Cell Death and Differentiation (1999) 6, 1133–1142

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A comparison of the cytoplasmic domains of the Fas receptor and the p75 neurotrophin receptor

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Received 15.7.98; revised 20.8.99; accepted 23.8.99
Edited by C Thiele

Abstract

The p75 neurotrophic receptor (p75) shares structural features with the Fas receptor (FasR). Both receptors contain extracellular cysteine-rich repeats, a single transmembrane domain, and intracellular death domains. However, it has not been clearly established whether their death domains are equivalent in their ability to mediate apoptosis. To understand better the role of p75 during apoptosis, we constructed chimeric receptors that contained the extracellular portion of the FasR and the intracellular portion of p75. These chimeric receptors, one containing the p75 transmembrane domain and the other containing the FasR transmembrane portion, as well as wild-type p75 and Fas receptors, were transiently transfected into human U373 glioma cells and human embryonic kidney 293 cells (293 cells), which are both responsive to Fas-mediated apoptosis. Whereas expression of FasR was sufficient to induce apoptosis in U373 and 293 cells, expression of p75 and the chimeric receptors induced only minimal levels of cell death compared to FasR. The results indicate that the magnitudes of FasR- and p75-induced killing are different and suggest that the death domain of p75 does not function in the same manner as the FasR death domain.

Keywords: neurotrophins; p75 receptor; death domain; Fas receptor

Abbreviations: DRG, dorsal root ganglion; FADD, Fas-associated death domain protein; FLICE, FADD-like ICE; FasR, Fas receptor; GFP, green fluorescent protein; JNK, c-jun kinase; LIF, leukemia inhibitory factor; MACH, MORT1-associated CED-3 homolog; MORT, mediator of receptor-induced toxicity; NGF, nerve growth factor; NTR, neurotrophin; PE, phycoerythrin; TNF, tumor necrosis factor; TRAF, TNF receptor associated factor; TRADD, TNF-R1-associated death domain protein; TUNEL, TdT-mediated dUTP Nick End Labeling

Introduction

The p75 receptor is the founding member of the TNF receptor superfamily.[1] Structural features shared by several members of this family include a cysteine-rich extracellular domain repeated two to six times and an intracellular motif termed the 'death domain'. The term 'death domain' was coined from its functional role in mediating apoptosis, particularly via the p55 TNF receptor and FasR.[2,3] The death domain is a protein association motif[4,5] that binds to cytoplasmic proteins, which can trigger the caspase protease cascade or other signal transduction pathways. Multiple adaptor and death effector proteins that bind to the death domains of the p55 TNF and Fas receptors have been identified.

Considerable attention has been focused upon the possibility that p75 acts as a cell death receptor.[6–10] Apoptosis has been detected in p75-positive retinal precursor cells during embryonic development[11,12] and in transgenic mice overexpressing the cytoplasmic domain of p75.[13] In differentiated oligodendroglial cells *in vitro*, nerve growth factor (NGF), a ligand for p75, induces a rapid apoptotic response.[14] A cell death role for p75 has been implicated in several p75-expressing cell lines[15,16] and also in sensory[17] and sympathetic neurons.[18] Following nerve injury and other traumatic conditions, p75 is frequently found to be upregulated in a variety of different cell types. In fact, apoptosis, of Schwann cells[19] and neuronal cells after seizure[20] have been correlated with high levels of p75 receptor expression.

Despite evidence that p75 participates in apoptosis, the mechanisms responsible for this activity remain undefined. Elucidation of the actions of p75 is complicated by the expression of Trk receptor tyrosine kinases, which exert a prominent survival or proliferative signal. The TrkA, TrkB, and TrkC tyrosine kinases are receptors for NGF, BDNF and NT-4, and NT-3, respectively.[21] The p75 receptor serves as a receptor for all of the neurotrophins and can enhance Trk signaling functions.[22–24] Additionally, activation of TrkA prevents p75-mediated apoptosis when both receptors are co-expressed.[25]

The existence of a death domain sequence at the C-terminus of p75[26] suggests that p75-mediated apoptosis may be mechanistically similar to Fas and the p55 TNF receptors. Given the structural homology between p75 and the other TNF receptor family members, it is conceivable that p75 may initiate apoptosis through a mechanism involving receptor oligomerization and recruitment of cell death proteins. In this study, we have tested whether p75 acts like the FasR by generating chimeric receptors with p75 and FasR and assessed the functional consequences of their expression in cultured cells.

Results

The nuclear magnetic resonance (NMR) structures of the cytoplasmic domains of both FasR and p75 have been reported.[26,27] The death domains of both proteins are composed of six alpha helices which form a globular structure. Given this overall structural similarity, we were interested in determining whether the death domain of p75 is competent to induce cell death in a manner similar to the FasR. The FasR binds to homotrimeric or higher order oligomeric forms of the Fas ligand. Ligand-induced receptor aggregation leads to the association of the FasR death domain with downstream death effector molecules, which subsequently trigger cell death.[28] In addition, an agonist antibody, Jo2, can trigger the same Fas-mediated death observed with the naturally occurring Fas ligand in immune cells.[29]

To determine whether the p75 cytoplasmic domain behaves in a way similar to the FasR intracellular domain, chimeric cDNAs which contain murine FasR extracellular sequences linked to the cytoplasmic domain of rat p75 were constructed. This strategy allowed the death domain of p75 to replace the FasR death domain and permitted receptor oligomerization of the p75 cytoplasmic domain by FasR agonists. Two different constructs were generated, A7, which contains the transmembrane and cytoplasmic sequences of p75, and B9, which contains the cytoplasmic sequence of p75 and the transmembrane sequence of the FasR (Figure 1). Using these chimeric receptors, we investigated whether the p75 death domain is competent to induce cell death.

