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TNF-Related Apoptosis-Inducing Ligand Mediates Tumoricidal Activity of Human Monocytes Stimulated by Newcastle Disease Virus

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The Newcastle disease virus (NDV) is used as an antineoplastic and immunostimulatory agent in clinical tumor therapy (1). Several phase II trials of postoperative tumor vaccination with NDV-modified, patient-derived, live tumor cells or viral oncolytes indicated clinical benefits (2, 3). The therapy is well tolerated, and to date no serious side effects have been observed in any of the trials. Recently, it was demonstrated that NDV infection of human melanoma cells induces a B7-1/B7-2-independent, T cell costimulatory activity in the tumor cell (4).

As an avian paramyxovirus, NDV has a negative single-stranded RNA genome with only six genes. NDV binds to cells via the viral envelope protein hemagglutinin-neuraminidase, which attaches to sialic acid-containing host cell surface receptors. Binding is followed by fusion of the viral and cell surface membranes, a process that is mediated by a separate viral fusion protein. The viral RNA is then released into the cytoplasm of the tumor cell, where viral replication occurs. This involves transcription into positive strand RNA, replication, and translation of viral proteins in the rough endoplasmic reticulum and at free ribosomes. New virions are produced by budding. The tumor selectivity of NDV replication is of special interest (5). Normal cells were found to be resistant to NDV replication with the exception of chicken chorioallantoic membrane cells. The apathogenic strain NDV-Ulster used in this study replicates in tumor cells via an abortive replication cycle that is monocyolic, because the newly produced virions are noninfectious (6).

NDV was reported to be a strong inducer of type I IFNs and TNF-α in both mouse and human macrophages and lymphocytes (7–10). When applying NDV Ulster for tumor therapy in nude mice, a pronounced bystander effect was observed when mixing virus-infected human melanoma cells with noninfected tumor cells (11). Recently, it was demonstrated that the nonlytic NDV strains LaSota and Ulster activate a tumoricidal activity of murine macrophages both in vitro and in vivo (12).

A central mechanism in the antitumor activity of immune cells is induction of apoptosis in the target cells. Three apoptosis-inducing members of the TNF family, TNF-α, CD95 ligand (CD95L/FasL/APO-1L), and TRAIL/APO-2L, have been shown to kill various tumor cell lines in vitro (13–15). In addition, TRAIL exerts antitumor activity in vivo (16–19). CD95L, TNF-α, and TRAIL induce apoptosis by binding to CD95, TNF-R1, TRAIL-R1, and TRAIL-R2, respectively. TRAIL also interacts with TRAIL-R3 and TRAIL-R4, which do not mediate apoptosis due to complete or partial absence of an intracellular death domain, and the soluble receptor OPG (20).

Functional expression of TRAIL was observed on the surface of different cells of the immune system, such as type I (IFN-α) and type II (IFN-γ) IFN-stimulated monocytes (Mφ) (21) and dendritic cells (22). In addition to up-regulation by IFNs, which are elevated during viral infection, TRAIL induction has been shown on CMV-infected fibroblasts (23) and on measles virus-stimulated dendritic cells (DC) (24). Recently, mouse liver NK cells were shown to up-regulate TRAIL after IFN-γ stimulation in vivo, which contributed to NK cell cytotoxicity in the reduction of liver metastases (25, 26).

To date the antitumor mechanisms of NDV-stimulated Mφ are largely unknown. TNF-α was suggested to be involved in the tumoricidal activity of NDV-activated murine macrophages and human PBMC (7, 10, 12). In the present study we tested whether the three death receptor-ligand systems mentioned above are crucial for tumor cell killing by NDV-stimulated Mφ and, if so, which one(s) plays a major role.

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Dedicated to the memory of Dr. Harald zur Hausen who has made important contributions to our understanding of viral oncogenesis.

1Division of Cellular Immunology and 2Division of Apoptosis Regulation, Tumor Immunology Program, German Cancer Research Center, Heidelberg, Germany; and 3Department of Anesthesiology, University of Heidelberg, Heidelberg, Germany

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1 Abbreviations used in this paper: NDV, Newcastle disease virus; DC, dendritic cells; HU, hemagglutination unit; LZ-, leucine zipper; Mφ, monocyte.

