Acta Neuropathol (1999) 97 : 1–4

EXPRESS COMMUNICATION

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Human astrocytic brain tumors express APO2L/TRAIL

Received: 19 August 1998 / Accepted: 14 September 1998

Abstract APO2 ligand (APO2L) is a CD95 ligand (CD95L)-related cytokine of the tumor necrosis factor family that interacts with agonistic (DR4, DR5) and antagonistic (DcR1, DcR2) receptors. Cultured malignant glioma cells preferentially express agonistic receptors and are susceptible to APO2L-induced apoptosis. Here, we report that 8 of 8 human glioma cell lines expressed APO2L mRNA and protein in vitro. Immunohistochemistry using a monoclonal antibody to APO2L revealed that all 23 primary astrocytic brain tumors analyzed, including low-grade astrocytomas and glioblastomas, express APO2L in vivo. With the exception of reactive astrocytes, non-neoplastic glia and neurons in the cerebrum lacked immunoreactivity of APO2L. Thus, in addition to the CD95/CD95L system, a second death ligand/death receptor pair may regulate susceptibility to apoptosis in human glial neoplasms.

Key words APO2 ligand · CD95 ligand · Glioma

Introduction

APO2 ligand (APO2L), also known as TRAIL (TNF-related apoptosis-inducing ligand), is a novel member of the tumor necrosis factor (TNF) family [10, 17]. Like TNF and CD95 ligand (CD95L), APO2L has been shown to induce apoptosis in various cancer cell lines [10, 17] and specifically malignant glioma cell lines [11]. The apoptotic signalling cascade induced by APO2L is caspase dependent and seems to be similar to the death cascade induced by CD95 activation [4, 5, 11]. APO2L mRNA has been detected in most human tissues, including peripheral blood leukocytes, spleen and thymus.

Two agonistic receptors for APO2L have been identified: DR4 [7] and DR5 (TRAIL-R2) [8, 13, 14]. Both contain a highly conserved death domain which is essential for the transmission of the death signal. DR4 and DR5 are widely expressed in human tissues, including those in which APO2L is expressed. Furthermore, tumor cells are susceptible to APO2L-induced apoptosis, whereas APO2L does not exhibit systemic toxicity (Ashkenazi, personal communication). These findings can be explained by the identification of two antagonistic decoy receptors: decoy receptor 1 (DcR1) (also called TRID or TRAIL-R3) [1, 8, 13] and decoy receptor 2 (DcR2) (also called TRUNDD) [6, 9]. DcR1 has an extracellular APO2L binding region with two cysteine-rich domains and a transmembrane domain but no intracellular domain. DcR2 also has an APO2L-binding domain and a transmembrane domain. The cytoplasmic region of DcR2 contains a truncated death domain which does not transduce the death signal. Both receptors do not induce apoptosis upon forced expression but protect cells from APO2L-induced cell death. DcR1 is mainly expressed in spleen, peripheral blood lymphocytes, placenta and lung, whereas expression of DcR2 mRNA can be found in most human tissues. In contrast, most tumor cell lines lack expression of antagonistic APO2L receptors [8, 11, 13].

Interestingly, despite the widespread tissue distribution of APO2L receptors, no expression of DR5, DcR1 or DcR2 was detected in the human brain [8, 13]. To our knowledge, DR4 expression in brain has not been investigated. However, APO2L receptors are expressed in human malignant glioma cell lines [11]. The cell lines predominantly express the agonistic receptors DR4 and DR5 and are susceptible to APO2L-induced apoptosis.

Similiarly to APO2L receptors, no APO2L mRNA was detected in human brain, although APO2L mRNA is expressed in nearly all other human tissues [17]. Therefore,

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we investigated whether, similar to CD95L [2, 12, 15], APO2L is expressed by malignant gliomas in vitro and in vivo.

Materials and methods

Cell culture

T98G human malignant glioma cells were obtained from the American Type Culture Collection (Rockville, Md.). LN-18, LN-308, LN-319, LN-428, U138MG, U251MG and U373MG human malignant glioma cells were kindly provided by Dr. N. de Tribolet (Lausanne, Switzerland). The glioma cells were cultured in DMEM containing 10% FCS, 2 mM glutamine, and 1% penicillin/streptomycin.

