[CANCER RESEARCH 64, 2684–2691, April 15, 2004]

Caspase-8-Dependent HER-2 Cleavage in Response to Tumor Necrosis Factor α Stimulation Is Counteracted by Nuclear Factor κB through c-FLIP-L Expression

Valérie Benoit,[1] Alain Chariot,[1] Laurence Delacroix,[2] Valérie Deregowski,[1] Nathalie Jacobs,[2] Marie-Paule Merville,[1] and Vincent Bours[1]

[1]Laboratory of Medical Chemistry and Human Genetics, Center for Molecular and Cellular Therapy and [2]Center for Research in Experimental Cancerology, University of Liege, Liege, Belgium

ABSTRACT

The oncoprotein HER-2/*neu* is a prosurvival factor, and its overexpression has been correlated with poor prognosis in patients with breast cancer. We report that HER-2 is a new substrate for caspase-8 and that tumor necrosis factor α (TNF-α) stimulation leads to an early caspase-8-dependent HER-2 cleavage in MCF7 A/Z breast adenocarcinoma cells defective for nuclear factor κB (NFκB) activation. We show that the antiapoptotic transcription factor NFκB counteracts this cleavage through induction of the caspase-8 inhibitor c-FLIP. Our results also demonstrate that this HER-2 cleavage contributes to the TNF-α-induced apoptosis pathway because ectopic expression of an uncleavable HER-2 protects NFκB-defective cells against TNF-α-mediated cell death. Therefore, we propose an original model in which NFκB exerts a new antiapoptotic function by counteracting TNF-α-triggered cleavage of the HER-2 survival factor.

INTRODUCTION

Tumor necrosis factor α (TNF-α) plays a pivotal role in the control of cell proliferation and inflammation by regulating proapoptotic and antiapoptotic signaling pathways through binding to two distinct receptors, TNF receptor 1 (TNF-R1) and TNF receptor 2 (TNF-R2; Refs. 1–5). TNF-α binding triggers receptor trimerization and subsequent recruitment of various signaling proteins to the receptor cytoplasmic domains. Depending on the nature of the receptor and the adaptor proteins, TNF-α can exert opposite effects (6–8). Activation of the TNF-R2 produces an antiapoptotic and proinflammatory cascade, whereas activation of the TNF-R1 leads to antagonist events, namely, apoptosis through recruitment and activation of caspase-8 (9) as opposed to the activation of the antiapoptotic and proinflammatory transcription factor nuclear factor κB (NFκB; Refs. 6–8). Numerous NFκB target genes counteract the TNF-α-activated apoptosis pathway and include caspase inhibitors such as c-IAP1 (10), c-IAP2 (11, 12), XIAP (13), and c-FLIP (14); Bcl-2 family members such as A1 (15–17) or Bcl-x_L (18, 19); and other proteins like A20 (20–22) or MnSOD (23–25). These opposite events can explain that TNF-α is a poor inducer of apoptosis in the absence of RNA or protein synthesis inhibitors (26–28).

Cancers are characterized by an increased proliferation and a decreased apoptotic rate (29). Among the proteins implicated in this dysregulation, HER-2/*neu* (ErbB-2) plays a major role in some ovarian and breast cancer cells. The *Her-2* oncogene encodes a transmembrane receptor protein structurally related to the epidermal growth factor receptor (30, 31). HER-2 is overexpressed in approximately one-third of the primary breast carcinomas (32) and in ovarian carcinomas (31, 33–35), and its expression is correlated with poor prognosis and decreased overall and disease-free survival (36–38). HER-2 overexpression has been demonstrated to enhance proliferative, metastatic, and prosurvival signals in breast cancer cell lines (39–41) and to induce resistance to hormonal therapy, paclitaxel, and TNF-α (31, 39, 42, 43), although one report did not find any modification of TNF response in relation with HER-2 expression (44). Moreover, the anti-HER-2 antibody trastuzumab has clinical activity either alone or combined with chemotherapy in HER-2-expressing breast cancers (45–50).

Because the link between HER-2 overexpression and NFκB currently is unclear, we studied the effect of TNF-α treatment and NFκB activation on HER-2 expression in MCF7 A/Z breast adenocarcinoma cells. We demonstrated that TNF-α stimulation leads to HER-2 cleavage through a caspase-8-dependent pathway in cells defective for NFκB activation. Therefore, our results provide evidence for HER-2 being a newly described caspase-8 substrate and demonstrate that NFκB-dependent inhibition of HER-2 cleavage could be a novel mechanism for NFκB antiapoptotic role.

MATERIALS AND METHODS

Reagents. Human recombinant TNF-α was purchased from Roche (Mannheim, Germany). Cycloheximide and actinomycin D were from Sigma (St. Louis, MO), and BAY 11–7085 was obtained from Biomol (Plymouth Meeting, PA). Recombinant caspase-8 and caspase inhibitors were purchased from Calbiochem (La Jolla, CA).

Cell Cultures and Transfections. MCF7 A/Z breast cancer cells (supplied by Dr. Mareel, University of Ghent, Belgium) were maintained in RPMI 1640 medium without phenol red supplemented with 10% stripped fetal bovine serum, 1% L-glutamine (200 mM), penicillin (100 IU), and streptomycin (100 μg/ml). For the stably transfected cell lines (pcDNA3 and IκBα MT), culture medium was supplemented with geneticin (G418, 500 μg/ml; Roche).

For DNA transfection, cells were plated at 7×10^5 cells per 35-mm-diameter well culture dishes and transfected 24 h later with FuGENE, according to the protocol provided by the manufacturer (Roche).

Protein Extraction and Western Blot Analysis. Whole cell extracts were obtained by resuspending the PBS-washed cellular pellets in SDS 1%. The lysates then were boiled for 10 min, and protein amounts were quantified with Micro BCA Protein Assay reagent (Pierce, Rockford, IL) using a BSA standard solution as reference.

Protein extracts were separated on SDS-PAGE gels and blotted onto an Immobilon P membrane (polyvinylidene diflouride; Millipore, Bedford, MA). The membranes then were blocked in Tris-buffered saline/Tween 20% buffer plus 5% nonfat dry milk, incubated for 2 h with the first antibody, washed with Tris-buffered saline/Tween 20%, and incubated for 1 h with the second horseradish peroxidase-conjugated antibody (DAKO, Glostrup, Denmark). The reaction was revealed with the enhanced chemoluminescence detection method (ECL kit; Amersham Pharmacia Biotech, Piscataway, NJ).

The following antibodies were used for Western blot analysis: rabbit poly-

Received 9/16/03; revised 1/8/04; accepted 2/9/04.

Grant support: This research was supported by the Leon Fredericq Foundation, the Centre Anticancéreux près l'Ulg (Liege, Belgium), and by grants from Télévie and the National Fund for Scientific Research (Belgium).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: V. Benoit is Research Assistant, N. Jacobs is Senior Research Assistant, and A. Chariot and M-P. Merville are Research Associates at the National Fund for Scientific Research (Belgium).

Requests for reprints: Vincent Bours, Laboratory of Medical Chemistry and Human Genetics, CHU B35, Sart Tilman, 4000 Liege, Belgium. Phone: 32-43-66-81-44; Fax: 32-43-66-81-46; E-mail: vbours@ulg.ac.be.

clonal anti-HER-2 (Upstate Biology, Lake Placid, NY), mouse monoclonal antiactin (Sigma), mouse monoclonal anti-poly(ADP-ribose) polymerase (PARP; PharMingen, San Diego, CA), and mouse monoclonal XIAP (R&D, Minneapolis, MN). Mouse monoclonal anti-c-FLIP (sc-5276), rabbit polyclonal anti-c-IAP-1 (sc-7943), and anti-c-IAP-2 (sc-7944) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Real-Time Quantitative PCR. Total RNA was extracted using RNeasy columns from Qiagen (Valencia, CA) according to the manufacturer's recommendations. After DNase treatment, RNAs were eluted and quantified using a spectrophotometer. One μg of RNA then was reverse transcribed using the first-strand cDNA synthesis kit for reverse transcription-PCR (Roche).