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We show that NDV efficiently induces functional surface expression of TRAIL on human Mφ and that this up-regulation does not require viral replication. TRAIL could be shown to be the main effector molecule responsible for tumoricidal activity of NDV-stimulated human Mφ on all tumor cell lines tested.

**Materials and Methods**

**Cell lines**

The human breast carcinoma cell lines BT20 and MCF-7 were obtained from the tumor cell bank (DKFZ) and cultured in DMEM/F-12 medium (Sigma-Aldrich, Taufkirchen, Germany) containing 10% FCS, 100 μg/ml streptomycin and penicillin, 10 mM glutamine, 1% nonessential amino acid solution (100×; Sigma-Aldrich). The human colon carcinoma line Colo205 and the esophagus carcinoma line Colo680N were also obtained from the tumor cell bank (DKFZ) and cultured in RPMI 1640 (In vitro, Groningen, The Netherlands) containing 10% FCS, 100 μg/ml streptomycin and penicillin, and 2 mM glutamine.

**Virus**

The avirulent, nonlytic strain NDV Ulster was obtained from Dr. P. Russell (London, U.K.) (27). A stock of infectious virus was propagated in embryonated chicken eggs, harvested from allantoic fluid, purified from debris by sucrose cushion at 88 × g for 30 min, 4°C, and then ultracentrifuged (50,000 × g, 60 min, 4°C). The sediment was resuspended in PBS and purified twice over sucrose (35%) via ultracentrifugation (97,000 × g, 60 min, 4°C). The virus was resuspended in PBS buffer containing 0.1% EDTA. NDV was quantified by a hemagglutination test in which one hemagglutination unit (HU) is defined as the smallest virus concentration leading to visible sheep erythrocyte agglutination. In some experiments the virus was inactivated with UV light for 5 min (254 nm, 2 mW/cm², 7-cm distance). Endotoxin (LPS) was not detectable in the NDV Ulster preparation measured by Limulus amebocyte assay (QKCL test; Serva, Heidelberg, Germany).

**Reagents**

Human IFN-α2A (200 IU/ml) was obtained from Hoffmann-La Roche (Nutley, NJ), and human TNF-α (2 μg/ml) was purchased from Upstate Biotechnologies (Lake Placid, NY). Leucine zipper (LZ)-TRAIL is a stable trimer of TRAIL and induces apoptosis upon binding to TRAIL-sensitive cells; it was produced as previously described (28). The mAbs specific for the different TRAIL receptors and TRAIL were generated by immunizing mice with TRAIL receptor-Fc fusion proteins or LZ-TRAIL, respectively. The specificity of the respective Abs was determined by staining cells transfected with expression plasmids for TRAIL-R1 to TRAIL-R4 or TRAIL. We used anti-TRAIL-R1 (clone HS101), anti-TRAIL-R2 (clone HS201), anti-TRAIL-R3 (clone HS301), anti-TRAIL-R4 (clone HS402), and biotinylated TRAIL-R2-Fc for FACS staining and anti-TRAIL (clone HS501) for Western blot analysis. The TRAIL- and TRAIL receptor-specific Abs are also available from Alexis (San Diego, CA). The soluble receptor-Fc proteins TRAIL-R2-Fc, TNF-R2-Fc (p75 TNF-R-Fc), and CD95-Fc bind to TRAIL, TNF-α/lymphotoxin α, and CD95L, respectively (28, 29). All receptor-Fc fusion proteins contain the same human IgG1 Fc portion.

**Mφ preparation and activation**

Human peripheral blood Mφ were isolated from 500 ml of fresh blood from healthy donors. PBMCs were purified via Ficoll-Paque (Amersham Pharmacia Biotech, Freiburg, Germany) and were cultured for 2 h at 37°C in plastic dishes. Nonadherent cells were washed away, and adherent cells were used. Mφ-enriched cells were >90% CD14+ as assessed by flow cytometric analysis using anti-human CD14-FITC (M5E2; BD Pharmingen, Hamburg, Germany). Mφ were stimulated for 16 h with 20 IU/ml NDV Ulster (live or UV-inactivated) or with IFN-α2A (200 IU/ml). Cells were gently scraped, washed twice, and used for flow cytometry or cytotoxicity assay, or supernatants were harvested, and IFN-α (CytoScreen Human IFN-α ELISA BioSource, Solingen, Germany) or IFN-β (Medgenix or EASIA Human IFN-β ELISA; BioSource) concentrations were determined by specific ELISA kits according to the manufacturers’ protocols.

