four-repeat domains (4R) in the C-terminal region, the binding domains, and the absence or presence of one or two inserts in the N-terminal region (Goedert et al. 1989). Each isoform is likely to have particular roles, since they are differentially expressed during development. In fact, it has been demonstrated that adult tau

isoforms with 4R are more efficient at promoting microtubule assembly than the fetal isoform with 3R (Goedert and Jakes 1990).

Tau is very hydrophilic, soluble, and natively unfolded. The functions of tau are regulated by phosphorylation, which can occur in multiple sites. For instance, it was demonstrated that tau is more efficient in promoting microtubule assembly when in a more dephosphorylated state. Interestingly, the longest form of human tau contains 80 serine or threonine residues and five tyrosine residues, which represents almost 20% of the protein with potential for phosphorylation (Stoothoff and Johnson 2005). Several kinases have been shown to phosphorylate tau. The glycogen synthase kinase 3 (GSK3β) is the main kinase, responsible for phosphorylation of tau at numerous sites. GSK3β may also play a role in regulating the degradation of tau, via the proteasome (David et al. 2002). In addition to GSK3β, the cycline-dependent kinase 5 (CDK5), and the microtubule affinity-regulating kinase (MARK) are also known to regulate the physiological role of tau through phosphorylation. In contrast, to counterbalance the action of kinases, tau is readily dephosphorylated by numerous phosphatases, including protein phosphatase 1 (PP1), 2A (PP2A), 2B (PP2B), and 5 (PP5) (Tian and Wang 2002).

The underlying mechanisms contributing to the pathological processing of tau in neurodegenerative conditions have not been entirely elucidated. Nevertheless, it is clear that tau becomes characteristically altered both functionally and structurally in AD and other tauopathies. Supporting a central role for tau in dementia, genetic studies showed that missense mutations in the *tau* gene induce the development of neurodegenerative disorders such as the frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (Hutton et al. 1998). Interestingly, in FTDP-17 and other sporadic tauopathies, pronounced tau pathology was observed, without amyloid plaque formation. Moreover, the distribution and abundance of tau pathology in AD is well correlated

with neuronal degeneration and clinical symptoms, underscoring the relevance of tau (Braak and Braak 1991). The majority of tau mutations are associated with either reduced ability of the protein to interact with microtubules, or increased capacity to aggregate (Hasegawa et al. 1998). Nevertheless, there are no identified mutations associated with AD pathology.

The abnormal phosphorylation of tau has been proposed as the primary modification of tau in AD brains. For example, phosphorylation of Ser205, Ser396, Ser404, Thr205, and Thr212 enhance the polymerization of tau into filaments (Necula and Kuret 2005). Proteolysis of tau is another posttranslational event that may influence the aggregation in NFT. In fact, truncation at both the N- and C-terminal regions directly influences fibril formation of tau. Cleavage of tau by caspase-3 at Asp421 in the C-terminal region has been detected in AD brains and was shown to promote tau assembly *in vitro* (Gamblin et al. 2003b). This fragment apparently causes neuronal death, with a significant role in the nucleation-dependent filament formation (Rissman et al. 2004). In contrast, the removal of the N-terminal region greatly inhibits the aggregation of tau, although this specific truncation occurs later in the NFT formation process (Gamblin et al. 2003a). Thus, aggregation of tau within neurons appears to involve an ordered series of events. First, the microtubule binding function needs to be neutralized. Then, tau molecules must self-associate to promote oligomerization, which leads to conformational changes and increased insolubility of tau. Finally, these oligomers induce nucleation and formation of NFT, which accumulate in neuron cell bodies.

Amyloid plaques and NFT are often present in the same area of the brain, but the relationship between these two entities is not known. It was recently demonstrated that Aβ-induced caspase-3 activation leads to tau cleavage, and aggregation in NFT (Rissman et al. 2004). In addition, hyperphosphorylation of tau was detected in mature hippocampal neurons treated with Aβ (Ferreira et al.

1997). Thus, NFT pathology may represent a downstream effect of Aβ accumulation probably triggered by apoptosis, among other toxic mechanisms.

3.2.3. Neuronal dysfunction and degeneration

In the AD brain, multiple mechanisms occur simultaneously and it is not always easy to distinguish changes that promote the disease from the consequences of the disease. It is established, however, that amyloid plaques and NFT are extra- and intracellular aggregates that are toxic to neurons (Fig. 5).

Several reports have suggested that Aβ activates microglia, inciting an inflammatory response and release of neurotoxic cytokines. In fact, prominent activation of inflammatory processes and the innate immune response are observed in AD brains (Wyss-Coray and Mucke 2002). Neuroinflammation would then occur in the early stages of the disease, driving the pathology. However, other studies suggest that inflammation may even be beneficial and responsible for triggering survival pathways (Wyss-Coray 2006). Thus, the exact role of inflammation in AD is still controversial.

Aβ can also trigger excessive release of excitatory amino acids such as glutamate from glial cells, injuring nearby neurons and inducing the production of ROS. Indeed, increasing evidence supports a role for oxidative stress in vulnerable brain regions of AD patients. Oxidative damage begins early in the disease and can even be responsible for a decline in proteasome function (Cecarini et al. 2007). Moreover, Aβ may directly or indirectly injure mitochondria, thus decreasing the brain metabolism. Aβ is involved in disruption of calcium homeostasis, via ER-mediated stress, inducing mitochondrial dysfunction (Pereira et al. 2004). In addition, Aβ blocks the respiratory complex I and the pyruvate dehydrogenase, inducing a decline in ATP and enhancing ROS production (Casley et al. 2002). In fact, as regulators of both energy metabolism and apoptotic pathways, mitochondria and their consequent dysfunction have a key role in AD (Moreira et

al. 2006). Moreover, because synaptic function has a high energy demand, mitochondrial failure is a major factor of synaptic failure in AD. In addition, cholinergic transmission and synaptic density are considerably decreased in AD patients and probably contribute to memory loss and cognitive deficits. Finally, Aβ and tau may be responsible for paralyzing axonal and dendritic transport.

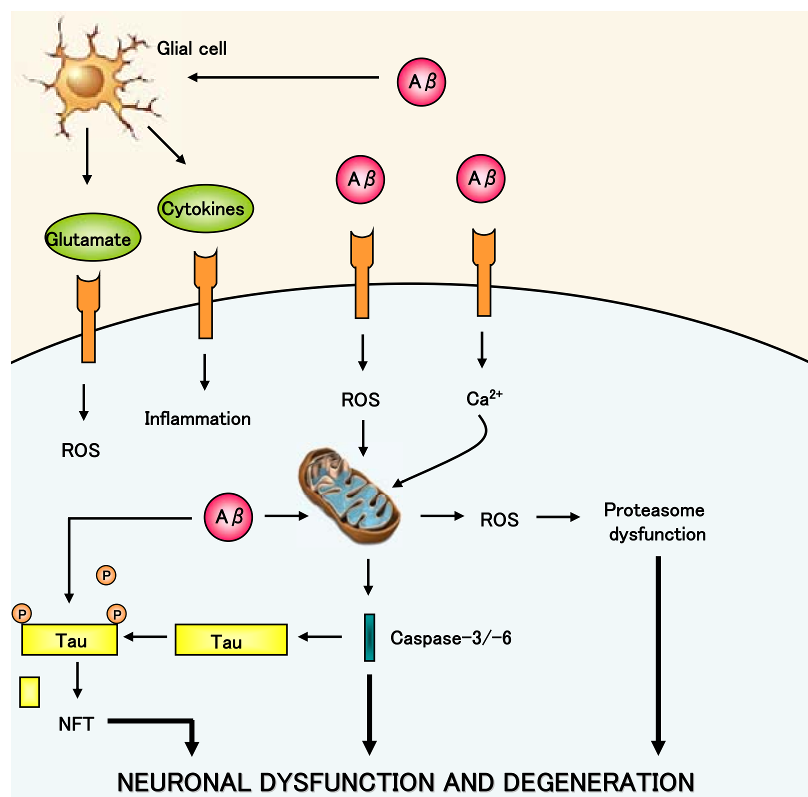

Fig. 5. Proposed mechanism of citotoxicity induced by Aβ and tau. Soluble and/or aggregated Aβ interacts with glial cells, inducing the release of glutamate and inflammatory cytokines. Aβ triggers signal transduction cascades that culminate in increased production of ROS and disruption of calcium homeostasis. Moreover, Aβ may directly or indirectly injure mitochondria, enhancing ROS production and inducing

proteasome failure. Effector caspases are activated, which can be responsible for the cleavage of tau. In addition, abnormal phosphorylation of tau might be enhanced by Aβ. Tau aggregates into NFT and redistributes in neuron cell bodies, paralyzing axonal and dendritic transport. All together, these abnormal mechanisms in AD brains are thought to culminate in neuronal dysfunction and degeneration.

In conclusion, mitochondrial dysfunction and consequent energy deficits contribute to impaired clearance of protein aggregates and neuronal dysfunction, affecting neurotransmission and neuronal transport in AD brains. In this scenario, neurons no longer have the ability to survive and massively die in the affected brain regions. However, the primary mechanism(s) by which neurons die is still not clear. Nevertheless, apoptosis has been the focus of intense research in the last few years as a pivotal phenomenon in AD progression.

3.2.4. The role of apoptosis and cell cycle proteins in AD

First hints that apoptosis could be involved in AD came from studies performed in the mid-90's, where cultured neurons were incubated with fibrillar Aβ (Loo et al. 1993). Since then, both *in vitro* and *in vivo* models have supported apoptosis as a general mechanism of cell death in AD. Analysis of post-mortem brain tissue has also provided evidence for nuclear DNA fragmentation and activation of apoptotic mechanisms in humans. Studies in the brain of AD patients showed an upregulation of 50-fold in the levels of apoptosis, as compared with age-matched controls (Colurso et al. 2003). Nevertheless, evidence for frank cellular apoptosis in AD brains is controversial, since clear detection of apoptotic cells is difficult and problematic. In this regard, apoptosis in the pathogenesis of AD is still an issue of debate, and it has even been suggested that it provides a protective role in removing damaged cells from the brain.

Studies of human AD tissue demonstrated that the expression of the antiapoptotic Bcl-2 and proapoptotic Bax proteins are regulated in brain areas showing increased apoptosis (Su et al. 1997). In addition, c-Jun, a protein of the JNK-pathway and an immediate-early proapoptotic molecule was found to be colocalized in neurons with fragmented DNA (Anderson et al. 1996). Moreover, recent evidence suggests that mitochondrial dysfunction and ER-induced stress have pivotal roles in the execution of apoptosis in AD (Takuma et al. 2005). In fact, together with Aβ-induced toxicity, many other stimuli, such as oxidative stress, neurotrophic factor deprivation, low energy metabolism, and mitochondrial failure, known to induce apoptosis in cultured neurons, are also prominent in AD brains.

Activation of caspases-3 in AD has been reported using specific antibodies to the activated form of the enzyme (Su et al. 2001). Caspases-3 was detected in more than 50% of hippocampal neurons showing granulovascular degeneration and often colocalized with tau protein. In addition, an increase in brain fodrin proteolysis in sprouting neurons was observed (Peterson et al. 1991). Caspase-6 was also found to be activated in AD brains (Guo et al. 2004). The activation of caspase-6 occurs primarily in the frontal and temporal cortex, and is colocalized with pre-tangles, suggesting that it may be an early event in AD pathogenesis. Despite the clear activation of effector caspases, coincident morphologic evidence of apoptosis are not always easy to detect and levels of apoptosis can not *per se* explain the massive neuronal degeneration observed in AD brains. Nevertheless, since AD is a chronic disease, and the apoptotic process short-lived, the detection of substantial apoptotic neurons in AD brains could be difficult. Moreover, the apoptotic mechanisms may play an important role in disease pathogenesis, even in the absence of clear apoptosis. In fact, caspases-3 can cleave APP and presenilins, and the product of APP cleavage, a C-terminal peptide called C31, is a potent inducer of cell death (Lu et al. 2000). Importantly, caspases-3 appears to be

responsible for cleaving tau in its C-terminal region early in disease pathogenesis. In AD brains, caspases-3-cleaved tau colocalizes with both intracellular Aβ and activated caspases-3, mainly in tangle-bearing neurons (Rissman et al. 2004). Recent studies have suggested that caspases-6 is also capable of cleaving tau and promotes its aggregation in NFT (Guo et al. 2004). While caspases-6-cleaved tau was found in amyloid plaques and NFT, caspases-6 was located primarily in neurites. This suggests that apoptosis-like mechanisms can damage synapses, axons and dendrites without causing overt neuronal death.

Expression of cell cycle markers in AD neurons suggests their potential involvement in the disease process. In postmitotic neurons, which lack the required components to complete the cell cycle, the presence of mitotic markers indicates a breach of cell cycle check points. This activated but uncoordinated cell cycle activity can induce deleterious effects and result in many pathological events in AD, including apoptosis. For example, as a response to DNA damage and ROS production, cyclin D-CDK was found to be upregulated in AD (Park et al. 1998). In addition, phosphorylation and dysfunction of pRb resulting in E2F-1 release may occur in neuronal cells (Phillips and Vousden 2001).

Evidence for a pivotal function of p53 in neuronal death has been provided by data from cell culture models documenting increased p53 levels in affected neurons (Morrison et al. 2003). Coincidently, accumulation of both $A\beta_{1-42}$ and p53 was found in degenerating neurons in both transgenic mice and human AD brain. Intracellular $A\beta_{1-42}$ may cause p53-dependent neuronal apoptosis through activation of the p53-promoter, thus demonstrating an alternative pathway of cell death in AD (Ohyagi et al. 2005). The inhibition of p53 attenuates Aβ-induced apoptosis, associated with preserved mitochondrial function and reduced activation of caspases-3 (Culmsee et al. 2001). These findings suggest that p53 is an important mediator of ROS-induced signaling upstream of the mitochondrial dysfunction. Moreover, upregulation of p53 is also associated with indirect induction of tau

abnormal phosphorylation in HEK293a cells, underscoring the relevance of p53 in AD pathology (Hooper et al. 2007).

Several lines of evidence confirm the pivotal role of apoptosis in AD. Either by inducing effective neuronal death or enhancing mechanisms that culminate in neuronal dysfunction, the importance of apoptosis in the pathogenesis of AD should not be ignored. Indeed, therapeutic interventions targeting apoptosis and specific molecular intervenients, such as p53, are expected to play a role in AD treatment.

3.3. Clinical diagnosis and treatment of AD

After the first symptoms appear, the diagnosis of AD is based on the medical history, together with clinical, neurological and psychiatric examinations. Given the complexity of the disease and a possible overlapping of clinical and pathological features with other dementias, the diagnosis of AD is not always evident. Absolute diagnosis requires microscopic examination of the cerebral cortex, and the identification of amyloid plaques and NFT (McKhann et al. 1984). Cortical biopsy can provide efficient results during patient's life but is not routinely performed. Thus, a definitive diagnosis of AD is typically obtained in post mortem analysis. Neuroioimaging, including magnetic resonance imaging (MRI) and computed tomography (CT), is important to exclude alternative causes of dementia, such as brain tumor and subdural hematoma. MRI and CT are valuable tools to detect cerebral atrophy, specifically in the hippocampus and entorhinal cortex. Positron emission tomography imaging of glucose is a promising method and it can be used to differentiate with high sensitivity patients with AD from cognitively normal elderly people or with other dementias (Silverman et al. 2001). Furthermore, the identification of cerebrospinal fluid

biological markers, such as total and hyperphosphorylated tau and $A\beta_{1-42}$, for the prediction of incipient AD shows great promise (Blennow and Hampel 2003).

Unfortunately for the millions of patients that are diagnosed with AD, the efficacy of the available treatment is far from satisfactory. Currently, approved drugs can only slow progression of disease, albeit with some improvement in quality of life. They typically ameliorate the neurotransmitter disturbances that account for the characteristic memory decline and other cognitive symptoms. Acetylcholinesterase inhibitors, including donopezil, galantamine, and rivastigmine are designed to combat impairment of cholinergic neurons by slowing degradation of acetylcholine after its release at synapses. Memantine is another drug that is currently used in AD treatment, which prevents overstimulation of the *N*-methyl-D-aspartate subtype of glutamate receptors. To manage the behavioral signs, such as aggression, agitation and psychosis, many AD patients also receive antipsychotics and antidepressants.

Over the last decade, many pathways have been identified that may contribute to the pathogenesis of AD. Thus, substantial efforts are underway to translate this knowledge into novel therapeutic interventions that prevent or arrest the disease. The major focus has been to inhibit Aβ production and aggregation, and to increase Aβ clearance from the brain. Currently, inhibitors of β- and γ-secretases to reduce brain Aβ concentrations are being tested in transgenic mice as well as phase I studies (Chang et al. 2004; Siemers et al. 2005). In addition, α-secretase stimulation is also being tested for APP processing into non-amyloidogenic peptides production and consequent Aβ reduction (Etcheberrigaray et al. 2004). On the other hand, Aβ immunotherapy is being studied and has been shown to markedly reduce AD pathology (Schenk et al. 2004). A glycosaminoglycan mimetic and the metal chelator clioquinol have been shown to reduce Aβ fibrillization *in vitro* and in transgenic mice (Cherny et al. 2001; Sadowski et al. 2004). Inhibitors of tau phosphorylation are under investigation

despite the involvement of multiple kinases and phosphatases. Finally, different types of drugs and supplements, such as anti-inflammatory and cholesterol-lowering drugs, estrogens, and antioxidants have been suggested as having a protective role on AD. Nevertheless, the results are controversial and the beneficial effects have been difficult to prove.

It will probably take several decades before a cure for AD is discovered. However, we now know that for an effective treatment, this devastating disease requires a complete therapeutic intervention that targets several cellular and molecular deregulated mechanisms. Given the growing evidence that apoptosis contributes significantly to neuronal death and dysfunction in AD, a strategy of antiapoptotic therapy is expected to provide some beneficial results.

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Objectives

The research presented in this thesis was motivated by three major objectives. First, our main goal was to characterize the role of apoptosis in AD. By using *in vitro* and *in vivo* models of sporadic and familial AD, we confirmed the relevance of apoptosis in a neurodegenerative disorder that affects millions of people every year. Second, we investigated the potential antiapoptotic function of TUDCA in AD. Given its efficacy at modulating several models of non-hepatic diseases, such as neurological disorders, the role of TUDCA as modulator of apoptosis was further expanded to AD models. Finally, we intended to characterize the molecular pathways by which TUDCA inhibits apoptosis in AD. In this regard, the role of apoptosis and cell cycle-related proteins in TUDCA antiapoptotic effects has emerged as challenging targets for further therapeutic intervention.

The specific questions addressed in this thesis are:

1. Is apoptosis a relevant mechanism of cell death in AD and how can it contribute to the pathogenesis of the disease?
2. Is TUDCA capable of reducing the levels of apoptosis in several models of AD?
3. Does modulation of apoptosis and cell-cycle related proteins play a role during AD-associated apoptosis and can they be targeted for future therapeutic intervention?

Ultimately, our overarching goal is to achieve a better understanding of the pathologic function of apoptosis in AD and to characterize the antiapoptotic mechanisms of TUDCA. The identification of specific targets and modulators of the bile acid will help design effective therapeutic strategies.