The quantitative PCR reaction samples involved 2 μl of 20× diluted cDNAs, 2 μl of 10× SYBR Green PCR mix buffer, 1.6 μl $MgCl_2$ 25 mM and 7 μM of each primer. The number of cycles was selected to allow linear amplification of the cDNAs under study. For quantitative PCR, the GAPDH housekeeping gene was used as a control. Quantification was performed with the LightCycler PCR Technology (Roche). The primer sequences were as follows: *GAPDH*, 5′-ATGGGGAAGGTGAAGGTGGTC-3′ and 5′-TGATGGCATGGACTGTGG-3′; and *HER-2*, 5′-AGACGAAGCATACGTGA-3′ and 5′-GTACGAGCCGCACATC-3′.

Caspase-8 Activity. To evaluate caspase-8 activity, pcDNA3 MCF7 A/Z cells pretreated or not with cycloheximide (CHX) and IκBα MT cells were stimulated with TNF-α. Cell lysates then were incubated for 3 h at 37°C with a caspase-8 fluorogenic substrate, Ac-IEPD-AMC (Alexis Biochemicals, Lausen, Switzerland). The fluorescence of the generated cleaved product then was measured using a spectrofluorometer (380 nm and 460 nm). The experimental procedure followed the manufacturer's recommendations.

FLIP Antisense Experiments. FLIP antisense, 5′-ACTTGTCCCTGCTCCTTGAA-3′; sense, 5′-TTCAAGGAGCA GGGACAAGT-3′; or scrambled, 5′-ATCACGTATCGTCGCTTCTC-3′ oligonucleotides bearing phosphorothioate linkages were delivered into cells by lipofection with FuGENE (Roche) at a final concentration of 10 μM for 8 h before TNF-α treatment.

Generation of HER-2 Mutants. We screened the HER-2 amino acid sequence for aspartate residues and identified six putative caspase-8 cleavage sites. For each site, we mutated the aspartate residue into an alanine using the Quick Change XL site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) following the manufacturer's instructions. Twenty-seven nucleotide-long oligonucleotides carrying a single nucleotide mutation in the middle were generated in this purpose, and the mutations were GCT (A) instead of GAT (D) for the mutations D837A, D1012A, D1019A, D1087A, and D1125, and GCC (A) instead of GAC (D) for the mutation D115A.

***In Vitro* Proteolysis Assays.** *In vitro* proteolysis assays were performed as described previously (51). HER-2 coding sequence (a gift from Dr. Di Fiore, Bethesda, MD) was subcloned by PCR in a PCR-XL-TOPO vector using the TOPO XL PCR cloning kit (Invitrogen, Carlsbad, CA). This plasmid was mutated *in vitro* using the Quick Change XL site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions.

C-termini were amplified by PCR and translated *in vitro* in the presence of [^{35}S]methionine using the TNT T7 Quick for PCR DNA kit (Promega, Madison, WI) and incubated with recombinant caspase-8 (100 units) or cellular extracts (50 μg) at 37°C for 2 h.

After TNF-α stimulation, cells were harvested, washed with PBS, and centrifuged. The cellular pellets then were resuspended in hypotonic buffer (10 mM HEPES, 40 mM glycerophosphate, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 0.5 μg/ml aprotinin). After freezing and thawing, the lysates were cleared by centrifugation. The extracts were incubated in the presence of the ^{35}S-labeled HER-2 C-termini with or without a 15-min pretreatment with caspase-8 inhibitor. The samples then were boiled and analyzed by SDS-PAGE.

Thymidine Incorporation. MCF7 A/Z IκBα cells were seeded in 96-well plates (5000 cells/well). Twenty-four h later, cells were transfected with the pcDNA3 empty vector or with an expression vector coding for HER2 WT or MT. Twenty-four h after transfection, [H^3]thymidine (0.4 μCi/well; Sigma) was added in the culture medium, and cells were incubated for 5 h. Cells then were lysed, and DNA was transferred on a Unifilter GC plate (Perkin Elmer, Boston, MA) using a Packard Filter Mate. After plate drying, Microscint O (Perkin Elmer) was added, and [H^3]thymidine incorporation was assessed using a microplate scintillation counter (Top count; Perkin Elmer).

RESULTS

TNF-α-Mediated HER-2 Degradation in NFκB-Defective Cells. To study the effects of TNF-α and NFκB on HER-2 expression levels, we used MCF7 A/Z mammary adenocarcinoma cells stably transfected either with an empty vector (pcDNA3 or control cells) or with an expression vector encoding IκBα mutated on serines 32 and 36 (IκBα MT or MT cells), an NFκB super-repressor that inhibits NFκB nuclear translocation and biological activity and leads to increased apoptotic response to TNF-α (52).

Both cell lines were stimulated with TNF-α for up to 24 h. HER-2 expression was investigated by Western blot analysis and remained constant in the control cell line. By contrast, an important decrease of HER-2 levels was observed in the cell overexpressing the mutated form of IκBα (Fig. 1).

To determine the influence of transcriptional regulation on decreased HER-2 protein, MCF7 A/Z pcDNA3 or IκBα MT was treated with TNF-α for 2 or 4 h, and levels of *HER-2* transcripts were assessed by quantitative real-time PCR using *HER-2*-specific primers and *GAPDH* housekeeping gene as controls. No difference in *HER-2* mRNA levels was observed when both cell lines were stimulated by TNF-α (Fig. 2*A*). Moreover, TNF-α did not modify the transcriptional activity on the *HER-2* proximal promoter as judged by luciferase assays (data not shown). To inhibit RNA neosynthesis, control and IκBα MCF7 A/Z cells were preincubated with actinomycin D for 1 h before a TNF-α time course stimulation and real-time PCR experiment were performed with *HER-2* and *GAPDH* primers. We observed that the degradation rate of *HER-2* mRNA was identical in both cell lines (Fig. 2*B*). Therefore, these results demonstrate that in our experimental conditions, TNF-α-mediated HER-2 down-regulation was not the consequence of decreased RNA levels.

To investigate whether a difference in HER-2 protein stability was involved in the TNF-α-mediated HER-2 disappearance in IκBα MT MCF7 cells, we first incubated both cell lines with CHX before TNF-α stimulation. HER-2 protein levels were assessed using Western blot analysis. In both cell lines, HER-2 protein was not detectable beyond 4 h of TNF-α stimulation (Fig. 3*A*). However, HER-2 protein is highly stable because we detected its expression >24 h after CHX alone in both cell lines (Fig. 3*B* and data not shown).

TNF-α-stimulated control cells then were preincubated with CHX or BAY 11–7085, a NFκB inhibitor, and HER-2 protein levels were assessed by Western blot analysis. In agreement with our previous results, we observed a decrease in HER-2 protein levels in response to TNF-α only after NFκB or protein synthesis inhibition (Fig. 3*B*), indicating that an NFκB-dependent protein synthesis occurs in MCF7 A/Z control cells and counteracts the TNF-α-mediated HER-2 degradation.

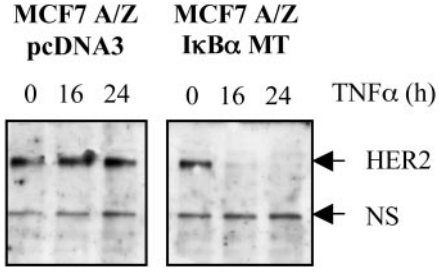

Fig. 1. Tumor necrosis factor α (*TNF-α*)-mediated HER-2 degradation in nuclear factor κB (NFκB)-defective cells. pcDNA3 or IκBα MT MCF7 A/Z cells were treated with TNF-α (100 units/ml) for the indicated times. Ten μg of total protein extracts were analyzed by Western blot analysis with a specific anti-HER-2 COOH-terminal antibody. A nonspecific band is shown as loading control.

