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Mice overexpressing human caspase 3 appear phenotypically normal but exhibit increased apoptosis and larger lesion volumes in response to transient focal cerebral ischaemia

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Abstract

Caspase 3 activation has been implicated in cell death following a number of neurodegenerative insults. To determine whether caspase genes can affect the susceptibility of cells to neurodegeneration, a transgenic mouse line was created, expressing human caspase 3 under control of its own promoter. The human gene was regulated by the murine homeostatic machinery and human procaspase 3 was expressed in the same tissues as mouse caspase 3. These novel transgenic mice appeared phenotypically and developmentally normal and survived in excess of 2 years. Behavioural assessment using the 5-choice serial reaction time task found no differences from wild-type littermates. Caspase activity was found to be tightly regulated under physiological conditions, however, significantly larger lesions were obtained when transgenic mice were subjected to focal cerebral ischaemia/reperfusion injury compared to wild-type littermates. These data demonstrate that mice overexpressing human caspase 3 are essentially normal, however, they have increased susceptibility to degenerative insults.

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Abbreviations: 5-CSRTT, 5-choice serial reaction time task; FISH, fluorescence *in situ* hybridization; MCAo, middle cerebral artery occlusion; Tg, transgenic; WT, wild type; YAC, yeast artificial chromosome

Introduction

Caspases are a large family of cysteine aspartyl-specific proteases that play a pivotal role in apoptotic cell death.[1,2] They are the mammalian orthologues of the *Caenorhabditis elegans* death gene, CED-3 that is essential for cell death in the development of the worm.[3,4] Caspases exist as inactive proenzymes which undergo proteolytic processing and dimerization to form the active enzymes. They are activated in a sequential manner and play a central role in the execution phase of apoptotic death cleaving many vital structural and regulatory proteins (reviewed by Fischer *et al.*[5]). Numerous biochemical and cell biological studies on the activation of caspases have been reported leading to the ordering of the proapoptotic proteases into a hierarchical cascade: with the apical or initiator caspases 8 and 9 cleaving and activating the effector caspases 3, 6 and 7.[6–8] Using cell-free, immunodepleted extracts, caspase 3 was shown to be the primary executioner caspase.[9] Although thymocytes and hepatocytes from caspase 3 knockout mice retained normal susceptibility to various apoptotic stimuli,[10,11] brain development was profoundly affected in these animals.[10] The decreased apoptosis observed in the brain suggested a critical role for caspase 3 in the development of the central nervous system. Caspase 3 has also been implicated in cell death following a number of neurodegenerative insults including global ischaemia,[12,13] focal ischaemia,[14–19] transient ischaemia of the spinal cord,[20] neonatal cerebral hypoxia–ischaemia,[21–23] traumatic brain injury,[24–26] Alzheimer's disease,[27–29] Huntington's disease,[30–32] and Parkinson's disease.[33–37] It was therefore hypothesized that overexpression of caspase 3 may sensitize the animal to normal (physiological) apoptotic processes that occur during development and natural ageing and confer particular susceptibility to neurodegenerative insults. To test this hypothesis, a caspase 3 transgenic mouse line was generated using a yeast artificial chromosome (YAC) containing the human genomic DNA spanning the full-length gene including all regulatory elements. The development of these transgenic mice and their sensitivity to a neurodegenerative insult (focal cerebral ischaemia) were investigated.

Results

Generation of transgenic lines and expression of caspase 3 transgene

A human genomic YAC clone (35EB2) which had previously been shown to encompass the caspase 3 gene,[38] was used for the construction of transgenic mice. Southern blot analysis, PCR and pulsed-field electrophoresis showed that YAC 35EB2 contained all seven exons and the 3′ untranslated

region (UTR) of human caspase 3, as well as 80 kb of upstream and 50 kb of downstream regulatory sequences (Figure 1a). Fluorescence *in situ* hybridization (FISH) studies on human neutrophils confirmed that the YAC was nonchimeric and mapped to the telomeric region of human chromosome 4, which is coincident with the location of the caspase 3 gene[38] (Figure 1b). Genotyping of 89 offspring born after microinjection by PCR analysis identified 10 transgenic founders, five of which transmitted the transgene. Southern blotting and PCR confirmed that the transgenic lines had integrated one to three copies of the YAC (data not shown). Two transgenic lines were characterized extensively – C57BL/6J-Tg(CASP3)F18Fine and C57BL/6J-Tg(CASP3)F57Fine. Mice were backcrossed to C57BL/6J for at least 10 generations to ensure congenicity and were born in expected ratios as predicted from Mendelian genetics. Northern analysis and RT-PCR of P6 brains demonstrated that the human gene was transcribed in transgenic mice but not wild-type (WT) littermates during development (Figure 2a, b). Western blotting with a human-specific antibody confirmed expression of human caspase 3 zymogen in these mice (Figure 2c). The spatial expression of the human form in the adult mouse was essentially identical to that of the murine protein with highest levels being detected in the spleen, thymus, liver, kidney and brain (Figure 3).

Histological analysis

Transgenic mice appeared to be phenotypically and developmentally normal and were essentially indistinguishable from their WT littermates throughout life which extended beyond 26 months. The anatomical organization of the brain and the cerebrovasculature were examined in detail using serial horizontal sections stained for acetyl cholinesterase (modified Karnovsky and Roots) and Nissl bodies (thionin) in both young and aged (24-month-old) mice. Comparable horizontal brain sections of transgenic and WT mice were examined in terms of organization and dimensions (Figure 4). Detailed inspection of the aged brains by four independent scorers failed to identify any differences between WT and transgenic brains. There were no significant differences in any of the measurements taken including cortical mantel size, cerebellar and hippocampal dimension and overall brain size. It was concluded that the caspase 3 transgenic mice were histologically normal.