To verify and compare the expression levels of the A7 and B9 chimeric receptors, the receptor cDNAs were transiently transfected into COS-1 cells. Immunofluorescence experiments with Jo2, an agonist antibody against the murine FasR, indicated that the two chimeras were expressed at the cell surface, at levels comparable to the wild-type FasR (Figure 2A). Cell lysates from COS-1 and 293 cells transfected with the Fas and p75 receptor constructs, along with A7 and B9 cDNAs, were also analyzed by Western blot analysis. Using an antibody made against the cytoplasmic domain of p75 (9992), chimeric receptors A7 and B9 were easily detected (Figure 2B). No immunoreactive proteins were detected in lysates from untransfected or wild-type FasR-transfected cells. As expected, the size of the chimeric receptors migrated at a smaller size than p75 due to the shorter extracellular domain of the FasR. A similar pattern of expression was detected in transfected 293 cells (Figure 2C).

Although each receptor was expressed at high levels in COS-1 cells, no evidence of apoptosis was observed, even in the presence of the Jo2 agonist antibody (data not shown), which is an effective inducer of Fas-mediated cell death.[29] These observations suggest that COS-1 cells are refractory to apoptosis by FasR overexpression, unlike other cells such as U373, 293, MCF7, and Jurkat cells.

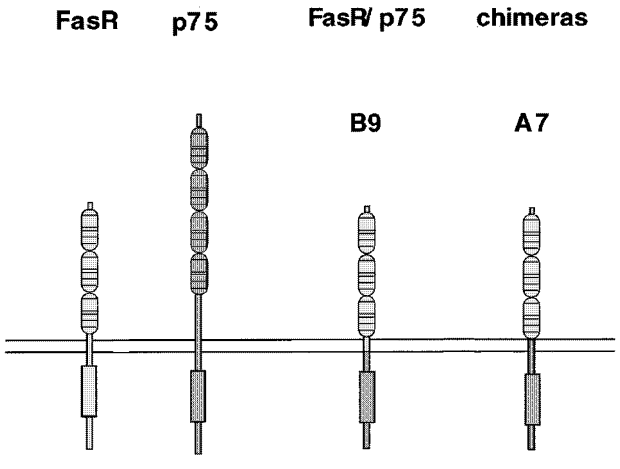

Figure 1 Schematic diagram of the FasR, p75 neurotrophin receptor, and chimeric receptors, A7 and B9. Each chimeric construct consists of the extracellular domain of the FasR fused to the intracellular domain of p75. A7 and B9 differ in their transmembrane (TM) domains, with A7 containing the p75 TM region and B9 containing the FasR TM region. Construction of the A7 and B9 chimeric cDNAs by PCR is described in the Materials and Methods section

U373 cells

For recipient cells, we turned to U373 glioma and 293 cells, which are sensitive to Fas- and TNF-mediated cell killing. U373 cell death can be induced by treatment with human anti-Fas antibodies.[30] These cells can be routinely transfected at an efficiency of 5%. To assess the cell death activities of the chimeric receptors, we transiently transfected the receptor cDNAs together with pRC-lacZ into U373 cells. In the presence of the β-galactosidase substrate, X-gal, we could quantitate transfected cells undergoing FasR-induced cell death. Within a few hours after transfection (3–6 h), FasR-transfected cells exhibited condensed nuclei by Hoechst 33342 staining, a hallmark of apoptosis (Figure 3).

Twenty-four hours following removal of the DNA precipitate, nearly 90% of FasR-transfected cells underwent cell death, as evidenced by the detachment of dying cells from the culture dish (Figure 4). The percentage of viable cells remaining on the dish would be calculated relative to sister cultures that were transfected with the empty vector and pRC-lacZ. Cells transfected with the pcDNA3 vector or with pRC-lacZ alone displayed no changes in viability (Figure 4). Compared to vector controls, very few dying cells were observed after transfection of the wild-type p75 receptor or the A7 chimeric construct (data not shown). In contrast, 40–50% of U373 cells expressing the B9 receptor, which contains the FasR transmembrane region, appeared morphologically similar to those cells undergoing Fas-mediated apoptosis. In addition to differences in the magnitude of cell death, the time course of FasR- and B9-induced killing differed in that FasR-expressing cells died within $\sim$6 h of receptor expression, while B9-expressing cells required $>$24 h to die (data not shown).

Cell death mediated by p75 expression has been detected in several cell lines;[15,16] however, these results were obtained under conditions in which serum was

withdrawn from growing cells. Under normal growth conditions, expression of p75 receptors in many cell types, including U373 and 293 cells, does not activate death pathways[9] (data not shown). The FasR initiates apoptosis through receptor oligomerization, and clustering of FasRs can be achieved through agonist antibodies or the Fas ligand. Therefore, we tested the effects of a murine Fas agonist antibody, Jo2, in transfected U373 cells to determine whether aggregation of these receptors enhances apoptosis.

Treatment of transfected U373 cells with the Jo2 antibody, however, did not alter the number of cells undergoing cell death following transfection (Figure 4). Therefore, the killing activity of FasR and the B9 chimeric receptor in U373 cells appears to be independent of ligand-induced aggregation, presumably because the receptors are expressed at sufficiently high levels to function in the absence of ligand.

293 cells

A human embryonic kidney cell line, 293, was found to be sensitive to Fas-mediated apoptosis.[31] To compare further the effects of the chimeric receptors, we transiently transfected 293 cells with the receptor cDNAs. Western blot analysis (Figure 2C) and indirect immunofluorescence