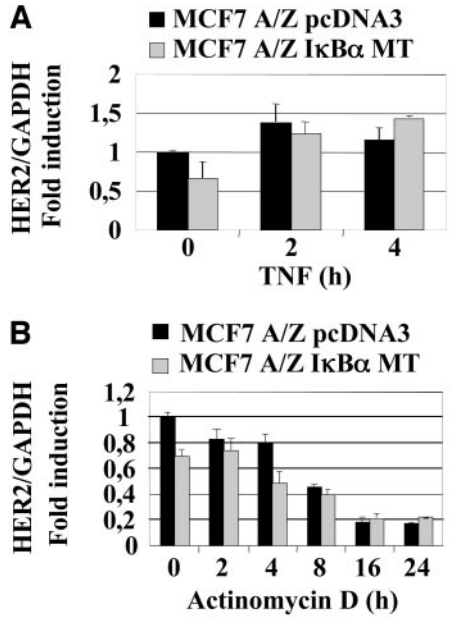

Fig. 2. *HER-2* mRNA expression and stability. *A*, pcDNA3 or IκBα MT MCF7 A/Z cells were stimulated by tumor necrosis factor α (*TNF-α*; 100 units/ml) for the indicated times. Quantitative real-time PCR was performed using *HER-2*-specific oligonucleotides and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as internal control. *B*, control or MT cells were treated with actinomycin D (5 μg/ml) for the indicated times. Quantitative real-time PCR was performed as in *A*.

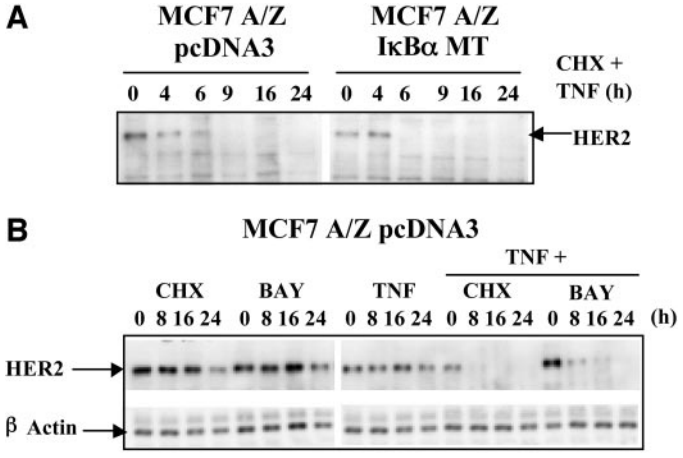

Fig. 3. HER-2 protein stability. *A*, control or MT cells were incubated with cycloheximide (*CHX*; 50 μg/ml) for 1 h before tumor necrosis factor α (*TNF-α*; 100 units/ml) time course stimulation. Ten μg of total cellular extract were subjected to anti-HER-2 Western blot analysis. *B*, pcDNA3 MCF7 A/Z cells were incubated up to 24 h with CHX (50 μg/ml), BAY 11–7085 (10 μM), or TNF-α (100 units/ml) either alone or in combination. For combined treatment, cells were preincubated with CHX or BAY 11–7085 for 1 h before TNF-α stimulation. Ten μg of total cellular extracts were used for Western blot analysis with a specific anti-HER-2 antibody or an antiactin antibody for loading control.

HER-2 Cleavage by α-Activated Caspase-8 in NFκB-Defective Cells. We hypothesized that decreased HER-2 levels in response to TNF-α might be a consequence of protease activation. To test such a hypothesis, we preincubated cells with protease inhibitors and tested their ability to counteract the TNF-α-mediated HER-2 decay. Phenylmethylsulfonyl fluoride (a serine protease inhibitor) and hydroxamate (a specific inhibitor of some metalloproteases) did not prevent HER-2 degradation in response to TNF-α stimulation in cells overexpressing the mutated IκBα (data not shown).

Because it has been demonstrated recently that HER-2 may be a caspase substrate in geldanamycin and staurosporin-treated cells, we investigated this pathway (53). We observed endogenous caspase-8 cleavage and activation following TNF-α stimulation in MT cells and CHX-pretreated control cells (Fig. 4, *A* and *B*), and the kinetics of this activation perfectly paralleled HER-2 decay and PARP cleavage (compare Fig. 4 and Fig. 5).

Moreover, z-IETD-fmk, a specific caspase-8 inhibitor, blocked TNF-α- and CHX-induced HER-2 degradation in control cells, whereas caspase-3 or caspase-9 inhibitors did not have any effect (Fig. 5*A* and data not shown). Furthermore, MCF7 A/Z MT cells or CHX-pretreated control cells were stimulated with TNF-α in the presence of DMSO or z-IETD-fmk, and the kinetics of HER-2 protein degradation and PARP cleavage were compared. Fig. 5, *B* and *C*, shows that in all of our experimental conditions, HER-2 and PARP cleavages occurred simultaneously and were inhibited similarly by the caspase-8 inhibitor. The efficiency of the z-IETD-fmk inhibitor was confirmed by Western blot analysis revealed with a caspase-8-specific antibody (data not shown).

Taken together, these data indicated that caspase-8 is responsible for the TNF-α-induced HER-2 degradation in NFκB-deficient cells.

Inhibition of Caspase-8-Mediated HER-2 Cleavage by NFκB-Regulated c-FLIP. Our data indicated that caspase-8-induced HER-2 cleavage only occurred in cells in which NFκB activation or protein synthesis was inhibited. This cleavage is prevented in control cells through a TNF-α-induced NFκB pathway leading to the expression of caspase-inhibiting proteins.

To identify the protein products of these NFκB-regulated genes, control and MT cells were treated with TNF-α, and total cellular lysates were analyzed by Western blot analysis revealed with specific antibodies directed against well-known NFκB-regulated caspase inhibitors. As shown in Fig. 6, c-IAP-1, c-IAP-2, and XIAP protein levels were similar in untreated or TNF-α-stimulated control or MT MCF7 A/Z cells. However, c-FLIP-L protein amounts were increased markedly in TNF-α-stimulated control cells, whereas the expression of this protein decreased rapidly following treatment of MT cells (Fig. 6).

Because c-FLIP-L is a well-known caspase-8 inhibitor and an

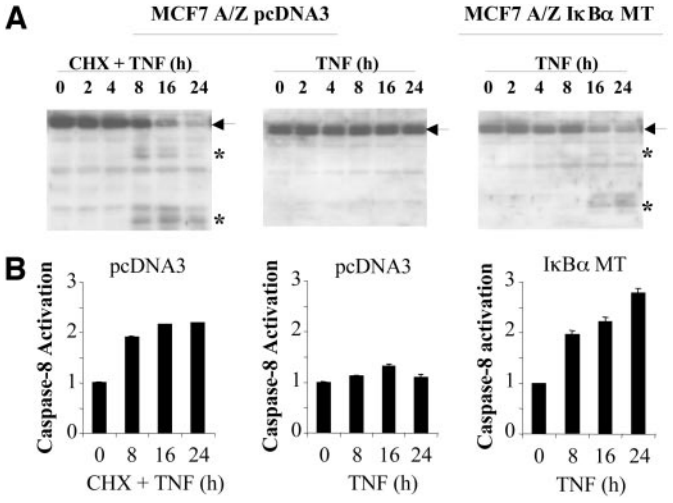

Fig. 4. Caspase-8 cleavage and activation in response to tumor necrosis factor α (*TNF-α*). *A*, caspase-8 cleavage. pcDNA3 MCF7 A/Z cells were stimulated with cycloheximide (*CHX*; 50 μg/ml) and TNF-α (100 units/ml; *left*) or TNF-α only (*middle*), and IκBα MT MCF7 A/Z cells were stimulated with TNF-α. Proteolysis of caspase-8 in total cellular extracts (10 μg) was assessed by Western blot analysis. Caspase-8 proform is indicated by an arrow, and asterisks show the cleaved fragments. *B*, caspase-8 activity. pcDNA3 MCF7 A/Z cells were stimulated with CHX (50 μg/ml) and TNF-α (100 units/ml) or TNF-α only, and IκBα MT MCF7 A/Z cells were stimulated with TNF-α as indicated. Cellular extracts were incubated with a fluorogenic caspase-8 substrate. Fluorescence of the generated cleaved product was assessed with a spectrofluorometer. The results are expressed in fold activation compared with untreated cells. A representative experiment out of three reproducible measurements is represented.