2

Inhibition of the E2F-1/p53/Bax pathway by tauroursodeoxycholic acid in amyloid β-peptide-induced apoptosis in PC12 cells

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Abstract

Amyloid β-peptide (Aβ)-induced cell death may involve activation of the E2F-1 transcription factor and other cell cycle-related proteins. In previous studies, we have shown that tauroursodeoxycholic acid (TUDCA), an endogenous bile acid, modulates Aβ-induced apoptosis by interfering with crucial events of the mitochondrial pathway. In this study, we examined the role of E2F and p53 activation in the induction of apoptosis by Aβ, and investigated novel molecular targets for TUDCA. The results showed that despite Bcl-2 upregulation, PC12 neuronal cells underwent significant apoptosis after incubation with the active fragment $A\beta_{25-35}$, as assessed by DNA fragmentation, nuclear morphology and caspase-3-like activation. In addition, transcription through the E2F-1 promoter was significantly induced and associated with loss of the retinoblastoma protein. In contrast, levels of E2F-1, p53 and Bax proteins were markedly increased. Overexpression of E2F-1 in PC12 cells was sufficient to induce p53 and Bax proteins, as well as nuclear fragmentation. Notably, TUDCA modulated Aβ-induced apoptosis, E2F-1 induction, p53 stabilization, and Bax expression. Further, TUDCA protected PC12 cells against p53- and Bax-dependent apoptosis induced by E2F-1 and p53 overexpression, respectively. In conclusion, the results demonstrate that Aβ-induced apoptosis of PC12 cells proceeds through a E2F-1/p53/Bax pathway, which in turn can be specifically inhibited by TUDCA, thus underscoring its potential therapeutic use.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive memory loss and deficit of cognitive skills. The pathological hallmarks of AD include selective damage of synapses and neurons, neurofibrillary tangles, activated glia, and presence of senile plaques (Selkoe 2001). Amyloid β-peptide (Aβ) is the major constituent of senile or amyloid plaques found in the brains of AD patients. Aβ is derived from the processing of the amyloid precursor protein (Haas and Selkoe 1997), and is thought to play a critical role in the onset or progression of AD. Previous studies have shown that Aβ-induced cytotoxicity involves oxidative stress, inflammation and perturbation of calcium homeostasis (Selkoe 2001). In fact, both necrosis and apoptosis are thought to occur in primary neurons and neuronal cell lines after exposure to Aβ, as well as in brains of AD patients (Yankner et al. 1990; Loo et al. 1993; Behl et al. 1994; Su et al. 1994; Mark et al. 1995).

Cell cycle-related molecules are upregulated in post-mitotic neurons within effected brain regions during AD (Mcshea et al. 1997; Vincent et al. 1997; Busser et al. 1998). However, it is unclear whether deregulation of cell cycle events contributes to neurodegeneration in AD. E2F-1 is the best-characterized member of the E2F family of transcription factors that regulates genes involved in cell cycle, proliferation and apoptosis (Phillips and Vousden 2001). Under certain stress conditions and during the cell cycle, E2F-1 is released from the retinoblastoma protein (pRb) (Mittnacht 1998), thus transactivating its target genes. Interestingly, E2F-1 expression is increased in Aβ-treated cells suggesting that this transcription factor may also promote neuronal apoptosis via E2F-1 transcriptional activation (Giovanni et al. 1999; Giovanni et al. 2000; Hou et al. 2000). Activation of E2F-1 induces cells to undergo apoptosis that may occur

through stabilization of the tumor suppressor protein p53 via the transcription of $p14^{ARF}$, transcriptional activation of the p53 homologue p73, and inhibition of the anti-apoptotic signaling of nuclear factor κB (Phillips and Vousden 2001).

Although the role of p53 in suppressing cell cycle progression has been extensively described, less is known about the mechanism by which p53 induces apoptosis. Nevertheless, both E2F-1 and p53 can upregulate apoptotic proteins such as Bax and the apoptosis protease-activating factor 1 (Apaf-1), resulting in caspase activation and death in several cell types, including neuronal cells (Miyashita et al. 1994; Moroni et al. 2001; O´Hare et al. 2000; Fortin et al. 2001). The involvement of p53 in Aβ-induced apoptosis was initially thought to be negligible (Blasko et al. 2000). However, it has recently been demonstrated that p53 participates in apoptosis of primary human neurons triggered by Aβ peptide, probably through modulation of Bax expression (Zhang et al. 2002). During apoptosis, cytosolic Bax is translocated to the mitochondrial membrane where it induces cytochrome *c* release. Once in the cytosol, cytochrome *c* is a coactivator of Apaf-1 in the cleavage of procaspase-9 and execution of apoptosis through the mitochondrial pathway (Green 2000). Exposure of cells to Aβ peptide has been shown to result in mitochondrial perturbation and subsequent caspase activation (Paradis et al. 1996; Selznick et al. 2000; Xu et al. 2001; Luo et al. 2002; Solá et al. 2003). In addition, we have reported that Aβ induces cytochrome *c* release via direct mitochondrial membrane permeabilization (Rodrigues et al. 2000a), which appears to be associated with profound changes on membrane lipid and protein structure (Rodrigues et al. 2001).

Ursodeoxycholic acid (UDCA) and its taurine-conjugated derivative TUDCA are endogenous bile acids that increase the apoptotic threshold in several cell types (Rodrigues et al. 1998). We have previously shown that TUDCA stabilizes the mitochondrial membrane and prevents Aβ-induced apoptosis in primary rat neurons (Solá et al. 2003). TUDCA acts by inhibiting mitochondrial

membrane depolarization and channel formation, production of reactive oxygen species, release of cytochrome *c*, and caspase activation (Rodrigues et al. 2000b; Rodrigues et al. 2003a). Interestingly, we have also demonstrated that TUDCA inhibits E2F-1-induced apoptosis, in part, through a caspase-independent mechanism (Solá et al. 2003). Finally, TUDCA is neuroprotective in a transgenic mouse model of Huntington's disease (Keene et al. 2002), and in rat models of ischemic and hemorrhagic stroke (Rodrigues et al. 2002; Rodrigues et al. 2003b). Here, we further characterized the anti-apoptotic effects of TUDCA in Aβ-induced death of PC12 neuronal cells. Our results suggest that TUDCA specifically inhibits the activation of E2F-1, p53 and Bax triggered by Aβ peptide, thus modulating the apoptotic threshold.

Material and methods

Cell culture and induction of apoptosis

PC12 cells were grown in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% heat-inactivated horse serum (Sigma Chemical Co.), 5% fetal bovine serum (Invitrogen Corp., Grand Island, NY, USA) and 1% penicillin/streptomycin, and maintained at 37ºC in a humidified atmosphere of 5% CO_2. Cells were plated at either 2 x 10^5 cells/cm^2 for morphologic assessment of apoptosis and viability assays, or 4 x 10^5 cells/cm^2 for transfection assays and protein extraction, and differentiated in the presence of nerve growth factor as described previously (Park et al. 1998). PC12 neuronal cells were then incubated in medium supplemented with 100 μM TUDCA (Sigma Chemical Co.), or no addition (control) for 12 h, and exposed to 25 μM $A\beta_{25-35}$ peptide active fragment (Bachem AG, Bubendorf, Switzerland) for 1, 8, 24, and 48 h. Cells were fixed for microscopic assessment of apoptosis or processed for cell viability assays. In

addition, total and cytosolic proteins were extracted for immunoblotting and caspase activity.

Evaluation of apoptosis and caspase activation

Viability of PC12 neuronal cells was analyzed by the metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) bromide (Sigma Chemical Co.), and by trypan blue dye exclusion. Hoechst labeling of cells was used to detect apoptotic nuclei. In brief, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 10 min at room temperature, incubated with Hoechst dye 33258 (Sigma Chemical Co.) at 5 mg/ml in PBS for 5 min, and then washed with PBS and mounted using PBS:glycerol (3:1, v/v). Fluorescent nuclei were scored blindly by laboratory personnel and categorized according to the condensation and staining characteristics of chromatin. Normal nuclei showed non-condensed chromatin dispersed over the entire nucleus. Apoptotic nuclei were identified by condensed chromatin, contiguous to the nuclear membrane, as well as nuclear fragmentation of condensed chromatin. Three random microscopic fields per sample of ~ 150 nuclei were counted and mean values expressed as the percentage of apoptotic nuclei. In addition, for the terminal transferase-mediated dUTP-digoxigenin nick end-labeling (TUNEL) assay, cells were fixed in 4% paraformaldehyde in PBS, pH 7.4, for 10 min at room temperature, and post-fixed in precooled ethanol:acetic acid (2:1, v/v) for 5 min at -20ºC. Digoxigenin-nucleotide residues were added to 3'-OH ends of double or single-stranded DNA by the terminal deoxynucleotidyl transferase. Reactions were performed according to the manufacturer's recommendations (Serologicals Corp., Norcross, GA, USA), and the specimens were then coverslipped with mounting medium before analysis by phase-contrast microscopy.

Finally, caspase activity was determined in cytosolic protein extracts after harvesting and homogenization of cells in isolation buffer, containing 10 mM Tris-HCl buffer, pH 7.6, 5 mM $MgCl_2$, 1.5 mM KAc, 2 mM DTT, and protease inhibitor cocktail tablets (Complete^{TM}; Roche Applied Science, Mannheim, Germany). General caspase-3-like activity was determined by enzymatic cleavage of chromophore *p*-nitroanilide (pNA) from the substrate *N*-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA; Sigma Chemical Co.). The proteolytic reaction was carried out in isolation buffer containing 50 µg cytosolic protein and 50 µM DEVD-pNA. The reaction mixtures were incubated at 37°C for 1 h, and the formation of pNA was measured at 405 nm using a 96-well plate reader.

Transfections and CAT assays

Transfections were performed using reporter constructs E2F-1CAT and 4xE2FCAT, and four expression constructs, pCMVE2F-1, pCMVE2F-1Δ53, pCMVp53 and pCMVp53(143Ala). E2F-1CAT consisted of the entire human E2F-1 promoter fused to the chloramphenicol acetyltransferase (CAT) gene (Johnson et al. 1993); and 4xE2FCAT was generated by insertion of a synthetic promoter containing four E2F consensus binding sites (Ohtani and Nevins 1994) upstream of the CAT reporter gene. Overexpression plasmids were generated by cloning either wild type E2F-1 (pCMVE2F-1) and p53 (pCMVp53) or mutant E2F-1 (pCMVE2F-1Δ53) and p53 (pCMVp53(143Ala)), all under CMV enhancer/promoter control (Qin et al. 1992). PC12 cells at 40% confluence were transfected with 4 and 8 µg of reporter and expression plasmids, respectively, using Lipofectamine^{TM} 2000 (Invitrogen Corp.). To assess transfection efficiency, cells were cotransfected with the luciferase construct, PGL3-Control vector (Promega Corp., Madison, WI, USA). Based on this reporter, transfection efficiencies were ~ 70% and did not differ between wild-type and dominant negative plasmids. Twelve hours after E2F-1CAT or 4xE2FCAT transfection,

vehicle or 100 μM of TUDCA was added to cells. After an additional 12 h, 25 μM Aβ was included in the cultures. The cells were incubated with Aβ for 24 h after which all cells were harvested for CAT ELISA (Roche Applied Science) and luciferase assays (Promega Corp.), according to the manufacturers' instructions. Twelve hours prior to transfection with the expression plasmids, PC12 neuronal cells were treated with vehicle or 100 μM of TUDCA. At 48 and 60 h post-transfection, all cells were harvested, and total protein extracts analyzed for p53 or Bax expression, respectively. In parallel experiments, cells were also fixed for morphologic detection of apoptosis.

Immunoblotting

Steady-state levels of E2F-1, pRb, Mdm-2, p53, Bcl-2, Bax, and Bcl-x_L proteins were determined by Western blot analysis. Briefly, 150 μg of total protein extracts were separated on 8 or 12% SDS-polyacrylamide electrophoresis gels. Following electrophoretic transfer onto nitrocellulose membranes, immunoblots were incubated with 15% H_2O_2 for 15 min at room temperature. After blocking with 5% milk solution, the blots were incubated overnight at 4ºC with primary mouse monoclonal antibodies reactive to E2F-1, Mdm-2, p53, Bax and Bcl-2, or primary rabbit polyclonal antibodies to pRb and Bcl-$x_{S/L}$ (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and finally with secondary antibodies conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA, USA) for 3 h at room temperature. The membranes were processed for protein detection using Super Signal™ substrate (Pierce, Rockford, IL, USA). β-actin was used as a loading control. Protein concentrations were determined using the Bio-Rad protein assay kit according to the manufacturer's specifications.

Densitometry and statistical analysis

The relative intensities of protein bands were analyzed using the ImageMaster 1D Elite v4.00 densitometric analysis program (Amersham Biosciences, Piscataway, NJ, USA). All data were expressed as mean ± SEM from at least three separate experiments. Statistical analysis was performed using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA) for the Student's *t* test. Values of $p < 0.05$ were considered significant.

Results

TUDCA inhibits Aβ-induced apoptosis in PC12 cells

Numerous studies have shown that both $A\beta_{1-40}$ or $A\beta_{1-42}$ and its active fragment $A\beta_{25-35}$ are highly toxic to primary neurons and a variety of neuronal cell lines. In addition, TUDCA prevents cell death caused by apoptotic stimuli in a multiplicity of cell types (Solá et al. 2003; Rodrigues et al. 1998; Rodrigues et al. 2000b), suggesting that it could also modulate Aβ toxicity in a neuronal cell line. We used an *in vitro* model of Aβ-induced death of neuronal PC12 cells which has provided potentially valuable insight into Aβ signaling events. Apoptosis was assessed by changes in nuclear and DNA fragmentation, and by caspase-3-like activation (Fig. 1). Increased levels of apoptosis were observed in PC12 cells after incubation with active fragment $A\beta_{25-35}$, with a maximum apoptotic response at 48 h ($p < 0.01$). In contrast, the control reverse peptide $A\beta_{35-25}$ was not toxic to PC12 cells (data not shown). TUDCA prevented both nuclear fragmentation and caspase-3-like activation by 60-75% ($p < 0.05$). A marked protection by TUDCA was also confirmed using cell viability assays (data not shown). Taken together, these findings indicate that PC12 neuronal cells undergo apoptosis when exposed to Aβ, which in turn is markedly prevented by TUDCA.

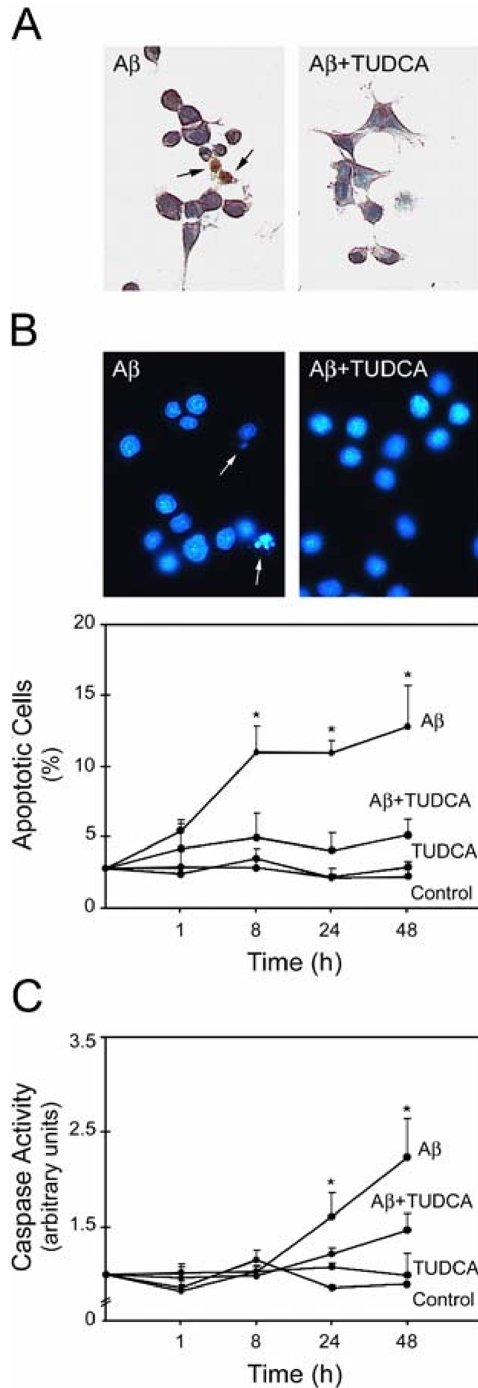

Fig. 1. TUDCA inhibits apoptosis induced by Aβ in PC12 neuronal cells. Cells were incubated with 25 μM $A\beta_{25-35}$, or no addition (control), ± 100 μM TUDCA for 1, 8, 24 and 48 h. In coincubation experiments, TUDCA was added 12 h prior to incubation with Aβ. Cells were fixed and stained for microscopic assessment of apoptosis, and cytosolic proteins were extracted for caspase activity as described in "Materials and Methods". **A:** TUNEL staining in cells incubated with Aβ ± TUDCA for 24 h. **B:** Fluorescence microscopy of Hoechst staining (*top*) and percentage of apoptosis in cells exposed to Aβ ± TUDCA at the indicated times (*bottom*). **C:** DEVD-specific caspase activity in cytosolic fractions after incubation with Aβ ± TUDCA. Cells pretreated with TUDCA showed less nuclear fragmentation and caspase activation compared to Aβ alone ($p < 0.05$). The results are expressed as mean ± SEM of at least 3 different experiments. *$p < 0.01$ from controls at the same time point.

E2F-1 expression and pRb loss are modulated by TUDCA

The E2F-1 transcription factor controls cell cycle progression as well as induction of apoptosis. It has been suggested that E2F-1 participates in Aβ-induced apoptosis of neuronal cells (Giovanni et al. 2000). In addition, TUDCA inhibited Aβ-associated apoptosis in PC12 cells, indicating that E2F-1 could be an important regulatory factor targeted by TUDCA. PC12 cells were transfected with CAT transcription reporter plasmids under the control of the E2F promoters, and incubated with Aβ for 48 h. CAT activity assays showed that Aβ-induced apoptosis was associated with a mild increase in transcription by E2F-1 at 24 h ($p < 0.01$) (Fig. 2). Pretreatment with TUDCA caused a significant decrease in Aβ-driven transcriptional activation of E2F-1 ($p < 0.05$). In contrast, CAT activity was unchanged with the 4xE2F promoter at 24 h, suggesting that Aβ specifically induces E2F-1 within the E2F family (data not shown).

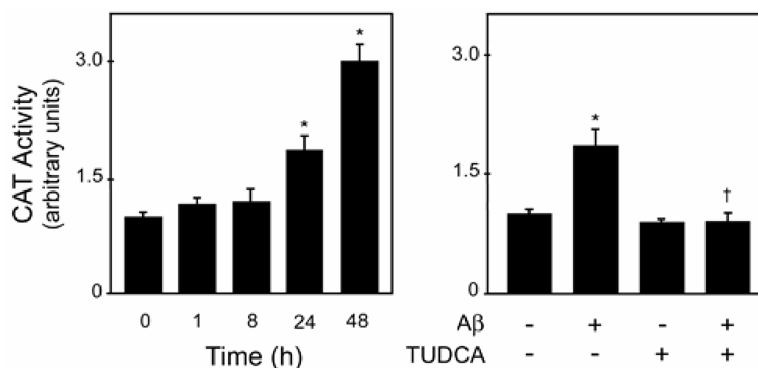

Fig. 2. TUDCA effect on E2F-1-mediated transcription in PC12 cells incubated with Aβ. Cells were cotransfected with a CAT transcription reporter plasmid under the E2F-1-dependent promoter E2F-1CAT, and a luciferase control construct as described in "Materials and Methods." After 12 h, vehicle or 100 μM of TUDCA was added to cells. At 24 h, 25 μM Aβ was included in the cultures and cells harvested for the CAT ELISA and luciferase assays. E2F-1-mediated transcription in cells exposed to Aβ for 1, 8, 24 and

48 h (*left*), or incubated with Aβ ± TUDCA for 24 h (*right*). CAT activity (absorbance/mg protein) was normalized to control luciferase expression, and the results are expressed as mean ± SEM arbitrary units of at least 3 different experiments. *$p < 0.05$ from control; †$p < 0.05$ from Aβ.

