Brief Definitive Report

Neu Differentiation Factor (NDF), a Dominant Oncogene, Causes Apoptosis In Vitro and In Vivo

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Summary

Neu differentiation factor (NDF, also called neuregulin) is a potent inducer of epithelial cell proliferation and has been shown to induce mammary carcinomas in transgenic mice. Notwithstanding this proliferative effect, we have shown that a novel isoform of NDF can induce apoptosis when overexpressed. Here we report that this property also extends to other NDF isoforms and that the cytoplasmic portion of NDF is largely responsible for the apoptotic effect, whereas the proliferative activity is likely to depend upon the secreted version of NDF. In accordance with these contradictory properties, we find that tumors induced by NDF display extensive apoptosis in vivo. NDF is therefore an oncogene whose deregulation can induce transformation as well as apoptosis.

Key words: Neu differentiation factor • neuregulin • apoptosis • tumors • TUNEL assay

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β2b Neu differentiation factor (NDF), a novel isoform of NDF, was recently isolated in a screen for dominant, apoptosis-inducing genes (1). NDF comprises a gene family of differentially spliced isoforms. All NDF isoforms encode membrane-anchored precursor proteins from which the mature growth factor is proteolytically released (2, 3). The β2b isoform of NDF can cause apoptosis when overexpressed in tissue culture cells (1). Interestingly, both extra- and intracellular domains of the β2b NDF precursor are required for apoptosis induction. This indicates that only cells overexpressing the precursor can undergo apoptosis and that this effect is not due to the secreted NDF molecule. Several other lines of evidence suggest that this apoptosis is a cell-autonomous effect. Chief among them is the observation that cells lacking NDF-binding erbB receptors are still sensitive to apoptosis induction (1). In this report we address the sequence requirements of NDF for apoptosis induction. We also investigate the apparently contradictory finding that NDF overexpression can lead to tumor formation in a mouse model (4) as well as induce apoptosis in cells (1).

Materials and Methods

Quantitative Apoptosis Assay. Quantification of apoptosis induction was performed as previously described (1). In brief, the indicated amounts of expression plasmid were transfected into baby hamster kidney (BHK) cells together with 1 μg of a β-galactosidase (β-gal) expression construct. 24 h later the cells were stained for β-gal activity and the percentage of blue and morphologically apoptotic cells with respect to all blue and transfected cells was determined.

Cell Transfections. BHK cells were transfected using calcium phosphate coprecipitation as previously described (1).

Expression Constructs. Constructs for expressing the NDF fusion proteins with the signal peptide of the human erbB-3 receptor (residues 1–29) were generated with recombinant PCR. All other mutants of β2b NDF were likewise engineered by PCR. For each PCR expression construct, two independently generated clones were used in the transfection experiments. All constructs were control sequenced. For all PCR reactions the thermostable enzyme Pwo (Boehringer Mannheim, Indianapolis, IN) that has proofreading activity was used. The α2c NDF cDNA was a gift from Amgen Inc. (Thousand Oaks, CA). The human TGF-α cDNA was a gift from Dr. Merlino (National Cancer Institute, Fredericktown, MD; reference 5). Both cDNAs were subcloned into the plasmid pcDNA3 (Invitrogen, San Diego, CA). All constructs were in vitro–translated and yielded proteins of the correct size. In addition, for each of the inactive constructs shown in Fig. 1 (Δ 2-262, Sig Δ 2-262, and Δ 2-198), myc-tagged versions were made and tested to ensure that appropriate protein product was synthesized as a result of the transfection. Protein of an appropriate mobility on SDS gel was detected by anti-myc-tag antibody in each case.

Apoptosis in Tumor Tissue. Paraffin sections were obtained from tumors originating in transgenic mice that harbored an NDF gene, v-Ha-ras, or a myc gene under the control of the MMTV promoter (4, 6, 7). Subsequently, sections were stained with the TUNEL (Tdt-mediated dUTP–biotin nick end labeling) technique (Boehringer Mannheim), which marks the DNA ends generated in apoptosis (8), or the Annexin V stain (PharMingen, San Diego, CA), which detects phosphatidylserine on the outer membrane of apoptotic cells (9).