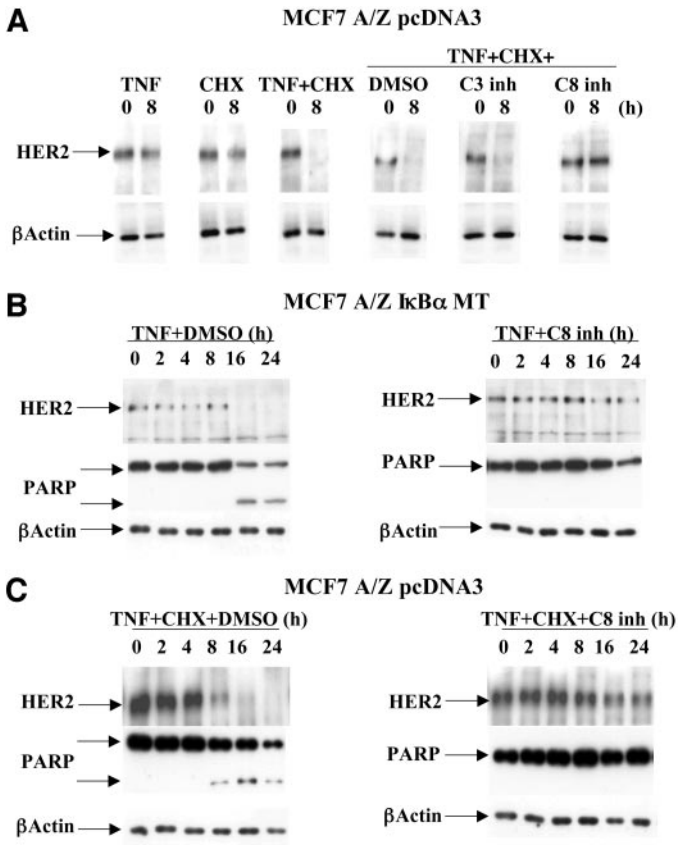

Fig. 5. HER-2 cleavage by caspase-8. *A*, pcDNA3 MCF7 A/Z cells were stimulated by TNF-α (100 units/ml), cycloheximide (*CHX*; 50 ìg/ml), or both for 8 h (*left*). CHX- and TNF-α-costimulated cells were preincubated with a caspase-3- or caspase-8-specific inhibitor (20 μM) or with vehicle (DMSO) for 1 h (*right*). HER-2 expression was evaluated by Western blot analysis performed with total protein extracts (10 μg). *B*, IκBα MT MCF7 A/Z cells were stimulated with tumor necrosis factor α (*TNF-α*; 100 units/ml) for indicated times in the absence (*left*) or presence (*right*) of the caspase-8 inhibitor (20 μM). Western blot analysis was carried out with 10-μg total extracts. Proteins were probed with anti-HER-2, anti-poly(ADP-ribose) polymerase (*PARP*), and antiactin antibodies as indicated. *C*, pcDNA3 MCF7 A/Z cells were costimulated with TNF-α (100 units/ml) and CHX (50 μg/ml) for the indicated times in the absence (*left*) or presence (*right*) of caspase-8 inhibitor (20 μM). Western blot analysis was performed as described in *B*.

NFκB target gene product, we investigated whether modulation of its expression affects HER-2 cleavage (14, 54, 55). Specific antisense oligonucleotides targeting *c-flip* mRNA efficiently decreased c-FLIP protein expression in control cells, whereas scrambled or sense oligonucleotides did not (Fig. 7*A*). The inhibition of c-FLIP expression in control cells was correlated with TNF-α-induced PARP cleavage and HER-2 degradation (Fig. 7*A*). Interestingly, the inhibition of c-FLIP expression by the same antisense oligonucleotides also led to TNF-α-induced HER-2 degradation in BT-474 and SKBR3 breast cancer cells, which are known to overexpress HER-2 (Fig. 7*B*).

As additional evidence for c-FLIP-dependent inhibition of HER-2 cleavage, the opposite experiment was performed by reintroducing c-FLIP-L exogenous transient expression in IκBα MT cells. We demonstrated that such ectopic c-FLIP expression partially inhibited TNF-α-mediated HER-2 cleavage (Fig. 8). IκBα MT cells were transfected with either an empty vector or a c-FLIP expression vector and stimulated with TNF-α. Cellular extracts then were analyzed by Western blot analysis for c-FLIP and HER-2 expression. In mock-transfected cells (Fig. 8*A*), HER-2 completely disappeared after 16 h of TNF-α treatment, whereas it still could be observed in c-FLIP-expressing cells (Fig. 8*B*).

These data clearly indicated that c-FLIP expression was induced through NFκB activation in TNF-α-stimulated control cells and that this protein inhibited the caspase-8-mediated HER-2 cleavage.

Identification of HER-2 Cleavage Sites Targeted by Caspase-8. HER-2 degradation by caspase-8 did not generate any detectable cleavage product on immunoblot analyses performed with cellular extracts, even in the presence of proteasome inhibitors (data not shown). Therefore, HER-2 cleavage also was investigated *in vitro*. The HER-2 cytoplasmic domain was translated *in vitro*, and *in vitro* proteolysis assays were performed in the presence of hypotonic cellular extracts from control or MT cells, either untreated or TNF-α stimulated for 10 h. As shown in Fig. 9*A*, S^{35}-labeled HER-2 COOH-terminus domain was cleaved in two fragments after incubation with cellular extracts from TNF-α-treated IκBα MT cells. This cleavage was inhibited by preincubation of these extracts with the caspase-8 inhibitor, and similar cleavage fragments were generated after HER-2 COOH-terminus domain incubation with recombinant caspase-8 (Fig. 9*A*).

We localized six potential caspase cleavage sites in the HER-2 COOH-terminal domain, and the aspartate residues from these sites were mutated into alanines (Fig. 9*B*). The mutants MT1, MT2, and MT3 designated proteins where the residues 1012, 1019, and 1125 were mutated, respectively. *In vitro* proteolysis assays showed that the D1125A (MT3) mutant was partially resistant to proteolysis by recombinant caspase-8 (Fig. 9*C*). Therefore, we proceeded with additional mutations and generated double mutants carrying the D1125A

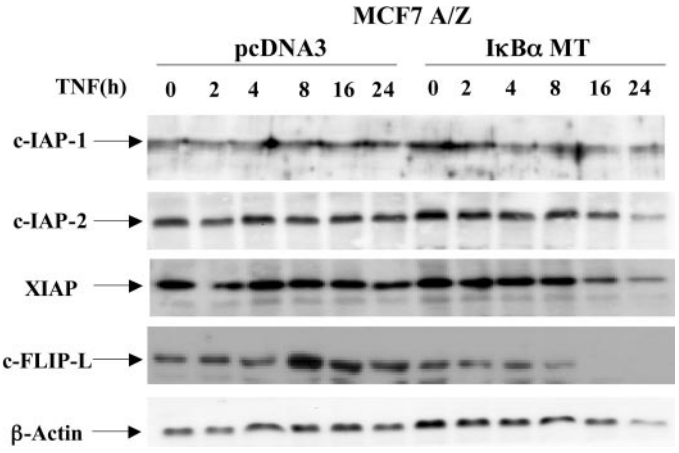

Fig. 6. Expression of nuclear factor κB (NFκB)-controlled caspase-8 inhibitors. pcDNA3 and IκBα MT MCF7 A/Z cells were stimulated with tumor necrosis factor α (*TNF-α*; 100 units/ml) for up to 24 h. Total extracts (10 μg) were analyzed by Western blot analysis with anti-c-IAP-1, anti-c-IAP-2, anti-XIAP, anti-c-FLIP-L, and antiactin antibodies, respectively.