Next, we evaluated E2F-1 protein levels by immunoblot analysis to assess whether transcriptional changes were associated with altered protein expression. PC12 cells exhibited a marked increase in E2F-1 protein levels, which was significant at 1 h (2.5-fold, $p < 0.05$) through 48 h (3.5-fold, $p < 0.01$) of incubation with Aβ peptide (Fig. 3A). Notably, TUDCA reduced Aβ-induced E2F-1 upregulation by ~ 60% ($p < 0.05$) (Fig. 3B). Thus, Aβ-induced apoptosis in PC12 neuronal cells appears to involve the E2F-1 transcription factor. Interestingly, it appears that E2F-1 is required for reactivation of the cell cycle, which is a prerequisite for Aβ-induced neuronal death (Copani et al. 1999).

The retinoblastoma protein regulates both the transactivation and function of the E2F family of transcription factors. Thus, we determined whether Aβ induced E2F-1 activation by modulating pRb function. Indeed, the results showed that Aβ caused a marked loss of pRb by 30-50% throughout the time course of 48 h ($p < 0.01$) (Fig. 3A). Further, the inhibition of E2F-1 expression by TUDCA was also dependent on changes in pRb expression. TUDCA prevented Aβ-associated loss of pRb by ~ 65% at 24 h ($p < 0.05$) (Fig. 3B). Therefore, these data suggest that TUDCA reduces E2F-1 activation induced by Aβ peptide not only by directly modulating E2F-1 levels, but also by inhibiting loss of pRb.

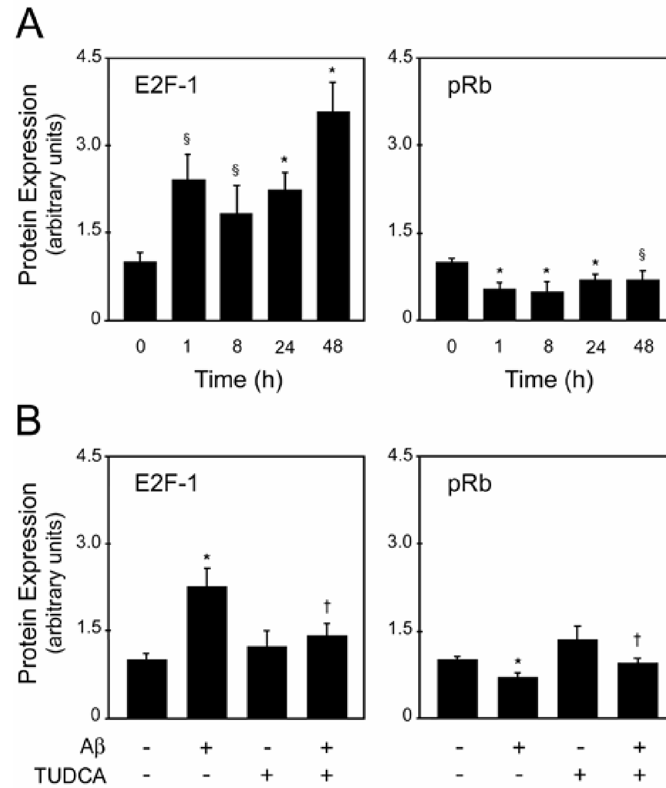

Fig. 3. Modulation of E2F-1 and pRb expression in PC12 cells. Cells were incubated with 25 μM Aβ, or no addition (control), ± 100 μM TUDCA, which was added to the incubation media 12 h prior to incubation with Aβ. Total proteins were extracted for Western blot analysis as described in "Materials and Methods". **A:** Protein levels of E2F-1 and pRb in cells incubated with Aβ for 1, 8, 24 and 48 h. **B:** Protein levels of E2F-1 and pRb in cells exposed to Aβ ± TUDCA for 24 h. The results are normalized to endogenous β-actin production and expressed as mean ± SEM arbitrary units of at least 4 different experiments. §$p < 0.05$ and *$p < 0.01$ from control; †$p < 0.05$ from Aβ.

TUDCA inhibits Aβ-induced p53 stabilization via the Mdm-2 protein

Many cellular genes contain E2F sites that contribute to transcriptional regulation. The p53/Mdm-2 pathway is a potential target of the E2F-1 transcription factor, which activates p53 through inhibition of the Mdm-2 protein. Immunoblot analysis of total protein extracts showed that Aβ induced a swift and stable increase in p53 levels up to 5-fold at 24 h ($p < 0.01$) (Fig. 4A). This was accompanied by increased activation of p53, since its inhibitor Mdm-2 was also markedly diminished during Aβ exposure (~ 60% at 8 h, $p < 0.01$). Incubation with TUDCA alone produced no significant changes in p53 and Mdm-2 protein levels (Fig. 4B). However, coincubation with TUDCA reduced the observed Aβ increase in p53 by 60%, and almost completely inhibited the decrease in Mdm-2 ($p < 0.05$).

TUDCA modulates Aβ-induced expression of Bcl-2 family proteins

p53 has been shown to regulate the expression of Bcl-2 family proteins (Miyashita et al. 1994). Our data also suggest that E2F-1-mediated p53 stabilization may modulate Bcl-2 family protein expression. Exposure to Aβ led to a prompt increase in pro-apoptotic Bax levels which was maintained throughout the time course (Fig. 5A). At 24 h of incubation, Bax protein production was enhanced by > 2-fold ($p < 0.01$). Anti-apoptotic Bcl-2 was also increased after incubation of PC12 cells with Aβ ($p < 0.05$). In contrast, the anti-apoptotic Bcl-x_L protein remained relatively unchanged. Coincubation with TUDCA reduced Aβ-associated Bax increase by ~ 80% at 24 h ($p < 0.05$) (Fig. 5B). Similarly, TUDCA prevented Bcl-2 protein changes. These results suggest that TUDCA prevents Aβ-induced changes in Bcl-2 family protein expression, restoring the equilibrium between pro- and anti-apoptotic members.

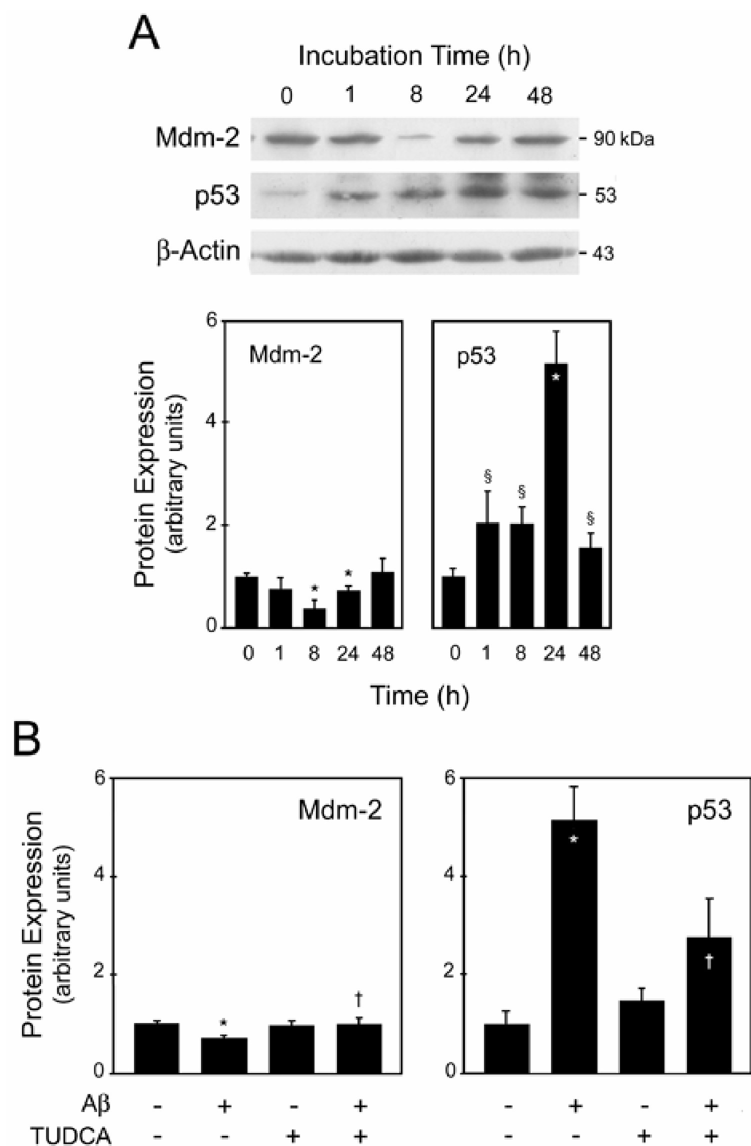

Fig. 4. Effects of TUDCA on Aβ-induced modulation of Mdm-2 and p53. PC12 neuronal cells were incubated with 25 μM Aβ, or no addition (control), ± 100 μM TUDCA. In coincubation experiments, TUDCA was added to cells 12 h prior to incubation with Aβ. Total proteins were extracted and subjected to Western blot analysis as described in "Materials and Methods". **A:** Protein levels of Mdm-2 and p53 in cells incubated with Aβ

for 1, 8, 24 and 48 h. **B:** Protein levels of Mdm-2 and p53 in cells exposed to Aβ ± TUDCA for 24 h. The results are normalized to endogenous β-actin production and expressed as mean ± SEM arbitrary units of at least 4 different experiments. §$p < 0.05$ and *$p < 0.01$ from control; †$p < 0.05$ from Aβ.

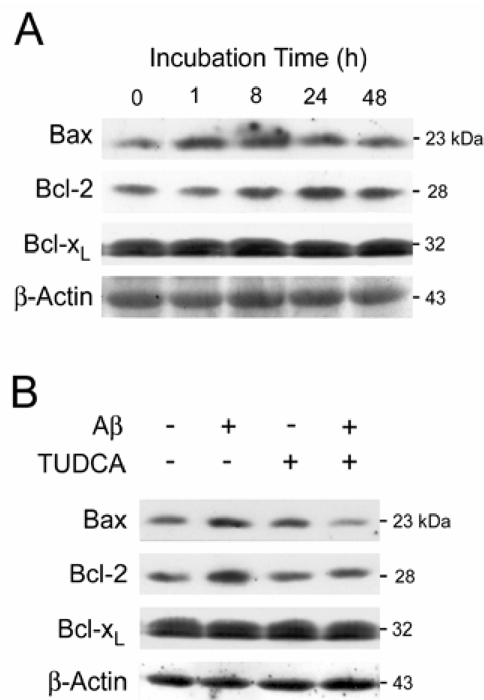

Fig. 5. Modulation of Bcl-2 family members in PC12 neuronal cells. Cells were incubated with 25 μM Aβ, or no addition (control), ± 100 μM TUDCA that was added to hepatocytes 12 h prior to incubation with Aβ. Total proteins were extracted for Western blot analysis as described in "Materials and Methods". **A:** Representative immunoblots of Bax, Bcl-2, and Bcl-x_L proteins in cells exposed to Aβ for 1, 8, 24 and 48 h. **B:** Immunoblots of Bax, Bcl-2, and Bcl-x_L proteins in cells incubated with Aβ, ± bile acids for 24 h. The blots were normalized to endogenous β-actin protein levels.

The E2F-1/p53/Bax pathway is modulated by TUDCA

To further characterize the mechanism by which TUDCA modulates the p53-regulated apoptosis pathway, we investigated its effects within the E2F-1/p53/Bax pathway. PC12 cells were transfected with plasmids to overexpress either wild-type or mutant E2F-1, or p53. In the absence of Aβ, cells transfected with wild-type E2F-1 and p53 showed ~ 40 and 65% of apoptosis, respectively, compared with only 10 and 19% of cells transfected with the corresponding mutant plasmids ($p < 0.01$) (Fig. 6A). Interestingly, TUDCA markedly reduced apoptosis after transfection with wild-type plasmids to almost control levels ($p < 0.01$).

Overexpression of E2F-1 led to an ~ 2-fold increase in p53 levels, when compared with cells expressing the E2F-1 mutant plasmid ($p < 0.05$) (Fig. 6B). Pretreatment with TUDCA completely prevented the increase in p53 induced by E2F-1 ($p < 0.01$), suggesting a direct effect at the level of E2F-1. This action was supported by a mild transcriptional repression of E2F-1 by TUDCA (data not shown). However, because overexpressed wild-type E2F-1 is regulated differently than native E2F-1, the protective effect of TUDCA could involve other mechanisms, such as pRb modulation. Cells overexpressing wild-type p53 showed a 2-fold direct increase in target protein Bax relative to cells expressing mutant p53 ($p < 0.05$). p53 overexpression was also accompanied by an increase in its degradation, detected by several intense bands with a molecular weight < 53 kDa (data not shown), which may have been because p53 induces its specific inhibitor, Mdm-2. Nevertheless, TUDCA completely prevented the p53-induced Bax expression ($p < 0.01$). Thus, our results indicate that TUDCA controls p53 function directly, and not only by interfering with upstream factors of the E2F-1/p53/Bax pathway.

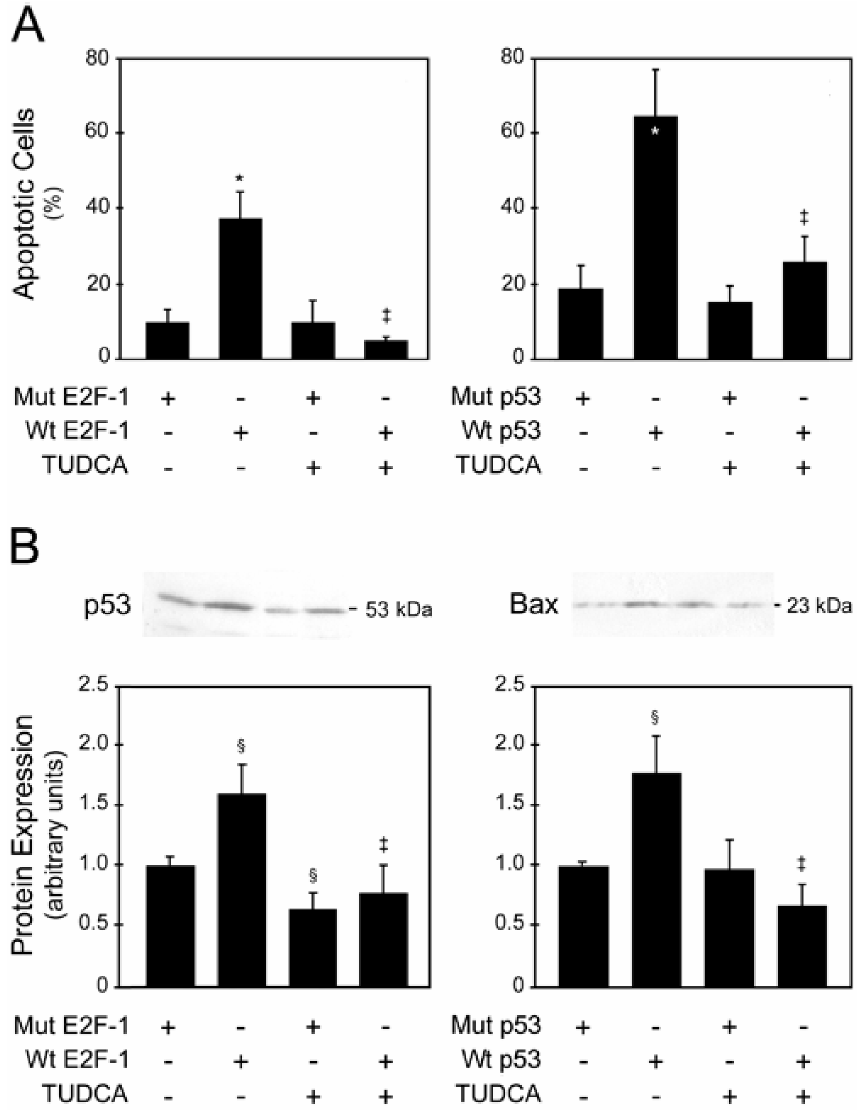

Fig. 6. TUDCA specifically inhibits the E2F-1/p53/Bax pathway. Twelve hours after incubation with 100 μM TUDCA, PC12 cells were transfected with the constructs pCMVE2F-1 and pCMVE2F-1Δ53, or with pCMVp53 and pCMVp53(143Ala) plasmids. At 48 and 60 h post-transfection, cells were fixed and stained for morphological detection of apoptosis. In addition, total proteins were extracted and subjected to Western blot

analysis of p53 and Bax as described in "Materials and Methods". **A:** Percentage of apoptosis in cells exposed to bile acids and transfected with the constructs pCMVE2F-1 and pCMVE2F-1Δ53 (*left*), or with pCMVp53 and pCMVp53(143Ala) plasmids (*right*). The results are expressed as mean ± SEM of at least 4 different experiments. **B:** p53 expression in cells transfected with the constructs pCMVE2F-1 and pCMVE2F-1Δ53 (*left*) and Bax expression in cells tranfected with pCMVp53 and pCMVp53(143Ala) plasmids (*right*). The results were normalized to luciferase expression and expressed as mean ± SEM arbitrary units relative to mutant E2F-1 or p53 of at least 3 different experiments. §$p < 0.05$ and *$p < 0.01$ from respective mutant; ‡$p < 0.01$ from respective wild-type.

Discussion

The precise molecular mechanisms responsible for AD-associated neurodegeneration are not fully understood; however, it has been proposed that Aβ peptide plays a crucial role in the pathogenesis of the disease. Aβ-induced toxicity is a multifactorial process that is thought to involve generation of reactive oxygen species, alteration of intracellular calcium homeostasis, mitochondrial perturbation, and caspase activation. We have previously reported that TUDCA prevents Aβ-induced apoptosis of cortical neurons by inhibiting the mitochondrial pathway of cell death (Solá et al. 2003). The present study provides evidence that Aβ-induced apoptosis of PC12 neuronal cells involves activation of the E2F-1/p53/Bax pathway, which in turn is significantly altered by TUDCA. Thus, our results suggest that TUDCA can interfere with alternate molecular targets.

Previous studies have reported that a variety of cyclins, cyclin-dependent kinases, and pRb are activated in neurons following exposure to Aβ (Giovanni et al. 1999) and in the brains of AD patients (McShea et al. 1997; Vincent et al. 1997; Busser et al. 1998). Thus, proteins that normally control cell cycle progression in

proliferating cells may also modulate neuronal death. Further, neurons lacking E2F-1, a transcription factor regulated by pRb, are significantly protected from death due to Aβ peptide (Giovanni et al. 2000). In the present study, we further investigated the role of E2F-1 in Aβ-induced neurotoxicity. PC12 neuronal cells treated with active fragment Aβ (25–35) showed elevated levels of apoptosis, together with a significant increase in E2F-1 promoter-driven transcription and E2F-1 protein production. The role of E2F-1 in apoptosis was also demonstrated in overexpression experiments, where a significant percentage of cells showed increased nuclear fragmentation. In addition, pRb levels were markedly decreased after Aβ exposure, probably allowing release and activation of E2F-1. This is consistent with previous findings where loss of pRb is associated with increased cell death (Liu and Kitsis 1996; Shan et al. 1996) and overexpression of pRb results in enhanced survival (Fan et al. 1996; Macleod et al. 1996; Berry et al. 1996). Finally, mice lacking pRb show massive neuronal loss during development (Jacks et al. 1992).

Activation of E2F-1 may result in modulation of proteins involved in the apoptotic process such as p53, Bax, and Apaf-1, or in the G1/S transition of the cell cycle (Moroni et al. 2001; O´Hare et al. 2000; Phillips et al. 1997). Interestingly, Aβ peptide appears to act as a proliferative signal for differentiated neurons, driving cells into the cell cycle (Copani et al. 1999). It was suggested that neurons must cross the G1/S transition before Aβ induces apoptosis. The mechanism by which Aβ promotes transcription of E2F-1 is still not clear. Nevertheless, TUDCA efficiently prevented E2F-1 upregulation and pRb loss, thus modulating activity of the transcription factor. This effect may be related to a recently described interaction between unconjugated UDCA and steroid nuclear receptors (Miura et al. 2001), linking cytoprotection to modulation of transcription and gene expression.