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Results

Previous mapping data (1) indicated that both the extra- and the intracellular domains of β2c NDF are necessary for apoptosis. Since NDF does not contain a genuine signal sequence (2, 3), we speculated that deletions in the NH_2-terminal, extracellular domain might cause a mislocalization of these deletion proteins that could be responsible for their inactivity. To test this, we fused the signal sequence of the erbB-3 receptor to two deletion versions of β2b NDF that were by themselves not able to induce apoptosis: the cytoplasmic domains (Fig. 1, *Sig Δ 2-262*) and a construct also containing the transmembrane domain as well as the β and the 2 exon sequences (Fig. 1, *Sig Δ 2-198*). A quantitative apoptosis assay revealed that the cytoplasmic domain, even when fused to the signal sequence (Sig Δ 2-262), remains inactive, whereas the construct containing the transmembrane domain (Sig Δ 2-198) regains its activity for apoptosis induction (Fig. 1). Its activity is even higher than that of the wild-type β2b NDF (24 vs. 13.2% apoptotic cells). A fusion construct of the complete β2b NDF with the signal sequence is likewise more efficient than wild-type β2b NDF (data not shown). To demonstrate that the "inactive" constructs actually synthesized the protein, myc-tagged versions of each construct (Fig. 1, *Δ 2-262*, *Sig Δ 2-262*, and *Δ 2-198*) were transfected into BHK cells and analyzed for protein using SDS gels and anti-myc-tag antibody. Each transfection produced cross-reacting protein of appropriate mobility (data not shown).

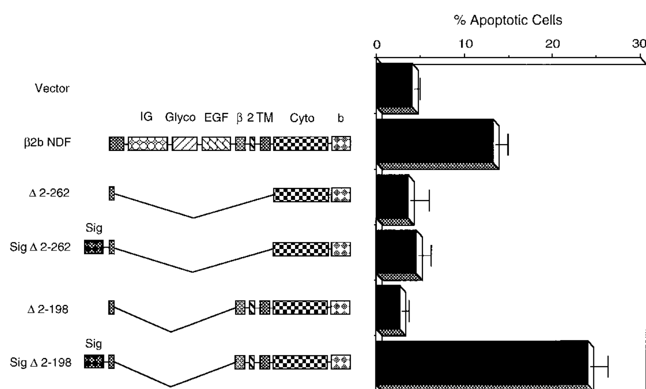

Figure 1. Apoptosis induction of NDF deletion mutants with and without a signal sequence. The domains of wild-type β2b NDF are indicated on top of the diagrammatic representation. *IG*, immunoglobulin homology domain; *Glyco*, glycosylated domain; *EGF*, epidermal growth factor domain; *β*, sequences of β exon; *2*, sequences of exon 2; *TM*, transmembrane domain; *Cyto*, cytoplasmic domain; *b*, sequences of b exon; *Sig*, signal sequence of erbB-3. The names of the deletion constructs denote the deleted amino acids of wild-type β2b NDF. As a control in the case of the constructs that did not induce apoptosis (Δ 2-262, Sig Δ 2-262, and Δ 2-198), myc-tagged versions were transfected into BHK cells and the protein products were analyzed on SDS gels and detected with anti-myc-tag antibody. In each case, protein of appropriate mobility was detected (data not shown). 1.5 μg of each expression construct were transfected into BHK cells with 1 μg of a β-gal expression vector. 24 h after transfection the cells were stained for β-gal activity and the percentage of apoptotic and blue cells of all blue cells was determined using morphological inspection. Shown are the means and SD of at least 1,000 counted cells of four independent transfections.

Since the construct sig Δ 2-198 still contains the combination of the exons β, 2, and b that define the specificity of this particular isoform, we wanted to test whether other isoforms could also lead to apoptosis. Fig. 2 shows that exchanging the β exon for the α exon in the intracellular domain of NDF does not alter the extent of cell death. As shown previously (1), the c isoform of β2 NDF is considerably less efficient in apoptosis induction. A c isoform containing the α exon instead of the β exon is also slightly less potent in apoptosis induction (Fig. 2). We also tested TGF-α in this assay. Like NDF, TGF-α contains an epidermal growth factor homology domain (5) and encodes a ligand for a receptor that is first synthesized as a membrane-bound precursor protein. Despite this similarity, overexpression of TGF-α is unable to induce apoptosis in this assay (Fig. 2).

The Fas receptor is another apoptosis inducer that resides on the membrane. This receptor is activated by overexpression or by cross-linking through its cognate ligand and concomitant aggregation of its intracellular "death domain" (10). To test whether β2b NDF is also activated by clustering, we generated a fusion protein with the extracellular sequences of the IL-4 receptor and the cytoplasmic domain of β2b NDF. However, even when cross-linked with an antibody against the IL4R, this construct was unable to induce apoptosis (data not shown).