To determine the role of IFN-α/β in the induction of TRAIL surface expression, IFN-α (10,000 IU/ml) rat anti-human IFN-α polyclonal anti-serum; BioSource) or IFN-β (4,000 IU/ml) goat anti-human IFN-β polyclonal antiserum; BioSource) was blocked during Mφ activation with specific neutralizing Abs.

**Preparation of cell lysates and Western blot analysis**

Cells were harvested by centrifugation at 300 × g for 10 min at 4°C and washed twice with ice-cold PBS, and lysates were prepared by resuspending the resulting cell pellets in 100 μl of lysis buffer/1 × 106 cells (30 mM Tris-HCl (pH 7.5), 120 mM NaCl, 10% glycerol, and 1% Triton X-100) supplemented with Complete protease inhibitors (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. After 30-min incubation on ice, the lysates were centrifuged once at 15,000 × g at 4°C to remove nuclei.

For Western blot analysis the resulting postnuclear supernatants were supplemented with 2-fold concentrated standard reducing sample buffer. Subsequently, lysates containing 20 μg of protein, as determined by the bicinchoninic acid method (Pierce, Rockford, IL), were separated on 4–12% NuPage Bis-Tris gradient gels (NOVEX, San Diego, CA) in MOPS buffer according to the manufacturer’s instructions. After protein transfer onto nitrocellulose membranes (Amersham Pharmacia Biotech) by electroblotting, and membranes were blocked with 5% nonfat dry milk in PBS/Tween (PBS containing 0.05% Tween20) for at least 1 h, washed with PBS/Tween, and incubated in PBS/Tween containing 3% nonfat dry milk and 1 μg/ml primary Abs against human TRAIL (clone HS501). After three washes for 5 min each time in PBS/Tween, the blots were developed by ECL using SuperSignal West Dura substrate following the manufacturer’s protocol (PeroBio Science, Bonn, Germany).

**Flow cytometry**

Cells were incubated with mAbs of the same isotype (mlgG1) against the four different TRAIL-expressing TRAIL receptors (anti-TRAIL-R1 clone HS101, anti-TRAIL-R2 clone HS201, anti-TRAIL-R3 clone HS301, anti-TRAIL-R4 clone HS402), CD95 (anti-AP0-1 IgG1 isotype), or control mlgG1, followed by biotinylated secondary goat anti-mlgG1 Abs (Southern Biotechnology Associates, Birmingham, AL) and streptavidin–PE (BD Pharmingen). TRAIL surface expression was determined by incubating cells with biotinylated TRAIL-R2-Fc (human IgG1, followed by strepavidin–PE. As a control, biotinylated human IgG1 (Sigma-Aldrich, Deisenhofen, Germany) was used. TRAIL-R2-Fc was further transfected with EZ-Link biotin-hydrazide (Pierce) according to the manufacturer’s instructions. Surface staining was determined on a FACS can cytometer (BD Biosciences, Heidelberg, Germany).

**Cytotoxicity assay**

Tumor cells (2 × 10⁶) were labeled with 200 μCi of ³¹Cr for 1 h at 37°C, washed four times, and resuspended in Complete medium. To quantify TRAIL-induced cell death, ³¹Cr-labeled tumor cells (10⁴ cells/well) were incubated with varying numbers of Mφ for 8 h. As a positive control, LZ-TRAIL (2 μg/ml) was added to the target cells. Cells were labeled with 37 μCi of ⁵¹Cr for 1 h prior to addition of tumor cell targets. All cytotoxicity assays were performed in 96-well, round-bottom plates in triplicate, and the percent specific lysis was calculated as: 100 × (experimental cpm − spontaneous cpm)/ (total cpm − spontaneous cpm). Spontaneous and total ³¹Cr release values were determined in the presence of either medium alone or 10% Triton X-100. In some experiments BT20 and Colo205 cells were stimulated with 200 IU/ml IFN-α2A (Hoffmann-La Roche) for 24 h before using them in cytotoxicity assays.

The viability of NDV-stimulated Mφ cultivated in parallel experiments for 8 h in 24-well, round-bottom plates in the absence or presence of TRAIL-R2-Fc, CD95-Fc, or TNF-R2-Fc (20 μg/ml) was >95% and was similar in all samples tested as determined by propidium iodide exclusion (data not shown).