Reverse transcription-polymerase chain reaction

Total RNA was isolated by guanidinium thiocyanate-phenol-chloroform extraction and treated with RNase-free DNase I (10 U/ml) for 30 min at 25° C to remove genomic DNA contamination. cDNA was synthesized from 5 mg RNA in a final volume of 20 µl using SuperScript TM II (Gibco-BRL, Gaithersburg, Md.) and oligo(dT) priming (Pharmacia, Uppsala, Sweden). The PCR conditions were as follows: APO2L 35 cycles, 45 s at 95° C, 45 s at 58° C, 45 s at 72° C, primer sequences AGACCTGCGTGCTGATCGTG (nucleotides 131–150) and TTATTTTGCGGCCCAGAGCC (nucleotides 525–544); β-actin 35 cycles, 45 s at 95° C, 45 s at 55° C, 45 s at 72° C, primer sequences TGTTTGAGACCTTCAACACCC (nucleotides 409–429) and AGCACTGTGTTGGCGTACAG (nucleotides 918–937). The PCR fragments were separated in 1% agarose gels and visualized by ethidium bromide. A cDNA plasmid encoding full-length APO2L served as a positive control. The β-actin cDNA fragment was amplified as an internal control for equal amplification. A water control was run in each amplification to control for cross-contamination between tubes. No signal was obtained when reverse transcription (RT) was omitted during the RT step, confirming that the PCR signals were not derived from genomic DNA contamination.

Immunoblot analysis

The cells were lysed in lysis buffer containing 50 mM TRIS-HCl, ph 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet-P40, 100 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 2 µg/ml aprotinin. After 15 min on ice, the lysates were centrifuged for 10 min at 13 000 rpm. The soluble supernatant fraction was used for immunoblot analysis. Protein concentrations were measured by Bio-Rad assay, and 20 µg protein were loaded per lane and separated on 12% gels. The proteins were electroblotted onto nitrocellullose membranes, the membranes were blocked and incubated overnight with rabbit polyclonal anti-APO2L antibody (pAb 1) (10 µg/ml) generated by immunization with the synthetic peptide GTRGRSNTLSSPNSKN derived from the extracellular APO2L sequence. The membranes were washed three times and were incubated with secondary anti-rabbit alkaline phosphatase-conjugated antibody for 1 h, again washed three times in PBS, and stained with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate in 0.1 M TRIS-HCl pH 9.5 containing 50 mM $MgCl_2$ and 10 mM NaCl.

Immunohistochemistry

The surgical specimens of diffuse astrocytomas were obtained from patients treated in the Department of Neurosurgery, University Hospital of Zürich, Switzerland. A total of 23 diffuse astrocytomas were graded according to the WHO classification into low-grade astrocytoma (grade II, 3 males and 4 females), anaplastic astrocytomas (grade III, 2 males and 3 females) and glioblastomas (grade IV, 8 males and 3 females). Mean ages of patients were 39.6 years for low-grade astrocytoma, 42 years for anaplastic astrocytomas, and 54 years for glioblastomas. Tumor tissues were fixed in buffered formalin at 4° C overnight and embedded into paraffin.

Monoclonal antibody 5C2.4.9 against human APO2L was generously provided by Dr. A. Ashkenazi [10] and dissolved in antibody diluent (TRIS-HCl buffer containing carrier proteins and 0.015 M NaN_3, Dako) at 0.1 µg/µl. After deparaffinization, sections were boiled in a high-pressure oven in 10 mM sodium citrate pH 6.0 for 10 min. Sections were allowed to cool for 30 min, rinsed twice in distilled water, incubated in PBS for 5 min, and then reacted overnight at 4° C with APO2L antibody (1 : 500 in PBS). The reaction was visualized using a Vecstain ABC kit and diaminobenzidine (Vector laboratories, Burlingame, Calif.). Sections were conterstained with hematoxylin.

Results

First, we investigated APO2L mRNA expression in 8 human malignant glioma cell lines by RT-PCR. Figure 1 A shows that APO2L is expressed by all cell lines examined. Next, we asked whether the cell lines also expressed APO2L protein. Immunoblot analysis revealed an immunoreactive band of approximately 32 kDa in all cell lines, which corresponds to the predicted molecular mass of full-length APO2L. Soluble APO2L examined as a control migrated at approximately 24 kDa, as expected from the predicted molecular mass [10].

Second, we investigated whether APO2L was expressed in primary human astrocytic gliomas in vivo.