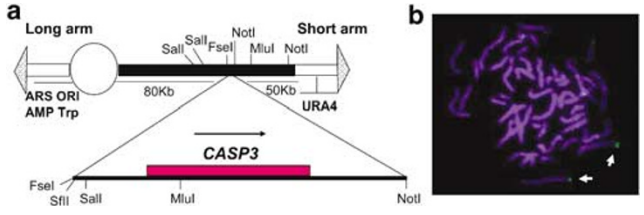

Figure 1 Characterization of YAC for generation of human caspase 3 transgenic mice. (**a**) Schematic representation of YAC 35EB2 which encompasses the human caspase 3 gene and approximately 80 and 50 kb of upstream and downstream regulatory elements, respectively. (**b**) FISH analysis of YAC 35EB2 on metaphase chromosomes of human neutrophils demonstrated that the YAC was nonchimeric and mapped to the telomeric region of human chromosome 4 (arrows)

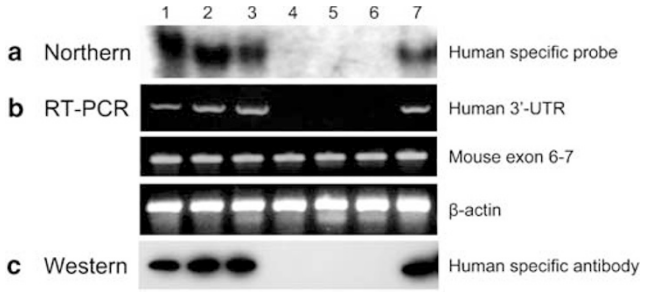

Figure 2 Expression of human caspase 3 in developing transgenic mouse brain. The expression of caspase 3 was analysed in a litter of seven pups at postnatal day 6. Transgenic mice are in lanes 1, 2, 3 and 7 and WT littermates are in lanes 4, 5 and 6. (**a**) Northern blot analysis showing expression of the human caspase 3 gene. The probe used corresponds to a 0.6 kb fragment of the human 3′ UTR region. (**b**) RT-PCR analysis performed using human-specific caspase 3 primers (top panel), mouse-specific caspase 3 primers (middle panel) or mouse β-actin primers (bottom panel). (**c**) Western blot analysis of total brain lysate showing expression of the human procaspase 3 in transgenic brains but not those of WT littermates

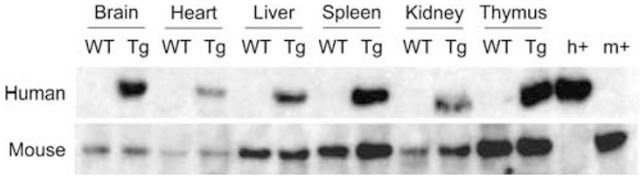

Figure 3 Similarities of spatial expression of mouse and human caspase 3 proteins in adult mice. The expression of human and mouse procaspase 3 in brain, heart, liver, spleen, kidney and thymus of wild-type (WT) and transgenic (Tg) mice was characterized by Western blot using antibodies recognizing specific for human (top panel) or mouse (bottom panel) caspase 3

Behavioural assessment using 5-choice serial reaction time test

The normal ageing process is associated with impairments in sustained attention as assessed in humans by the continuous performance test and in animals by the 5-choice serial reaction time task (5-CSRTT).[39,40] As it was hypothesized that overexpression of human caspase 3 would exacerbate the cognitive decline associated with the normal ageing process, transgenic mice and age-matched WT littermates were assessed in the 5-CSRTT at 14, 20 and 27 months of age. In order to receive a liquid reward, mice were required to make a response at one of five locations following a brief light pulse. The initial training schedule lasted until the mice had attained asymptotic performance at 1 s stimulus duration or 40 daily sessions had elapsed. Transgenic mice acquired the 5-CSRTT as readily as their WT littermates (Figure 5a). ANOVA confirmed no significant main effect of genotype on the cumulative number of trials taken to reach asymptotic performance ($F_{(1,42)} = 0.14$, $P = 0.71$). Asymptotic performance, measured in terms of mean accuracy from three consecutive sessions revealed no effect of ageing ($F_{(2,23)} = 0.98$, $P = 0.39$) nor of transgene ($F_{(1,23)} = 0.91$, $P = 0.35$; Figure 5b). These data suggest that overexpression of human caspase 3 is not deleterious and that under normal physiological conditions, caspase 3 activity is tightly regulated. However, an alternative explanation is that the mouse

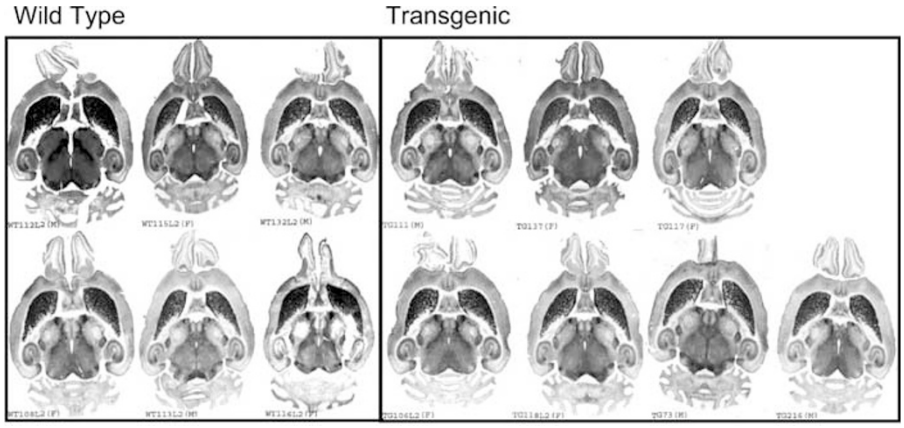

Figure 4 Histological assessment of caspase 3 transgenic mice. Horizontal brain sections from seven transgenic mice and six WT littermates aged 24 months were stained for acetyl cholinesterase. Multiple measurements were compared but no differences between the mice were found

machinery is not able to process the human proenzyme. In order to verify that the transgenic human proenzyme could be cleaved and activated, further biochemical experiments were performed.