BT20 and Colo205 were stimulated with LZ-TRAIL (2 μg/ml), anti-APO-1 (1 μg/ml anti-APO-1, IgG3-isotype cross-linked with protein A (10 ng/ml; Sigma-Aldrich), or TNF-α (2 μg/ml).

**RT-PCR**

RNA was prepared using the RNeasy kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. For each isolation, 5 × 10⁶ cells were used. For generation of cDNA 1 μg of total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Grand Island, NY) with oligo(dT)₁₅ primers (Roche, Mannheim, Germany) in 20 μl containing 10 mM DTT and 500 μM dNTPs for 45 min. Five microliters of a 1/5 dilution of this cDNA-containing solution was used for DNA amplification in a DNA thermocycler (Gene Amp PCR System 9700; PerkinElmer, Wellesley, MA) with 0.5 U of
To test whether NDV-stimulated Mφ/H9278 were incubated for 8 h with unstimulated Mφ/H9278, tumor cell targets lines. As a negative control, tumor cell targets lines.

A–D, yielding a PCR product of 390 bp. and antisense, 5'-CCC GGC CAG CCA GGT AGG CCC AGA GCA-3'. From the graphs, but were twice with similar results. SD bars were omitted tures, and experiments were repeated at least tuers. Data points represent the mean of triplicate cul-

Results

NDV-stimulated Mφ kill various human tumor cell lines

To test whether NDV-stimulated Mφ are capable of killing human tumor cells, Mφ were stimulated by NDV for 16 h and then incu-
bated with different tumor cell targets in an 8-h chromium re-
lease assay. All tumor cell lines tested were killed by NDV-stim-
ulated Mφ. There were differences in the sensitivity of the tumor cell lines for the tumoricidal activity of NDV-stimulated Mφ (Fig. 1). The breast carcinoma cell line BT20 and the colon carcinoma cell line Colo205 were much more sensitive to Mφ-mediated cy-
totoxicity than the breast carcinoma line MCF-7 and the esophagus carcinoma cell line Colo680N. Unstimulated Mφ exhibited only minimal tumoricidal activity toward all four different tumor cell lines (Fig. 1). To investigate the mechanism of NDV-stimulated Mφ-mediated cytotoxicity in more detail, Colo205 and BT20, the two more sensitive of the four cell lines, were studied further.

Surface expression of death receptors and sensitivity to death receptor triggering

To examine possible mechanisms of tumor cell killing, BT20 and Colo205 cells were first tested for surface expression of the apoptosis-related TNF receptor family members CD95, TRAIL-R1 to TRAIL-R4, TNF-R1 (p55 TNF-R), and TNF-R2 (p75 TNF-R). Both cell lines expressed CD95, TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4 (Fig. 2A). Colo205 showed heterogeneous expression of TNF-R1 and TNF-R2, with a fraction of cells not ex-
pressing the TNF receptors and another fraction expressing them at low density, while expression of these two receptors on BT20 was only marginally detectable (data not shown).

In previous experiments we found that NDV is a strong inducer of IFN-α in cocultures of Mφ and tumor cells (our unpublished observation). We therefore tested whether IFN-α can influence death receptor expression after stimulation of the tumor cells for 24 h with IFN-α. We found that preincubation of the tumor cells with IFN-α did not change the surface expression of any of these receptors (Fig. 2A).

In addition to death receptor staining on tumor cells, we exam-
ined surface expression of the different TRAIL receptors on Mφ. Unstimulated Mφ expressed TRAIL-R1 and TRAIL-R2, while TRAIL-R3 and TRAIL-R4 could not be detected (Fig. 2B). Stim-
ulation with either IFN-α or NDV resulted in a sharp down-reg-
ulation of TRAIL-R1 and TRAIL-R2, whereas TRAIL-R3 and TRAIL-R4 remained unaltered.

We next investigated the sensitivity of the tumor cells to ago-

nists of the various death receptor systems. Chromium-labeled target cells were stimulated with TRAIL, the CD95-reactive mAb anti-APO-1, or TNF-α for 8 h and then tested for specific lysis. Both BT20 and Colo205 cells were sensitive to TRAIL-mediated killing (Fig. 2C). We were also interested in finding out whether preincubation of tumor cells with IFN-α could enhance the sensi-
tivity for death receptor triggering. Fig. 2C shows that preincu-
bation of BT20, but not Colo205, with IFN-α resulted in enhanced TRAIL-mediated killing. Both cell lines were also sensitive to anti-
APO-1 and to TNF-α, but to a lesser extent, and this cytotoxicity was only slightly enhanced by tumor cell preincubation with IFN-α. Taken together, these data indicate that BT20 and Colo205 cells exhibited the highest sensitivity to cytotoxicity mediated by TRAIL.