To test whether NDF used the Fas pathway, a dominant negative version of FADD, one of the downstream molecules in the Fas receptor complex, was used to block Fas receptor–mediated apoptosis (11). Under these conditions, we were not able to detect any effect on NDF-induced apoptosis upon cotransfection of the mutant FADD (data not shown).

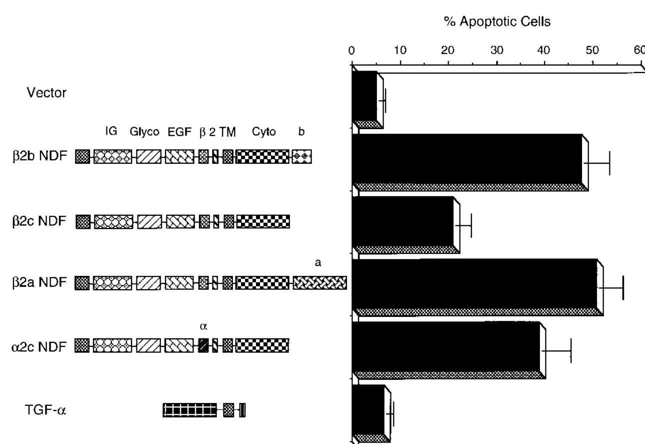

Figure 2. Apoptosis induction by different NDF isoforms. Expression plasmids (4 μg) of the indicated NDF isoforms or of TGF-α were transfected together with a β-gal vector (1 μg) into BHK cells. Apoptosis induction was measured as in Fig. 1. The structure of each NDF isoform is schematically depicted in the diagram. Each subdomain is named as in Fig. 1. α and a denote the α or the a exon in the extra- or intracellular domain, respectively.

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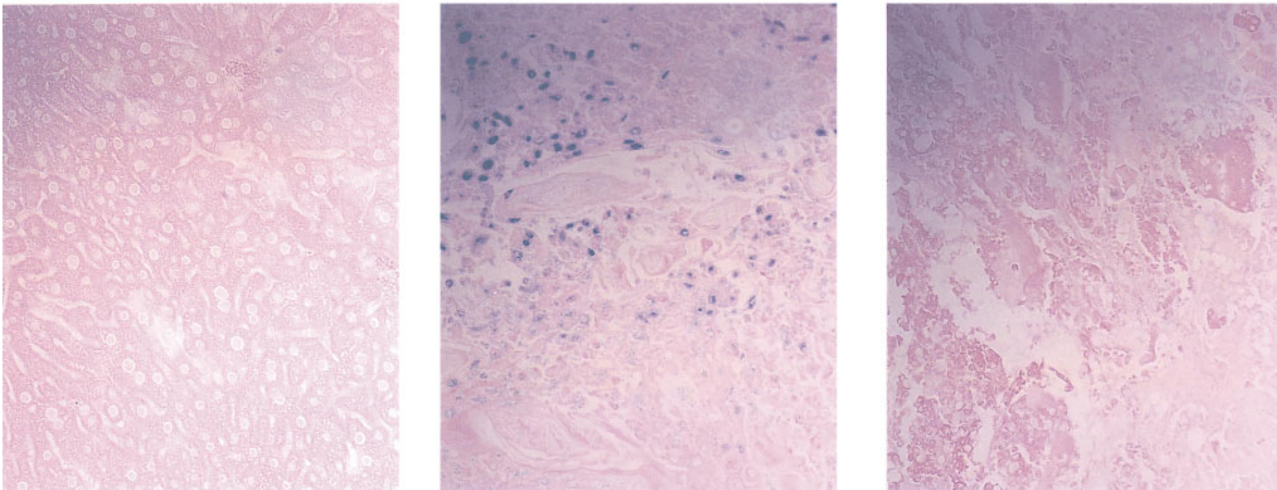

Figure 3. Apoptosis induction in NDF-caused tumors. Hematoxylin and eosin–stained tissues from an adenocarcinoma in the mammary gland of NDF-transgenic mice, WT mammary gland, and a myc-induced tumor were probed with the TUNEL technique that detects DNA ends that are generated during apoptosis.