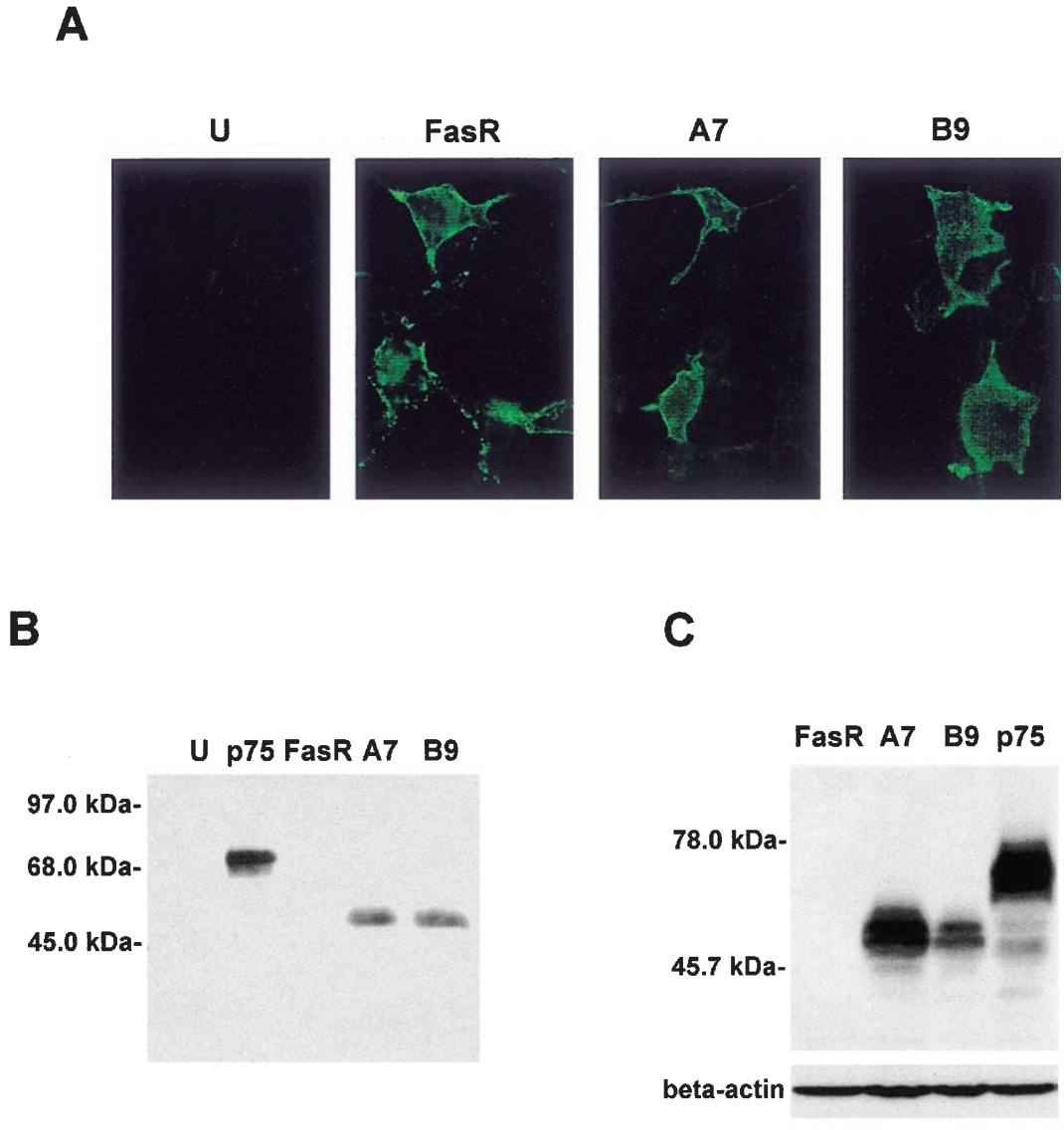

Figure 2 Expression of the Fas receptor (FasR), p75, and A7 and B9 chimeras. (**A**) Transfected COS-1 cells were subjected to indirect immunofluorescence with Jo2, an antibody that recognizes the extracellular domain of the murine Fas receptor.[29] (**B**) Cell lysates prepared from COS-1 cells transiently transfected with FasR, p75, A7, and B9 constructs were immunoblotted with 9992, an antibody specific for the intracellular domain of p75. U=untransfected. (**C**) Cell lysates were prepared from 293 cell transiently transfected with FasR, p75, A7, and B9 expression constructs and then subjected to Western blot analysis with the anti-p75 antibody, 9992

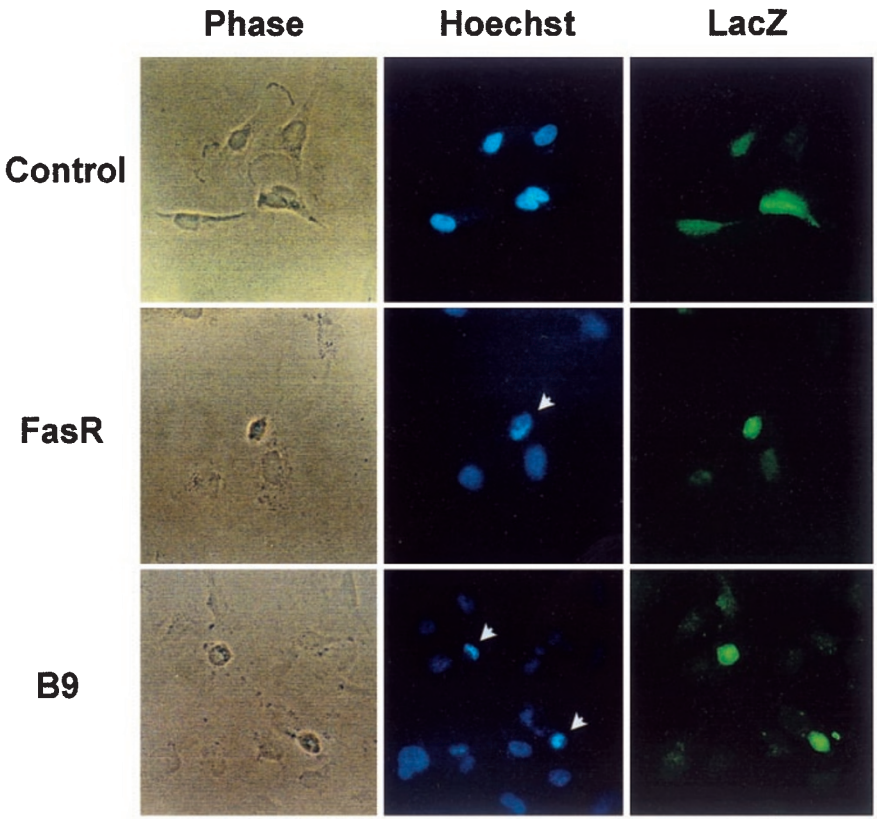

Figure 3 Morphology of U373 cells transiently transfected with the FasR and B9 chimeric receptor. After transfection, cells were plated into 24-well plates and processed for staining. Vector (pcDNA3) and receptor-transfected cells were identified with β-galactosidase antibody staining (LacZ column), and nuclei were examined by Hoechst 33342 staining (Hoechst column). Control=vector alone