NDV-stimulated Mφ kill tumor cells via TRAIL

To test whether one or several of the apoptosis-inducing ligands may in fact be involved in the tumoricidal activity of NDV-stim-
ulated Mφ, we performed blocking experiments with soluble Fc fusion proteins for TNF, TRAIL, and CD95. First, we used Colo205 cells that were not preincubated with IFN-α as target cells.

FIGURE 1. NDV stimulates tumoricidal activity of human Mφ toward different tumor cell lines. A–D, Mφ were stimulated with 20 HU/ml NDV for 16 h in RPMI 1640 with 10% FCS. 51Cr-labeled Colo680N (A), Colo205 (B), MCF-7 (C), and BT20 (D) tumor cell targets were cultured with Mφ for 8 h at the indicated E:T cell ratios. As a negative control, tumor cell targets were incubated for 8 h with unstimulated Mφ. Data points represent the mean of triplicate cultures, and experiments were repeated at least twice with similar results. SD bars were omitted from the graphs, but were <10% of the value of all points.

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FIGURE 2. Surface expression of CD95 and TRAIL receptors on tumor cells and Mφ and sensitivity of tumor cells to LZ-TRAIL, anti-APO-1, and TNF-α. A. BT20 and Colo205 cells were incubated for 24 h in the absence or the presence of IFN-α (200 IU/ml) and then analyzed for surface expression of TRAIL-R1 to TRAIL-R4 and CD95. Solid bold lines represent stainings with HS201 (TRAIL-R2), HS402 (TRAIL-R4), and anti-APO-1 (CD95). Solid lines represent stainings with HS101 (TRAIL-R1) and HS301 (TRAIL-R3). Dotted lines represent stainings with isotype control mAbs. Histograms represent 10^4 tumor cells, and viability was >95% as assessed by propidium iodide exclusion. B. Mφ were activated with IFN-αA2 (200 IU/ml) or NDV (20 HU/ml) for 16 h and then analyzed for surface expression of TRAIL-R1 to TRAIL-R4. Solid bold lines represent stainings with HS201 (TRAIL-R2) and HS402 (TRAIL-R4). Solid lines represent stainings with isotype control mAbs. C. Tumor cells were used with or without 24-h IFN-αA2 preincubation. ^51Cr-labeled tumor cells were cultured for 8 h in the presence of LZ-TRAIL (2 μg/ml), anti-APO-1 (1 μg/ml with 10 ng/ml protein A), or TNF-α (2 μg/ml). Bars represent the mean of triplicate cultures, and experiments were repeated at least twice with similar results. SD values were omitted from the graph, but were <10% the value of all points.
in an 8-h chromium release assay. Fig. 3A shows that TRAIL-R2-Fc almost completely blocked the killing of Colo205 by NDV-stimulated Mφ. In contrast, neither CD95-Fc nor TNF-R2-Fc affected the respective Mφ-mediated tumor cell killing. Next we used BT20 cells preincubated with IFN-α to enhance the TRAIL sensitivity of these cells. The results in Fig. 3B demonstrate that TRAIL-R2-Fc also blocked the killing of IFN-α-pretreated BT20 breast carcinoma cells by NDV-stimulated Mφ almost completely. Yet, in these cells CD95-Fc seemed to exert an inhibitory effect, albeit rather small, while TNF-R2-Fc showed almost no effect.

To investigate whether viral replication competence is required for the induction of tumoricidal activity by NDV-stimulated Mφ, we first tested the up-regulation of viral envelope proteins on the surface of NDV-stimulated Mφ by FACS analysis as a marker for intracellular viral replication. When human Mφ were stimulated with live NDV, no shift of viral hemagglutinin-neuraminidase Ag expression toward higher cell surface density was observed with time of incubation (data not shown), suggesting that there was no active viral replication in these cells. To ultimately determine whether replication competence is required for the induction of tumoricidal activity, we tested the inducibility of anti-tumor cytotoxicity by replication-incompetent UV-inactivated NDV (NDV-UV) to induce Mφ anti-tumor cytotoxicity. Mφ were stimulated with NDV-UV, and receptor-specific Fc proteins were used for blocking. NDV-UV-stimulated Mφ killed IFN-α-pretreated BT20 cells to a similar extent as Mφ activated by live NDV (Fig. 3C). TRAIL-R2-Fc completely blocked this cytotoxic activity, while CD95-Fc showed only a small inhibitory effect, and TNF-R2-Fc did not show any inhibitory effect.