Activation of human procaspase 3 *in vitro*

Human procaspase 3 in brain extracts from transgenic mice could be biochemically activated by treatment with recombinant active human caspase 6 or 8 *in vitro* (Figure 6). The intrinsic murine apoptotic machinery could also process the human proenzyme in cultured cells. Cerebellar granule cells were cultured from transgenic mice and WT littermates and apoptosis was induced with serum deprivation under conditions of low potassium. Western blotting studies with a human-specific antibody confirmed that the human procaspase 3 was cleaved under these conditions (Figure 7). This processing was not confined to neuronal cells as caspase 3 cleavage was also detected in cultured splenocytes after the induction of apoptosis with Fas ligand (Figure 6). Furthermore, caspase 3-like activity was measured by DEVD cleavage in P6 brain extracts and no differences could be detected between transgenic and WT mice (data not shown). These data demonstrated that the human proenzyme can be cleaved and activated by the mouse machinery but under normal physiological conditions, caspase 3 activity appears to be tightly regulated. However, it was hypothesized that overexpression of human caspase 3 proenzyme may sensitize mice to degenerative insults.

Caspase 3 activation *in vivo* following cerebral ischaemia

To test this hypothesis that overexpression of caspase 3 would sensitize mice to ischaemic brain damage, transgenic and WT littermate mice were subjected to 30, 45 and 60 min of middle cerebral artery occlusion (MCAo). WT mice exhibited qualitatively different patterns of damage with increasing ischaemic duration and the pattern of damage in transgenic mice was also qualitatively different from that observed in WT littermates (Figure 8a). A 30 min MCAo failed to produce damage in the majority of WT animals examined while the majority of those subjected to 45 min MCAo exhibited lesions. At both time points the lesions were restricted to the striatum, in those WT animals showing ischaemic damage. In contrast, all transgenic mice subjected to 30 and 45 min MCAo displayed ischaemic damage to the striatum. Moreover, at 45 min the majority of transgenic mice also exhibited cortical damage. All transgenic and WT animals subjected to 60 min MCAo had large infarcts with both cortical and striatal components. No damage was observed outwith the vasculature territory of the MCA in any of the animals investigated. Quantitative histopathology demonstrated that transgenic animals developed larger hemispheric lesions than their WT littermates following each of the ischaemic insults (Figure 8b). This was confirmed by two-way ANOVA which revealed a significant effect of both ischaemic duration ($F_{(2,35)} = 7.9$; $P = 0.002$) and transgene ($F_{(1,35)} = 21.9$; $P < 0.001$).

Immunohistochemistry of cleaved caspase-3 and TUNEL staining

Immunocytochemistry was performed on sections from transgenic and WT animals 24 h after a 30 min ischaemic insult (Figure 9a). Cleaved caspase 3- and TUNEL-positive cells were quantified in the ischaemic striatum of transgenic and WT animals. These data showed that transgenic animals exhibited a significantly higher number of active caspase 3- and TUNEL-positive cells within the ischaemic striatum than their WT littermates ($P = 0.028$; Figure 9b).

Discussion

A transgenic mouse line overexpressing human caspase 3 was made using a previously characterized human genomic YAC clone.[38] This YAC encompassed the human caspase 3 gene and, as expected, hybridized to the telomeric region of human chromosome 4, the chromosomal location of the caspase 3 gene.[38,41] Although YAC transgenic mice usually show position-independent expression of the encoded genes,[42] mice were bred to congenicity with C57BL/6J by backcrossing for at least 10 generations and characterized.