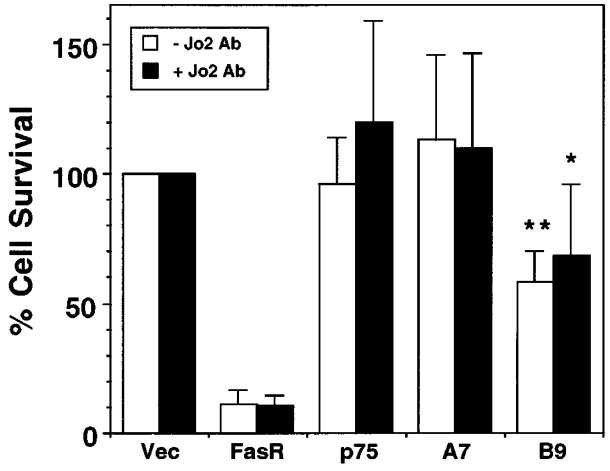

Figure 4 U373 cell viability following transient transfection with receptor constructs. Transfected cells were incubated for 36–48 h in serum-free medium in the absence or presence of 50 ng/ml anti-Fas Jo2 agonist antibody, after which cells were fixed and stained in the presence of X-gal, the β-galactosidase substrate. The number of viable blue cells were counted and calculated as a percentage of vector-transfected cells. All bars depict mean $\pm$ S.E.M. from five independent experiments. **Indicates a significant difference between untreated vector and untreated B9 at $P<0.002$, and *indicates a significant difference between treated vector and treated B9 at $P<0.02$, both according to Student's *t*-test

(Figure 6) verified the expression of the transfected receptors. In addition, assessment of FasR, A7 and B9 cell surface expression by flow cytometry indicated that the different receptors were expressed at similar levels in a comparable percentage of transfected cells (Table 1). For these measurements, an antibody (Jo2) was used that detected cell surface expression of FasR and the chimeric constructs. The percentage of FasR-expressing cells was found to be slightly lower than that of the chimera-expressing cells. Overall, no significant differences in expression level among the constructs was detected by Western blot or flow cytometry. Since the efficiency of transfection, 60–70%, was considerably higher than in U373 cells, the 293 cell system was used to quantitate cell death.

As a first measure of cell death, Annexin V staining was performed on transfected 293 cultures. Annexin V is a naturally occurring protein which avidly binds to phosphatidylserine (PS). During the early stages of apoptosis, PS translocates from the inner to the outer leaflet of the plasma membrane, allowing detection by Annexin V. As a marker of transfection, a GFP construct was co-transfected with the receptor constructs. The percentage of Annexin V- and GFP-double positive cells among GFP-positive cells for each condition was assessed by flow cytometry (Figure 5).

Table 1 Assessment of FasR and chimeric receptor expression by flow cytometry

Construct	% Jo2-positive cells	Mean Jo2-fluorescence (arbitrary FL2 units)
FasR	28.4	294.3
A7	39.3	255.5
B9	37.9	203.1

Two hundred and ninety-three cells were transiently transfected for 24 h with the indicated receptor cDNAs. Afterwards, cells were resuspended in PBS containing 1% BSA and 0.05% sodium azide and incubated at 4°C for 30 min with 0.5 μg anti-FasR Jo2 antibody (phycoerythrin (PE)-conjugate) per 10^6 cells. For each condition, 5000 events were counted by flow cytometry. Mean Jo2-fluorescence represents the average intensity of Jo2-PE-fluorescence per event in the Jo2-positive subpopulation. FL2 refers to the flow cytometry channel used to measure Jo2-PE fluorescence

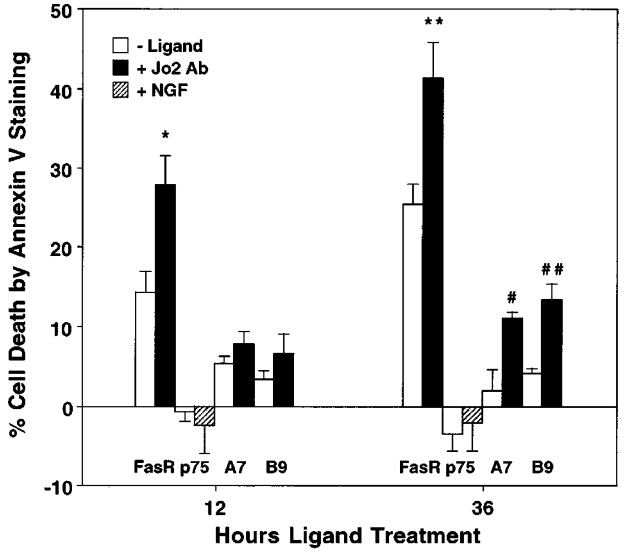

Figure 5 Cell death of 293 cells following transient transfection with receptor constructs as assessed by Annexin V-PE flow cytometry. After co-transfecting cells with a GFP construct and cDNAs encoding pcDNA3, FasR, p75, A7, or B9, cells were incubated in DMEM plus 5% FBS with or without ligand for 12 or 36 h. FasR-, A7, and B9-transfected cells were exposed to the anti-Fas agonist antibody Jo2 (500 ng/ml) while p75-transfected cells were exposed to NGF (100 ng/ml). pcDNA3 cells were exposed for 12 or 36 h to DMEM plus 5% FBS with (1) no ligand, (2) Jo2 antibody, or (3) NGF. Following ligand exposure, cells were stained with Annexin V-PE and assessed by flow cytometry for the percentage of Annexin V- and GFP-double positive cells among total GFP-positive cells. To assess receptor-specific cell death, Annexin V percentages obtained under the different pcDNA3-transfected conditions were subtracted from the values measured under receptor-transfected conditions with corresponding ligand and time conditions. Final Annexin V-positive percentages were expressed as (condition-control)/(100-control). All bars represent mean$\pm$S.E.M. from three independent experiments. * and ** indicate significant differences between FasR and FasR+Jo2 at 12 and 36 h respectively ($P<0.03$). #Indicates a significant difference between A7 and A7+Jo2 at 36 h ($P<0.03$). ##Indicates a significant difference between B9 and B9+Jo2 at 36 h ($P<0.006$). Statistical analysis was performed by Student's *t*-test