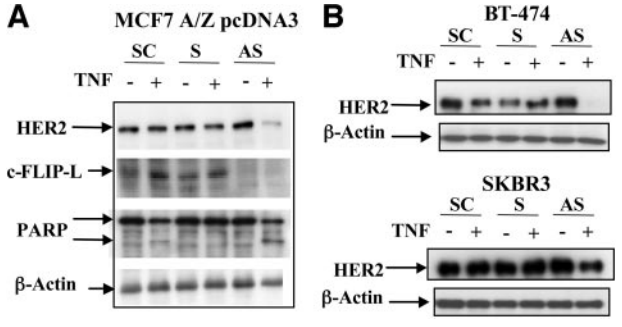

Fig. 7. Increased HER-2 cleavage by inhibition of c-FLIP expression. *A*, pcDNA3 MCF7 A/Z cells were transfected with c-FLIP-L antisense (*AS*), sense (*S*), or scrambled (*SC*) oligonucleotides. Eight h after transfection, cells were left untreated or were stimulated with tumor necrosis factor α (*TNF-α*; 100 units/ml) for an additional 24 h. Total extracts (10 μg) were evaluated by Western blot analysis with anti-HER-2, anti-c-FLIP-L, anti-poly(ADP-ribose) polymerase (*PARP*), and antiactin antibodies, respectively. *B*, BT-474 and SKBR3, two HER-2-overexpressing cell lines, were handled as above. Total cellular extracts (5 μg) were submitted to an HER-2 and actin Western blot analysis.

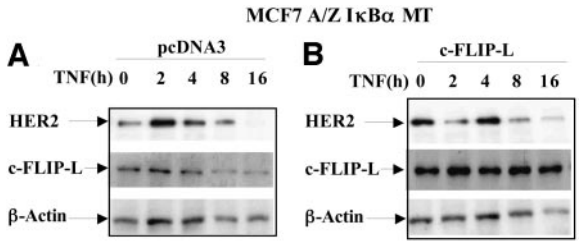

Fig. 8. Inhibition of HER-2 cleavage by c-FLIP expression. IκBα MT MCF7 A/Z cells were transfected with pcDNA3 (*A*) or c-FLIP-L (*B*) expression vectors. Twenty-four h after transfection, cells were stimulated with TNF-α (100 units/ml) for indicated times. Western blot analysis was performed with total extracts (10 μg) using anti-HER-2, anti-c-FLIP-L, and antiactin antibodies.

mutation plus a mutation of aspartate 837 (MT34), 1087 (MT35), or 1115 (MT36; Fig. 9*B*). The double-mutant MT35, carrying D1125A and D1087A substitutions, was resistant completely to recombinant caspase-8-induced proteolysis (Fig. 9*D*).

The wild-type and the HER-2 double mutants then were cloned into a pcDNA3 expression vector, and we verified that the mutated form was still uncleavable by recombinant caspase-8 during *in vitro* proteolysis assay (data not shown). The HER-2 wild-type and MT35 double mutant then were expressed exogenously in MCF7 A/Z IκBα MT cells by transient transfection. Total cellular extracts from TNF-α-treated cells were evaluated by immunoblot analyses, and these experiments showed that exogenously overexpressed wild-type HER-2 was cleaved partially in response to TNF-α treatment, whereas the MT35 mutant was not (Fig. 9*E*).

Involvement of HER-2 Cleavage in TNF-α-Induced Cell Death. To test the biological significance of these observations, IκBα MT MCF7 A/Z cells were transfected transiently with an empty expression vector, an expression vector coding for the wild-type HER-2, or with an expression vector coding for the MT35 HER-2. Cells then were stimulated for 2 days with TNF-α and living cells counted by trypan blue exclusion. As shown in Fig. 10*A*, most control pcDNA3-transfected cells were killed by TNF-α treatment. Exogenously expressed wild-type HER-2 clearly protected MCF7 A/Z cells from TNF-α-induced cytotoxicity because the proportion of living cells was increased from 26 to 38% of the control. Interestingly, cell survival was additionally and reproducibly increased following exogenous expression of the MT35 HER-2 mutant (49% of the untreated control). These transfected cells also were pretreated with the anti-HER-2 monoclonal antibody trastuzumab before TNF-α stimulation. This antibody clearly suppressed HER-2-mediated cytoprotection against TNF-α-induced cell death because it reduced the percentages of living cells to 26% after transfection of the wild-type HER-2 vector and to 30% after transfection of the mutated HER-2 vector (Fig. 10*A*). For this experiment, transduction efficiency was controlled by transfecting in parallel an expression vector coding for the green fluorescent protein and counting positive cells by fluorescence-activated cell sorting. In representative experiments, ~50% of the cells were transfected (data not shown).

We checked that the increased living cell numbers were not related to HER-2-induced proliferation by measuring thymidine incorporation following transfection of MCF7 A/Z cells with the same expression vectors. As shown in Fig. 10*B*, there was not any significant difference in thymidine incorporation between control and HER-2-overexpressing cells.

These data proved that HER-2 expression protected IκBα MT cells against TNF-α-induced cell death. Therefore, the TNF-α-mediated HER-2 cleavage in these cells may participate in the apoptotic pathway.

DISCUSSION

The apoptosis relies on the activity of caspases, a growing family of aspartyl-specific cysteine proteases that are essential for death-receptor proximal events and the execution of apoptosis by cleavage of broad-spectrum substrates. Among these "death substrates" are molecules involved in DNA repair like PARP, structural proteins, lamin, and focal adhesion kinase (56), oncoproteins such as Bcl-2 (57), and signaling proteins (58). Caspase-mediated cleavage of signaling molecules such as caspase-activated DNase (59, 60), STAT-1 (61), Raf-1, Akt (62), and c-Abl (63) leads to either their activation or inactivation. Because caspases are responsible for the onset of apoptosis, identification of their cellular substrates is essential and provides insights into the downstream events involved in apoptosis signaling. Among potential caspase substrates, a recent study showed that geldanamycin or staurosporin treatment of SKBR-3 human breast cancer cells led to HER-2 proteolytic cleavage, which was inhibited partially by a pleiotropic caspase inhibitor (53). Our results clearly identified the oncoprotein HER-2 as a caspase-8 substrate. Interestingly, HER-2 cleavage by caspase-8 occurred early after TNF-α treatment, suggesting that this cleavage might be involved in proapoptotic signal progression.