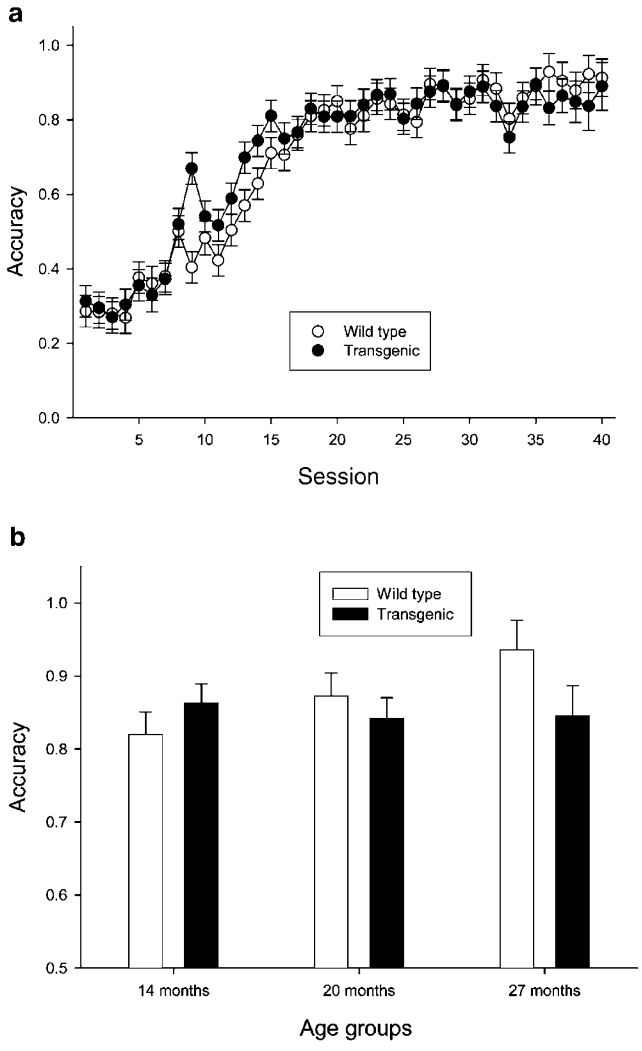

Figure 5 Behavioural assessment using 5-choice serial reaction time test (**a**) Acquisition of the 5-CSRTT in transgenic mice and age-matched WT littermates. Data are presented as mean accuracy ± S.E.M. from the initial training period. (**b**) Asymptotic performance assessed at 14, 20 and 27 months of age. Data are presented as mean ± S.E.M. accuracy derived from three consecutive sessions once asymptotic performance had been established. Data were analysed by two-way ANOVA and show no significant differences between groups during acquisition ($F(1,42) = 0.14$, $P = 0.71$) or at final levels of performance either by age ($F(2,23) = 0.98$, $P = 0.39$) or genotype ($F(1,23) = 0.91$, $P = 0.35$)

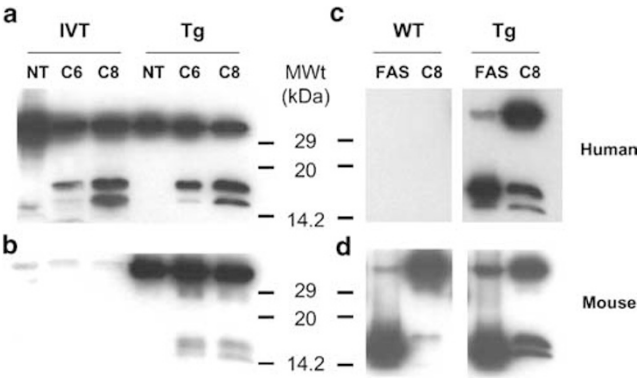

Figure 6 Biochemical activation of human caspase 3 transgene *in vitro*. (**a**) Human procaspase 3 expressed *in vitro* (IVT) or in transgenic brain extracts (Tg) was cleaved by addition of recombinant active human caspase 6 (C6) or 8 (C8). No cleavage products were obtained in untreated caspase 3 lanes (NT). (**b**) A similar blot probed with an antibody specific for murine caspase 3 demonstrated that mouse caspase 3 was also cleaved by the addition of recombinant active human caspase 6 or 8. (**c**) Cleavage of human caspase 3 was detected in splenocytes treated with Fas (Fas) or cerebellar brain tissue extracts treated with recombinant human caspase 8 (C8) from transgenic (Tg) but not WT littermate mice. (**d**) A similar blot probed for mouse caspase 3 demonstrated that mouse caspase 3 was also cleaved by these treatments in both types of mouse

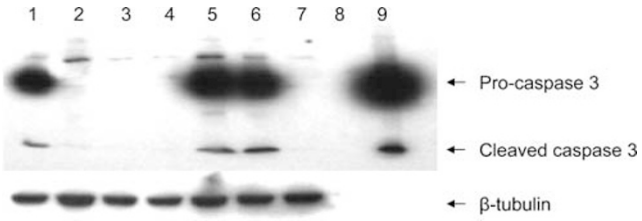

Figure 7 Human caspase 3 activation in cultured cells. Cerebellar granule cells were cultured and apoptosis induced with potassium serum deprivation. Western blot analysis with a human caspase 3-specific antibody demonstrated processing of the human procaspase 3 to its cleaved product in transgenic samples (lanes 1, 5 and 6) but not in WT littermates (lanes 2, 3, 4 and 7). No sample was loaded in lane 8 and lane 9 contained a positive control sample (SH-SY5Y human neuroblastoma cell line treated with staurosporine). Equal loading of lanes was confirmed with β-tubulin (bottom panel)

The human caspase 3 transgenic mice were essentially normal as no obvious phenotypic, developmental or histological differences were observed. The human protein was expressed in the same tissues as its murine counterpart. Highest expression levels were found in the spleen, thymus, liver, kidney and brain in agreement with published data for caspase 3 mRNA[43,44] and protein[44] levels. This demonstrated that *in vivo* the human gene was subject to similar control to the mouse gene. Although detailed analysis of the mouse promoter has not been reported, the mouse and human caspase 3 cDNA sequences are 83% identical (HomoloGene database[45]) while at the protein level they are 87% identical (UniGene database[45]). Therefore, it is likely that the gene promoters are similar in these two species also. Supporting this theory, the human and rat caspase 3 promoters show 61.8% identity[46] suggesting that the regulatory regions of the caspase 3 gene are conserved between mammalian species and likely to be subject to the same regulatory controls.

Caspase 3 has been termed the 'common executioner' for various cell death pathways and is therefore produced as an inactive proenzyme, which has to undergo proteolytic cleavage and dimerization to produce the active enzyme. As an executioner caspase, procaspase 3 can activated by both the extrinsic (death-receptor-mediated) and the intrinsic (mitochondrial) pathways of programmed cell death (reviewed by Ashe and Berry[47]). In the extrinsic pathway, binding of a ligand (such as Fas ligand) to its death receptor (Fas receptor) activates the signalling pathway resulting in caspase 8-mediated cleavage of procaspase 3. It was possible to cleave the human proenzyme by addition of recombinant human caspase 8 *in vitro* and by Fas ligand treatment of splenocyte cultures from transgenic mice. Processing of the human procaspase 3 was also initiated by withdrawal of trophic support from cultured cerebellar granule cells and by direct treatment with active human caspase 6 as had previously