Transfection with the FasR yielded low to moderate levels of cell death, 14.5 and 25.7%, at 12 and 36 h post-transfection. In contrast, p75-transfected cells were not significantly different from vector-transfected cells at both time points. Expression of A7 and B9 chimeric receptors 12 h following transfection elicited very low levels of cell death, which did not increase at 36 h.

To determine whether receptor oligomerization after ligand treatment would augment the effects of receptor-mediated cell death of 293 cells, FasR-, A7-, and B9-transfected cells were treated with the Jo2 antibody. Also, p75-transfected cells were treated with NGF. At 12 and 36 h post-transfection, ligand treatment increased FasR-mediated apoptosis by 92.9 and 61.2%, respectively, compared to FasR transfection alone. NGF treatment of p75-transfected cells did not alter p75's inability to induce 293 cell death, even with increasing time. However, after transfection with the chimeric A7 and B9 receptors, Jo2 treatment resulted in a small increase in Annexin V staining at 12 h and a higher augmentation of staining at 36 h. At 36 h post-transfection, Jo2 exposure increased A7-induced cytotoxicity 5.4-fold and B9-induced cytotoxicity 3.2-fold compared to untreated cells expressing the transfected receptors. These results are consistent with the results obtained in U373 cells, in that a low magnitude and slow time course of B9-induced killing was observed compared to FasR-mediated death.

As an independent measure of cell death and to confirm further the apoptotic nature of A7- and B9-mediated killing, TUNEL staining on 293 cells transfected with FasR, A7, and B9 constructs was performed. The TUNEL reaction labels free termini of fragmented DNA, which is indicative of the late stages of programmed cell death. Vector-transfected cells did not give rise to receptor expression 36 h after transfection, as detected by Jo2 immunofluorescence, or any TUNEL-positive cells (Figure 6). Vector-transfected cells treated for 36 h with Jo2 antibody similarly showed little or no evidence of TUNEL positivity (data not shown). On the other hand, FasR transfection yielded numerous TUNEL-positive cells that displayed simultaneous Jo2 staining 36 h following transfection, and the number of TUNEL-positive cells increased with Jo2 antibody treatment. Consistent with the Annexin V results, A7 and B9 transfection resulted in few TUNEL- and Jo2-double positive cells 36 h post-transfection. As with the FasR, Jo2 antibody treatment potentiated both A7- and B9-mediated cell death.

Using GFP as marker for co-transfection, the percentage of TUNEL- and GFP-double positive cells among total GFP-positive cells for each receptor transfection was determined after a 36 h incubation, with or without Jo2 antibody treatment (Figure 7). Exposure to the Jo2 antibody potentiated the apoptosis induced by FasR transfection by 1.78-fold. The response of A7- and B9-induced apoptosis was considerably lower but was enhanced by exposure to Jo2. Taken together, these results indicate that engagement of FasR by Jo2 induced a greater level of apoptosis, compared to the A7 and B9 chimeric constructs that contain the p75 cytoplasmic domain. Nevertheless, we did observe an increase in chimera-induced cell death with agonist antibody compared to receptor transfection alone, suggesting that a small apoptotic effect could be mediated by aggregation of the p75 cytoplasmic domain.