NDF has been shown to lead to tumor formation when overexpressed in the mammary gland of transgenic mice (4). Data presented here and previously (1) showed that overexpressed NDF can also induce cell death. Since these two effects are seemingly contradictory, we tested the tumors that express high amounts of NDF (4) for apoptosis. 10 advanced adenocarcinomas from 5 different transgenic mice were examined for apoptosis by the TUNEL technique or by staining with Annexin V. Seven tumors (64%) were strongly positive for apoptosis. The stained cells showed cytoplasm shrinkage typical of apoptotic cells. Every transgenic mouse had at least one highly apoptosis-positive tumor. This cell death was not a consequence of insufficient blood supply since apoptotic cells were evenly distributed in the tumor mass and could also be detected at the edge of the tumor tissue (Fig. 3). Surrounding normal tissue was apoptosis free. In contrast, three myc- and three ras-induced tumors were apoptosis negative. Furthermore, we have established two cell lines from NDF-induced tumors. These tumor cell lines exhibited strong apoptosis as evident by Annexin V staining after having been in cell culture for as long as 25 passages (data not shown).

Discussion

Previously we described a novel isoform of NDF isolated in a screen for dominant, apoptosis-inducing genes (1). Here we show that this property is shared by other isoforms of NDF and we establish structural requirements for this activity. For example, the c exon that places a stop codon at the end of the cytoplasmic domain of β2 NDF results in decreased apoptosis (1). This is also the case for the α2c isoform, although in a less pronounced manner (Fig. 2). Since the uncleaved precursor of the c isoform possesses a longer half-life than the precursor of other isoforms (12), one could assume that these unprocessed precursors are inactive with respect to apoptosis and that only cleaved, membrane-bound NDF forms can induce it. This would explain the reduced apoptotic activities of c exon–containing isoforms. In addition, extracellular sequences might influence the kinetics of the processing of NDF precursors, since these are cleaved at position 228 or 223 in the α or β exons,

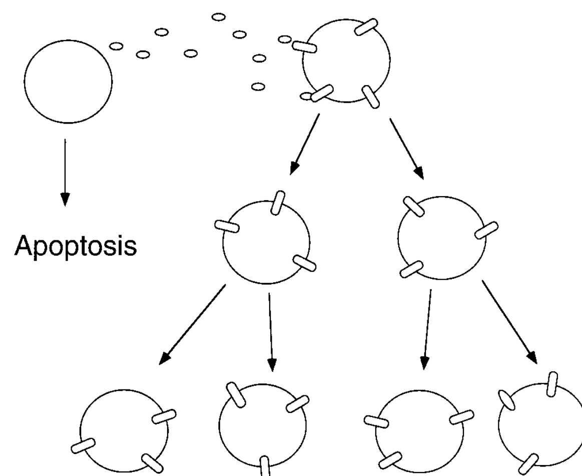

Figure 4. Model for the apoptotic and tumorigenic activity of NDF. A cell that overexpresses the NDF-precursor releases high amounts of the secreted NDF form. Its interaction with erbB receptors can lead to the subsequent proliferation of neighboring cells and might constitute the first step towards transformation. NDF's apoptosis-inducing activity is concomitantly activated when NDF is overexpressed and thereby kills this potentially dangerous cell.

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respectively (3, 4). This might account for the differences of apoptotic induction by α2c and β2c NDF (Fig. 2). Furthermore, the efficient apoptosis induction of the construct sig Δ 2-198 NDF (Fig. 1) might be explained by the fact that this construct mimics an already processed precursor molecule.

We found that attaching a signal sequence to a construct of NDF lacking its secreted domains was sufficient for induction of cell death (Fig. 1). This suggests that the inactivity of the deletion mutants could be due to mislocalization on the membrane. This experiment shows that the apoptotic activity of NDF can be separated from its oncogenic function that appears to be mediated by its growth factor moiety (4). Therefore, these data also corroborate our notion (1) that NDF causes apoptosis cell autonomously when overexpressed. As a next step, it will be important to isolate proteins that interact with the cytoplasmic domain of NDF and that transmit this apoptotic signal. However, we have also found that the transmembrane domain of NDF is important for its induction of apoptosis. A fusion construct with only the cytoplasmic domain of NDF and the IL-4R does not lead to cell death when overexpressed or crosslinked with an antibody. Although this might be due to an incorrectly folded cytoplasmic domain, it is noteworthy that the transmembrane domain is the most conserved sequence between NDF and its recently isolated paralog, NRG-2 (13, 14). Therefore, this might be the crucial element in the loss of function of this construct.

Although we isolated NDF in a screen for dominant, apoptosis-inducing genes in tissue culture cells (1), NDF has also been shown to function as an oncogene (4). In this report we demonstrate that both effects can also be seen in vivo. NDF overexpression induces tumors in the mammary gland that nevertheless display extensive apoptosis. Therefore, NDF has a dual role in apoptosis and tumorigenesis.