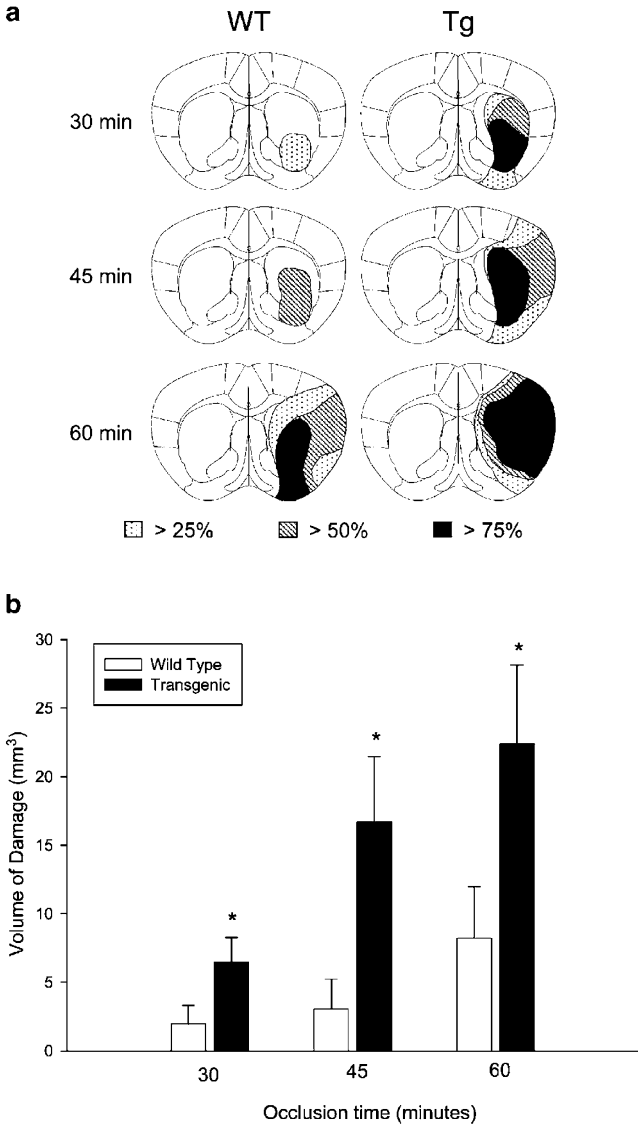

Figure 8 Caspase 3 overexpressing mice exhibit larger lesions than nontransgenic littermates following MCA occlusion. (**a**) Incidence maps showing the patterns of ischaemic damage obtained in transgenic (Tg) mice and WT littermates at the level of the striatum (Bregma + 0.74) following 30, 45 or 60 min of transient MCA occlusion. (**b**) Measurement of total lesion volume produced by transient MCA occlusion (30, 45 or 60 min) measured (over 9 levels) at 24 h using thionin staining. Two-way ANOVA showed that there was a significant effect of time ($F(2,35) = 7.9$, $P = 0.002$) and genotype ($F(1,35) = 21.9$, $P < 0.001$). Data are mean ± S.E.M., $n = 5$–9 mice per group

been shown for rodent caspase 3.[48,49] Taken together, these data demonstrated that the transgene contained the full-length human gene and that once produced, the human proenzyme could be cleaved and activated by both human and murine machinery. However *in vivo*, the human gene and protein appeared to be regulated as normal for their murine counterparts suggesting that caspase activity is tightly regulated under physiological conditions. This is hardly surprising considering the high degree of homology between the human and mouse caspase 3 amino-acid sequences which are 87% identical (UniGene database[45]).

It was hypothesized that the increased levels of procaspase 3 may sensitize the mice to the normal ageing process and to acute pathophysiological insults such as stroke. However, the present data provide little evidence that the presence of the human proenzyme affected the normal ageing process: transgenic mice lived for more than 2 years and were histologically normal when brains were analysed at 24 months. Moreover, these transgenic mice were indistinguishable from their WT littermates when tested at 14, 20 and 27 months of age in the 5-CSRTT. This test was used because it is an attentional task similar to the continuous performance test used to assess attentional function in man. The normal ageing process in humans is associated with impairments in sustained attention[50,51] and similar age-related deficits have been detected in rats using the 5-CSRTT.[39,52,53] Although this test has been used in mice,[54,55] no ageing studies have previously been reported. Interestingly no age-related deficit was found in either the transgenic or WT mice and all ages of mice performed the task equally well. As no age-related deficits were seen in this test of sustained attention, other tests of cognitive function (e.g. of spatial memory) need to be performed to fully elucidate what, if any, impairments are revealed due to ageing in the transgenic compared to WT mice.

Several lines of evidence point to caspase 3 as a mediator of ischaemic cell death. Caspase 3 activation has been detected following focal (and global) ischaemic insults to the brain[14–19] where it coincides with areas of enhanced apoptotic cell death[14] and treatment with caspase 3 inhibitors has been reported to reduce infarct size following MCA occlusion.[14,56–58] Moreover, studies in caspase 3-deficient mice indicate that these animals are relatively resistant to ischaemic insults with smaller lesions than their WT littermates.[59] In keeping with these literature reports, the studies presented here demonstrate that mice overexpressing human caspase 3 exhibit more apoptotic cell death and larger cerebral lesions than their WT littermates. Similarly, heart-targeted overexpression of caspase 3 resulted in increased lesion size following myocardial ischaemia–reperfusion.[60]

To test the hypothesis that caspase 3 overexpression would sensitize the brain to ischaemic damage, we chose to examine three different ischaemic insults. Brief occlusion of the MCA in C57BL/6J mice has been reported to produce an ischaemic lesion that is restricted to the striatum while more prolonged ischaemia results in damage to both striatum and overlying cortex, although there is evidence that the precise threshold for induction of cortical damage may differ from one laboratory to another.[61–63] In the present study, human caspase 3 overexpressing mice exhibited larger and more consistent lesions at each of the three time points investigated. Moreover, in the 45 min occlusion group, where damage was restricted to the striatum of WT littermates, the transgenic mice exhibited consistent damage to both cortex and striatum. No differences in cerebrovascular anatomy were detected between transgenic and WT mice; therefore, the increased lesion volumes could not be attributed to this factor. These results suggest that human caspase 3 overexpression does sensitize the brain to ischaemic damage.