Although tumoricidal activity of NDV-stimulated Mφ was blocked by TRAIL-R2-Fc to a large extent, the inhibition was not complete (Fig. 3, A–C). Therefore, we tested whether the combination of the Fc fusion proteins used in this study is able to completely block tumor cell killing by NDV-stimulated Mφ. The results presented in Fig. 3D show that only TRAIL-R2-Fc inhibited the killing of IFN-α-pretreated BT20 breast carcinoma cells by NDV-stimulated Mφ. Moreover, neither CD95-Fc nor TNF-R2-Fc exerted an additional inhibitory effect on Mφ-mediated tumor cell killing when combined with TRAIL-R2-Fc.

Taken together, these results clearly demonstrate that NDV induces tumoricidal activity in human Mφ, that viral replication competence is not required for this phenomenon, and that TRAIL acts as the main effector molecule of this tumoricidal effect in the tumor systems tested.

**NDV induces TRAIL surface expression on human Mφ**

After having identified TRAIL as the effector molecule, we investigated how its activity was up-regulated upon NDV stimulation of Mφ. First, TRAIL mRNA expression was analyzed by RT-PCR. Mφ were stimulated with NDV for 1, 2, and 4 h before TRAIL-specific RT-PCR was performed (donor 1). Fig. 4A shows that TRAIL mRNA was induced after 2 h and increased up to 4 h after incubation with NDV. Unstimulated Mφ expressed only low basal levels of TRAIL mRNA that did not result in appreciable surface expression of TRAIL. Additionally, Mφ were stimulated with NDV and NDV-UV for 4 h and compared with IFN-α-stimulated Mφ as a positive control for TRAIL-specific mRNA induction (donor 2). In all three cases Mφ showed a clear up-regulation of TRAIL-specific mRNA after stimulation with IFN-α, NDV, and NDV-UV for 4 h. These results demonstrate that NDV induces TRAIL mRNA in a time-dependent manner and that viral replication competence is not required for this effect.

Second, expression of TRAIL was analyzed by Western blot analysis (Fig. 4B). We found that TRAIL protein was expressed.
16 h after stimulation with NDV in Mφ of four different donors in an inducible fashion. TRAIL expression was even higher after stimulation with NDV than after IFN-α stimulation, which we used as a positive control (donor 5). Interestingly, stimulation with NDV-UV resulted in the same strong induction of TRAIL expression as observed with non-UV-inactivated NDV (donor 6).

Third, TRAIL surface expression on Mφ was tested by FACS analysis after stimulation with NDV and NDV-UV for 16 h (Fig. 4C). We again used IFN-α stimulation as a positive control (21). Stimulation with NDV (donor 1) and NDV-UV (donor 2) induced TRAIL expression on the surface of Mφ to the same extent as observed after stimulation with IFN-α.

As stated above, NDV is a strong inducer of IFN-α in cocultures of Mφ and tumor cells (our unpublished observation) and stimulation of Mφ with IFN-α leads to an up-regulation of TRAIL surface expression (Fig. 4C). Therefore, we tested whether NDV-induced TRAIL induction on Mφ could be blocked by neutralizing IFN-α. However, we found that neutralization of IFN-α alone did not alter NDV-stimulated TRAIL surface expression on Mφ (Fig. 4D, left panel) and that neutralization of IFN-β alone only minimally reduced TRAIL surface expression (Fig. 4D, middle panel). In contrast, when Mφ were stimulated with NDV in the presence of both neutralizing antisera for IFN-α and IFN-β, up-regulation of TRAIL by NDV was almost completely inhibited (Fig. 4D, right panel).