The tumor suppressor p53 appears to play an important role in E2F-1-associated apoptosis. Given its importance, p53 is a tightly regulated molecule, mainly at the level of protein stability. In normal cells, p53 activity is restrained through a negative feedback loop in which the tumor suppressor induces its specific inhibitor, Mdm-2, which in turn binds to p53 and targets its proteosomal degradation (Haupt et al. 1997; Kubbutat et al. 1997). However, in cells under stress, p53 is activated in part through inhibition of its degradation by Mdm-2 (Ashcroft et al. 2000). p53 stabilization can also be achieved through an E2F-1-dependent mechanism, in which E2F-1 induces the tumor suppressor protein $p14^{ARF}$ (Bates et al. 1998) that binds to Mdm-2, thus preventing p53 degradation (Kamijo et al. 1998). Interestingly, it has been recently shown that Aβ can promote p53 stability by activating the c-Jun N-terminal protein kinase 1 pathway, and increasing the levels of p53 independent of its affect on phosphorylation (Fogarty et al. 2003). Our results indicate that Aβ markedly induced E2F-1 activity, which then resulted in Mdm-2 degradation, p53 stabilization and apoptosis. In contrast, TUDCA significantly suppressed the Aβ peptide effects on p53 and Mdm-2 proteins, thereby inhibiting the Aβ-activated E2F-1/p53 pathway. Further, TUDCA inhibited E2F-1-induced p53 activation and the marked levels of apoptosis with E2F-1 overexpression. Our results suggest that TUDCA inhibition of Aβ-induced apoptosis likely involves pathways for both E2F-1 transcription factor and the tumor suppressor, pRb.

Aβ peptide appears to also modulate protein levels of Bcl-2 family members in PC12 cells, resulting in upregulation of pro-apoptotic Bax and increased expression of anti-apoptotic Bcl-2. This is not surprising since p53 is known to modulate Bcl-2 family gene expression (Miyashita et al. 1994). p53 is highly expressed in cells after accumulation of Aβ and in the brains of AD patients (Zhang et al. 2002; LaFerla et al. 1996; Kitamura et al. 1997), and p53 inhibitors can prevent neuronal cell death induced by Aβ (Culmsee et al. 2001). Further, Aβ

has been shown to either upregulate pro-apoptotic Bax expression or require Bax to mediate neurotoxicity (Paradis et al. 1996; Seznick et al. 2000; Culmsee et al. 2001). Although controversial, Bax protein levels have also been reported to increase in AD brain (MacGibbon et al. 1997; Nagy et al. 1997; Su et al. 1999). Finally, we have demonstrated that Bax protein increases in mitochondria during Aβ-induced apoptosis of neurons, thus providing a mechanism for cytochrome *c* release, and subsequent caspase-3 activation and nuclear fragmentation (Solá et al. 2003). In this study, the results of transgene overexpression suggest that upregulation of Bax was indeed a result of p53 activation. TUDCA, by decreasing E2F-1 transcriptional activation, prevented the downstream events of Aβ-induced cell death associated with Bax production. Further, TUDCA can specifically modulate the E2F-1/p53/Bax pathway, abrogating E2F-1-induced p53 and p53-associated Bax expression. Of note is that phosphorylated Bad was upregulated following Aβ incubation (data not shown), thereby possibly sequesting Bad from its mitochondrial targets, and increasing the availability of Bcl-2.

In conclusion, this study suggests that TUDCA strongly abrogates Aβ-induced apoptosis of PC12 neuronal cells. The bile acid specifically inhibited the E2F-1/p53 apoptotic pathway, thus modulating the expression of Bcl-2 family elements. The mechanism(s) by which TUDCA regulates the activation of E2F-1, or modulates sections of the E2F-1/p53/Bax pathway remains to be elucidated. Nevertheless, identification and validation of cellular targets that control life and death decisions may ultimately prove useful at developing new therapeutic interventions for diseases associated with deregulation of the apoptotic process.

Acknowledgments

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3

Tauroursodeoxycholic acid modulates p53-mediated apoptosis in Alzheimer's disease mutant neuroblastoma cells

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Abstract

Early onset familial Alzheimer's disease (FAD) is linked to autosomal dominant mutations in amyloid precursor protein (APP) and presenilin 1 and 2 (PS1, PS2) genes. These are critical mediators of total amyloid β-peptide (Aβ) production, inducing cell death through uncertain mechanisms. Tauroursodeoxycholic acid (TUDCA) modulates exogenous Aβ-induced apoptosis by interfering with E2F-1/p53/Bax. Here, we used mouse neuroblastoma cells that express either wild-type APP, APP with the Swedish mutation (APPswe), or double-mutated human APP and PS1 (APPswe/ΔE9), all exhibiting increased Aβ production and aggregation. Cell viability was decreased in APPswe and APPswe/ΔE9, but partially reversed by z-VAD.fmk. Nuclear fragmentation and caspase-2, -6, and -8 activation were also readily detected. TUDCA reduced nuclear fragmentation as well as caspase-2 and -6, but not caspase-8 activities. p53 activity, and Bcl-2 and Bax changes were also modulated by TUDCA. Overexpression of p53, but not mutant p53, in wild-type and mutant neuroblastoma cells was sufficient to induce apoptosis, which in turn was reduced by TUDCA. In addition, inhibition of the phosphatidylinositide 3'-OH kinase pathway reduced TUDCA protection against p53-induced apoptosis. In conclusion, FAD mutations are associated with the activation of classical apoptotic pathways. TUDCA reduces p53-induced apoptosis and modulates expression of Bcl-2 family.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder usually classified into sporadic, more frequent form, and familial AD (FAD), with rare gene mutations. Both sporadic and familial AD, however, present the same histopathological hallmarks, including deposition of amyloid β-peptide (Aβ), formation of neuritic plaques and neurofibrillary tangles, and loss of synapses (Masters et al. 1985; Selkoe 1990; Selkoe 1997). Aβ is derived from the processing of the amyloid precursor protein (APP), an integral membrane glycoprotein (Kang et al. 1997). A variety of APP mutations have been linked to autosomal FAD. In this regard, a double mutation at codons 670 and 671 (APPswe) has been identified in a Swedish family (Mullan et al. 1992). The β-secretase cleavage site of APP is mutated, resulting in very strong β-secretase activity and, consequently, increased Aβ production. The majority of early-onset FAD, however, is linked to mutations in presenilins genes, PS1 and PS2. Since its discovery, more than 35 different mutations have been described in the PS1 gene and all appear to be associated with high levels of Aβ production and aggregation (Schellenberg et al. 1992).

Previous studies have shown that Aβ-induced cytotoxicity involves oxidative stress, inflammation and perturbation of calcium homeostasis (Selkoe 2001). Both necrosis and apoptosis are thought to occur in primary neurons and neuronal cell lines after exposure to exogenous Aβ, as well as in brains of AD patients (Loo et al. 1993; Behl et al. 1994; Su et al. 1994). Further, cell cycle-related molecules are up-regulated in post-mitotic neurons within affected brain regions during AD (McShea et al. 1997; Vincent et al. 1997; Busser et al. 1998), but the specific role of these proteins in neurodegeneration is not clearly known. In fact, the involvement of the tumor suppressor protein p53 in Aβ-induced apoptosis was initially thought to be negligible (Blasko et al. 2000). It is now accepted that p53

can up-regulate apoptotic proteins such as Bax and the apoptosis protease-activating factor 1 (Apaf-1), resulting in caspase activation and death in several cell types, including neuronal cells (Miyashita et al. 1994; Fortin et al. 2001). In addition, we have demonstrated that p53 participates in Aβ-induced apoptosis of PC12 neuronal cells through modulation of Bax expression (Ramalho et al. 2004). Moreover, a recent study suggests a novel biological function of intracellular $A\beta_{1-42}$ as a transcription factor for the p53 promoter, enhancing p53-dependent neuronal apoptosis in AD (Ohyagi et al. 2005).

Exposure of cells to Aβ has been shown to result in mitochondrial perturbation and subsequent caspase activation (Xu et al. 2001; Luo et al. 2002; Solá et al. 2003a). On the other hand, recent studies in primary neurons suggest that activation of death receptors, such as the tumor necrosis factor type receptor 1 (TNF-R1) and Fas play a crucial role in Aβ-induced apoptosis (Li et al. 2004; Ethell and Buhler 2005). At least 7 different caspases (caspase-1, -2, -3, -6, -8, -9, and -12) have been implicated in regulating neuronal death in response to Aβ exposure *in vitro*, in animal models of neurodegenerative diseases, and in the human AD brain itself (Roth 2001). Nevertheless, despite all the data implicating caspases and apoptosis as etiologic factors in AD, the direct involvement of caspase-dependent neuronal apoptosis in AD pathogenesis remains uncertain. In essence, features of caspase-mediated neuronal apoptosis have been difficult to address *in vivo*, as well as extrapolating results from *in vitro* studies to animal models.

Ursodeoxycholic acid (UDCA) and its taurine-conjugated derivative, TUDCA, are endogenous bile acids that increase the apoptotic threshold in several cell types (Rodrigues et al. 1998; Rodrigues et al. 2000; Rodrigues et al. 2003a). Further, TUDCA inhibits E2F-1-induced apoptosis, upstream of the mitochondrial commitment, in part through a caspase-independent mechanism (Solá et al. 2003b). We have previously shown that TUDCA modulates mitochondrial function and

prevents Aβ-induced apoptosis (Ramalho et al. 2004; Solá et al. 2001a). Importantly, TUDCA was neuroprotective in a transgenic mouse model of Huntington's disease (Keene et al. 2002), reduced lesion volumes in rat models of ischemic and hemorrhagic stroke (Rodrigues et al. 2002; Rodrigues et al. 2003b), improved the survival and function of nigral transplants in a rat model of Parkinson's disease (Duan et al. 2002), and partially rescued a Parkinson's disease model of *C. elegans* from mitochondrial dysfunction (Ved et al. 2005). In addition, the subcutaneous administration of TUDCA every three days from 6 to 12 weeks of age increased significantly the levels of bile acid in the brain of transgenic mice with Huntington's disease (Keene et al. 2002).

The incubation of cells with Aβ represents a relatively inaccurate model of AD. In fact, the concentrations added to the culture medium are necessarily much higher than those found in the brains of AD patients. Therefore, it is important to carry out studies of Aβ-induced neurotoxicity under more physiological conditions that mimick the natural process of AD. Herein, we used a neuroblastoma cell model of AD, in which the cells were engineered to express wild-type APP, APP with the Swedish mutation, or the double-mutated human APP and PS1, thereby exhibiting endogenous Aβ aggregation at different levels (Thinakaran et al. 1996; Xu et al. 1997; Luo et al. 2002). Our results suggest that FAD mutations are associated with the activation of classical apoptotic pathways. In cell culture, TUDCA can interfere with the apoptotic mitochondrial pathway via p53 and inhibit the activation of caspase-2 and -6, thus modulating the apoptotic threshold.

Material and methods

Cell lines

N2a neuroblastoma control cells or the N2a cell lines with stable expression of APP695 (APPwt), Swedish mutant APP (APPswe) or the exon-9 deletion mutant

PS1 (APPswe/ΔE9) were grown in culture medium containing 45% Dulbecco's modified Eagle's medium (DMEM) and 50% OptiMEM (Invitrogen Corp., Grand Island, NY, USA), supplemented with 5% fetal bovine serum (Invitrogen Corp.), 1% L-Glutamine 200 mM (Merck & Co. Inc., NJ, USA) and 1% penicillin/streptomycin (Invitrogen Corp.). APPwt, APPswe and APPswe/ΔE9 medium was also supplemented with 0.2 μg/ml G418, and 0.4 μg/ml hygromycin for APPswe/ΔE9 only. Cells were plated at either 1 x 10^5 cells/cm^2 for morphologic assessment of apoptosis and viability assays or 2 x 10^5 cells/cm^2 for protein extraction. Neuroblastoma cells were incubated in medium supplemented with 100 μM TUDCA (Sigma Chemical Co., St. Louis, MO, USA), or no addition for 12 h. The expression of the transgenes was then induced by the addition of 1 μM butyric acid (sodium salt) (Sigma Chemical Co.) for 12 h in 1% serum medium, considered as non-toxic conditions (Kurita-Ochiai et al. 2001). In a subset of experiments, cells were incubated with 50 μM z-VAD.fmk (Sigma Chemical Co.), a general caspase inhibitor, for 1 h prior to transgene induction or with 250 μM H_2O_2 for 1 h after transgene induction. Cells were fixed for microscopic assessment of apoptosis or processed for cell viability assays. In addition, total and cytosolic proteins were extracted for immunoblotting and caspase activity assays, respectively.

Evaluation of apoptosis

Hoechst labeling of cells was used to detect apoptotic nuclei. In brief, cells were fixed in 4% paraformaldehyde in phosphate buffer (PBS), pH 7.4, for 10 min at room temperature, incubated with Hoechst dye 33258 (Sigma Chemical Co.) at 5 μg/ml in PBS for 5 min, and then washed with PBS and mounted using PBS:glycerol (3:1, v/v). Fluorescent nuclei were scored and categorized according to the condensation and staining characteristics of chromatin. Normal nuclei showed non-condensed chromatin dispersed over the entire nucleus. Apoptotic

nuclei were identified by condensed chromatin, contiguous to the nuclear membrane, as well as nuclear fragmentation of condensed chromatin. Three random microscopic fields per sample of ~ 150 nuclei were counted and mean values expressed as the percentage of apoptotic nuclei. In addition, for the terminal tranferase-mediated dUTP-digoxigenin nick end-labeling (TUNEL) assay, cells were fixed in 4% paraformaldehyde in PBS, pH 7.4, for 10 min at room temperature, and post-fixed in pre-cooled ethanol:acetic acid (2:1, v/v) for 5 min at -20ºC. Digoxigenin-nucleotide residues were added to 3'-OH ends of double- or single-stranded DNA by the terminal deoxynucleotidyl transferase. Reactions were performed according to manufacturer's recommendations (Serological Corp., Norcross, GA, USA). Finally, cell viability was also analyzed by the metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) bromide (Sigma Chemical Co.), and by trypan blue dye exclusion.

Transfections with expression plasmids

Transfections were performed using two expression constructs, pCMVp53 and pCMVp53(143Ala). Overexpression plasmids were generated by cloning either wild type p53 (pCMVp53) or mutant p53 (pCMVp53(143Ala)), both under CMV enhancer/promoter control (Qin et al. 1992). Neuroblastoma cells at 40% confluence were transfected with 4 μg of expression plasmids using Lipofectamine^{TM} 2000 (Invitrogen Corp.). To assess transfection efficiency, hepatocytes were cotransfected with the luciferase reporter construct, PGL3-Control vector (Promega Corp., Madison, WI). Based on this control, transfection efficiencies were approximately 70% and did not differ between wild-type and dominant negative plasmids. Twelve hours prior to transfection with expression plasmids, cells were treated with either vehicle or 100 μM of TUDCA. At 36 h post-transfection, cells were fixed for morphologic detection of apoptosis. In parallel experiments, wortmannin (Calbiochem, San Diego, CA, USA), an inhibitor

of phosphatidylinositide 3'-OH kinase (PI3K) phosphorylation, was added to cells 1 h prior to TUDCA at a final concentration of 200 nM.

Caspase activity assays

Caspase activity was determined in cytosolic protein extracts after harvesting and homogenization of cells in isolation buffer, containing 10 mM Tris-HCl buffer, pH 7.6, 5 mM magnesium chloride, 1.5 mM potassium acetate, 2 mM dithiothreitol, and protease inhibitor cocktail tablets (Complete^{TM}; Roche Applied Science, Mannheim, Germany). General caspase-2, -3, -6 and -8-like activities were determined by enzymatic cleavage of chromophore *p*-nitroanilide (pNA) from the substrates VDVAD-pNA, DEVD-pNA, VEID-pNA and IEPD-pNA (Sigma Chemical Co.), respectively. The proteolytic reaction was carried out in isolation buffer containing 50 μg cytosolic protein and 50 μM substrate. The reaction mixtures were incubated at 37°C for 1 h, and the formation of pNA was measured at 405 nm using a 96-well plate reader.

Immunoblot analysis

Steady-state levels of TNF-R1, Fas/CD95, p53, Bcl-2 and Bax proteins were determined by Western blot analysis. Briefly, 100 μg of total protein extracts were separated on 12% sodium dodecyl sulfate-polyacrylamide electrophoresis gels. Following electrophoretic transfer onto nitrocellulose membranes, immunoblots were incubated with 15% H_2O_2 for 15 min at room temperature. After blocking with 5% milk solution, the blots were incubated overnight at 4ºC with primary mouse monoclonal antibodies reactive to TNF-R1, Fas/CD95, p53, Bax and Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and finally with secondary antibody conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA, USA) for 3 h at room temperature. The membranes were processed for protein detection using Super Signal^{TM} substrate (Pierce, Rockford, IL, USA). β-

actin was used as a loading control. Protein concentrations were determined using the Bio-Rad protein assay kit according to the manufacturer's specifications.

Densitometry and statistical analysis

The relative intensities of protein bands were analyzed using the ImageMaster 1D Elite v4.00 densitometric analysis program (Amersham Biosciences, Piscataway, NJ, USA). All data were expressed as mean ± SEM from at least three separate experiments done on different days. Statistical analysis was performed using GraphPad InStat version 3.00 (GraphPad Software, San Diego, CA, USA) for the Student's *t* test. Values of $p < 0.05$ were considered significant.

Results

TUDCA reduces FAD-induced apoptosis

We investigated the role of apoptosis in FAD mutation-induced cytotoxicity and the protective effects of TUDCA, using cells expressing APPwt, APPswe or APPswe/ΔE9. In each case, they exhibited Aβ production and aggregation as well as cytotoxic cellular signaling pathways (Thinakaran et al. 1996; Xu et al. 1997; Luo et al. 2002). Cell viability was comparable between parental N2a cells and APPwt, APPswe and APPswe/ΔE9 cell lines prior to transgene induction (data not shown). Transgene expression resulted in at least 6-fold increased Aβ production in cells expressing FAD mutations as determined by control immunoprecipitation assays using the culture medium (data not shown). In addition, cell viability decreased by ~ 10% in APPswe and 30% in APPswe/ΔE9 compared with APPwt cells ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 1A). Pre-incubation with TUDCA and z-VAD.fmk pan-caspase inhibitor for 12 h and 1 h, respectively, reduced cell death by > 50% ($p < 0.05$) suggesting that caspase-dependent apoptosis may play a role in FAD mutation-induced cytotoxicity. In fact, nuclear fragmentation

characteristic of apoptosis was increased in APPswe and APPswe/ΔE9 cells by ~ 15 and 20%, respectively ($p < 0.01$) (Fig. 1B). As expected, apoptosis was further increased to 30% after additional oxidative stress by H_2O_2 (data not shown). TUDCA prevented morphologic changes of apoptosis in both APPswe and APPswe/ΔE9 overexpression ($p < 0.01$). These results were also confirmed using the TUNEL assay, where TUDCA was similarly protective (Fig. 1C). Finally, pre-incubation of cells with TUDCA for 1 h prior to transgene induction reduced cell death by only 6%.

In agreement with the above findings, caspase-2 and -8 activities increased to 40% in APPswe and 60% in APPswe/ΔE9 compared with APPwt cells ($p < 0.01$) (Fig. 2). Executioner caspase-6 activity was also increased in APPswe and APPswe/ΔE9 cells by 35 and 65%, respectively ($p < 0.01$). TUDCA significantly reduced caspase-2 and -6, but not caspase-8, activities. Surprisingly, caspase-3 was only marginally activated in neuroblastoma cells after transgene induction suggesting the involvement of potentially alternative pathways.

TUDCA modulates FAD-induced mitochondrial-dependent apoptosis

To provide a possible link between endogenous Aβ production, FAD mutation-induced cytotoxicity and activation of specific apoptotic pathways, we investigated expression of the death receptors TNF-R1 and Fas in neuroblastoma cells expressing APPswe or APPswe/ΔE9. FAD mutations resulted in significant up-regulation of TNF-R1 and Fas production ($p < 0.05$) (Fig. 3). However, co-incubation with TUDCA failed to modulate death receptor expression in neuroblastoma cells.