Initial studies reported that caspase 3 knockout mice have profound defects of apoptosis in the central nervous system

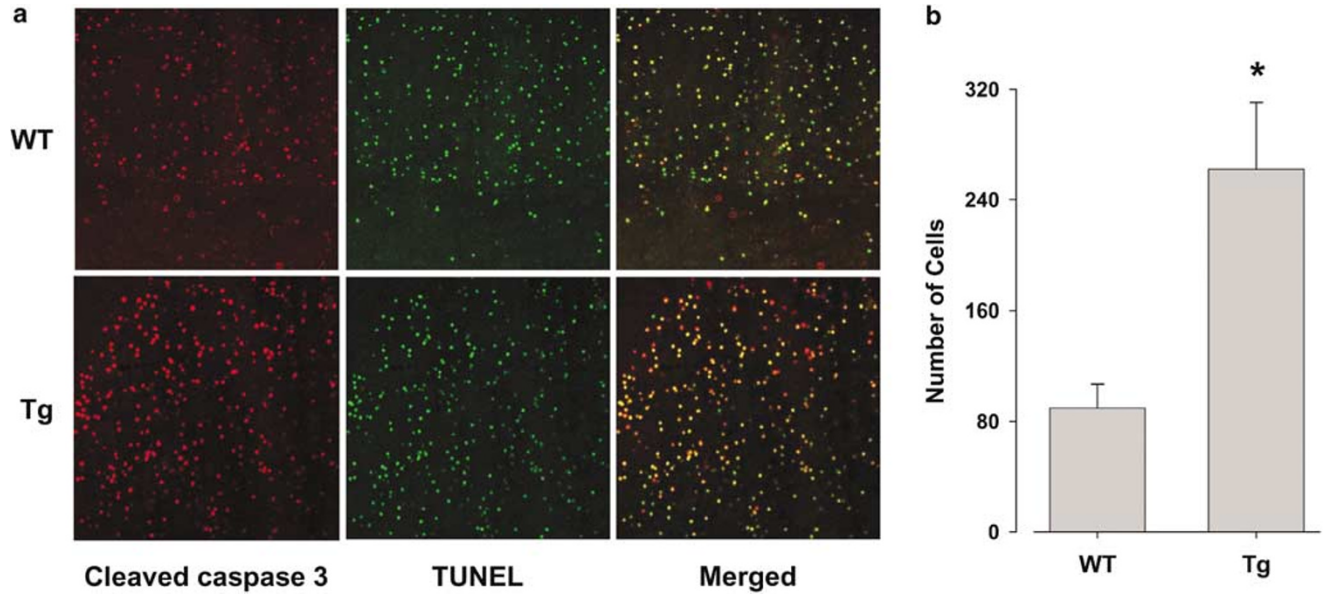

Figure 9 Detection of active caspase 3 in WT and transgenic ischaemic brains by confocal microscopy. Mice were subjected to 30 min MCA occlusion and brains processed for immunocytochemistry at 24 h. (**a**) Brain sections within the MCA territory were stained with an antibody directed against both mouse and human processed caspase 3 (red) and TUNEL (green) and representative images within the ischaemic striatum are shown. Superimposition of the two fluorescent images (right panels) indicate that there is a good correlation between caspase 3 activation and TUNEL staining (yellow). (**b**) Quantitative analysis demonstrated that transgenic animals exhibited a higher number of active caspase 3- and TUNEL-positive cells within the ischaemic striatum than their WT littermates. Data are mean $\pm$S.E.M., $n=3$ mice per group, $P=0.028$

showing decreased apoptosis in the brain resulting in hyperplasia and premature lethality with the mice dying at 1–3 weeks of age.[10] However, thymocytes and hepatocytes from these animals retained normal susceptibility to various apoptotic stimuli demonstrating the redundancy of caspases at least in these peripheral cells.[10,11] As the brains of the human caspase 3 transgenic mice also develop normally, the transgene must be regulated by the murine machinery so that the natural cell death occurring during brain development proceeds in its usual fashion. In contrast to the neurodegenerative stimulus response in the adult, overexpression of human caspase 3 did not seem to sensitize cells to cell death occurring during normal brain development. These data suggest that although caspase 3 participates in the cell death processes which occur during development of the brain and after a neurological insult, a fundamental difference exists perhaps in the activating pathways. However, it has been shown that the background strain may be important in determining the phenotype seen in genetically modified mice. The neurodevelopmental abnormalities seen in caspase 3-nullizygous mice have been shown to be strain dependent where mice on a 129/Sv background are severely affected while those on a C57BL/6 background are not affected and survive to adulthood suggesting the presence of a strain-specific genetic modifier.[64] Furthermore, in the forebrain of 129/Sv mouse embryos, numerous cells were found to be positive for cleaved caspase 3 immunoreactivity but only slight staining was seen in C57BL/6 embryos.[65] The transgenic human caspase 3 mice reported here have been bred to congenicity on a C57BL/6J background and this may be the reason why overexpression of human caspase 3 did not seem to sensitize cells to cell death occurring during normal brain development.

