Abstract The Fas/Fas ligand (L) signaling system has been implicated in the control of cell death and cell survival of T and B lymphocytes and in a variety of cell types under particular pathological conditions. In the present study we examined the expression of Fas and Fas-L, by Western blotting and immunohistochemistry, in the human frontal cortex and hippocampus of individuals with advanced Alzheimer’s disease (AD) and age-matched controls. Expression levels of Fas and Fas-L, as seen in Western blots, are preserved in the frontal cortex but decreased in the hippocampus in AD when compared with age-matched controls. Yet Fas and Fas-L immunoreactivity is found in remaining AD neurons in the frontal cortex and hippocampus. Moreover, Fas and Fas-L are expressed equally in tangle-bearing and non-tangle-bearing neurons, as revealed with double-labeling immunohistochemistry to Fas or Fas-L and tau or phosphorylated neurofilament epitopes. Dystrophic neurites of senile plaques are not stained with Fas and Fas-L antibodies. A moderate increase in Fas and a strong increase in Fas-L immunoreactivity occur in reactive astrocytes in AD. Yet there is no relationship between Fas or Fas-L expression and increased nuclear DNA vulnerability as revealed with double-labeling immunohistochemistry and in situ end-labeling of nuclear DNA fragmentation. Although the Fas/Fas-L system may have some effect in the control of reactive astrogliosis in AD, the present results show no evidence that Fas/Fas-L signals participate in specific processes of the disease, including neurofibrillary degeneration, dystrophic neurite formation, and cell death.

Keywords Fas · Fas ligand · Caspases · Alzheimer’s disease

Introduction

The Fas (CD95, APO-1) receptor is a member of the death receptor subfamily of the tumor necrosis factor (TNF)/nerve growth factor (NGF) superfamily. The receptor has an extracellular domain for ligand binding and an intracellular death domain (DD). Following binding to its specific ligand Fas-L, trimerization of Fas recruits the Fas-associated death domain (FADD) via interactions between the death domains of Fas and FADD. This is followed by FADD-like IL-1β-converting enzyme (FLICE)/caspase-8 binding via interactions between the death-effector domains of FADD and caspase-8, and by activation of caspase-8. Activation of caspase-8, in turn, activates the caspase cascade leading to apoptosis [10, 17, 22, 40, 41, 43, 59, 62]. The Fas/Fas-L signaling systems plays a pivotal role in the control of cell death and survival of T and B lymphocytes, in the regulation of the immune system, and in the progression of autoimmune diseases [1, 7, 28, 51]. Fas-L-expressing lymphocytes may also kill Fas-bearing cells, thus damaging those tissues that express Fas [24]. The Fas/Fas-L signaling pathway may also act in other systems in which Fas is expressed by target cells, including a variety of epithelial cells, thymus, lung, spleen, uterus, placenta, testicles, seminal vesicle and prostate [21, 30]. Fas and Fas-L are also expressed, and control cell death, in both hematopoietic and non-hematopoietic malignancies [58].

Fas-L is expressed in the nervous system of the embryo [21], and in the normal rat and human brain [6]. Fas-L immunoreactivity is found in astrocytes and neurons in vivo, and Fas-L-positive astrocytes induce apoptosis of Fas-expressing Jurkat cells in vitro [6]. For these reasons,
interest has emerged in the study of the Fas/Fas-L signaling system in normal and pathological nervous tissue [4]. Increased Fas and Fas-L expression has been found in chronic inflammatory lesions in multiple sclerosis [15], and serum levels of soluble Fas are elevated in patients with a relapsing-remitting course [64]. Immunohistochemical studies have shown elevated Fas expression in oligodendrocytes and intense Fas-L immunoreactivity in microglia and infiltrating lymphocytes in chronic active and chronic silent lesions, thus suggesting Fas-mediated signaling in oligodendrocyte injury in multiple sclerosis [8, 16]. Similarly, Fas-mediated direct and bystander killing of innocent Fas-positive targets has been postulated in experimental encephalomyelitis [55].

Positive Fas staining is observed in degenerating neurons and astrocytes of the brain stem in neonatal mice infected with ts1, the neuropathogenic mutant of the Moloney murine leukemia virus. Fas-L is observed in sites where spongiform degeneration occurs in these infected animals, thus suggesting that neural cell death induced by ts1 infection is due to Fas-mediated mechanisms [11]. Immunoreactivity to Fas antigen is induced in motor neurons that are committed to die following spinal cord ischemia in rabbits [46]. Fas mRNA is induced in post-ischemic murine forebrain as revealed with Northern blotting and in situ hybridization [36, 37]. Moreover, Fas-L is induced in neurons of apoptotic areas after reversible middle cerebral artery occlusion in the rat [34]. Furthermore, recombinant FAS-L produces apoptosis in primary neurons and neuron-like cells in vitro [34]. These results suggest an involvement of Fas-L in the pathophysiology of post-ischemic damage in the nervous system.