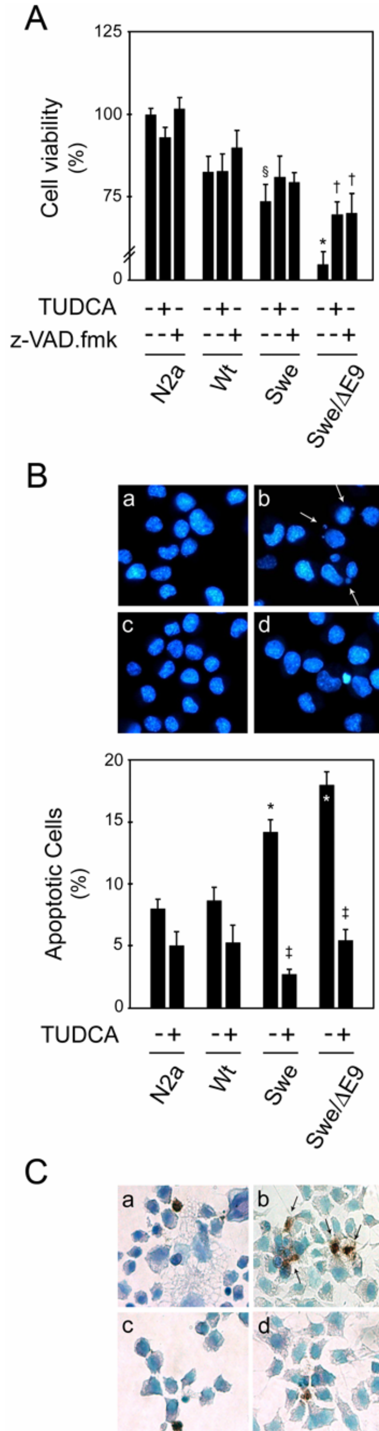

Fig. 1. TUDCA inhibits apoptosis in mutant APPswe and APPswe/ΔE9 neuroblastoma cells. Cells were treated with either no addition (control), 100 μM TUDCA for 12 h, or 50 μM z-VAD.fmk for 1 h prior to transgene induction. Cell viability was assessed by the MTT metabolism assay 12 h after initiating transgene expression. In addition, cells were fixed and stained for morphological assessment of apoptosis. **A:** MTT assay in cells after transgene induction ± TUDCA or z-VAD.fmk. **B:** Fluorescence microscopy of Hoechst staining *(top)* in APPwt (a), APPswe/ΔE9 (b), APPwt + TUDCA (c) and APPswe/ΔE9 + TUDCA (d), and percentage of apoptosis (*bottom*). Apoptotic nuclei were identified by condensed chromatin as well as nuclear fragmentation (arrows). **C:** TUNEL staining in APPwt (a), APPswe/ΔE9 (b), APPwt + TUDCA (c) and APPswe/ΔE9 + TUDCA (d) treated cells. Apoptotic cells were identified by condensed nucleus with brownish staining (arrows); normal cells were counterstained with methyl green. The results are expressed as mean ± SEM of at least 3 different experiments. *$p < 0.01$ and §$p < 0.05$ from APPwt; ‡$p < 0.01$ and †$p < 0.05$ from cells without TUDCA or z-VAD.fmk.

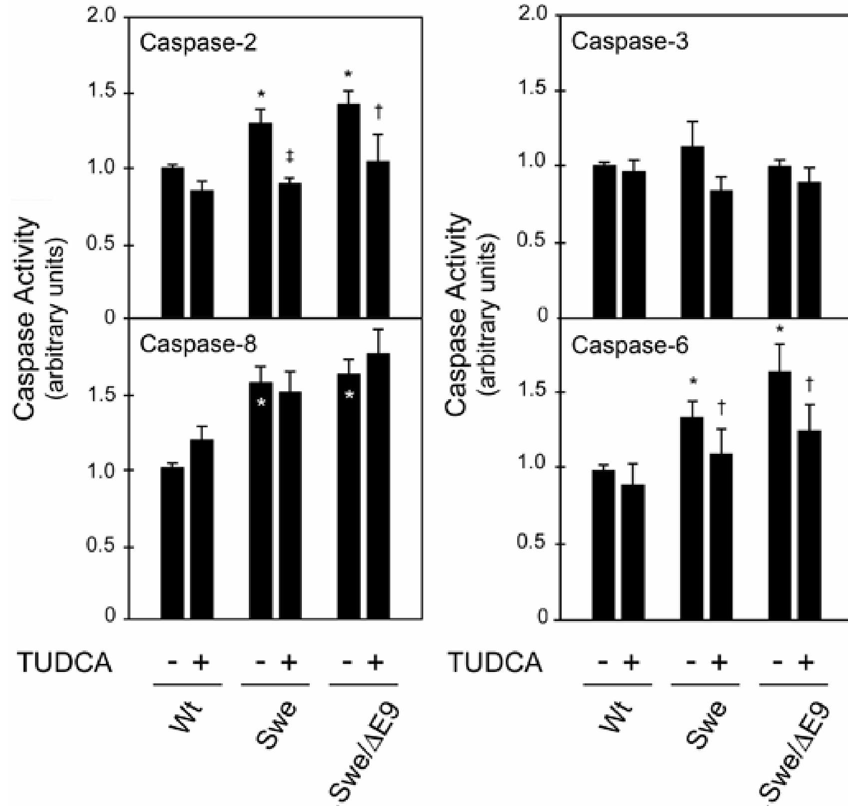

Fig. 2. TUDCA reduces caspase activity in mutant APPswe and APPswe/ΔE9 neuroblastoma cells. Cells were treated with either no addition (control) or 100 μM TUDCA for 12 h before transgene induction. Cytosolic proteins were extracted for caspase-2, -3, -6 and -8 activity assays 12 h after transgene expression. The results are expressed as mean ± SEM of at least 3 different experiments. *$p < 0.01$ from APPwt; ‡$p < 0.01$ and †$p < 0.05$ from cells without TUDCA.

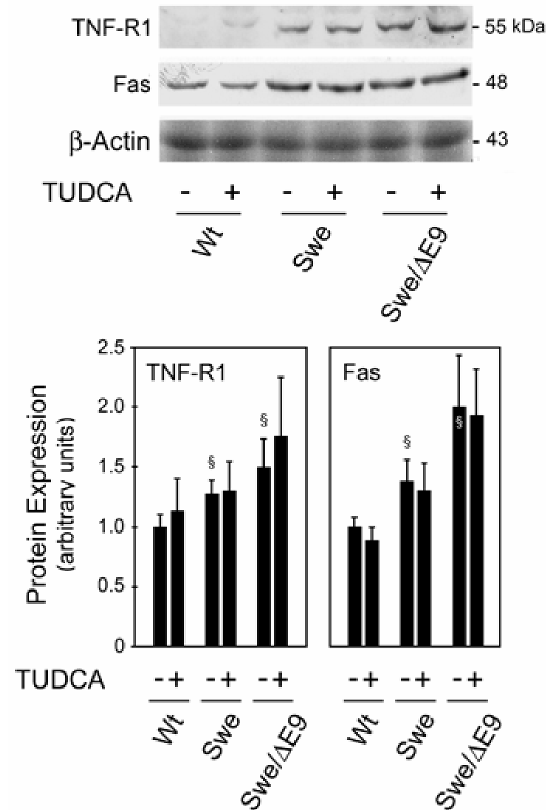

Fig. 3. Death receptor expression in mutant APPswe and APPswe/ΔE9 neuroblastoma cells. Cells were treated with either no addition (control) or 100 μM TUDCA for 12 h prior to transgene induction. Total proteins were extracted and subjected to Western blot analysis 12 h after transgene expression. Representative immunoblots of TNF-R1 and Fas, and corresponding histograms. The blots were normalized to endogenous β-actin protein levels. Results are expressed as mean ± SEM arbitrary units of at least 4 different experiments. §$p < 0.05$ from APPwt.

The transcription factor p53 is also a key modulator of cellular stress responses, and activation of p53 can trigger apoptosis in many cell types, including

neurons. Evidence for a pivotal function of p53 in neuronal death in many neurodegenerative diseases, including AD, is provided by data from both *in vitro* and *in vivo* models of increased p53 cell levels (Morrison et al. 2003). In fact, our own studies with neuronal PC12 cells indicated that exogenous Aβ markedly induced E2F-1 activity, which then resulted in Mdm-2 degradation, p53 stabilization and apoptosis (Ramalho et al. 2004). In the present study we evaluated the levels of p53 in cells expressing FAD mutations. Interestingly, p53 expression increased ~ 20% in APPswe and 50% in APPswe/ΔE9 cells ($p < 0.05$) (Fig. 4). Coincident with p53 up-regulation, anti-apoptotic Bcl-2 protein expression decreased 25-50% in APPswe and APPswe/ΔE9 cells ($p < 0.01$) (Fig. 4). Transgene expression also led to increased pro-apoptotic Bax levels in both APPswe and APPswe/ΔE9 cells ($p < 0.05$). In contrast, coincubation with TUDCA reduced p53 protein expression to control levels and modulated Bcl-2 and Bax protein changes. These results suggested that TUDCA prevents neuronal apoptosis associated with FAD mutations by interfering with mitochondrial-mediated cell death, perhaps through modulation of p53 and its downstream targets.

To further characterize the mechanism by which TUDCA modulates p53-regulated mitochondrial apoptosis, we investigated its specific effects on p53-induced cell death. Neuroblastoma cells were transfected with plasmids to overexpress either wild-type or mutant p53. In the absence of transgene expression, neuroblastoma cells transfected with wild-type p53 showed morphologic signs of apoptosis after Hoechst staining in 15-20% of cells, compared with only 3-5% in cells transfected with the mutant plasmid ($p < 0.01$) (Fig. 5).

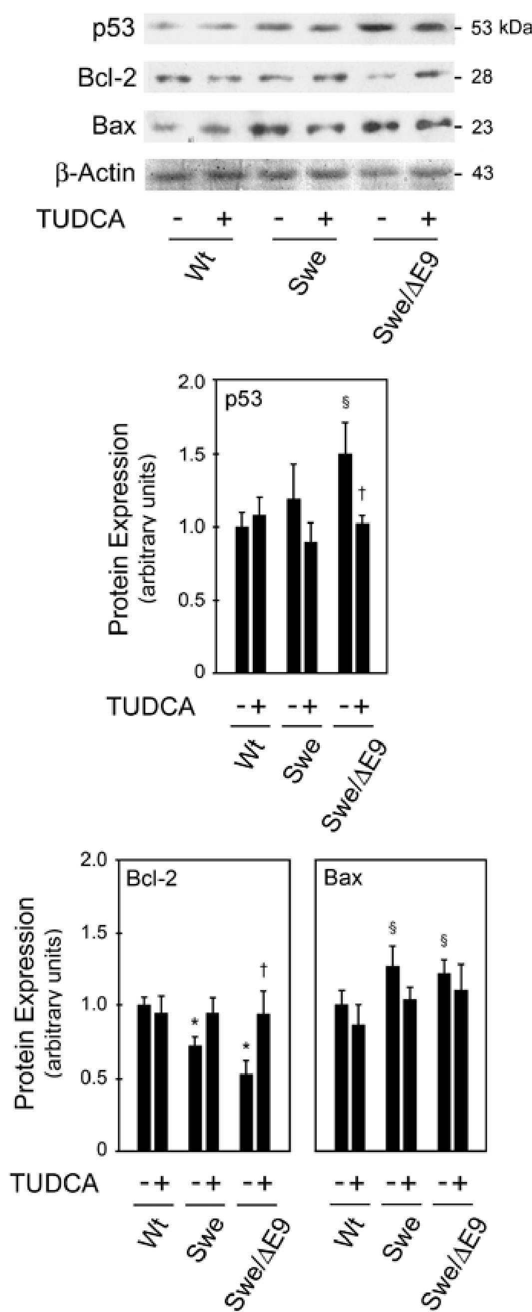

Fig. 4. Modulation of p53 and Bcl-2 family members in mutant APPswe and APPswe/ΔE9 neuroblastoma cells. Cells were treated with either no addition (control) or 100 μM TUDCA for 12 h before transgene induction. Total proteins were extracted and subjected to Western blot analysis 12 h after transgene expression. Representative immunoblots of p53, Bcl-2 and Bax, and corresponding histograms are shown. The blots were normalized to endogenous β-actin protein levels. Results are expressed as mean ± SEM arbitrary units of at least 4 different experiments. *$p < 0.01$ and §$p < 0.05$ from APPwt; †$p < 0.05$ from cells without TUDCA.

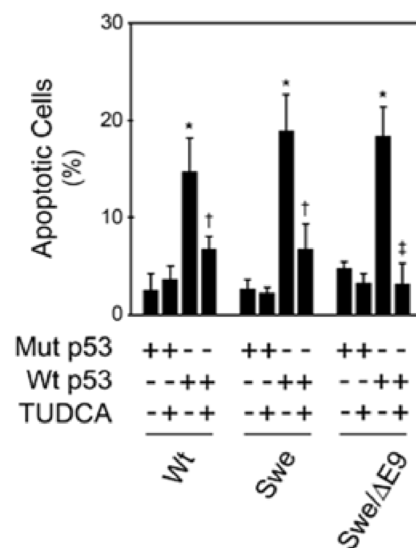

Fig. 5. Modulation of p53 and Bcl-2 family members in mutant APPswe and APPswe/ΔE9 neuroblastoma cells. Cells were treated with either no addition (control) or 100 μM TUDCA for 12 h before transgene induction. Total proteins were extracted and subjected to Western blot analysis 12 h after transgene expression. Representative immunoblots of p53, Bcl-2 and Bax, and corresponding histograms are shown. The blots were normalized to endogenous β-actin protein levels. Results are expressed as mean ± SEM arbitrary units of at least 4 different experiments. *$p < 0.01$ and §$p < 0.05$ from APPwt; †$p < 0.05$ from cells without TUDCA.

Interestingly, TUDCA markedly reduced apoptosis after transfection with wild-type p53 to almost control levels ($p < 0.05$). In addition, we have recently shown that TUDCA can activate a PI3K survival pathway in neurons (Solá et al. 2003a). Wortmannin, a specific inhibitor of the PI3K pathway, blocked serine/threonine protein kinase Akt activation, reducing the protective effect of TUDCA against Aβ-induced cell death in neurons. Wortmannin alone was not significantly cytototoxic in APPwt cells (data not shown). Notably, the PI3K

inhibitor reduced TUDCA protection in APPwt cells transfected with wild-type p53 by almost 100% ($p < 0.01$). These results suggest that TUDCA interferes with the activation of p53-related apoptotic events in a PI3K-dependent manner.

Discussion

The role of Aβ in the pathogenesis and progression of AD-associated neurodegeneration has not been firmly established. Aβ-induced toxicity is a multifactorial process that is thought to involve the generation of reactive oxygen species, alteration of intracellular calcium homeostasis, mitochondrial perturbation, and caspase activation (Selkoe 2001). Recent studies corroborated the involvement of apoptosis by showing that Aβ alters expression of the Bcl-2 family of apoptosis-related genes (Yao et al. 2005), and that survival signaling pathways are required for protection of Aβ-mediated neuronal apoptosis (Watson and Fan 2005). In addition, we have previously reported that TUDCA prevents Aβ-induced apoptosis by inhibiting the mitochondrial pathway of cell death and regulating cell cycle-related proteins (Ramalho et al. 2004; Solá et al. 2003a). The present study shows that TUDCA inhibits p53-induced toxicity in cells expressing FAD mutations. In stably transfected neuroblastoma cells, the endogenous non-toxic bile acid modulates p53 up-regulation, expression of Bcl-2 family members, and caspase activation. These results indicate that attenuation of p53-mediated apoptosis may underlie the protective effects of TUDCA, possibly via a PI3K-dependent manner.

A major hallmark of AD is the progressive neuronal loss associated with reduced memory function. Results from histopathological studies using *post mortem* brain tissue of AD patients, as well as a variety of *in vitro* studies suggest

that Aβ induces apoptosis and that cell death plays a relevant role in AD pathogenesis (Loo et al. 2003; Su et al. 1994). In the present study, apoptosis increased in mutant APPswe and APPswe/ΔE9 overexpressing cells. Moreover, TUDCA inhibited apoptosis, confirming the importance of this bile acid in the modulation of FAD mutation-induced cell death.

Apoptosis induction is an essential function of the transcription factor p53. This protein is highly expressed in cells after accumulation of Aβ and in the brains of AD patients. Furthermore, p53 inhibitors can prevent neuronal cell death induced by Aβ (Culmsee et al. 2001). Our results indicate that FAD mutations, particularly the expression of the APPswe/ΔE9 transgene, resulted in p53 up-regulation. Moreover, overexpression of p53 in neuroblastoma cells induced apoptosis, suggesting that it may have an important role in FAD mutation-induced cell death. Interestingly, TUDCA prevented p53-induced apoptosis suggesting a specific modulation of p53 expression and/or pathways in which p53 is involved. Moreover, co-incubations with wortmannin resulted in inhibition of TUDCA protection, suggesting an involvement of the PI3K pathway.

Studies in three different neuronal cell types showed that Aβ-induced cell death requires the activation of caspase-2 (Troy et al. 2000). Caspase-2, an initiator caspase that triggers apoptosis via activation of the mitochondrial pathway, can be a target of the c-Jun N-terminal kinases signaling pathway, which is known to be involved in Aβ-induced citotoxicity (Troy et al. 2000). In addition, p53 is also known to activate caspase-2, via the PIDDosome (Tinel and Tschopp 2004). Our results showed that caspase-2 activity was increased in APPswe and APPswe/ΔE9 cells, and this was coincident with p53 up-regulation. Curiously, TUDCA prevented caspase-2 activation; it remains to be determined as to whether the effect was direct or indirect.

p53 has additional recognized apoptotic targets, including death receptors located at the plasma membrane, such as Fas (Kannan et al. 2001). Fas and TNF-

R1 expression was increased in both APPswe and APPswe/ΔE9, compared with APPwt cells. Moreover, caspase-8 was readily activated. TUDCA, however, did not prevent these modifications, suggesting that the bile acid does not modulate FAD mutation-induced apoptosis by the extrinsic pathway. Bcl-2 family members represent other well-known targets of p53 (Miyashita et al. 1994). In addition, Aβ has been shown to either up-regulate pro-apoptotic Bax expression or require Bax to mediate neurotoxicity (Paradis et al. 1996; Selznick et al. 2000). Our results demonstrated that expression of FAD mutations, Aβ production, and subsequent p53 stabilization, resulted in up-regulation of pro-apoptotic Bax and decreased expression of anti-apoptotic Bcl-2. Interestingly, TUDCA significantly tempered the observed changes in Bcl-2 and Bax protein levels, confirming our previous results in primary neurons (Solá et al. 2003a) and in PC12 neuronal cells (Ramalho et al. 2004).

Effector caspase-3 was only slightly activated in cells expressing FAD mutations. The precise role of caspase-3 in AD is not fully understood. There are several evidences that caspase-3 might be involved in the neuronal death of neurodegenerative diseases, including AD (Shimohama et al. 1999; Mashiah et al. 1998). Other results, however, suggest that caspase-3 activation is only slightly up-regulated in AD patients, and therefore can not justify the massive loss of neurons in this pathology. A study in human neurons showed that microinjection with recombinant active caspase-3 did not induce cell death (Zhang et al. 2002). In contrast, caspase-6 induced a protracted TUNEL-positive cell death. Another study failed to detect the active fragment of caspase-3 in *post mortem* tissue from AD patients but could confirm the presence of caspase-6 active protein (LeBlanc et al. 1999). In contrast with caspase-3, caspase-6 was significantly up-regulated in APPswe and APPswe/ΔE9 cells, confirming the importance of caspase-6 in AD. Caspase-6 can cleave the nuclear envelope protein lamin A, several transcription factors, poly (ADP-ribose) polymerase, and APP generating potentially

amyloidogenic fragments (Galande et al. 2001; Fernandes-Alnemri et al. 1995). Nevertheless, the precise role of caspase-6 in the pathogenesis of AD remains to be determined. In addition, although caspase-3 has been implicated, it is not clearly known how caspase-6 is activated. Curiously, a recent study identified caspase-6 as a downstream target of p53 in the apoptotic process (MacLachlan and El-Deiry 2002). This may explain the marginal activation of caspase-3 but a robust activation of caspase-6 in APPswe and APPswe/ΔE9 cells, which in turn was prevented by TUDCA.