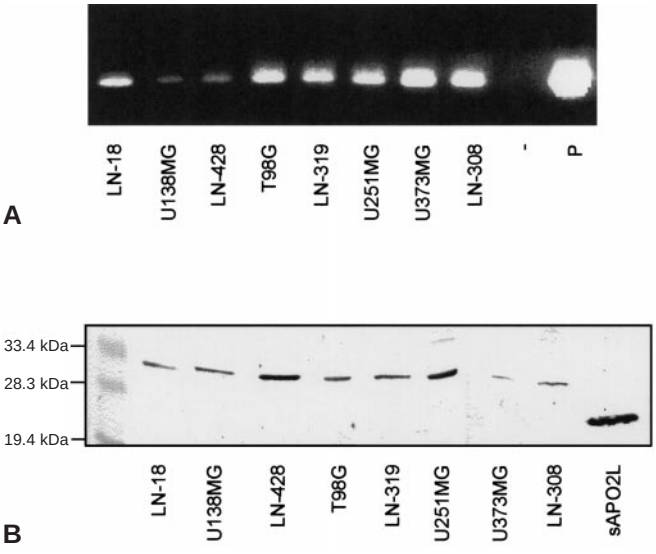

Fig. 1 A, B Expression of APO2L in human malignant glioma cell lines. **A** APO2L mRNA expression was analyzed by RT-PCR as described in Materials and methods (- water control, *P* cDNA plasmid control). RNA quality was ascertained by actin amplification (data not shown). The amplified fragment size for APO2L was 413 bp. **B** Immunoblot analysis of APO2L protein expression; 20 µg protein were loaded per lane (sAPO2L, 400 ng sAPO2L), and APO2L was detected using a polyclonal rabbit anti-APO2L antibody (10 µg/ml) (for details, see Methods)