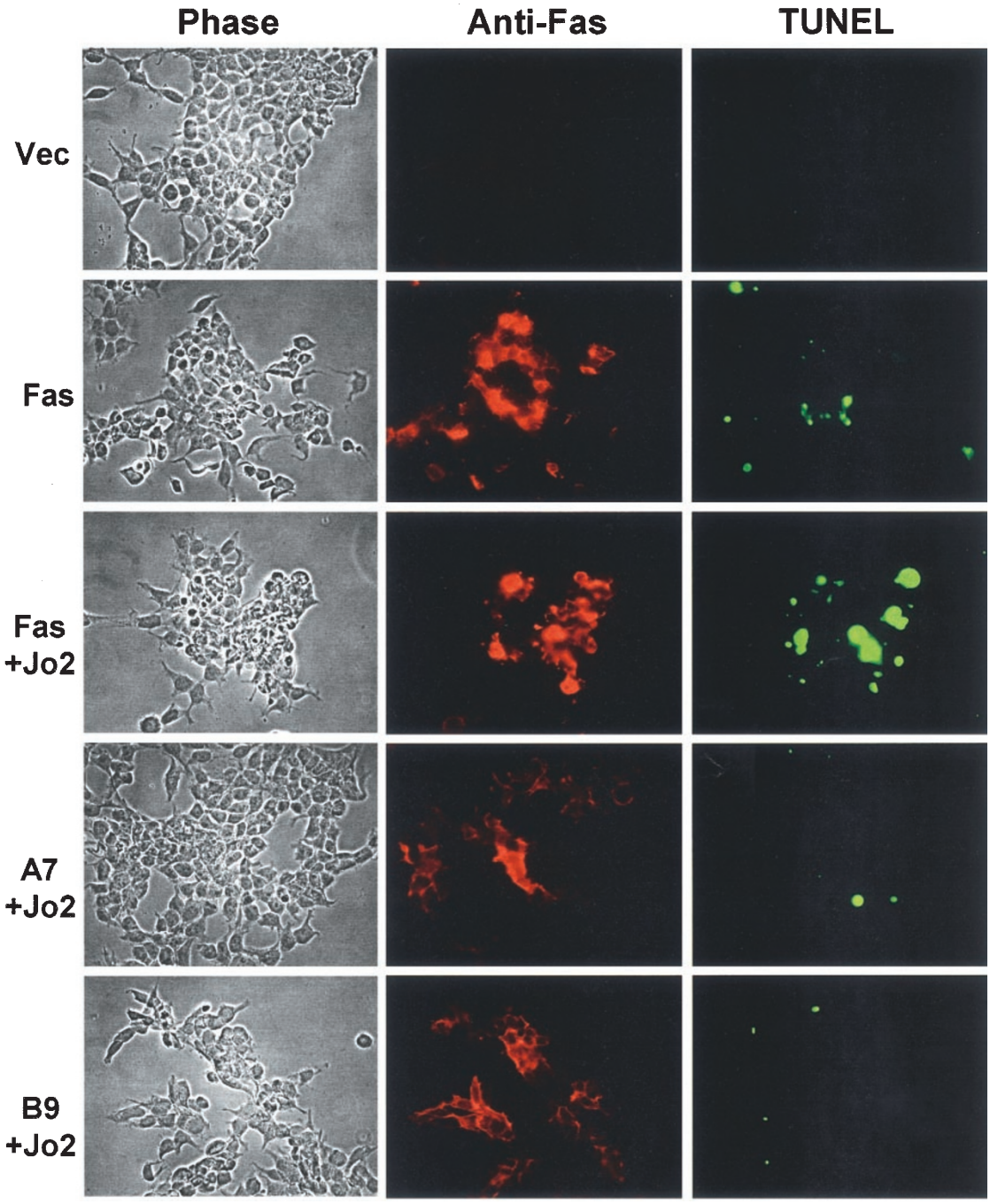

Figure 6 Phase-contrast microscopy, immunostaining, and TUNEL assays of transiently transfected 293 cells. Following 1 day transfection with pcDNA3 vector, FasR, A7, and B9 constructs, cells were exposed for 36 h to DMEM plus 5% FBS with or without 500 ng/ml Jo2 antibody. Cells were then subjected to both anti-Fas Jo2 immunofluorescence (second column) and TUNEL staining (third column). Vec=vector

Discussion

Structural analysis of the C-terminal 80 amino acid sequence of the p75 receptor reveals features similar to the death domain of the FasR.[26,27] However, the results here indicate that the abilities of these two receptors to induce cell death in U373 cells are markedly different. A comparison of cell death activities of p75 and the FasR indicates that expression of the FasR intracellular region results in much greater cell death than expression of the p75 intracellular region. These results were further supported in transfected 293 cells. Transfection of FasR resulted in apoptosis, as measured by Annexin V staining and TUNEL reactivity, whereas introduction of p75 did not alter cell viability. A clear effect of the agonist antibody, Jo2, could be observed after treatment of FasR- compared to vector-transfected cells but not with 293 cells overexpressing

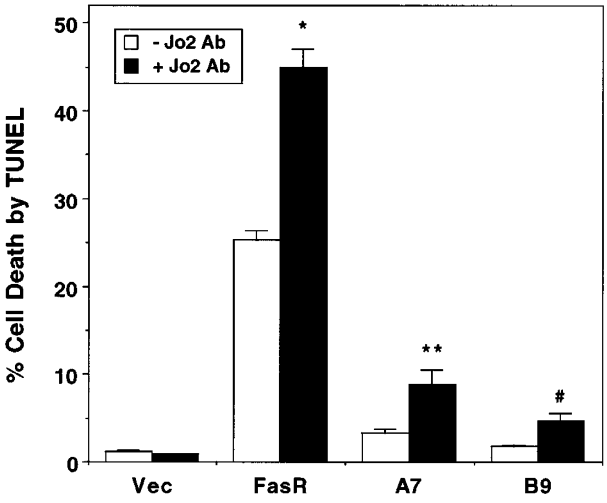

Figure 7 Quantitation of TUNEL-positive 293 cells following transient transfection with receptor constructs. Cells were co-transfected for 1 day with a GFP construct and one of the following cDNAs: pcDNA3 vector, FasR, A7 or B9. Afterwards, cells were incubated for 36 h in DMEM plus 5% FBS with or without 500 ng/ml Jo2 antibody, processed for TUNEL staining, and counted. Per cent TUNEL-positive cells represents the number of TUNEL- and GFP-double positive cells divided by the number of GFP-positive cells. All bars represent mean $\pm$ S.E.M. from three to four independent experiments. *Indicates significant difference between FasR and FasR+Jo2 at $P<0.001$. **Indicates significant difference between A7 and A7+Jo2 at $P<0.04$. #Indicates significant difference between B9 and B9+Jo2 at $P<0.01$. Statistical analysis was performed by Student's *t*-test. Vec=vector

p75. A similar ligand enhancement of cell death was observed in 293 cells transfected with the chimeras, although to a diminished degree compared to ligand enhancement in FasR-transfected cells.

We infer from these experimental results that the mechanism of p75 killing is different from that of FasR. Several features may contribute to the differences between p75 and FasR. For example, the TNF and Fas receptor death domains easily self-aggregate,[32] whereas self-association has not been observed with the death domain of p75 (unpublished results). This may be due to amino acid sequence differences or to slight alterations in structure. For example, the first α-helix of the death domain is oriented in different positions for the Fas and p75 receptors. Also, the death domain of p75 resembles a death effector domain structure,[33] raising the possibility that protein-protein interactions relevant to cell death are mediated by this domain.