It was reported previously that HER-2 proteolytic fragments were degraded rapidly but could be observed in the presence of proteasome inhibitors (55). Nevertheless, in our experimental conditions, fragments derived from HER-2 cleavage were not detectable even in the presence of various protease inhibitors. Therefore, we decided to investigate HER-2 cleavage by *in vitro* proteolysis assays. This technique allowed us to observe cleaved fragments, identify two sites

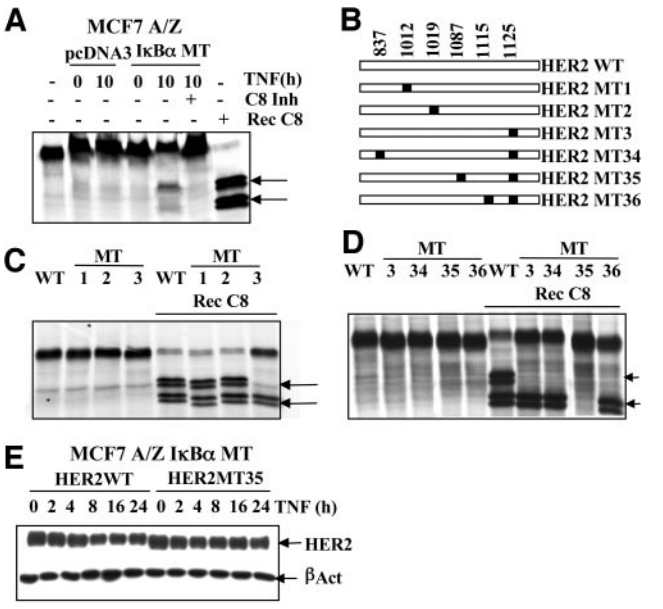

Fig. 9. Identification of the HER-2 cleavage sites. *A*, pcDNA3 and IκBα MT MCF7 A/Z cells were stimulated with TNF-α (100 units/ml) for 10 h or left untreated, and hypotonic extracts were prepared. ^{35}S-labeled *in vitro* translated HER-2 COOH-terminus was incubated at 37°C for 2 h with either recombinant caspase-8 or hypotonic extracts in the presence or absence of caspase-8 inhibitor as indicated. Samples then were separated using SDS-PAGE. Cleaved bands are marked with an arrow. *B*, schematic representation of the *in vitro* mutagenesis performed using the HER-2 COOH-terminus template. White rectangles represent the COOH-terminal part of HER-2. Black squares and numbers indicate the aspartate to alanine substitutions. *C* and *D*, wild-type (*WT*), single-mutant (*MT*; *C*), or double-mutant (*D*) ^{35}S-labeled *in vitro* translated HER-2 C-termini were loaded untreated or were incubated with recombinant caspase-8 for 2 h at 37°C. Samples then were separated using SDS-PAGE. Arrows indicate the cleaved bands. *E*, IκBα MT MCF7 A/Z cells were transfected with expression vectors encoding wild-type HER-2 (*HER-2WT*) or the double-mutant 35 (*HER-2MT35*). Eight h after transfection, cells were stimulated by TNF-α (100 units/ml), and total extracts (10 μg) were analyzed by Western blot analysis using specific anti-HER-2 and antiactin antibodies.

targeted by caspase-8, and generate a mutant resistant to proteolytic cleavage *in vitro* and in cells.

It was shown previously that TNF-α treatment led to a decrease in *HER-2* mRNA synthesis (64) or in protein level expression (65). We did not observe any effect of TNF-α on *HER-2* transcription level. These discrepancies probably rely on differences in cellular models. Nevertheless, for the first time, we report a caspase-dependent cleavage of the HER-2 protein following TNF-α treatment.

HER-2 expression enhances proliferative, metastatic, and prosurvival signals in breast cancer cells and is correlated with a poor prognosis in breast and ovarian adenocarcinomas (39–41). Furthermore, HER-2 expression induces a resistance to cancer therapy, and its inhibition by a specific antibody, trastuzumab, has clinical activity either alone or in combination with chemotherapy in HER-2-expressing breast cancers (45–49). Moreover, it has been demonstrated that HER-2 overexpression can induce resistance to TNF-α stimulation (43) and that trastuzumab can restore the cytotoxic response (50). Our results raise the hypothesis that a caspase-8-mediated HER-2 cleavage following TNF-α stimulation could participate in the subsequent apoptosis. Overexpression of a wild-type HER-2 protein significantly inhibits TNF-α-induced cell death, and survival is further increased following overexpression of an uncleavable HER-2 protein, demonstrating that HER-2 cleavage contributes to apoptosis.

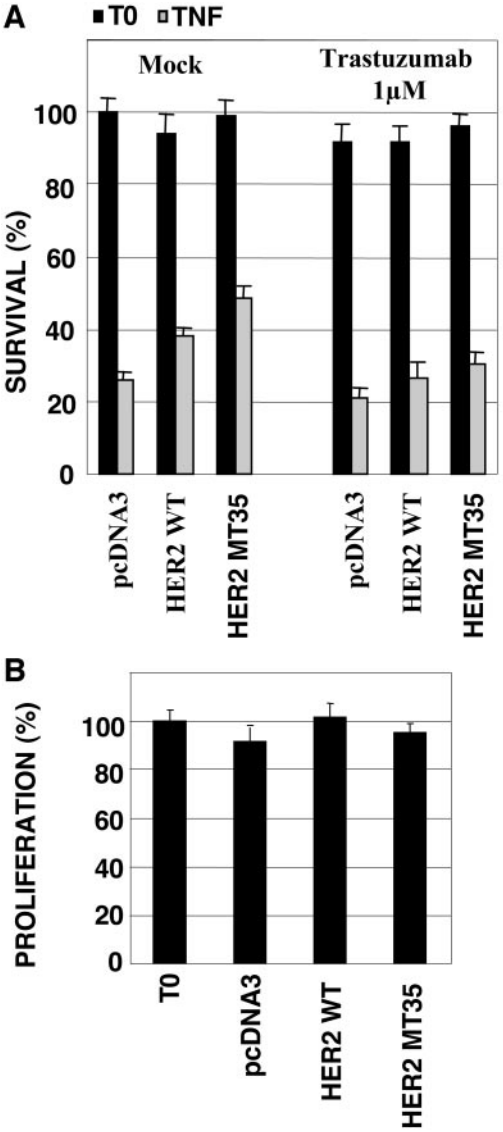

Fig. 10. HER-2 expression and cell survival. *A*, IκBα MT MCF7 A/Z cells were transfected with an empty vector or expression vectors encoding wild-type HER-2 (*HER-2WT*) or the double mutant (*HER-2MT35*). Eight h after transfection, cells were treated with TNF-α for 24 h, and a trypan blue exclusion counting of the living cells was performed. In the same experiment, cells also were pretreated for 24 h with the trastuzumab monoclonal antibody before TNF-α stimulation, as indicated in the figure. The data are representative of three independent experiments and are expressed as percentage of the values observed with control cells. *B*, IκBα MT MCF7 A/Z cells were transfected as in *A* or left untransfected (T0). Twenty-four h after transfection, [H^3]thymidine was added in culture medium for 5 h. [H^3]thymidine incorporation then was evaluated and expressed as percentage of the values observed in the control cells.

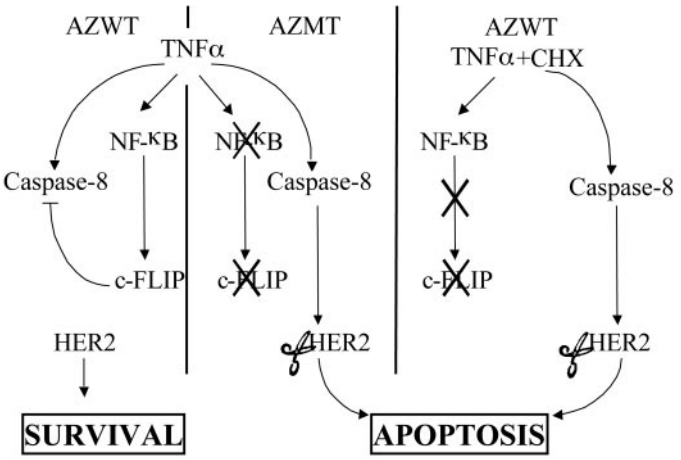

Fig. 11. A new model for proapoptotic and antiapoptotic pathways in response to tumor necrosis factor α (*TNF-α*). In TNF-α-stimulated MCF7 A/Z cells, nuclear factor κB (*NFκB*) activation leads to c-FLIP expression, inhibition of the caspase-8-mediated HER-2 cleavage, and cell survival. In contrast, in IκBα MT and cycloheximide (*CHX*)-pretreated pcDNA3 MCF7 A/Z cells, c-FLIP expression in response to TNF-α is prevented by NFκB or protein synthesis inhibition, and TNF-α stimulation triggers caspase-8 activation, subsequent HER-2 cleavage, and apoptosis.