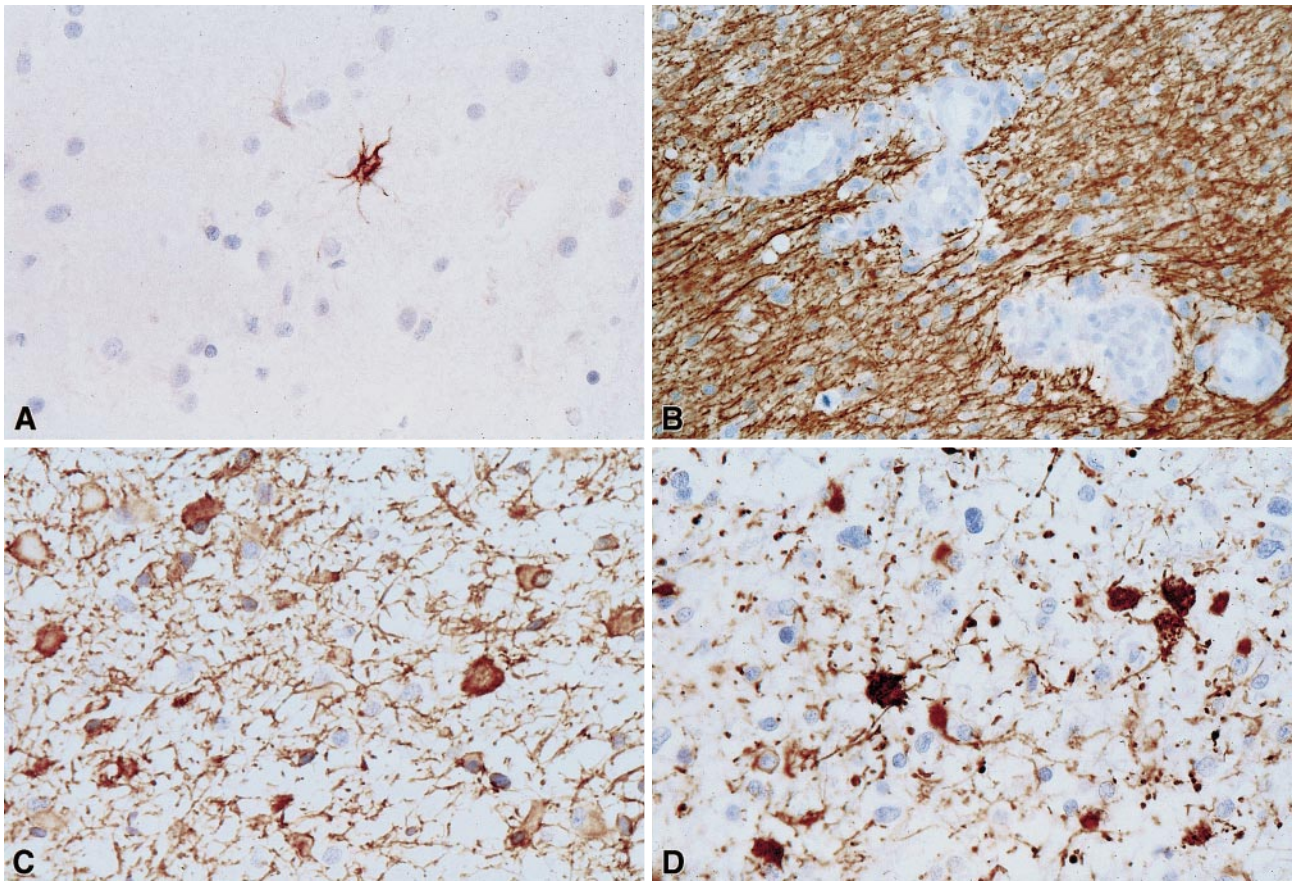

Fig. 2 A–D Expression of APO2L in human malignant gliomas in vivo. **A** Normal cerebral tissue lacks APO2L immunoreactivity, except for reactive astrocytes. **B** APO2L is expressed by most glioblastoma cells, but not by proliferating microvessels. Neoplastic astrocytes show a preferential expression of APO2L at and below the plasma membrane (**C**) and in their cell processes (**D**). **A**, **C**, **D** × 310, **B** × 155

Normal cerebral tissue was negative for APO2L expression except for reactive astrocytes present in surrounding brain areas of tumor tissues, which showed a strong signal in their perikaryon and cell processes (Fig. 2 A). In contrast, all 23 astrocytic brain tumors investigated (7 low-grade astrocytomas, 5 anaplastic astrocytomas and 11 glioblastomas) exhibited APO2L expression. With the exception of some areas with advanced anaplasia, most tumour cells were immunoreactive, causing a staining pattern similar to that by glial fibrillary acidic protein (GFAP) (Fig. 2 B). Immunoreactivity to APO2L monoclonal antibody prevailed in the cytoplasm below the plasma membrane (Fig. 2 C) and in astrocyte processes (Fig. 2 D). Proliferating vascular endothelial cells lacked immunoreactivity for APO2L (Fig. 2 B).

Discussion

The expression of cytokines and cytokine receptors in human brain tumors has attracted a lot of interest because of their potential significance for tumor immunology. Specifically, the death ligand/receptor pair CD95L/CD95 has been explored as a therapeutic target for glioblastoma multiforme [16].

The biological role of APO2L has not been clearly defined. The existence of at least four receptors suggests a highly regulated system with important biological functions. This is supported by the widespread tissue distribution of both APO2L and its receptors. High expression of APO2L in peripheral blood leukocytes and in lymphoid tissues such as spleen and thymus has led to the assumption that APO2L might serve a similar role as CD95L in the control of peripheral immune responses and in maintaining immune privilege [3]. However, in contrast to the limited expression of CD95L, APO2L mRNA has been detected in nearly all tissues examined [17]. In this context, it is of interest to note that normal brain tissue is negative for APO2L (Fig. 2 A). This is in agreement with the lack of detectable APO2L mRNA levels in human brain [17]. However, we find that malignant glioma cells express APO2L mRNA and protein in vitro (Fig. 1) and in vivo (Fig. 2). This is unexpected in light of strong pro-apoptotic properties of soluble APO2L on glioma cells [11] and provokes the question as to the biological function of APO2L expressed by tumor cells. Since APO2L is suggested to have negative regulatory functions in the immune system, one might speculate that APO2L expressed on tumor cells may mediate tumor immune escape, as suggested for CD95L [2, 12, 15, 16].

Although no expression of DR5, DcR1 and DcR2 mRNA was observed in Northern blots of normal brain tissue [8, 13], their expression needs to be studied at the protein and single-cell level as soon as suitable specific receptor antibodies available. There is mRNA for all four receptors in malignant glioma cell lines, in particular for the agonistic receptors [11], also indicating that APO2L and its receptors are coexpressed in glioma cells in the absence of autocrine suicide or paracrine fratricide.

Further investigations of the expression of APO2L receptors in human gliomas are necessary to answer the critical question of whether a specific loss of decoy receptors in glioma compared with non-neoplastic cells may allow the selective therapeutic elimination of glioma cells via APO2L/DR4 and APO2L/DR5 interactions [11].

Acknowledgements This work was supported by the Deutsche Forschungsgemeinschaft (We 1502/5-2) and the Foundation for Promotion of Cancer Research, Tokyo, Japan. The authors thank A. Ashkenazi (Department of Molecular Oncology, Genentech, Inc., South San Francisco, CA) for generously providing APO2L antibodies.

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