FIGURE 4. Induction of TRAIL mRNA and surface expression in NDV- and NDV-UV-stimulated Mφ. A, Mφ were stimulated with NDV (20 HU/ml) for 1, 2, 4, and 8 h or with IFN-αA2 (200 IU/ml) or NDV-UV (20 HU/ml) for 4 h. RNA was isolated, and TRAIL-specific RT-PCR was performed. β-Actin was used as a control over the same time course. B, Mφ were stimulated with IFN-αA2 (200 IU/ml), NDV (20 HU/ml), or NDV-UV (20 HU/ml) for 16 h. Cells were lysed, and TRAIL-specific Western blot analysis was performed. C, Mφ were stimulated with IFN-αA2 (200 IU/ml), 20 HU/ml NDV, or NDV-UV for 16 h. Flow cytometric analysis of TRAIL expression was performed. Solid bold lines represent cells stained with TRAIL-R2-Fc, and solid lines represent cells stained with control IgG1. Experiments were repeated at least three times with different donors and led to similar results each time. Due to limited amounts of Mφ obtainable from 500 ml of donor blood, not all stimulations in A–D were performed with each donor. D, Mφ were stimulated with NDV (20 HU/ml) for 16 h, and neutralizing antisera for IFN-α (10,000 NU/ml) and/or IFN-β (4,000 NU/ml) were added. TRAIL expression was determined by flow cytometry. Gray lines represent TRAIL expression on unstimulated Mφ. Solid lines represent TRAIL expression on NDV-stimulated Mφ. Solid bold lines represent TRAIL expression on NDV-stimulated Mφ with neutralizing antisera against IFN-α and/or IFN-β. Histograms represent 10^4 gated Mφ, and viability was >95% as assessed by propidium iodide exclusion. E, Mφ were stimulated with different concentration of NDV (0.02, 0.2, 2, and 20 HU/ml) for 16 h. Supernatants were harvested, and concentrations of IFN-α (picograms per milliliter) and IFN-β (international units per milliliter) were determined by ELISA.
Having identified the critical role of IFN-α and IFN-β in NDV-stimulated TRAIL up-regulation on human Mφ, we determined the production of these cytokines by human Mφ after stimulation with NDV. As shown in Fig. 4E, NDV dose-dependently stimulated the release of IFN-α and IFN-β into the culture supernatant.

In conclusion, our results show that TRAIL is induced on human Mφ after NDV stimulation independently of viral replication. Induced TRAIL is the main stimulator of the death receptor systems and is primarily responsible for the tumoricidal activity seen with NDV-stimulated Mφ in the tested human tumor lines.

Discussion

NDV has been used as an antitumor and immunostimulatory agent in different tumor vaccine strategies. It has been shown that NDV induces NF-κB activation and NO production in murine macrophages (30) and that murine macrophages stimulated by NDV can kill tumor cells in vitro (7, 10) and in vivo (12). However, the mechanism of the NDV-mediated antitumor effect has not yet been solved at the molecular level. We show here that human NDV-stimulated Mφ exert a potent tumoricidal effect in vitro by induction of apoptosis in target cells. Apoptosis induction via cell-to-cell contact is mainly mediated by interaction of members of the TNF superfamily on the effector cell with their cognate receptors on the target cell. In the present study we therefore tested whether the TNF, the CD95 (Fas/APO-1), and/or the TRAIL death receptor/ligand systems may be responsible for or contribute to the tumoricidal effects mediated by NDV. Using soluble receptor fusion proteins for these three death receptor/ligand systems we could demonstrate that the TRAIL/TRAIL receptor pathway acts as a potent effector system employed by NDV-stimulated Mφ to induce apoptosis in tumor target cells. These data suggest that in vivo also the anti-tumor effects of NDV may be mediated by TRAIL.