HER-2 expression inhibits cell death by inducing antiapoptotic pathways, such as Bcl-2 and Bcl-XL up-regulation (66), or by activation of the Akt/NFκB prosurvival cascade (67–69). NFκB is an extensively described antiapoptotic transcription factor whose nuclear DNA binding is potently and rapidly induced by TNF-α in almost all of the cell lines (70). Constitutive NFκB activation has been observed in a wide variety of cancers and is associated with a resistance to apoptosis because many of its target genes code for antiapoptotic molecules (10–25). In our model, TNF-α stimulation leads to NFκB activation and subsequent expression of one of its target genes, *c-FLIP* (14, 54). c-FLIP is a caspase-8 inhibitor and therefore counteracts HER-2 cleavage triggered by TNF-α stimulation (55). This c-FLIP-mediated cleavage inhibition does not occur when NFκB activity is blocked by IκBα MT expression or a chemical inhibitor like BAY 11–7085, demonstrating that it requires the integrity of the NFκB pathway.

Because we demonstrated that HER-2 cleavage might participate in TNF-α-induced apoptosis, our results suggest that NFκB-dependent inhibition of HER-2 cleavage is a novel mechanism for NFκB antiapoptotic role. Interestingly, we also observed a HER-2 cleavage in response to daunorubicin through an NFκB-independent mechanism, suggesting that this cleavage could be a general feature of apoptosis.[3] These results currently are under investigation.

In conclusion, we provide evidence, for the first time, that caspase-8 cleaves the HER-2 oncoprotein in response to TNF-α stimulation, and we propose an original model in which NFκB exerts a new antiapoptotic function through c-FLIP-induced expression and subsequent inhibition of TNF-α-triggered cleavage of the HER-2 survival factor (Fig. 11). Therefore, this antiapoptotic mechanism

[3] Unpublished observations.

underlines the opportunity to evaluate the NFκB activation status and the HER-2 expression level in breast cancer cells and opens new ways to develop combined anticancer therapies for HER-2-overexpressing cancers. Because NFκB inhibitors currently are being evaluated as anticancer therapeutic agents, our results indicate a putative novel activity for these agents. Anti-HER-2 antibodies and NFκB inhibitors possibly may be combined with chemotherapy or biological modifiers for the management of HER-2-overexpressing cancers. It also would be most interesting to determine whether a similar effect could be observed with other receptors belonging to the EGFR family.

ACKNOWLEDGMENTS

We thank Dr. Jacques Piette for the critical reading of the manuscript.

REFERENCES

1. Barbara JA, Smith WB, Gamble JR, et al. Dissociation of TNF-α cytotoxic and proinflammatory activities by p55 receptor- and p75 receptor-selective TNF-α mutants. EMBO J 1994;13:843–50.
2. Lewis M, Tartaglia LA, Lee A, et al. Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific. Proc Natl Acad Sci USA 1991;88:2830–4.
3. Heller RA, Song K, Fan N, Chang DJ. The p70 tumor necrosis factor receptor mediates cytotoxicity. Cell 1992;70:47–56.
4. Loetscher H, Pan YC, Lahm HW, et al. Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor. Cell 1990;61:351–9.
5. Schall TJ, Lewis M, Koller KJ, et al. Molecular cloning and expression of a receptor for human tumor necrosis factor. Cell 1990;61:361–70.
6. Karin M, Lin A. NF-κB at the crossroads of life and death. Nat Immunol 2002;3: 221–7.
7. Sartorius U, Schmitz I, Krammer PH. Molecular mechanisms of death-receptor-mediated apoptosis. Chembiochem 2001;2:20–9.
8. Baud V, Karin M. Signal transduction by tumor necrosis factor and its relatives. Trends Cell Biol 2001;11:372–7.
9. Rath PC, Aggarwal BB. TNF-induced signaling in apoptosis. J Clin Immunol 1999; 19:350–64.
10. Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS Jr. NF-κB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 1998;281:1680–3.
11. Chu ZL, McKinsey TA, Liu L, Gentry JJ, Malim MH, Ballard DW. Suppression of tumor necrosis factor-induced cell death by inhibitor of apoptosis c-IAP2 is under NF-κB control. Proc Natl Acad Sci USA 1997;94:10057–62.
12. Hong SY, Yoon WH, Park JH, Kang SG, Ahn JH, Lee TH. Involvement of two NF-κB binding elements in tumor necrosis factor α-, CD40-, and Epstein-Barr virus latent membrane protein 1-mediated induction of the cellular inhibitor of apoptosis protein 2 gene. J Biol Chem 2000;275:18022–8.
13. Stehlik C, de Martin R, Kumabashiri I, Schmid JA, Binder BR, Lipp J. Nuclear factor (NF)-κB-regulated X-chromosome-linked iap gene expression protects endothelial cells from tumor necrosis factor α-induced apoptosis. J Exp Med 1998;188:211–6.
14. Kreuz S, Siegmund D, Scheurich P, Wajant H. NF-κB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. Mol Cell Biol 2001; 21:3964–73.
15. Grumont RJ, Rourke IJ, Gerondakis S. Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. Genes Dev 1999;13:400–11.
16. Lee HH, Dadgostar H, Cheng Q, Shu J, Cheng G. NF-κB-mediated up-regulation of Bcl-x and Bfl-1/A1 is required for CD40 survival signaling in B lymphocytes. Proc Natl Acad Sci USA 1999;96:9136–41.
17. Zong WX, Edelstein LC, Chen C, Bash J, Gelinas C. The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-κB that blocks TNFα-induced apoptosis. Genes Dev 1999;13:382–7.
18. Khoshnan A, Tindell C, Laux I, Bae D, Bennett B, Nel AE. The NF-κB cascade is important in Bcl-xL expression and for the anti-apoptotic effects of the CD28 receptor in primary human CD4+ lymphocytes. J Immunol 2000;165:1743–54.
19. Chen C, Edelstein LC, Gelinas C. The Rel/NF-κB family directly activates expression of the apoptosis inhibitor Bcl-x(L). Mol Cell Biol 2000;20:2687–95.
20. Krikos A, Laherty CD, Dixit VM. Transcriptional activation of the tumor necrosis factor α-inducible zinc finger protein, A20, is mediated by κB elements. J Biol Chem 1992;267:17971–6.
21. Sarma V, Lin Z, Clark L, et al. Activation of the B-cell surface receptor CD40 induces A20, a novel zinc finger protein that inhibits apoptosis. J Biol Chem 1995;270: 12343–6.
22. Jaattela M, Mouritzen H, Elling F, Bastholm L. A20 zinc finger protein inhibits TNF and IL-1 signaling. J Immunol 1996;156:1166–73.
23. Jones PL, Ping D, Boss JM. Tumor necrosis factor α and interleukin-1β regulate the murine manganese superoxide dismutase gene through a complex intronic enhancer involving C/EBP-β and NF-κB. Mol Cell Biol 1997;17:6970–81.
24. Delhalle S, Deregowski V, Benoit V, Merville MP, Bours V. NF-κB-dependent MnSOD expression protects adenocarcinoma cells from TNF-α-induced apoptosis. Oncogene 2002;21:3917–24.
25. Bernard D, Monte D, Vandenbunder B, Abbadie C. The c-Rel transcription factor can both induce and inhibit apoptosis in the same cells via the upregulation of MnSOD. Oncogene 2002;21:4392–402.
26. Beg AA, Baltimore D. An essential role for NF-κB in preventing TNF-α-induced cell death. Science 1996;274:782–4.
27. Wang CY, Mayo MW, Baldwin AS Jr. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. Science 1996;274:784–7.
28. Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM. Suppression of TNF-α-induced apoptosis by NF-κB. Science 1996;274:787–9.
29. Krammer PH. CD95(APO-1/Fas)-mediated apoptosis: live and let die. Adv Immunol 1999;71:163–210.
30. Coussens L, Yang-Feng TL, Liao YC, et al. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science 1985;230:1132–9.
31. Slamon DJ, Godolphin W, Jones LA, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 1989;244:707–12.
32. Press MF, Pike MC, Chazin VR, et al. Her-2/neu expression in node-negative breast cancer: direct tissue quantitation by computerized image analysis and association of overexpression with increased risk of recurrent disease. Cancer Res 1993;53: 4960–70.
33. Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol 1995; 19:183–232.
34. Tyson FL, Boyer CM, Kaufman R, et al. Expression and amplification of the HER-2/neu (c-erbB-2) protooncogene in epithelial ovarian tumors and cell lines. Am J Obstet Gynecol 1991;165:640–6.
35. Zhang X, Silva E, Gershenson D, Hung MC. Amplification and rearrangement of c-erb B proto-oncogenes in cancer of human female genital tract. Oncogene 1989;4: 985–9.
36. Berchuck A, Kamel A, Whitaker R, et al. Overexpression of HER-2/neu is associated with poor survival in advanced epithelial ovarian cancer. Cancer Res 1990;50:4087–91.
37. Ross JS, Fletcher JA. The HER-2/neu oncogene in breast cancer: prognostic factor, predictive factor, and target for therapy. Stem Cells 1998;16:413–28.
38. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987;235:177–182.
39. Hung MC, Zhang X, Yan DH, et al. Aberrant expression of the c-erbB-2/neu protooncogene in ovarian cancer. Cancer Lett 1992;61:95–103.
40. Ignatoski KM, Maehama T, Markwart SM, Dixon JE, Livant DL, Ethier SP. ERBB-2 overexpression confers PI 3′ kinase-dependent invasion capacity on human mammary epithelial cells. Br J Cancer 2000;82:666–74.
41. Tzahar E, Yarden Y. The ErbB-2/HER2 oncogenic receptor of adenocarcinomas: from orphanhood to multiple stromal ligands. Biochim Biophys Acta 1998;1377: M25–37.
42. Yu D, Jing T, Liu B, et al. Overexpression of ErbB2 blocks Taxol-induced apoptosis by upregulation of p21Cip1, which inhibits p34Cdc2 kinase. Mol Cell 1998;2: 581–91.
43. Hudziak RM, Lewis GD, Shalaby MR, et al. Amplified expression of the HER2/ERBB2 oncogene induces resistance to tumor necrosis factor α in NIH 3T3 cells. Proc Natl Acad Sci USA 1988;85:5102–6.
44. Egeblad M, Jaattela M. Cell death induced by TNF or serum starvation is independent of ErbB receptor signaling in MCF-7 breast carcinoma cells. Int J Cancer 2000;86: 617–25.
45. Sliwkowski MX, Lofgren JA, Lewis GD, Hotaling TE, Fendly BM, Fox JA. Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin). Semin Oncol 1999;26:60–70.
46. Baselga J, Norton L, Albanell J, Kim YM, Mendelsohn J. Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. Cancer Res 1998;58:2825–31.
47. Baselga J, Tripathy D, Mendelsohn J, et al. Phase II study of weekly intravenous trastuzumab (Herceptin) in patients with HER2/neu-overexpressing metastatic breast cancer. Semin Oncol 1999;26:78–83.
48. Pegram MD, Slamon DJ. Combination therapy with trastuzumab (Herceptin) and cisplatin for chemoresistant metastatic breast cancer: evidence for receptor-enhanced chemosensitivity. Semin Oncol 1999;26:89–95.
49. Pegram MD, Lipton A, Hayes DF, et al. Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER2/neu monoclonal antibody plus cisplatin in patients with HER2/neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. J Clin Oncol 1998;16:2659–71.
50. Hudziak RM, Lewis GD, Winget M, Fendly BM, Shepard HM, Ullrich A. p185HER2 monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor. Mol Cell Biol 1989;9:1165–72.
51. Tang G, Yang J, Minemoto Y, Lin A. Blocking caspase-3-mediated proteolysis of IKKβ suppresses TNF-α-induced apoptosis. Mol Cell 2001;8:1005–16.
52. Delhalle S, Deregowski V, Benoit V, Merville MP, Bours V. NF-κB-dependent MnSOD expression protects adenocarcinoma cells from TNF-α-induced apoptosis. Oncogene 2002;21:3917–24.
53. Tikhomirov O, Carpenter G. Caspase-dependent cleavage of ErbB-2 by geldanamycin and staurosporin. J Biol Chem 2001;276:33675–80.
54. Micheau O, Lens S, Gaide O, Alevizopoulos K, Tschopp J. NF-κB signals induce the expression of c-FLIP. Mol Cell Biol 2001;21:5299–305.