Collectively, our studies further expand the neuroprotective effects of TUDCA. The results demonstrate that TUDCA modulates p53 stabilization and Bcl-2 family expression, reduces caspase-2 and -6 activation, and inhibits nuclear fragmentation. Thus, modulation of apoptosis by TUDCA in APPswe and APPswe/ΔE9 cells appears to result from its specific effects at preventing p53 activation. Finally, identification and validation of more cellular targets are likely to provide new therapeutic interventions for modulation of neuronal apoptotic.death in Alzheimer's disease.

Acknowledgments

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4

Apoptosis in transgenic mice expressing the P301L mutated form of human tau

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Abstract

The rTg4510 mouse is a tauopathy model, characterized by massive neurodegeneration in Alzheimer's disease (AD)-relevant cortical and limbic structures, deficits in spatial reference memory, and progression of neurofibrillary tangles. In this study, we examined the role of apoptosis in neuronal loss and associated tau pathology. The results showed that DNA fragmentation and caspase-3 activation are common in the hippocampus and frontal cortex of young rTg4510 mice. These changes were associated with cleavage of tau into smaller intermediate fragments, which persist with age. Interestingly, active caspase-3 was often co-localized with cleaved tau. *In vitro*, fibrillar $A\beta_{1-42}$ resulted in nuclear fragmentation, caspase activation, and caspase-3-induced cleavage of tau. Notably, incubation with the antiapoptotic molecule tauroursodeoxycholic acid abrogated apoptosis-mediated cleavage of tau in rat cortical neurons. In conclusion, caspase-3-cleaved intermediate tau species precede cell loss in rTg54510 brains and Aβ-exposed cultured neurons. These results underscore the potential role of apoptosis in neurodegeneration.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by accelerated neuronal death leading to dementia. Hallmark pathologic features include extracellular plaques of amyloid β (Aβ) and intracellular aggregations of tau protein (Selkoe 2001). Tau is a microtubule-associated protein, abundant in the central nervous system, and predominantly expressed in axons (Binder et al. 1985). It plays fundamental roles in both stabilizing neuronal microtubules, and establishing cellular polarity and intracellular transport (Drewes et al. 1998). Under physiological conditions, tau is a highly soluble, hydrophobic and unfolded protein, tightly regulated by phosphorylation at specific serine and threonine residues (Johnson and Stoothoff 2004). In AD and other tauopathies, tau looses its capacity to bind microtubules, migrates to the cell body, and aggregates into neurofibrillary tangles (NFT) (Mandelkow and Mandelkow 1998). Post-translational conformational changes of tau, such as abnormal hyperphosphorylation and proteolysis increase its ability to aggregate (Alonso et al. 2001, Gamblin et al. 2003a). Curiously, NFT accumulate with well defined spatial and temporal progression (Santacruz et al. 2005).

The role of apoptosis in AD and other tauopathies is still controversial. Indeed, synaptic loss and electrophysiological abnormalities typically precede cell loss. Nevertheless, apoptosis is increased (Su et al. 1994) and caspase-3 is activated (Selznick et al. 1999) in the brain of AD patients. Interestingly, tau can be cleaved by caspase-3 at Asp421 in its C-terminal region, resulting in a N-terminal product that can be detected in cultured neurons, in AD brains (Rissman et al. 2004), and in other tauopathies (Newman et al. 2005). Truncated tau plays a role in nucleation-dependent filament formation of tau and induces neuronal death. Moreover, caspase-3-cleaved tau is often colocalized with Aβ peptide deposition. Caspase-3 activation is a well established event in Aβ-induced neuronal apoptosis,

suggesting a link between amyloid plaques and NFT formation (Gamblin et al. 2003b).

The rTg4510 mouse model expresses human tau containing the P301L mutation associated with frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17). It is thought to represent a robust model of tauopathy (Santacruz et al. 2005, Ramsden et al. 2005, Spires et al. 2006). In fact, transgenic mice showed massive neurodegeneration in specific cortical and limbic structures, leading to forebrain atrophy and brain weight loss. In addition, the progression of NFT pathology and neuronal loss was also correlated with deficits in spatial reference memory. Nonetheless, the onset of memory deficits at 2.5-4 months preceded NFT pathology and neuronal loss at 5.5 months. The suppression of transgene expression, after initial NFT formation, prevented further loss of neurons and enabled partial recovery of cognitive functions, but did not inhibit the progression of NFT. These findings suggest that NFT formation is not directly responsible for neurodegeneration and memory loss in rTg4510. Instead, toxic intermediate tau species may trigger further neurodegeneration.

Ursodeoxycholic acid (UDCA) and its taurine-conjugated derivative, tauroursodeoxycholic acid (TUDCA), are endogenous bile acids that increase the apoptotic threshold in several cell types (Rodrigues et al. 2000, Rodrigues et al. 1998). We have previously shown that TUDCA stabilizes mitochondrial function and prevents Aβ-induced apoptosis in primary rat neurons and in neuronal cell lines (Solá et al. 2003, Ramalho et al. 2006, Ramalho et al. 2004). Furthermore, TUDCA was neuroprotective in a transgenic mouse model of Huntington's disease (Keene et al. 2002), reduced lesion volumes in rat models of ischemic and hemorrhagic stroke (Rodrigues et al. 2002, Rodrigues et al. 2003), improved the survival and function of nigral transplants in a rat model of Parkinson's disease (Duan et al. 2002) and partially rescued a Parkinson's disease model of *Caernohabditis elegans* from mitochondrial dysfunction (Ved et al. 2005).

Using rTg4510 mice, we investigated the role of apoptosis in neuronal loss and tau associated pathology. Our results suggest that apoptosis is an early event associated with tau cleavage in the hippocampus and the frontal cortex. Cleaved tau, in turn, appears to represent a toxic form of tau. In cultured cortical neurons, apoptosis and caspase-3 cleavage of tau induced by fibrillar $A\beta_{1-42}$ were significantly inhibited by TUDCA. Thus, caspase-3-cleaved intermediate tau species are responsible, in part, for cell loss in rTg4510 brains and Aβ-exposed cultured neurons. These results underscore the potential role of apoptosis in neurodegeneration.

Material and methods

Generation of transgenic mice

The rTg4510 is a recently developed mouse model of tauopathy in which expression of human tau, containing the frontotempotal dementia-associated P301L mutation can be suppressed with doxycycline administration (Santacruz et al. 2005). Briefly, the method for generating rTg4510 mice utilized a system of responder and activator transgenes. Mice expressing the activator transgene, consisting of a four-repeat human tau with the P301L mutation placed downstream of a tetracycline operon responsive element were successively backcrossed at least five times onto a 129S6 background strain. Responder mice, consisting of a tet-off open reading frame placed downstream of *Ca^{2+}-calmodulin kinase II* promoter elements were maintained in the FVB/N strain. From this, double transgenic mice were generated in which expression of the P301L tau expression was restricted to the forebrain structures. Mice were screened by PCR using the primer pairs 5'-GAT TAA CAG CGC ATT AGA GCT G-3' and 5´-GCA TAT GAT CAA TTC AAG GCC GAT AAG-3' for activator transgenes, and 5'-TGA ACC AGG ATG GCT GAG CC-3' and 5'-TTG TCA TCG CTT CCA GTC CCC G-3' for responder

transgenes. Tau-expressing mice and littermate control mice (lacking either the tau responder transgene or the activator transgene) between 2.5 and 8.5 months were used. Animals were sacrificed with an overdose of ketamine xylazine cocktail via an intraperitoneal cavity injection. The chest cavity was then opened by cutting through the diaphragm and rib cage laterally. The right atrium was cut to drain blood; and the left ventricle punctured for cannula placement. PBS was flushed through the circulatory system using a pressure of 60 mmHg for 3-4 min or until the right atrium was cleared. The brain was removed and rapidly frozen in dry ice. Cryostat brain sections of 10 µm were prepared for analysis of transgenic and control mice. In addition, different areas of the brain were isolated, including the olfactory bulb, frontal cortex, sensorimotor cortex, medial septal nucleus, hippocampus, and entorhinal cortex.

All animals were housed and tested accordingly to standards established by the American Association for the Accreditation of Laboratory Animal Care and Institutional Animal Care and Use Committee guidelines, with every effort made to minimize the number of animals used.

Isolation and culture of rat cortical neurons

Primary cultures of rat cortical neurons were prepared from 17- to 18-day-old fetuses of Wistar rats as previously described (Brewer et al. 1993) with minor modifications. In short, pregnant rats were ether-anesthetized and decapitated. The fetuses were collected in Hank's balanced salt solution (HBSS-1; Invitrogen, Grand Island, NY, USA) and rapidly decapitated. After removal of meninges and white matter, the brain cortex was collected in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS-2). The cortex was then mechanically fragmented, transferred to a 0.025% trypsin in HBSS-2 solution, and incubated for 15 min at 37ºC. Following trypsinization, cells were washed twice in HBSS-2 containing 10% fetal calf serum (FBS) and re-suspended in Neurobasal medium (Invitrogen),

supplemented with 0.5 mM L-glutamine, 25 µM L-glutamic acid, 2% B-27 supplement (Invitrogen), and 12 mg/mL gentamicin. Neurons were then plated on tissue culture plates, precoated with poly-D-lysine at 1 x 10^6 cells/mL, and maintained at 37ºC in a humidified atmosphere of 5% CO_2. All experiments were performed on cells cultured for 4 days in fresh medium without glutamic acid and B-27 supplement. Cells were characterized by phase contrast microscopy and indirect immunocytochemistry for neurofilaments and glial fibrillary acidic protein. Neuronal cultures were >95% pure. After 4 days in culture, isolated rat neurons were incubated with 20 µM $A\beta_{1-42}$ (Bachem AG, Budendorf, Switzerland) that had been induced to form fibrils by preincubation in culture medium. In short, 0.45 mg of $A\beta_{1-42}$ peptide was dissolved in 20 µl of DMSO and diluted to a 100-µM stock solution in medium, which was then incubated with gentle shaking at room temperature for 4 days. Fibrillar $A\beta_{1-42}$ was then diluted to 20 µM and applied to neuron cultures; 0.2% DMSO was added to control cultures. Cortical neurons were incubated with fibrillar $A\beta_{1-42}$ for 24 h, with or without 100 µM TUDCA (Sigma Chemical, St. Louis, MO, USA), or no addition. In co-incubation experiments, TUDCA was added to neurons 12 h prior to incubation with $A\beta_{1-42}$.

Evaluation of apoptosis and caspase-3 activity

DNA fragmentation in brain sections of both transgenic and control mice was detected using an ApopTag® peroxidase *in situ* apoptosis detection kit (Serologicals Corp., Norcross, GA) for transferase mediated dUTP-digoxigenin nick-end labelling (TUNEL) staining. In brief, tissue sections were fixed in 4% paraformaldehyde in PBS, pH 7.4, for 10 min at room temperature, post-fixed in precooled ethanol:acetic acid (2:1, v/v) for 5 min at -20ºC, and treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. After adding the equilibration buffer, sections were treated with terminal deoxynucleotidyltransferase (TdT) and digoxigenin-dNTPs for 60 min at 37ºC.

Specimens were then treated with anti-digoxigenin-peroxidase for 30 min at 37ºC, colorized with 3,3'-diaminobenzidine (DAB) substrate, and counterstained with 0.5% methyl green. Finally, slides were rinsed, dehydrated, and mounted. A negative control was prepared by omitting the TdT enzyme to control for non-specific incorporation of nucleotides or binding of enzyme-conjugate. The specimens were examined using a bright-field microscope (Zeiss Axioskop; Carl Zeiss GmbH, Jena, Germany) and the data expressed as the number of TUNEL-positive cells/high-power field (x400).

Cell viability of cortical neurons was assessed using trypan blue dye exclusion and confirmed by lactate dehydrogenase viability assays (Sigma-Aldrich). In addition, Hoechst labeling of cells was used to detect apoptotic nuclei. Briefly, the medium was gently removed to prevent detachment of cells. Attached neurons were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 10 min at room temperature, incubated with Hoechst dye 33258 (Sigma-Aldrich) at 5 μg/ml in PBS for 5 min, washed with PBS and mounted using PBS:glycerol (3:1, v/v). Fluorescent nuclei were scored blindly and categorized according to the condensation and staining characteristics of chromatin. Normal nuclei showed non-condensed chromatin dispersed over the entire nucleus. Apoptotic nuclei were identified by condensed chromatin, contiguous to the nuclear membrane, as well as nuclear fragmentation of condensed chromatin. Three random microscopic fields per sample of ~ 250 nuclei were counted and mean values expressed as the percentage of apoptotic nuclei.

Finally, caspase activity was determined in cytosolic protein extracts from brain tissue and cell cultures. Samples were homogenized in isolation buffer, containing 10 mM Tris-HCl buffer, pH 7.6, 5 mM $MgCl_2$, 1.5 mM KAc, 2 mM DTT, and protease inhibitor cocktail tablets (Complete™; Roche Applied Science, Mannheim, Germany). General caspase-3-like activity was determined by enzymatic cleavage of chromophore *p*-nitroanilide (pNA) from the substrate *N*-

acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA; Sigma Chemical Co.). The proteolytic reaction was carried out in isolation buffer containing 50 µg cytosolic protein and 50 µM DEVD-pNA. The reaction mixtures were incubated at 37°C for 1 h, and the formation of pNA was measured at 405 nm using a 96-well plate reader.

Immunoblotting

Steady-state levels of total tau and caspase-3-cleaved tau proteins were determined by Western blot analysis. Briefly, 50 µg of total protein extracts were separated on 12% SDS-polyacrylamide electrophoresis minigels. Following electrophoretic transfer onto nitrocellulose membranes, immunoblots were incubated with 15% H_2O_2 for 15 min at room temperature. After blocking with 5% nonfat milk solution, the blots were incubated overnight at 4ºC with primary mouse monoclonal antibodies reactive to total tau (Zymed Laboratories Inc., San Francisco, CA, USA) and to caspase-3-cleaved tau (Chemicon, Millipore, Billerica, MA, USA) and finally with a secondary antibody conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA, USA) for 3 h at room temperature. The membranes were processed for protein detection using Super Signal^{TM} substrate (Pierce, Rockford, IL, USA). β-actin was used as a loading control. Protein concentrations were determined using the Bio-Rad protein assay kit according to the manufacturer's specifications.

Immunohistochemistry

Light-level immunohistochemistry was performed in fixed brain sections to detect caspase-3-cleaved tau. Briefly, slides were soaked in 3% hydrogen peroxide, 10% methanol for 10 min, washed, and incubated in serum blocking solution (Santa Cruz Biotechnology, Santa Cruz, CA) with 0.3% Triton X-100 for 1 h. Specimens were then incubated with primary antibody overnight at 4ºC. After rinsing,

specimens were incubated with biotinylated secondary antibody and a horseradish peroxidase-streptavidin complex, for 1 h each. Tissue samples were then colorized with DAB substrate, counterstained, mounted and visualized in a bright-field microscope (Zeiss Axioscope).

Immunofluorescence was performed to investigate the co-localization of caspase-3-cleaved tau and active caspase-3 in brain tissue and cultured cells. Fixed brain sections were soaked in 0.3% hydrogen peroxide for 5 min, washed and blocked (0.3% Triton X-100, 10% donkey serum, 1% FBS) for 1 h. In addition, cortical neurons were washed with PBS, incubated for 30 sec in microtubule stabilization buffer, and fixed for 10 min in 4% paraformaldehyde at room temperature. Fixed cultured cells were incubated with 1% nonfat milk in PBS containing 1% bovine serum albumin and 1.5% Triton X-100 for 1 h at room temperature. Specimens were then incubated with both monoclonal caspase-3-cleaved tau and polyclonal caspase-3 (Santa Cruz Biotechnology) antibodies overnight at 4ºC. After rinsing, specimens were then incubated with either fluorescently labeled anti-mouse or anti-rabbit (CyTM2 and CyTM5, respectively; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 2 h at room temperature. Samples were then mounted and visualized in a MRC1000 confocal microscope (Bio-Rad, Hercules, CA, USA). Finally, the percentage of caspase-3-cleaved tau and active caspase-3-positive cortical neurons with apoptotic nuclei was determined by staining with Hoechst 33258.

Densitometry and statistical analysis

The relative intensities of protein bands were analysed using the ImageMaster 1D Elite v4.00 densitometric analysis program (Amersham Biosciences, Piscataway, NJ, USA). All data were expressed as mean ± SEM from at least three separate experiments. Statistical analysis was performed using GraphPad InStat version

3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA) for the Student's *t* test. Values of $p < 0.05$ were considered significant.

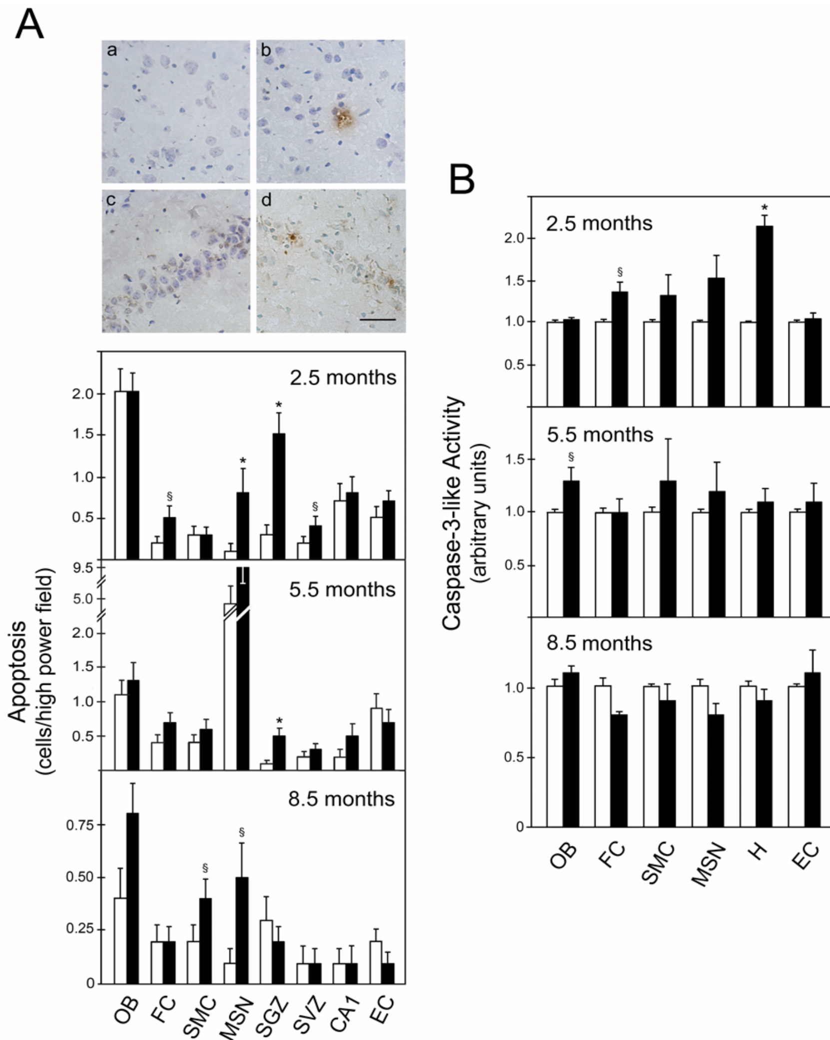
Results

TUNEL-positive cells and caspase activity in the frontal cortex and hippocampus of young rTg4510 animals

AD is a progressive neurodegenerative disease with well-defined spatial and temporal lesions that correlate with neuronal loss. The entorhinal cortex and hippocampus are particularly vulnerable (Braak and Braak 1991), while other cortical and subcortical areas are influenced as the disease progresses. In frontotemporal dementias, however, the most affected areas are the frontal and temporal cortex (Mirra and Hyman 2002). Previous studies using rTg4510 mice showed marked brain atrophy with massive neuronal loss, especially in the hippocampus (Santacruz et al. 2005). The dental gyrus showed the earliest onset of neuronal loss at 2.5 months of age (85% of total neuronal loss), followed by CA1 and CA2/3 regions at 5.5 months (69-82%), and cortex at 8.5 months (52%) (Spires et al. 2006). However, the role of apoptosis in neurodegeneration of rTg4510 mice remained unclear.

