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Guest Editorial

Stroke treatment enters the Fas lane

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Fas ligand (FasL or CD95L or Apo-1L) and tumour necrosis factor (TNF) are members of a superfamily of cytokines that play a primary role in the development and maintenance of the immune system and in inflammatory disease.[1,2] Genetic knockout experiments and studies of naturally occurring mutant mouse models have confirmed this important function. Typically, mice lacking FasL[3] or TNF[4] suffer from lymphoproliferative disorders resulting in a diminished immune response, caused by a severe disruption of FasL$^-$ or TNF$^-$ dependent apoptosis. In healthy individuals, FasL and TNF are largely restricted to immune tissues and to sites of immune privilege such as the eye[5] and the testes[6] where they can trigger apoptosis of invading immune cells.

The function of these death-inducing ligands in the healthy brain is not so clear, although it has been suggested that FasL may also function in the maintenance of immune privilege here.[7] On the other hand there is good evidence that FasL and TNF may be important factors in brain pathology. In particular, it is well documented that these death-inducing cytokines and their receptors are up-regulated following cerebral hypoxic-ischaemic injury in both the developing[8] and adult[9–12] brain. Moreover, a growing body of evidence indicates that TNF family cytokines are responsible at least in part, for neuronal loss following cerebral injury.[8,13–16] However, until now the exploitation of these findings for clinical benefit has been lacking.

In the present issue of *Cell Death and Differentiation*, Martin-Villalba and colleagues report significant protective effects of neutralising TNF and Fas signalling following stroke induced by middle cerebral artery occlusion.[17] In the first part of the study, it was found that infarct volume was reduced in FasL$^-$ (*gld*) and TNF$^-$ (knockout) mice by 54% and 67%, respectively, while hybrid mice lacking both cytokines showed a 93% reduction. Most impressive of all similar protective effects (70% reduction in infarct volume) were obtained in wild-type mice using a combination of antibodies to neutralise FasL and TNF.

How might these outstanding protective effects be explained? Perhaps the most obvious interpretation of the data is that both FasL and TNF are up-regulated on damaged neurons, (presumably along with their respective receptors) and so trigger apoptosis of themselves or neighbouring neurons (Figure 1). Alternatively since stroke induced brain damage has a significant inflammatory component, one could argue that the mice lacking FasL or TNF are protected from inflammatory injury by being immune compromised. This is certainly true to some extent as granulocyte infiltration following stroke was significantly lower in the mice lacking FasL and TNF and these animals showed virtually no infarct formation. Surprisingly, lymphocyte infiltration was more than three-fold higher in the same mice. These findings suggest that granulocytes are the major peripheral immune cells responsible for inflammatory damage and that perhaps the lack of TNF severely restricts their numbers or migration. However, the involvement of activated microglia in neuronal damage cannot be ruled out. Consistent with this possibility, FasL expression is increased in microglia following hypoxia-ischaemia *in vitro*.[18] Perhaps activated microglia (or peripheral lymphoid cells) are the true killers of stroke-damaged neural cells, but cannot execute this function if they lack FasL (Figure 1). The most likely explanation will probably involve a combination of the above.

It is generally accepted that following cerebral hypoxia-ischaemia, there are at least two phases of injury.[19] In the first phase, hypoxia-ischaemia leads to inadequate supplies of glucose and oxygen and so reduces cellular ATP levels and thus severely compromises those metabolic processes that require energy. ATP-dependent ion channels are disrupted causing cell membrane depolarisation and the activation of excitatory amino acid and neurotransmitter cascades and the subsequent generation of free radicals leading to cell necrosis. In the secondary (or reperfusion) phase of injury, or in areas of brain tissue less severely damaged such as in the ischaemic penumbra, many cells die by apoptosis. Later still, inflammatory processes are activated and immune mediated damage of neural tissue occurs.

The findings reported by Martin-Villalba and co-workers suggest that not only are FasL and TNF involved in both the reperfusion and inflammatory phases of injury, but antagonism of these cytokines also seems to afford protection from primary necrosis. While these results are dramatic, a discrepancy remains in the current literature between the TNF results using knockout animals. TNF is shown in some studies to be damaging in stroke[13,15,20] while others suggest that TNF is protective.[21–23] Exploring beyond the obvious explanations of species/strain differences or minor discrepancies in the injury model or cell types affected, the direct comparison of data from the TNF receptor knockout[22] with the TNF knockout in the present study does present an apparent paradox. This may be explained by a closer examination of TNF signalling. It is now clear that the classical p55 TNF receptor can deliver survival as well as apoptotic signals.[24] Perhaps other TNF receptors also exist in the brain that signal only apoptosis. In the case of stroke injury in the TNF receptor knockouts, cell survival may be