There is a growing list of substrates cleaved by caspases and suggested roles for caspases in other cellular processes such as cell cycle regulation and cellular differentiation have been proposed.[5] There is also mounting evidence that the apoptotic pathway is involved in normal neuronal plasticity[66] and a surprising role for caspase 3 activation as essential in neuroprotective preconditioning was recently demonstrated.[67] As the many diverse functions of caspases become apparent it would be interesting to see what effects (if any) caspase 3 overexpression had in these systems. Therefore, these novel human caspase 3 transgenic mice may help elucidate the role of caspase 3 in the many cellular processes in which it may be involved.

Materials and Methods

All animal work was performed under license by the UK Home Office subject to the Animal (Scientific Procedures) Act of 1986.

Construction of human caspase 3 YAC transgenic mice

YAC 35EB2 was selected from the ICI human YAC library[68] (UK HGMP Resource Centre, Cambridge, UK) because of its association with the human caspase 3 gene.[38] The YAC DNA was isolated from transformed yeast as described[69] and its integrity tested by restriction mapping and FISH on metaphase chromosomes of human neutrophils[70] using a Biotin-dUTP nick translation kit (Roche Diagnostics, Lewes, UK). A total of 840 F1 C57BL/6J $\times$ CBA fertilized eggs were microinjected[71] with a preparation of highly purified YAC DNA[72] (2 ng/μl) yielding 89 live births (10%), of which 10 were found to be transgenic (11%).

Genotyping and mouse breeding

Genotyping was performed on tail tips or ear punches. Tail tipping was performed under halothane/nitrous oxide anaesthesia and tail DNA was obtained by proteinase K (Promega, Southampton, UK) treatment.[73] Ear clips were heated at 95°C for 20 min in 25 mM NaOH/0.2 mM EDTA then neutralized by the addition of an equal volume of 40 mM Tris-HCl. Three primer pairs corresponding to both YAC ends (long arm: forward primer 5′-ATT GCT AAC GCA GTC AGG CAC C-3′, reverse primer 5′-TAG TGG CTC CAA GTA GCG AAG C-3′, producing 278 bp product; and short arm: forward primer 5′-TCT CCG AAC AGA AGG AAG AAC G-3′, reverse primer 5′-TGT TAC TTC TTC TGC CGC CTG C-3′, producing 568 bp product) and human caspase 3 (3′-UTR: forward primer 5′-TGA TGA TGT GGA AGA ACT TAG G-3′, reverse primer 5′-ACG GCT CCG CAC CTG CTG AGG C-3 producing 944 bp product) were initially used for PCR genotyping. Southern blotting confirmed the initial PCR results. Transgenic founders were crossed to C57BL/6J (Charles River, Margate, UK) to test for germline transmission of the full YAC DNA. Four transgenic lines were selected and assessed for expression of human caspase 3 by RT-PCR, Northern and Western blotting analysis. Of these lines, three (F18, F21 and F57) were found to express human caspase 3 and two lines (F18 and F57) were selected for further studies. The F18 and F57 lines were made congenic by successive backcrossing to C57BL/6J (Charles River) for at least 10 generations and named C57BL/6J-Tg(CASP3)F18-Fine and C57BL/6J-Tg(CASP3)F57Fine, respectively according to the guidelines set out by the International Committee on Standardized Genetic Nomenclature for Mice and implemented through the Mouse Genomic Nomenclature Committee (MGNC). Wild-type (WT) littermates were used as controls in all experiments.

RT-PCR and Northern blotting

Total RNA was isolated from mouse brains using TriReagent (Sigma-Aldrich, Poole, UK) according to the manufacturer's instructions. A measure of 1–5 μg of total brain RNA were reverse-transcribed (First Strand cDNA synthesis kit, Amersham Biosciences, Little Chalfont, UK) and used for subsequent PCR amplification. Primers specific for human caspase 3 were 5′-TGA TGA TGT GGA AGA ACT TAG G-3′ (forward primer) and 5′-ACG GCT CCG CAC CTG CTG AGG C-3′ (reverse primer) producing a product of 944 bp from the 3′-UTR; for mouse caspase 3 were 5′-GGC TTG CCA GAA GAT ACC GGT-3′ (forward primer) and 5′-GCA TAA ATT CTA GCT TGT GCG CGT A-3′ (reverse primer) producing a product of 150 bp from exons 6–7; and for β-actin were 5′-TCA TGA AGT GTG ACG TTG ACA TCC GT-3′ (forward primer) and 5′-CCT AGA AGC ATT TGC GGT GCA CGA TG-3′ (reverse primer) producing a product of 285 bp. For Northern blotting, 20 μg of total brain RNA were electrophoresed on 1.2% formaldehyde agarose gel in MOPS buffer and transferred overnight onto Hybond-N (Amersham Biosciences) in 20 $\times$ SSC.[73] Membranes were hybridized with a 600 bp human caspase 3-specific probe corresponding to the 3′-UTR (IMAGE EST 937366; UK HGMP Resource Centre, Cambridge, UK) using standard molecular procedures.[73]

Western blotting analysis

Total protein was isolated from mouse brain (cerebellum) using TriReagent (Sigma-Aldrich) according to the manufacturer's instructions. Human caspase 3 cDNA clone in pCRII TOPO (Invitrogen, Paisley, UK) was transcribed and translated *in vitro* (TnT Quick Coupled Transcription/Translation system, Promega). Treatment of brain extract or *in vitro* translated (IVT) caspase 3 with recombinant active human caspase 6 or 8 (BD Biosciences, Oxford, UK) was performed as previously described.[49] Cerebellar granule neuron cultures were prepared from postnatal day 6 (P6) transgenic and WT littermates and apoptosis induced on the 7th day of *in vitro* culture in low K^+ medium (serum free Basal medium Eagles, 5 mM KCl) as previously described.[49] Splenocytes were cultured from adult caspase-3 transgenic mice and WT littermates[74,75] and apoptosis induced with anti-mouse Fas antibody (αCD3ε, BD Biosciences) as described.[74]