55. Krueger A, Baumann S, Krammer PH, Kirchhoff S. FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. Mol Cell Biol 2001;21:8247–54.
56. Wen LP, Fahrni JA, Troie S, Guan JL, Orth K, Rosen GD. Cleavage of focal adhesion kinase by caspases during apoptosis. J Biol Chem 1997;272:26056–61.
57. Kirsch DG, Doseff A, Chau BN, et al. Caspase-3-dependent cleavage of Bcl-2 promotes release of cytochrome c. J Biol Chem 1999;274:21155–61.
58. Nicholson DW. Caspase structure, proteolytic substrates, and function during apoptotic cell death. Cell Death Differ 1999;6:1028–42.
59. Sakahira H, Enari M, Nagata S. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 1998;391:96–9.
60. Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 1998;391:43–50.
61. King P, Goodbourn S. STAT1 is inactivated by a caspase. J Biol Chem 1998;273:8699–704.
62. Widmann C, Gibson S, Johnson GL. Caspase-dependent cleavage of signaling proteins during apoptosis. A turn-off mechanism for anti-apoptotic signals. J Biol Chem 1998;273:7141–7.
63. Barila D, Rufini A, Condo II, et al. Caspase-dependent cleavage of c-Abl contributes to apoptosis. Mol Cell Biol 2003;23:2790–9.
64. Kalthoff H, Roeder C, Gieseking J, Humburg I, Schmiegel W. Inverse regulation of human ERBB2 and epidermal growth factor receptors by tumor necrosis factor α. Proc Natl Acad Sci USA 1993;90:8972–6.
65. Kumar R, Mendelsohn J. Reduced expression of c-erbB2 gene product in human mammary carcinoma SK-BR-3 cells treated with interferon-γ and tumor necrosis factor-α. Anticancer Res 1994;14:1001–8.
66. Kumar R, Mandal M, Lipton A, Harvey H, Thompson CB. Overexpression of HER2 modulates bcl-2, bcl-XL, and tamoxifen-induced apoptosis in human MCF-7 breast cancer cells. Clin Cancer Res 1996;2:1215–9.
67. Zhou BP, Hu MC, Miller SA, et al. HER-2/neu blocks tumor necrosis factor-induced apoptosis via the Akt/NF-κB pathway. J Biol Chem 2000;275:8027–31.
68. Pianetti S, Arsura M, Romieu-Mourez R, Coffey RJ, Sonenshein GE. Her-2/neu overexpression induces NF-κB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of IκB-α that can be inhibited by the tumor suppressor PTEN. Oncogene 2001;20:1287–99.
69. Bhat-Nakshatri P, Sweeney CJ, Nakshatri H. Identification of signal transduction pathways involved in constitutive NF-κB activation in breast cancer cells. Oncogene 2002;21:2066–78.
70. Osborn L, Kunkel S, Nabel GJ. Tumor necrosis factor α and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor κB. Proc Natl Acad Sci USA 1989;86:2336–40.