We would like to suggest a biologic rationale that reconciles NDF's apoptotic and tumorigenic properties (Fig. 4). A cell might suffer a mutation that activates the endogenous NDF promoter. This would lead to the secretion of large amounts of NDF that then stimulate erbB receptors on neighboring cells (or by a paracrine mechanism on the same cell). This causes these cells to proliferate, which might be the first step in the transformation process. Thus NDF could use the apoptotic response as an autoregulatory event, eliminating tumorigenic signals from the organism. This mechanism might be especially important since secreted NDF could potentially induce proliferation in many neighboring cells. Furthermore, it has been shown that NDF-binding erbB receptors cannot be downregulated efficiently (15). Thus, overexpression of NDF would expose cells permanently to NDF's mitogenic activation. In tumors, NDF's apoptotic activity could be mitigated by secondary events like the activation of Bcl-2–like genes, which have been found to suppress NDF apoptosis (1).

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References

1. Grimm, S., and P. Leder. 1997. An apoptosis-inducing isoform of neu differentiation factor (NDF) identified using a novel screen for dominant, apoptosis-inducing genes. *J. Exp. Med.* 185:1137–1142.
2. Wen, D., E. Peles, R. Cupples, S.V. Suggs, S.S. Bacus, Y. Luo, G. Trail, S. Hu, S.M. Silbiger, R. Ben-Levy, et al. 1992. Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. *Cell.* 69:559–572.
3. Holmes, W.E., M.X. Sliwkoski, R.W. Akita, W.J. Henzel, J. Lee, J.W. Park, D. Yansura, N. Abadi, H. Paab, G.D. Lewis, et al. 1992. Identification of heregulin, a specific activator of p185 erbB2. *Science.* 256:1205–1209.
4. Krane, I.M., and P. Leder. 1996. NDF/Heregulin induced persistence of terminal end buds and adenocarcinomas in the mammary gland of transgenic mice. *Oncogene.* 12:1781–1789.
5. Jhappan, C., C. Stahle, R.N. Harkins, N. Fausto, G.H. Smith, and G.T. Merlino. 1990. TGF alpha overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. *Cell.* 61: 1137–1146.
6. Sinn, E., W. Muller, P. Pattengale, I. Tepler, R. Wallace, and

P. Leder. 1987. Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. *Cell.* 49:465–475.

7. Stewart, T.A., P.K. Pattengale, and P. Leder. 1984. Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. *Cell.* 38:627–637.
8. Gavrieli, Y., Y. Sherman, and S.A. Ben-Sasson. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell. Biol.* 119:493–501.
9. Koopman, G., C.P. Reutelingsperger, G.A. Kuiten, R.M. Keehen, S.T. Pals, and M.H. Oers. 1994. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood.* 84:1415–1420.
10. Nagata, S., and P. Goldstein. 1995. The fas death receptor. *Science.* 267:1449–1455.
11. Chinnaiyan, A.M., C.G. Tepper, M.F. Seldin, K. O'Rourke, F.C. Kischkel, S. Hellbard, P.H. Krammer, M.E. Peter, and V.M. Dixit. 1996. FADD/MORT1 is a common mediator of CD95 (Fas/Apo-1) and tumor necrosis factor receptor–induced apoptosis. *J. Biol. Chem.* 271:4961–4965.
12. Wen, D., S.V. Suggs, D. Karunagaran, D. Liu, R.L. Cupples, Y. Luo, A.M. Janssen, N. Ben-Baruch, D.B. Trollinger, V.L. Jacobson, et al. 1994. Structural and functional aspects of the multiplicity of Neu differentiation factors. *Mol. Cell Biol.* 14: 1909–1919.
13. Chang, H., D.J. Riese, Jr., W. Gilbert, D.F. Stern, and U.J. McMahan. 1997. Ligands for ErbB-family receptors encoded by a neuregulin-like gene. *Nature.* 387:509–512.
14. Carraway, K.L., III, J.L. Weber, M.J. Unger, J. Ledesma, N. Yu, M. Gassmann, and C. Lai. 1997. Neuregulin-2, a new ligand of ErbB3/ErbB4-receptor tyrosine kinases. *Nature.* 387:512–516.
15. Baulida, J., M.H. Krauss, M. Alimandi, P.P. Di Fiores, and G. Carpenter. 1996. All erbB receptors other than the epidermal growth factor receptor are endocytosis impaired. *J. Biol. Chem.* 271:5251–5257.

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