The Fas/Fas-L signaling system has been examined in Alzheimer’s disease (AD) and Parkinson’s diseases. Soluble Fas is elevated in nigrostriatal dopaminergic regions in parkinsonian patients, whereas no differences when compared with controls are observed in the cerebral cortex [39]. Fas antigen is elevated in the frontal and temporal lobes in AD [13, 14], and in the temporal lobe and cerebellum in adult Down syndrome patients with Alzheimer-like neuropathologic lesions [47]. Fas immunoreactivity has been observed in senile plaques and star-like cells in AD brains [42]. In the present study, Fas and Fas-L expression has been examined using Western blotting and single- and double-labeling immunohistochemistry in the frontal cortex and hippocampus of normal human brains and in advanced stages of AD. Special attention has been paid to the localization of antigens in tangle-bearing and non-tangle-bearing neurons, as well as in neuritic plaques, which are the hallmarks of AD. Likewise, special attention has been paid to Fas and Fas-L expression and increased nuclear DNA vulnerability, as visualized with the method of in situ end-labeling of nuclear DNA fragmentation in combination with Fas and Fas-L immunohistochemistry.

### Material and methods

#### Post-mortem effects

Adult male Sprague-Dawley rats (200–300 g) from our colony were killed under deep diethyl ether anesthesia, and the bodies were stored at 4°C for 0, 6 and 12 h ($n=2$ per time point). The brains were removed from the skulls, and processed for gel electrophoresis and Western blotting. The cerebral cortices were dissected, frozen on dry ice and stored at –80°C until use.

#### Middle cerebral artery occlusion in rats

Adult female Sprague-Dawley rats (200–300 g) were subjected to middle cerebral artery occlusion by electrocautery. Anesthesia was induced by 4% isoflurane in oxygen and maintained at 1–2% isoflurane during the procedure. The body temperature was maintained during and after the surgical procedure by placing the animals under heat lamps. Sham-operated (control) and ischemic rats were killed at 12 h and 24 h ($n=4$ per time point). For morphological studies, the rats were anesthetized with diethyl ether and perfused through the heart with saline followed by 4% paraformaldehyde phosphate-buffered saline (PBS). Immediately afterwards, the brains were removed from the skull and fixed with the same fixative solution for 24 h at 4°C. For gel electrophoresis and Western blotting, the rats were killed under deep diethyl ether anesthesia and the fresh brains were rapidly removed from the skulls. A cortical area of 2×2 mm comprising the infarcted tissue, or the corresponding region in controls, was dissected, frozen in liquid nitrogen and stored at –80°C until use.

Animal welfare was conducted according to the Real Decreto 223:1988 of the Spanish Government, which makes recommendations similar to those of the NIH report, Public Health Service Policy on the Human Care and Use of Laboratory Animals.

#### Human cases

Samples of the frontal cortex and hippocampus from ten patients with AD (ranging from 68 to 82 years, mean age: 72.1 years; four men, six women), nine age-matched controls with no neurological disease (ranging from 66 to 83 years, mean age 72.6 years; four men, five women), and three patients with old cortical infarcts (aged 73, 68 and 69 years; two men, one woman) were obtained at autopsy. The delay between death and tissue processing was between 2 and 6 h. All AD cases had suffered from long-lasting severe sporadic AD (mean duration 6.8 years), and all of them were categorized as stage VI of Braak and Braak [9] on neuropathological examination. In addition, three biopsy samples were recovered from our files. The biopsies were carried out for diagnostic purposes in three cases (two men, one woman) with early onset dementia (one of them familial). They were sent to our laboratory for morphological and immunohistochemical study after 4% formalin fixation for 24 h. In post-mortem cases, the samples were fixed with 4% paraformaldehyde in PBS for 48 h and embedded in paraffin for Fas and Fas-L immunohistochemical studies. A complete neuropathological examination was carried out in every case (AD cases and controls) in 10% formalin-fixed samples of selected brain regions. For this purpose, dewaxed paraffin sections, 7 µm thick, were stained with hematoxylin and eosin, Luxol fast blue-Klüver Barrera and methenamine silver (PAM), or processed for immunohistochemistry following the avidin-biotin-peroxidase method (ABC kit, Vectastain, Vector). Antibodies to phosphorylated neurofilaments of 170 kDa or 200 kDa (clones BF10 and RT97, Boehringer Mannheim) were used at dilutions of 1:100 or 1:50, respectively. Antibodies to tau (Sigma) were used at a dilution of 1:10. Antibodies to glial fibrillary acidic protein (GFAP, Dako, Dakopats), βA4-amyloid (Boehringer Mannheim) and ubiquitin (Dako) were used at dilutions of 1:250, 1:5 and 1:200, respectively. For gel electrophoresis and Western blotting, fresh samples of the frontal cortex and hippocampus from patients with