In this study, levels of apoptosis in brain slices of control and rTg4510 mice at 2.5, 5.5, and 8.5 months were evaluated using the TUNEL assay. At 2.5 months, transgenic animals showed significant levels of TUNEL-positive cells in specific regions of the brain, including the frontal cortex (FC) ($p < 0.05$), and the medial septal nucleus (MSN) ($p < 0.01$), subgranular zone (SGZ) ($p < 0.01$) and subventricular zone (SVZ) ($p < 0.05$) of the hippocampus (Fig. 1A, upper histogram). In addition, rTg4510 animals at 5.5 months showed increased apoptosis only in the MSN ($p < 0.05$) and SGZ ($p < 0.01$) (Fig. 1A, middle histogram). Finally, the number of apoptotic cells in transgenic animals at 8.5

months was similar to controls, except in the sensorimotor cortex (SMC) and MSN ($p < 0.05$) (Fig. 1A, lower histogram).



Fíg. 1. DNA fragmentation and caspase-3 activity occur early in the frontal cortex and hippocampus of rTg4510 mice. Brain slices were analyzed from both control (white bars) and transgenic animals (black bars) at 2.5, 5.5 and 8.5 months. DNA fragmentation was

assessed by the TUNEL assay, and the number of positive cells was counted in the olfactory bulb (OB), frontal cortex (FC), sensorimotor cortex (SMC), medial septal nucleus, subgranular zone (SGZ), subventricular zone (SVZ), CA1 and entorhinal cortex (EC). Cytosolic proteins were extracted for caspase-3-like activity assays from OB, FC, SMC, MSN, hippocampus (H) and EC of both control and transgenic animals at 2.5, 5.5 and 8.5 months. **A:** TUNEL staining in control FC (a); transgenic FC (b); control H (c); and transgenic H (d) (top) and number of apoptotic cells (bottom). Apoptotic nuclei were identified by a condensed nucleus with brown staining. **B:** DEVD-specific caspase activity. The results are expressed as mean ± SEM of at least 3 different experiments. $*p < 0.01$ and $§p < 0.05$ from control animals. Scale bar: 100 μm.

As a hallmark of apoptosis, caspase-3 activation was investigated in both control and transgenic animals. Consistent with TUNEL data, caspase-3-like activity was increased in FC ($p < 0.05$) and hippocampus ($p < 0.01$) at 2.5 months (Fig. 1B, upper histogram). At 5.5 months, despite the increase of TUNEL-positive cells, caspase-3 activity was not significantly altered in the hippocampus (Fig. 1B, middle histogram). Finally, no significant changes were detected at 8.5 months (Fig. 1B, lower histogram). Taken together, these findings indicate that DNA fragmentation and caspase-3 activation are early events in the frontal cortex and hippocampus of rTg4510, which precede massive neuronal loss (Santacruz et al. 2005).

Caspase-3 activation is associated with tau cleavage in rTg4510 animals

Tau protein is functionally and structurally altered in AD and other tauopathies. In fact, posttranslational changes such as abnormal phosphorylation and cleavage result in altered solubility and conformation, as well as aggregation (Alonso et al. 2001, Gamblin et al. 2003a). Next, we focused our study in the frontal cortex and hippocampus of rTg4510, which showed increased apoptosis, and investigated

patterns of tau expression with aging. Using the antibody T14 to detect total human tau, a fragment of ~ 50 kDa was evident in animals of all ages, although to a less extent in the hippocampus at 2.5 months (Fig. 2). This fragment corresponds to soluble and physiologic tau, which remains stable throughout the life of transgenic animals (Ramsden et al. 2005). A fragment of 64 kDa was only present in older animals, at 5.5 and 8.5 months, corresponding to the insoluble and pathological form of tau. Finally, small fragments of ~ 30 and 25 kDa were only present in the frontal cortex and hippocampus of younger animals. Cleaved fragments may represent pathological forms of tau and are associated with increased apoptosis. In fact, cleavage of tau at Asp421 of the C-terminal region by caspase-3 has been detected both in neurons and AD brains (Rissman et al. 2004).

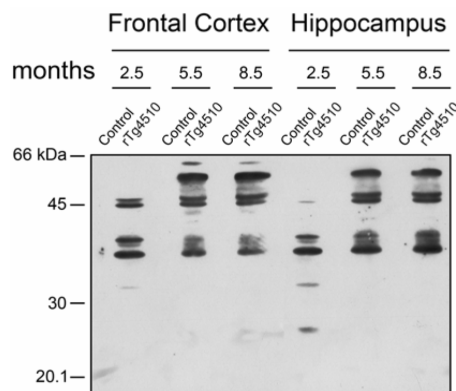


Fig. 2. Expression analysis of tau shows varying patterns with age of rTg4510 mice. Total proteins of frontal cortex and hippocampus in both control and transgenic animals at 2.5, 5.5 and 8.5 months were extracted and subjected to Western blot analysis. A representative immunoblot of total human tau is shown.

To determine if caspase-3 was responsible for cleavage of tau, we performed immunohistochemistry assays using a specific antibody against Asp421-cleaved

tau. At 2.5 months, the number of caspase-3-cleaved tau cells was significantly higher in FC and SGZ ($p < 0.01$) (Fig. 3A). The number of positive cells was also significantly higher in the olfactory bulb ($p < 0.01$), SMC ($p < 0.05$), and entorhinal cortex ($p < 0.01$) in transgenic animals. Consistently, western blot analysis showed that caspase-3-cleaved tau was already detected at 2.5 months (Fig. 3B). The ~ 50 kDa fragment was also evident in older animals, suggesting that tau persists during tangle maturation and neurodegeneration in older animals (Rissman et al. 2004, Santacruz et al. 2005, Spires et al. 2006).

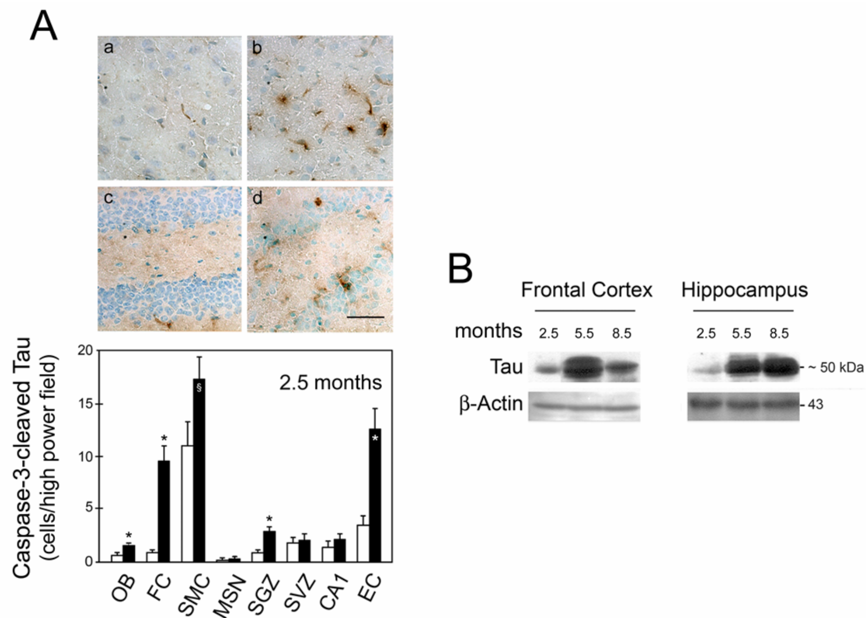


Fig. 3. Tau cleavage is an early event modulated by caspase-3 activation in rTg4510 mice. Brain slices were analyzed from both control (white bars) and transgenic animals (black bars) at 2.5 months. Caspase-3-cleaved tau cells were identified by immunohistochemistry and the number of positive cells was counted in the olfactory bulb (OB), frontal cortex (FC), sensorimotor cortex (SMC), medial septal nucleus, subgranular zone (SGZ), subventricular zone (SVZ), CA1 and entorhinal cortex (EC). Total proteins of frontal

cortex and hippocampus of both control and transgenic animals at 2.5, 5.5 and 8.5 months were extracted and analyzed by Western blot. **A:** Immunohistochemical staining in control FC (a); transgenic FC (b); control H (c); and transgenic H (d) (top) and number of caspase-3-cleaved tau positive cells (bottom). Positive cells were identified by brown staining. **B:** Representative immunoblots of caspase-3-cleaved tau. The results are expressed as mean ± SEM of at least 3 different experiments. $*p < 0.01$ and $§p < 0.05$ from control animals. Scale bar: 100 µm.

Finally, we performed co-immunohistochemistry analysis of brain slices to determine if cleaved tau co-localized with active caspase-3 in the frontal cortex and hippocampus. By confocal microscopy, the labeled cleaved tau was readily detectable (Fig. 4). Active caspase-3, however, was less obvious and showed more of a diffuse pattern. Nevertheless, cleaved tau was often co-localized with active caspase-3 in the frontal cortex and hippocampus of transgenic animals at 2.5 months. This suggests that active caspase-3 may be responsible for cleavage of tau in younger rTg4510 animals, specifically in the frontal cortex and hippocampus. Cleaved tau persists with age, possibly with a significant role in NFT formation.

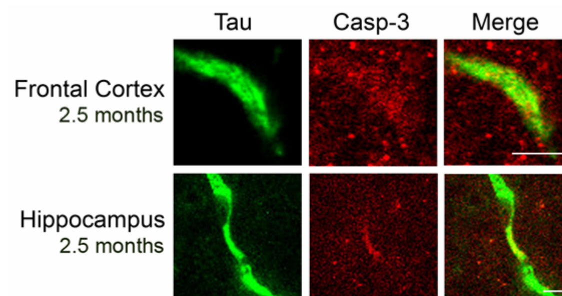


Fig. 4. Active caspase-3 is co-localized with cleaved tau in the frontal cortex and hippocampus of transgenic animals at 2.5 months. Brain slices were analyzed from both control and transgenic animals at 2.5 months. Co-localization of active caspase-3 and

caspase-3-cleaved tau was evaluated in the frontal cortex and hippocampus by fluorescent immunohistochemistry and confocal microscopy. Scale bar: 20 μm.

Modulation of Aβ-induced toxicity and cleavage of tau in cortical neurons

Amyloid plaques and NFT have been largely categorized as independent neuropathologic phenomena. In tauopathies, mutations in tau are thought to be sufficient to induce its aggregation in NFT and the consequent neuronal dysfunction, without the presence or aggregation of Aβ. However, in AD, there are no described mutations in tau and studies have suggested that tau is necessary for Aβ-induced cognitive dysfunction (Gotz et al. 2001). In fact, only a direct or indirect interaction between Aβ and tau could explain the mechanistic development of AD. A possible link would be Aβ-induced caspase-3 activation, followed by cleavage and subsequent aggregation of tau (Gamblin et al. 2003b). Thus, we examined this correlation in primary rat cortical neurons incubated with fibrillar $A\beta_{1-42}$ and investigated the potential modulation by incubation with the antiapoptotic bile acid, TUDCA. General cell death increased after fibrillar $A\beta_{1-42}$ incubation as evaluated by trypan blue dye exclusion and lactate dehydrogenase viability assays (data not shown). Nuclear fragmentation characteristic of apoptosis increased to ~ 30% ($p < 0.01$) in cells exposed to fibrillar $A\beta_{1-42}$ (Fig. 5A). In contrast, TUDCA abrogated nuclear condensation and fragmentation ($p < 0.01$). Similar results were obtained in caspase-3 activity assays ($p < 0.05$) (Fig. 5B).

Next, we investigated if $A\beta_{1-42}$-induced caspase activity was responsible for cleavage of tau. Immunofluorescence assays showed that nuclear fragmentation and active caspase-3 were often colocalized with Asp421 cleaved-tau in cortical neurons undergoing apoptosis (Fig. 6A). Interestingly, cleaved-tau was abnormally distributed within the neurons and detected in cell bodies. In addition,

immunoblotting revealed that cleaved-tau was upregulated by almost 10-fold after incubation with $A\beta_{1-42}$ ($p < 0.05$) (Fig. 6B). Notably, TUDCA reduced caspase-3-cleaved tau to control values ($p < 0.05$); and this was further confirmed by immunofluorescence. These results showed that fibrillar $A\beta_{1-42}$ decreased cell viability, and induced apoptosis with subsequent cleavage of tau, which may precede tau aggregation in NFT.

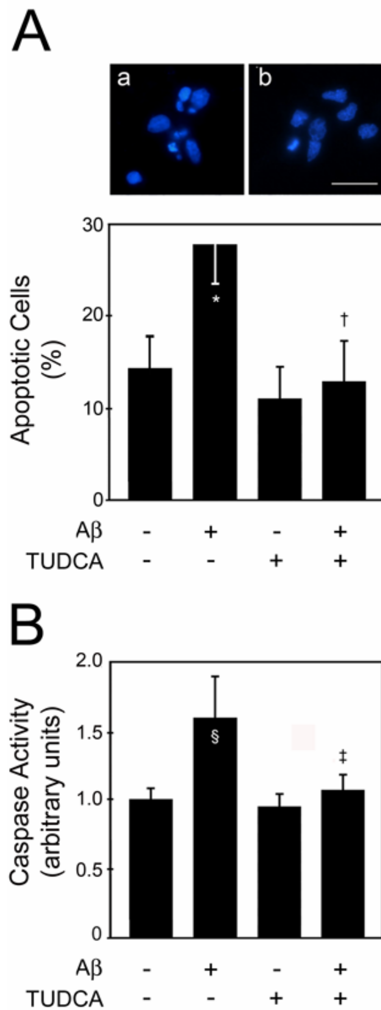


Fig. 5. TUDCA inhibits apoptosis and caspase-3 activity in primary cortical neurons incubated with fibrillar $A\beta_{1-42}$. Cells were incubated with 20 µM fibrillar $A\beta_{1-42}$, or no addition (control), ± 100 µM TUDCA for 24 h. In coincubation experiments, TUDCA was added 12 h prior to incubation with $A\beta_{1-42}$. Cells were fixed and stained for microscopy assessment of apoptosis. Cytosolic proteins were extracted for caspase-3-like activity assays. **A:** Fluorescence microscopy of Hoechst staining in controls (a); and in cells exposed to $A\beta_{1-42}$ (b) (top) and percentage of apoptosis (bottom). Apoptotic nuclei were identified by condensed chromatin as well as nuclear fragmentation. **B:** DEVD-specific caspase activity. The results are expressed as mean ± SEM of at least 3 different experiments. *$p < 0.01$ and §$p < 0.05$ from control; ‡$p < 0.01$ and †$p < 0.05$ from Aβ. Scale bar: 15 µm.

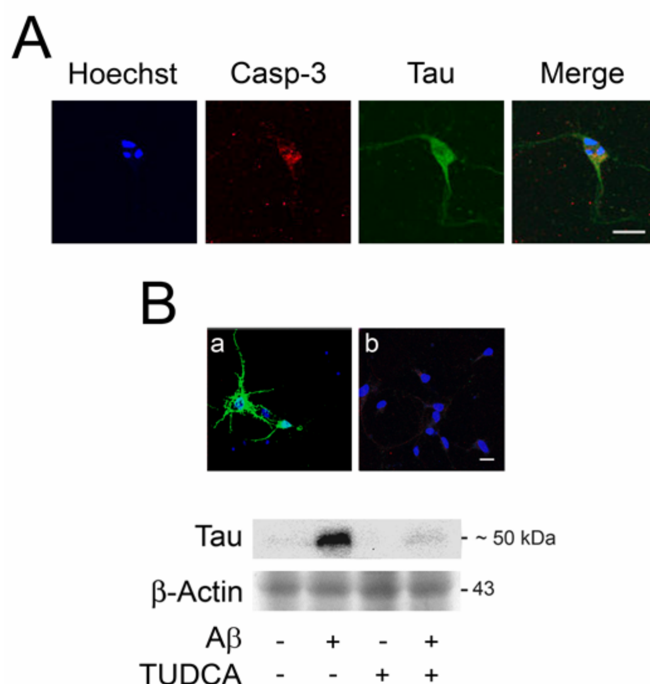


Fig. 6. TUDCA modulates cleavage of tau by caspase-3 in primary cortical neurons incubated with fibrillar $A\beta_{1\text{-}42}$. Cells were incubated with 20 μM fibrillar $A\beta_{1\text{-}42}$, or no addition (control), ± 100 μM TUDCA for 24 h. In coincubation experiments, TUDCA was added 12 h prior to incubation with $A\beta_{1\text{-}42}$. Colocalization of active caspase-3 and cleaved tau was evaluated by confocal immunofluorescence microscopy. Apoptotic nuclei were visualized with Hoechst staining. Total proteins were extracted and subjected to Western blot analysis. **A:** Confocal microscopy and colocalization of apoptotic nuclei, active caspase-3, and cleaved tau in cells exposed to $A\beta_{1\text{-}42}$. **B:** Confocal microscopy and colocalization of apoptotic nuclei and cleaved tau in cells exposed to Aβ (a) and Aβ + TUDCA (b) (top); representative immunoblot of caspase-3 cleaved tau in cells after treatment with Aβ ± TUDCA (bottom). The blot was normalized to endogenous β-actin protein levels. Scale bar: 20 μm.

Discussion

The precise molecular mechanisms involved in AD-related neuronal death and cognitive decline are not entirely understood. Aggregation of tau into insoluble NFT and formation of extracellular plaques by Aβ are the most characterized pathological features in AD. Nevertheless, the correlation between these abnormal entities and their role in neuronal death is still unclear. Implication of apoptosis as a general mechanism in many neurodegenerative disorders including AD has been supported by several studies (Fadeel and Orrenius 2005). In addition, we have previously reported that antiapoptotic treatment with TUDCA prevents Aβ-induced degeneration in neuronal cells (Solá et al. 2003, Ramalho et al. 2006, Ramalho et al. 2004). In the present study, we provide evidence that apoptosis is an early event in the frontal cortex and hippocampus of rTg4510 mice. Moreover, caspase-3 activation is associated with cleavage of tau in its C-terminal region, which precedes NFT formation. Cleaved tau represents potentially intermediate neurotoxic species that are correlated with initial neuronal dysfunction. Finally, antiapoptotic TUDCA prevents cleavage of tau from Aβ-induced apoptosis in cultured neurons, underscoring the role of modulation of apoptosis in tau cleavage.

Previous studies have shown that the rTg4510 mouse is a remarkable model of tauopathy. Tau expression induces age-dependent memory impairment, NFT formation and memory loss (Ramsden et al. 2005, Santacruz et al. 2005, Spires et al. 2006). Transgenic aged mice undergo massive neuronal loss, but the mechanisms by which neurons die are still not clear. In addition, although expressed in all forebrain structures, the transgene tau with the P301L mutation affects only specific brain regions. Neuronal loss is > 80% in hippocampal area CA1 and dentate gyrus, and ~ 50% in cortex by 8.5 months (Spires et al. 2006). These results are consistent with the regional specificity observed in tauopathies, including AD (Braak and Braak 1991).