In the TNF receptor superfamily, receptor activation occurs via clustering, which is mediated by binding of multimeric ligands.[34] Following oligomerization, the FasR recruits the adaptor molecule FADD/MORT1 via its death domain, which, in turn, leads to the recruitment and activation of the caspase proteases FLICE/MACH/caspase-8 and FLICE-2/caspase-10. These enzymes then cleave other caspases to initiate the apoptotic signal transduction pathway.[35,36] The p55 TNF receptor also uses this pathway, recruiting FADD/MORT1 via the adaptor protein TRADD.[37–39] Thus far, cleavage of FLICE/MORT1/caspase-8 has not been observed in oligodendrocytes undergoing p75-dependent apoptosis.[40] Neither is there evidence that FADD binds directly to p75.[41]

The p75 receptor can participate in the initiation or potentiation of the apoptotic process,[11,14–18] but the components responsible for signaling remain to be identified. Cell death mediated by p75 is not a general phenomenon but instead requires specific conditions, with regard to cell type, cell cycle stage, and developmental stage. Although there are examples in which p75 has been directly shown to be responsible for apoptotic cell death, it is also apparent that many cell types express p75 but do not undergo apoptosis. This suggests that cellular context and history are important determinants and that p75 alone is not sufficient for this pro-death activity. The pro-apoptotic effect of p75 may be influenced by age in culture, metabolic impairment, hypoxia, or other types of 'stress' signals, such as nerve injury or inflammation. In this respect, the cell death properties of this receptor may reflect its proposed function as a cytokine or stress receptor.[40]

Several mechanisms have been proposed to explain the actions of p75. The p75 receptor may act as an apoptotic receptor in the absence of ligand.[6,15,17,42] This hypothesis has been partially derived from experiments in which cell death is induced by either serum or NGF withdrawal. In this model, NGF acts to stop cell death by directly binding p75. These conditions may trigger changes in the structure or localization of p75 or in the protein substrates that interact with p75. Alternatively, this ligand-independent activity of p75 may be explained by recruitment of cellular death proteins under conditions of overexpression.

A ligand-dependent mechanism for cell death has been observed *in vitro* and *in vivo* for the p75 receptor, in which antibodies against NGF block apoptotic cell death, or NGF binding to p75 directly leads to cell death.[11,14] This leads to two diametrically opposed killing mechanisms involving NGF and p75, ligand-dependent and ligand-independent. Both of these mechanisms can be reconciled by the data presented in this study, if proapoptotic cellular proteins can interact with the p75 receptor and transduce competent signals in the absence of ligand. A prediction from the results derived by TNF receptor family members is that trimerization or higher order aggregation of p75 may be more effective in killing cells than dimerization, as is normally induced by the neurotrophins.

Mechanisms to explain p75 function have largely been extrapolated from the actions of the TNF receptor family members. The p75 receptor can activate ceramide production, NF-κB, and JNK,[7,43] depending upon the cell type and history. Although it is premature to define a complete biochemical pathway initiated by NGF binding to p75, it is plausible to make certain predictions on potential signaling candidates given the structural resemblance of p75 to the TNF receptor superfamily. The cytoplasmic region of p75 may serve as a module for recruiting cell death proteins, such as caspases. The structural data would predict that p75 may interact with common adaptor proteins, such as the TRAF family members, or other proteins similar to TRADD and FADD. Indeed, TRAF6 has been shown to be recruited to the p75 receptor upon treatment with neurotrophins and mediates p75-dependent

NF-κB activation in cultured Schwann cells.[44] Additionally, catalytically inactive caspases such as I-FLICE/CASH may provide a means for blocking cell death by binding to p75, as has been shown with TNF and Fas receptors.[45,46]

Although the death domain of p75 may associate with proteins that interact with other TNF receptor family members, the mechanisms of membrane recruitment may differ significantly for p75 compared to other TNF receptor family members. For instance, in primary DRG cultures maintained with LIF, p75 may transduce its death-inducing signals via its juxtamembrane region rather than its death domain.[41] Testing these and other signaling mechanisms will reveal how the p75 neurotrophin receptor differs from other TNF receptor members in promoting cell death.

Materials and Methods

Construction of FasR/p75 chimeras

A hybrid PCR-based strategy was used to construct two chimeras consisting of the extracellular domain of the mouse FasR and the intracellular domain of the rat p75 receptor. Chimera A7 cDNA encodes the extracellular portion of the FasR fused to the transmembrane and intracellular segments of p75, whereas chimera B9 consists of the extracellular and transmembrane portions of mFasR fused to the intracellular domain of p75NTR (Figure 1). The primers used in the construction of the chimeras were as follows. Primer **I**, 5′-ATTTGCGGCCGCCCACCATGCTGTGGATCTGGGC-3′; primer **II**, 5′-AGGAATGAGGCGATTTCTGGGACTTTG-3′; primer **III**, 5′-AGAAATCGCCTCATTCCTGTCTATTGCTC-3′; primer **IV**, 5′-TGAGGCAGTCTGCGTATGGGTC-3′; primer **V**, 5′-CCACCTCTTATATATAAATACAAGTGGAATTAAC-3′; primer **VI**, 5′-TTTATATATAAGAGGTGGAACAGCTGC-3′. Using pCDNA3-mFasR as the template in parallel reactions, the primer pair I and V and the primer pair I and II were used to amplify the extracellular portion of FasR with and without the FasR transmembrane domain, respectively. Likewise, with pBluescript-p75 as the template in parallel reactions, primer pair III and IV and primer pair IV and VI were used to amplify the intracellular portion of p75 with and without the p75 transmembrane domain, respectively. The PCR conditions used for the above reactions were as follows: 1 min at 94°C, 1 min at 48°C, and 1 min at 72°C for ten cycles; 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C for 20 cycles; 10 min at 72°C. The overlapping products of the primer I/II and primer III/IV amplifications were combined, denatured, annealed, and extended with Klenow in the presence of 250 μM dNTPs to yield template **A**. The I/V and VI/IV PCR products were treated in the same manner to yield template **B**. Finally, in a second set of PCRs, primers I and IV were used with templates **A** and **B** in separate reactions. The PCR conditions used for this second set of reactions were as follows: 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C for 25 cycles followed by 10 min at 72°C. These products were digested with *Not*I and *Kas*I and ligated into the corresponding sites of pcDNA3-p75 to give the final chimeric expression constructs, A7 and B9. Both constructs were verified by automated DNA sequencing (Rockefeller University DNA sequencing facility).