Stimulation of human Mφ with the nonlytic, avirulent NDV strain Ulster resulted in the killing of a number of different human target cells in vitro. Although we observed target cell killing by NDV-stimulated Mφ in all cell lines tested, Colo205 and BT20 were the two most sensitive of the four cell lines (Fig. 1). This sensitivity to NDV-stimulated Mφ correlated with TRAIL sensitivity (Fig. 2C) and functional expression of the apoptosis-inducing TRAIL receptors, TRAIL-R1 and TRAIL-R2, on the surface of these two cell types (Fig. 2A). However, although CD95 was expressed on the surface of both cell types (Fig. 2A), and TRF-R1 was expressed on the surface of Colo205 (data not shown), both cell lines were only marginally sensitive to CD95L and TRF (Fig. 2C). Nevertheless, potential contributions of these two death systems to the killing of tumor cells by NDV-stimulated Mφ should not be excluded. In that respect, human PBMC stimulated with the lytic NDV strain 73-T produced TNF-α (7, 10), and TNF-α mediated the killing of TNF-α-sensitive human tumor cells lines by NDV (73-T)-stimulated PBMCs. Also, murine macrophages stimulated with the nonlytic NDV strain LaSota killed different murine tumor cell targets in a TNF-α-dependent manner (12). The tumoricidal activity of NDV (73-T)-stimulated human PBMCs correlated with IFN-α induction (10). In the present study NO did not contribute to the tumoricidal activity of NDV-activated Mφ, because first, no NO production was detected in Mφ cultures stimulated by NDV, and, second, the addition of the NO inhibitor L-NAME to cytotoxicity assays did not result in any inhibition of tumor cell killing (data not shown).

TRAIL was both induced at the mRNA and protein levels (Fig. 4, A and B) and expressed on the surface of Mφ stimulated with NDV or UV-inactivated NDV (Fig. 4C). The up-regulation of TRAIL on NDV- and IFN-α-stimulated Mφ was accompanied by a significant down-regulation of the TRAIL death receptors (Fig. 2B), which could point to a protective mechanism against monocytic fratricide (21). Taken together, the cell-to-cell contact killing of tumor cells by NDV-stimulated Mφ is primarily mediated by TRAIL.

Apart from NDV and UV-inactivated NDV, IFN-α was also capable of up-regulating TRAIL expression on the surface of human Mφ (Fig. 4C) through induction of TRAIL mRNA (Fig. 4A). These data are in line with previously reported results obtained with both IFN-α and IFN-γ (21). Both IFN-α and IFN-β were strongly induced upon NDV stimulation of Mφ (Fig. 4E). Interestingly, neutralization of IFN-α and IFN-β together resulted in inhibition of up-regulation of TRAIL surface expression on Mφ (Fig. 4D), indicating a critical role of these cytokines for NDV-stimulated TRAIL induction.

The functional expression of TRAIL upon IFN stimulation has also been shown on other cell types of the immune system. In that respect, IFN-α and IFN-β induce TRAIL on CD4+ T cells (31), while IFN-α and IFN-γ stimulate the functional expression of TRAIL on DC (22). Recently, it was shown that IFN-γ stimulation of human Mφ led to the functional expression of both TWEAK and TRAIL, which cooperatively killed the human squamous cell carcinoma cell line HSC3 (32). Concerning cells outside the immune system, IFN-γ is capable of inducing TRAIL on the surface of human fibroblasts (23). Interestingly, Sedger et al. (23) also showed that the infection of fibroblasts with CMV resulted in functional expression of TRAIL on these fibroblasts. Yet, IFN-γ selectively induced TRAIL sensitivity in CMV-infected, but not in uninfected, fibroblasts, thus resulting in the selective killing of virus-infected cells. In the present study IFN-α sensitized BT20 cells for TRAIL-induced apoptosis (Fig. 2B) by an unknown mechanism. Interestingly, IFN-α was shown to potentiate TNF-induced apoptosis by suppression of NF-κB and AP-1 activation (33). A similar mechanism might result in sensitization of BT20 cells for TRAIL-induced apoptosis.

Recently, two other viruses have been shown to induce apoptosis via the TRAIL system. TRAIL was expressed on measles virus-infected DC killed the mammary adenocarcinoma cell line MDA231 (24), the same cell line that was shown to be sensitive to TRAIL treatment in vivo (16), while reovirus induced the auto- or paracrine TRAIL-mediated death of HEK293 cells (34). Thus, it seems that a more general theme is emerging: viral infection results in up-regulation of TRAIL and sensitization of either virally infected or transformed neoplastic cells to TRAIL. This translates into the potential use of viruses for tumor therapy. The lytic NDV strain 73-T was previously used to produce oncolysates for postoperative tumor vaccination in melanoma patients (2). In a different approach, a life-cell vaccine with autologous tumor cells modified with the nonlytic, avirulent NDV strain Ulster for postoperative active specific immunotherapy was established (2). Both strategies showed significant therapeutic effects in phase II clinical studies (2, 3). The results presented here demonstrate that one of the antineoplastic properties of the NDV strain Ulster is the activation of tumoricidal activity of human Mφ by induction of functional TRAIL.

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References