Here, we investigated whether apoptosis is primarily involved in neuronal loss of rTg4510 mice. DNA fragmentation and caspase activation were evident in transgenic animals at 2.5 months. In fact, TUNEL-positive cells were easily detected in the frontal cortex, subgranular zone of the dentate gyrus, and subventricular zone. Interestingly, the subgranular zone gives rise to neural stem cells that ultimately differentiate into granule cell neurons. Therefore, apoptosis seen in the subgranular zone may account for the loss of granule cells. Levels of apoptosis were still slightly elevated in the subgranular zone at 5.5 months, but showed no changes at 8.5 months. Activation of caspase-3 was no longer evident at 5.5 and 8.5 months. Interestingly, apoptosis was increased in the medial septal nucleus at all ages, and in the sensorimotor cortex in older animals. We were also unable to detect significant levels of apoptosis in hippocampal CA1 of rTg4510 mice. Thus, caspase-dependent and -independent cell death is confined to specific areas, possibly signaling other brain regions.

Evidence of apoptosis in AD has been largely supported by tissue culture studies (Fadeel and Orrenius 2005). Contradictory results came from observations of human post-mortem brain tissue, where clear detection of apoptotic cells is difficult (Migheli et al. 1994). This may be explained by the fact that cell death in AD occurs over decades, while apoptosis is executed within a few hours. Thus, synchronous detection of a substantial number of apoptotic cells at any given time would be very difficult. In addition, there is strong evidence to suggest that apoptotic mechanisms may play an important role in disease pathogenesis, even in the absence of overt apoptosis (Dickson 2004). Our results suggest that apoptosis, rather than being responsible for extensive neuronal loss in older animals, triggers toxic events that ultimately contribute to cognitive deficits. In fact, activation of caspase-3 in the frontal cortex and hippocampus resulted in cleavage of tau in rTg4510 mice at 2.5 months. Neurons with active caspase-3 often showed Asp421-cleaved tau. Caspase-3-cleavage of tau is thought to induce abnormal

conformations by removing part of the C-terminal region, which is essential to inhibit tau aggregation *in vitro* (Abraha et al. 2000, Berry et al. 2003). Although early apoptosis may also represent a developmental-related phenomenon (Ramsden et al. 2005), it is clear that the cleavage of tau is a toxic process with potentially important neurodegenerative repercussions.

Importantly, the formation of NFT appears to be dissociated from neuronal loss in rTg4510 mice. It has been shown that, at 4 months of age, ~ 50% of neurons in the dentate gyrus were lost before any early conformational changes in tau were detectable (Spires et al. 2006). In addition, after transgene suppression, neuronal loss was prevented and memory decline reverted, but NFT continued to accumulate (Spires et al. 2006, Santacruz et al. 2005). Interestingly, rT4510 mice developed the first signs of cognitive decline at 2.5 months, coincident with cleavage of tau. Thus, it is possible that deficits in neurons occur in the early stages of disease, resulting from aberrant forms of tau, prior to massive neuronal loss and NFT formation. In this scenario, apoptosis and subsequent cleavage of tau are strong candidates to trigger the primary deleterious effects on neuronal function.

The identification of two forms of tau multimers with 140 and 170 kDa have recently been described in rTg4510 mice (Berger et al. 2007). The molecular weights suggest an oligomeric aggregate of the soluble and insoluble forms of tau, respectively. The aggregates accumulate early in the pathogenesis and appear to be associated with the development of functional deficits. Thus, it is tempting to speculate that cleavage of tau occurs prior to formation of the first oligomers, promoting their aggregation. Further, the fact that cleaved tau was found in older animals suggests that although cleavage may occur early in NFT formation, the resultant fragment persists throughout NFT evolution. It is possible that apoptosis continues beyond 2.5 months, and that other proteases such as calpains are also involved in tau cleavage (Park and Ferreira 2005). In this scenario, apoptosis may

represent the triggering event for the formation and aggregation of intermediate species of tau, which ultimately result in massive non-apoptotic neuronal death.

The efficacy of the rTg4510 mouse model relies on the capacity of mutated tau to induce neuronal modifications. Mutations of tau reduce its ability to interact with microtubules and are associated with tauopathies (Goedert et al. 1998). These disorders are distinct from AD because there is no evidence for Aβ, amyloid precursor protein (APP) or presenilin involvement. In AD, however, Aβ plays an important role in neuronal toxicity and cell death, and its involvement in tau pathology cannot be neglected. Indeed, both mutations of APP with Aβ deposition and intracranial injection of Aβ increased NFT formation in transgenic mice expressing an FTDP-17-causing mutant (Gotz et al. 2001, Lewis et al. 2001). Several evidences suggest that the link between Aβ and tau is predominantly, if not exclusively, unidirectional. In fact, tau has no effect on the onset and progression of Aβ accumulation (Oddo et al. 2007). Rather, tau accumulation occurs after amyloid plaques formation, further exacerbating the neurotoxicity induced by Aβ. In fact, it has been recently shown that overexpression of tau with the P301L mutation induces mitochondria dysfunction, increasing the production of reactive oxygen species and the vulnerability to Aβ insults (David et al. 2005). Moreover, reduction of endogenous tau levels proved to inhibit behavioural deficits in transgenic expressing human APP, without altering Aβ levels (Roberson et al. 2007).

Recent studies suggest that Aβ accumulation and caspase activation precedes and induces cleavage of tau and NFT formation (Gamblin et al. 2003b, Rissman et al. 2004). Our results confirmed that fibrillar $A\beta_{1-42}$ induces toxicity, nuclear fragmentation and caspase-3 activation, which was associated with cleavage of tau. Moreover, cleaved tau was often co-localized with active caspase-3 in neurons

undergoing nuclear modifications. Thus, apoptotic events and cleavage of tau occur almost simultaneously in neurons exposed to aggregated Aβ.

TUDCA prevented apoptosis and caspase-3 activation after incubations of neurons with fibrillar $A\beta_{1-42}$, thus reducing caspase-3-cleaved tau. This suggests that TUDCA may inhibit further aggregation of tau in NFT and promote cell survival. It would be interesting to investigate whether administrations of TUDCA to newborn or up to 2.5 months rTg4510 mice would prevent cleavage of tau, and subsequent massive neuronal loss, memory decline and NFT formation.

Collectively, the present study provides evidence that apoptosis may represent a crucial event in AD. Either by promoting further cell death or activating toxic effectors, apoptosis interferes with the pathogenesis of the disease. In rTg4510 mice, apoptosis is an early event in the frontal cortex and hippocampus, that preceeds NFT formation and massive neuronal death. Caspase-3-cleaved intermediate tau species were responsible for cell loss in rTg54510 brains and Aβ-exposed cultured neurons. These results underscore the importance of antiapoptotic agents in treating neurodegeneration associated with AD and other tauopathies.

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5

Concluding Remarks

The results presented in this thesis underscore the potential relevance of apoptosis in the pathogenesis of Alzheimer's disease (AD)-associated neurodegeneration in cell culture and *in vivo*. We suggest that apoptosis results in characteristic neuronal death and triggers additional neurotoxic mechanisms during neurodegeneration. Moreover, the neuroprotective role of tauroursodeoxycholic acid (TUDCA) has been extended to AD, which is considered the major cause of dementia. In fact, we have established that TUDCA inhibits amyloid β (Aβ)-induced neuronal death by modulating the transcription of genes involved in apoptosis and cell cycle. Aβ resulted in E2F-1 and p53 activation, associated with Bcl-2 family regulation, and further activation of effector caspases. TUDCA was a strong modulator of these changes. In addition, caspase-3 activation was responsible for cleavage of tau into a toxic fragment that facilitates aggregation in neurofibrillary tangles (NFT). These effects were also inhibited by TUDCA. Thus, our results further elucidate the signaling mechanisms involved in AD-associated neurodegeneration and neuroprotection by TUDCA. In this final Chapter, we integrate all findings, discuss some questions, and consider future perspectives.

Ursodeoxycholic acid (UDCA) is an endogenous bile acid used in the treatment of cholestatic liver diseases. The therapeutic effects of UDCA and its taurine-conjugated form, TUDCA, have been mainly attributed to their unique role in modulating the classical mitochondrial pathway of apoptosis (Botla et al. 1995; Rodrigues et al. 1998; Rodrigues et al. 1999). In fact, UDCA was shown to modulate mitochondrial membrane perturbation, channel formation, Bax translocation, cytochrome *c* release, caspase activation and subsequent substrate cleavage (Rodrigues et al. 1999). Importantly, UDCA appears to act in many cell types and in response to different toxic stimuli. The protective role of TUDCA has therefore been extended to non-hepatic diseases, including neurological disorders, such as Huntington's and Parkinson's diseases, as well as acute and hemorrhagic

stroke (Rodrigues et al. 2004). However, despite the increased relevance and applicability of UDCA and TUDCA, the precise mechanisms by which the bile acids initiate and transduce signals to the mitochondria remain a matter of discussion.

AD is a neurodegenerative disorder characterized by the presence of amyloid plaques and NFT, increased production of reactive oxygen species, disruption of calcium homeostasis, activation of inflammatory cytokines, and neuronal dysfunction (Selkoe 2001). Ultimately, neurons lose their ability to survive and undergo cell death in areas of the brain associated with memory and cognitive skills. However, the primary mechanism responsible for neuronal death in AD is still not clear. In the last several years, apoptosis has emerged as a key event in the pathogenesis of the disease. Its occurrence has been confirmed in AD brains (Colurso et al. 2003). Thus, the inhibition of apoptosis opens a new window of opportunities for therapeutic intervention in AD.

Aggregated and/or soluble Aβ are thought to trigger apoptosis in AD. Moreover, Aβ can also induce other cellular abnormalities, including cell cycle deregulation. In fact, in AD brains, cell cycle-related proteins are often upregulated, which may also be associated with increased levels of apoptosis (Busser et al. 1998). Using PC12 neuronal cells incubated with Aβ, we demonstrated increased DNA fragmentation, caspase-3 activation, as well as E2F-1, p53, and Bax expression. Importantly, TUDCA modulated Aβ-induced apoptosis by interfering with the E2F-1/p53/Bax pathway. These results suggest that TUDCA modulates neuronal apoptosis by both promoting mitochondrial stability, and regulating transcription of apoptosis- and cell cycle-related proteins (Fig. 1). This transcriptional function of TUDCA has already been demonstrated in DNA microarray analysis of bile acid-treated rat hepatocytes (Castro et al. 2005). Nevertheless, it remains to be determined how TUDCA regulates the transcriptional activity of p53 and E2F-1. A recent study suggested that nuclear

translocation of TUDCA, through a nuclear steroid receptor, transactivation-independent mechanism, is crucial for its antiapoptotic function in primary rat cortical neurons (Solá et al. 2006).

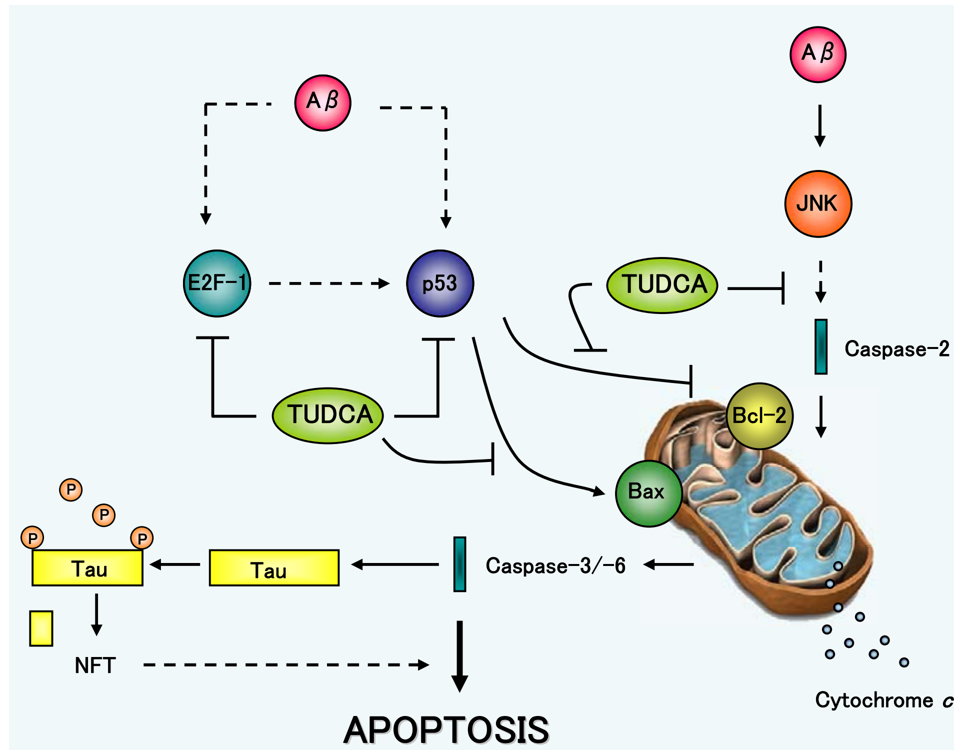

Fig. 1. Proposed model for the antiapoptotic function of TUDCA in AD. TUDCA prevents Aβ-induced mitochondrial apoptosis by modulating the expression of E2F-1, p53, and Bcl-2 family proteins, and subsequent activation of effector caspases. In addition, TUDCA prevents c-Jun N-terminal kinase-mediated caspase-2 activation, further inhibiting mitochondrial dysfunction. Finally, TUDCA promotes neuronal survival by reducing caspase-3 activation and subsequent cleavage of tau. See text for more complete description. JNK, c-Jun N-terminal kinase.

The incubation of cells with Aβ, however, is far from the ideal model of AD. In fact, the concentrations of Aβ added to culture media are usually much higher than those found in AD brains. Therefore, we pursued our studies using a model of familial AD consisting of neuroblastoma cells expressing hereditary mutations in the amyloid precursor protein (APP) and in presenilin 1. These cells exhibited endogenous Aβ aggregation, mimicking the natural process of AD. Similar to other studies, including our own, p53 played a pivotal function in neuronal death (Morrison et al. 2003). Indeed, apoptosis was associated with increased levels of p53, upregulation of Bax, and downregulation of Bcl-2. Importantly, TUDCA modulated p53 expression and Bcl-2 family changes. Moreover, we also showed that TUDCA modulated Aβ-induced neuronal apoptosis, in part, by activating a phosphatidylinositol 3-kinase (PI3K) signaling pathway. In fact, TUDCA interfered with the activation of p53-related apoptotic events in a PI3K-dependent manner, underscoring the pleiotropic effect of the bile acid.

As executioners of the apoptotic process, caspases have a pivotal role in AD-associated neuronal death. In fact, active caspase-3 and -6 have been detected in AD brains (Su et al. 2001; Guo et al. 2004). In our studies, caspase-3 was only marginally activated in the cell culture model of familial AD, while, caspase-6 was highly activated via a p53-dependent mechanism. These apparently contradictory results reflect the complexity of neuronal apoptosis in AD and underscore the importance of more accurate models of AD pathology. Interestingly, a recent study suggested that Aβ-induced cell death also requires the activation of caspase-2 (Troy et al. 200). Caspase-2 triggers apoptosis via activation of the mitochondrial pathway. It has also been described as a target of the c-Jun N-terminal kinase signaling pathway (Troy et al. 2000). Notably, TUDCA prevented caspase-2 activation in neuroblastoma cells expressing hereditary mutations associated with AD. Further investigations are warranted to elucidate the

mechanism(s) by which TUDCA interferes with this pathway linked to Aβ-induced toxicity.

The role of tau in AD and other tauopathies has recently attracted much attention. Although essential for physiological microtubule stability, tau distribution and abundance after posttranslational modifications is well correlated with neuronal degeneration and clinical symptoms in AD (Braak and Braak 1991). Using a transgenic model of tauopathy, the rTg4510 mice, with age-dependent memory impairment, NFT formation and memory loss (Santacruz et al. 2005), we further investigated the role of apoptosis in neurodegeneration. Our results showed that apoptosis is not primarily responsible for massive neuronal death. Rather, apoptosis triggers toxic mechanisms that precede NFT formation and memory loss. In fact, DNA fragmentation and caspase-3 activation were evident in the frontal cortex and hippocampus of young animals, which resulted in cleavage of tau into a toxic fragment that enhances NFT formation. Moreover, we explored a possible link between Aβ and tau, since tau is necessary for Aβ-induced cognitive dysfunction (Gotz et al. 2001). In our cell culture study, Aβ treatment of rat cortical neurons resulted in caspase-3 activation and subsequent cleavage of tau. Importantly, TUDCA markedly reduced levels of cleaved tau, by inhibiting apoptosis and, more specifically, caspase-3 activation. Thus, by interfering with apoptotic pathways, TUDCA not only increased the survival of neurons, but also prevented downstream conformation changes of tau, which may have positive consequences in slowing cognitive decline. To characterize the role of TUDCA in rTg4510 mice, it would be important to determine whether administration of the bile acid to newborn or young rTg4510 mice prevents apoptosis and cleavage of tau. In this regard, the effects of TUDCA on neuronal death, memory, and NFT formation warrant further experiments.

In vivo models are valuable tools to investigate the pathogenic mechanisms of a specific disease. However, a model that recapitulates all relevant aspects of AD

is not yet available. In an ideal model, Aβ and tau aggregation would mimic the neuronal abnormalities that are characteristic of AD. In this regard, a triple-transgenic model that expresses mutated forms of APP, presenilin 1, and tau has already been developed (Oddo et al. 2003). This may represent an efficient tool for understanding the pathology of AD and, more importantly, for developing effective therapeutic approaches.

As a final note, it is important to underscore the antiapoptotic properties of TUDCA. In this thesis, we showed that in addition to its direct effect at the mitochondria, TUDCA also appears to act upstream of mitochondria regulating apoptosis induced by Aβ at the level of transcription. Moreover, by modulating neuronal cell death, TUDCA may also interfere with subsequent downstream production of toxic factors responsible for AD-associated neuronal pathology. The ultimate challenge will be to characterize and integrate all the separate pathways in an orchestrated mechanism of TUDCA antiapoptic function, focusing on its potential clinical application in AD.

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Curriculum Vitæ

Rita Cruz Coelho de Mira Ramalho was born in Lisbon, Portugal, on 12 January 1979. In 1997, she graduated from High School and started her studies in Biology at the Faculty of Sciences, University of Lisbon, Portugal. In 2002, she received her B.Sc. degree, after completing a thesis on the identification of apoptotic markers in several models, after toxic insults. These studies were supervised by Professor Maria Celeste Lechner at the Unidade de Biologia Molecular, Faculty of Pharmacy, University of Lisbon, Portugal. In 2003, she was awarded a research fellowship supported by the project POCTI/BCI/44929/2002, from Fundação para a Ciência e Tecnologia, Lisbon, Portugal. The studies were directed to ascertain the role of tauroursodeoxycholic acid in amyloid β-induced apoptosis and supervised by Professor Cecília M. P. Rodrigues at the Centro de Patogénese Molecular, Faculty of Pharmacy, University of Lisbon, Portugal. In the same year, she was awarded a Ph.D. scholarship (SFRH/BD/12641/2003) from Fundação para a Ciência e Tecnologia. She started to work in the project that led to this thesis, under the supervision of Professor Cecília M. P. Rodrigues at the Centro de Patogénese Molecular, Faculty of Pharmacy, University of Lisbon, Portugal. During her Ph.D. studies, she spent a period of 3 months at the Departments of Medicine and Neurosurgery, University of Minnesota Medical School, Minneapolis, USA, under the guidance of Professor Clifford J. Steer and Professor Walter C. Low.

List of Publications

Articles in International Journals

1. **Ramalho RM**, Ribeiro PS, Solá S, Castro RE, Steer CJ, Rodrigues CMP. Inhibition of the E2F-1/p53/Bax pathway by tauroursodeoxycholic acid in amyloid β-peptide-induced apoptosis of PC12 cells. *Journal of Neurochemistry* 2004; 90: 567-575.

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