A measure of 30 μg of protein were resolved by 12% SDS-PAGE and electroblotted onto PVDF membranes (Immobilon-P; Sigma-Aldrich) for 2 h at a constant voltage of 10 V using semi-dry apparatus. Blots were blocked by overnight incubation in TBS (Tris-buffered saline, pH 8.0) containing 0.05% Tween-20, 5% nonfat milk powder. Caspase-3 was detected using commercial antibodies specific for human or mouse caspase 3 (both BD Biosciences 1/1000 dilution) or which recognized caspase 3 and cleaved caspase 3 from both species (both Cell Signaling Technology, Hitchin, UK; 1/1000 dilution). Bound antibody was detected using anti-mouse or anti-rabbit Ig conjugated to horseradish peroxidase (Amersham Biosciences, 1/2500 dilution) and enhanced chemiluminescence (ECL plus, Amersham Biosciences). To confirm equal loading of samples, blots were stripped of bound antibody (stripping buffer: 0.2 M glycine pH 2.2, 0.1% SDS, 0.1% Tween 20). Reincubation in ECL reagent confirmed that all bound antibody reagents were removed. Blots were blocked as described previously, incubated with an antibody against β-Tubulin (Sigma-Aldrich, 1/4000 dilution) and detected using anti-mouse Ig-HRP conjugate (Amersham Biosciences, 1/2500 dilution) and ECL.

Behavioural assessment using 5-choice serial reaction time test

Male transgenic animals ($n = 7$) and age-matched WT littermates ($n = 5$) were group-housed and tested 5 days a week during the light part of their cycle. Food was restricted to maintain animals at 85% of their free-feeding weight but water was available *ad libitum*. Training was carried out as previously described[55] using 12-month-old animals. In summary, subjects were required to sustain attention to an array of five stimulus locations over a period of 120 trials or 25 min. Subjects had to respond to brief (1 or 2 s) light pulses randomized across these locations in order to gain liquid reward. The initial training schedule continued for 40 sessions or until mice reached asymptotic performance at 1 s stimulus duration and performance was measured by accuracy, calculated as total correct responses/total responses. At the end of the initial training phase, animals were returned to free access to food and water. The two retraining phases were started 4 months after the previous phase ended and took approximately 2 months to complete. Retraining began with the 10 s stimulus duration and decreased to 2 s as previously described.[55] Therefore, asymptotic performance was assessed when mice were 14-, 20- and 27-month old. Group sizes reduced slightly over time: $n = 6$ and 5 for transgenic and WT mice, respectively, at 20 months and $n = 3$ per group at 27 months. As group sizes reduced over time, a repeated measures analysis was inappropriate and data were therefore analysed by two-way ANOVA using transgene and age as the main factors.

Cerebral ischaemia

Monofilament occlusion of the middle cerebral artery (MCA) was performed in adult male transgenic mice and WT littermates (25–30 g, approximately 10–12 weeks) using a modification of the method described

by Hata *et al*.[76] Briefly, the right common carotid (CCA), external carotid (ECA) and internal carotid (ICA) arteries and their branches were exposed through a midline cervical incision. A 6-0 silk suture was tied around the CCA proximal to the bifurcation of the ECA and ICA and a second suture tied around the ECA distal to the superior thyroid artery (STA). The STA and occipital artery (OA) were occluded by electrocoagulation. An 8-0 silicone-coated monofilament (diameter 220 μm) was introduced into the CCA and advanced 10 mm distal to the carotid bifurcation, occluding the origin of the MCA. Transgenic animals and their WT littermates were subjected to 30 ($n=8$–9 per group), 45 ($n=6$–7 per group) or 60 ($n=5$–6 per group) minutes of ischaemia and killed at 24 h by trans-cardiac perfusion with 4% paraformaldehyde under deep anaesthesia (pentobarbitone, 60 mg/kg i.p.). Brains were removed and processed for quantitative histological analysis. Cryostat sections (20 μm) were stained with thionin and examined for damage at nine stereotaxic levels by light microscopy. Image analysis (MCID, Interfocus Ltd, Haverhill, Suffolk, UK) was used to determine the volume of damage. Transgenic and WT littermate mice were compared using two-way analysis of variance. Genotyping was carried out at 4 weeks of age and confirmed prior to final analysis of the data.

Immunohistochemistry of cleaved caspase-3 and TUNEL staining

Caspase 3 transgenic ($n=3$) and WT animals ($n=3$) were subjected to 30 min of ischaemia. Animals were killed at 24 h and the brains removed and immediately frozen (-42°C) in M-1 Embedding Matrix (Lipshaw Co. Ltd, Pittsburgh, PA, USA). Cryostat sections (10 μm) were mounted onto TESPA slides and dried at 37°C for 1 h. The sections were acetone-fixed for 10 min at room temperature, air dried and then kept -70°C until processed. Slides were blocked for 1 h in 3% BSA, Tris-buffered saline, 1% Tween 20 then incubated at 4°C overnight in primary antibody against cleaved caspase 3 (Cell Signaling Technology; 1/20 dilution in block). After washing, bound antibody was detected using Cy-3 conjugated goat anti-rabbit (Amersham Biosciences; 1/200 in 1% BSA). TUNEL staining was performed with ApoTag In Situ Apoptosis Detection Kit (Appligene/Oncor, Chester-Le-Street, Co. Durham, UK). Caspase 3 and TUNEL-positive cells in the ischaemic striatum of transgenic and WT animals were counted in five brain sections per animal and analysed using Student's *t*-test.

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