Cell culture and transfection

COS-1 cells, 293 cells, and the glioblastoma-derived cell line U373 were cultured at 37% in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and penicillin/streptomycin (GIBCO BRL). For transient transfection of COS-1 and U373 cells, cells were cultured in 35 mm dishes and transfected with 2 μg total plasmid DNA (0.5 μg pRC-lacZ+1.5 μg receptor construct) using Lipofectamine (Gibco BRL) for U373 cells and the calcium phosphate method for COS-1 cells. Two hours post-transfection with Lipofectamine, U373 cells were split and allowed to recover in serum-containing medium for 12–24 h. COS-1 cells were split 12–24 h post-transfection. For transient transfection of 293 cells, cells plated in 6-well plates (1.3×10^6 cells/plate) were transfected for 24 h with 0.75 μg receptor construct and, for indicated experiments, co-transfected with 0.1 μg GFP construct using Fugene 6 (Roche).

Western blot analysis

To verify protein expression of the chimeras, COS-1 cells, a line unresponsive to Fas-induced killing, and 293 cells were used. COS-1 cells were plated in 10 cm dishes and transiently transfected with 20 μg of plasmid DNA. Cells (293) were cultured in 6-well plates and transiently transfected with 2 μg plasmid DNA per well. Cells were lysed in 0.2–0.5 ml Nonidet P-40 lysis buffer (1% Nonidet P-40, 20 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 25 μg/ml phenylmethylsulfonyl fluoride). Protein lysates were resolved on a 10% SDS-polyacrylamide gel under reducing conditions and transferred to PVDF membrane. The membrane was washed with 1 × TBS-T (10 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.1% Tween-20), blocked with 5% milk, incubated in 1% milk/TBS-T containing a 1 : 5000 dilution of the anti-p75 antibody 9992 for 1 h at room temperature, washed three times in TBS-T, and incubated for 1 h at room temperature in 1% milk/TBS-T with anti-rabbit secondary antibody conjugated to HRP (Roche). Protein was visualized by ECL (Amersham) and exposure to X-ray film.

Flow cytometry

Receptor expression was followed by flow cytometry after transfection in 293 cells. The FasR, A7, and B9 cDNAs (0.75 mg) were introduced into 293 cells plated in 6-well plates by Fugene 6-mediated transfection. Cells were suspended in PBS-EDTA (1 mM) and washed twice with PBS containing 1% BSA and 0.05% sodium azide. After incubating cells in 0.5 mg per 10^6 cells Jo2 antibody phycoerythrin (PE)-conjugate (Pharmingen), cells were washed twice and subjected to flow cytometry for Jo2-PE fluorescence.

Immunocytochemistry

For cell surface detection of Fas receptor, A7, and B9, COS-1 cells plated in 10 cm dishes were transfected with 20 μg plasmid DNA, split into 24-well plates, allowed to recover, and processed for staining as follows. Cells were fixed in 4% paraformaldehyde and incubated in PBS containing 1% normal goat serum (NGS) and 2 μg/ml anti-Fas Jo2 antibody (Pharmingen). A goat anti-hamster secondary antibody conjugated to FITC (Vector) was used for detection.

For simultaneous TUNEL-labeling and plasma membrane detection of receptors in 293 cells, cells were plated on 4-chamber plastic slides, transfected with 0.75 μg plasmid DNA, and stained after 36 h. Cells were first fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 for 2 min, and blocked in PBS containing 10% NGS for 1 h. Cells were then incubated in PBS containing 1% NGS and 2 μg/ml Jo2 antibody and incubated in TUNEL–FITC reaction solution (Roche) for 1 h at 37°C. Receptor expression was visualized by

incubation in PBS with 1% NGS and anti-rabbit secondary antibody conjugated to Texas Red (Jackson Immunoresearch).

For detection of β-galactosidase-positive cells, transfected U373 cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked in 1% NGS, incubated with a rabbit β-galactosidase antibody (5′-3′, Boulder CO, USA), and incubated with a biotinylated goat anti-rabbit secondary antibody and FITC-conjugated streptavidin. For Hoechst 33342 staining, U373 cells were incubated for 10 min at 37°C in Hoechst 33342 solution (Molecular Probes) at a 1 : 10000 dilution.

Cell death assays

For assessment of U373 cell death, cells were incubated for various times in serum-free medium $\pm$50 ng/ml anti-Fas Jo2 agonist antibody, after which cells were fixed in 4% paraformaldehyde and incubated in potassium ferrocyanide/potassium ferrous cyanide containing 1 mg/ml X-gal. The number of blue cells were counted and calculated as a percentage of vector-transfected control cells. Since dying cells detach from the cell culture dish, the remaining blue cells correspond to the number of viable transfected cells.

For Annexin V staining, 293 cells plated in 6-well plates were co-transfected with 0.1 μg GFP construct and 0.75 μg receptor construct for 24 h and exposed to no ligand, 500 ng/ml anti-Fas Jo2 agonist antibody, or 100 ng/ml NGF (Harlan) for 12 or 36 h. Cells were suspended using Hank's balanced salt solution (HBSS) containing trypsin-EDTA and transferred into 96-well V-bottom plates. After washing cells twice with PBS plus 1% BSA, cells were incubated for 15 min in Annexin V conjugated to phycoerythrin (Pharmingen) in the dark at room temperature and washed once with PBS plus 1% BSA. To assess the percentage of Annexin V-/GFP-double positive cells among GFP-positive cells, 2000 events were counted per condition by flow cytometry.

For quantitation of TUNEL labeling, 293 cells plated in 6-well plates were transfected as described for Annexin V staining and then exposed to no ligand or 500 ng/ml Jo2 antibody for 36 h. Cells were suspended with HBSS containing trypsin-EDTA and transferred to 96-well V-bottom plates. Cells were then fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 for 2 min, and incubated in TUNEL-TMR Red reaction solution (Roche) for 1 h at 37°C. After two washes with PBS plus 1% BSA, cells were transferred into flat-bottom 96-well plates for counting by fluorescence microscopy. For each condition, 100 GFP-positive cells were counted and assessed for simultaneous positive TUNEL reactivity.

Acknowledgements

This work was supported by grants provided by the NIH (H Kong, AH Kim and MV Chao) and a fellowship from the Tri-Institutional MD/PhD program and the Life and Health Insurance Medical Research Fund (JR Orlinick).

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