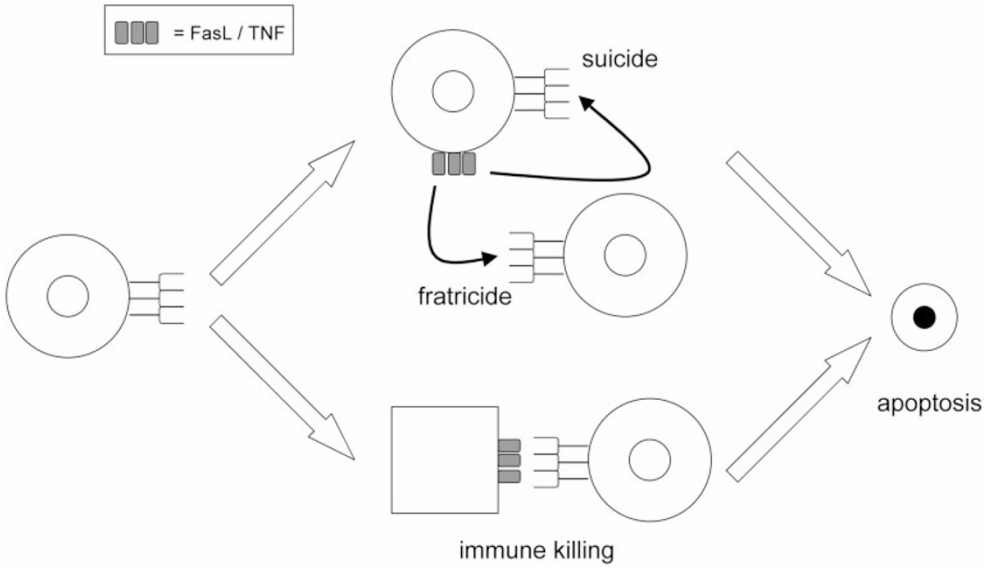

Figure 1 Two models explaining the role of FasL and TNF in stroke induced brain damage. Healthy neural cells (circular) express low levels of FasL/TNF. Following stroke injury, these cytokines (along with their receptors) are up-regulated and trigger apoptosis of damaged neurons (suicide) or of their healthy neighbours (fratricide). In the second model, activated granulocytes or lymphocytes cross the stroke damaged blood-brain barrier; alternatively, resident microglia may up-regulate FasL or TNF in situ following injury. These activated immune cells (square) can then trigger apoptosis in Fas or TNF receptor-bearing neural cells (immune killing)

compromised as increased TNF levels may activate these remaining members of the TNF receptor family. On the other hand TNF knockout mice will lack all TNF signalling (unless novel TNF receptor binding ligands also exist) and so will be protected.

To counter such criticism and to eliminate any subtle phenotypic differences in the genetically modified mice that may have been missed but could have accounted for the neuroprotective effects, the authors also investigated wild type animals and it was here that the most striking results were obtained. First it was found that neuronal apoptosis (using an *in vitro* model of oxygen-glucose deprivation) was significantly reduced by FasL and TNF blocking antibodies. *In vivo*, intraperitoneal injection of these neutralising antibodies into wild type mice 30 min after stroke injury also reduced granulocyte invasion into the brain. The combined rescue from reperfusion damage and inflammatory attack led to the most striking observation of all: the reversal of fate by the FasL/TNF neutralising antibody cocktail in animals that would have died 3 days after stroke injury. Not only did these mice survive, but also their performance in a locomotor test was indistinguishable from sham-operated controls. On closer inspection, mice lacking FasL/TNF signalling still showed signs of hippocampal damage and so may have learning or memory deficits not detected by the current study.

Although the *in vivo* data are truly impressive and suggest a direct and effective therapy for stroke, a few issues remain unresolved. In particular, there are clear differences between the *in vitro* and *in vivo* experiments. FasL or TNF antagonists each protected from neuronal apoptosis triggered by oxygen-glucose deprivation *in vitro* when added alone, but did not significantly reduce infarction after middle cerebral artery occlusion *in vivo*, despite a significant drop in granulocyte infiltration. The authors suggest that FasL exacerbates TNF induced damage and so both need to be blocked *in vivo*. However, in time-course studies they also show that TNF is up-regulated several hours before FasL. To confuse matters further, the combination of FasL and TNF blocking antibodies is toxic to neurons *in vitro* while they are clearly neuroprotective *in vivo*. Similarly, it is curious that FasL/TNF double negative mice show normal neuronal development, although their neurons cannot survive in culture. Finally, there is the issue of increased lymphocyte infiltration in the double negative mice, in spite of which there is virtually no neuronal loss. Perhaps, in antagonist-treated mice, the ability of lymphocytes to kill is neutralised in the periphery. However, in the absence of data showing uptake of intraperitoneally injected antibodies into the brain, one cannot exclude the possibility that the protective effects occur at the site of injury. While these apparently contradictory observations warrant further investigation, the overall clinical benefits of FasL/TNF antagonists are unambiguous.

Apart from heart disease and cancer, stroke is the major cause of death and disability in the developed world and it is estimated that stroke patients at any one time occupy more than 20% of all hospital beds.[25] Consequently, the data discussed here have implications of an enormous magnitude. With this in mind, perhaps the most prudent way forward is to follow the advice given to car drivers entering the fast lane: exciting as it may seem, proceed with caution.

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