Original Paper

Tumor Necrosis Factor α Stimulates Her-2 Cleavage by Activated Caspase-8

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Key Words

Caspase-8 • TNF- α • Her-2

Abstract

Background/Aim: Her-2 over-expression has been correlated with a poor prognosis in patients with breast cancer. Now, we explored the effect of TNF- α treatment and/or NF κ B activation on Her-2 expression in MCF-7 breast adenocarcinoma cells. **Methods:** Stably transfected MCF-7 cell lines with pcDNA3.0, IkB α MT, c-FLIP/control shRNA were established by FuGENE with the supplementation of G418 (500 μ g/ml). Western blot and Real-time PCR were applied to assess the expression levels of protein and mRNA of target gene. In addition, caspase-8 activity was evaluated by the incubation with a caspase-8 fluorogenic substrate, Ac-IEPD-AMC using a spectrofluorometer. **Results:** It was uncovered that Her-2 was a new substrate for caspase-8 and that tumor necrosis factor α (TNF- α) stimulation resulted in a caspase-8-dependent Her-2 cleavage in MCF-7 breast adenocarcinoma cells defective for nuclear factor κ B (NF κ B) activation. We demonstrated that the antiapoptotic transcription factor NF κ B counteracted this cleavage through the induction of caspase-8 inhibitor, c-FLIP. **Conclusion:** we propose a novel mechanism in which NF κ B functions as a new antiapoptotic factor by counteracting TNF- α -triggered Her-2 cleavage.

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Introduction

Tumor necrosis factor α (TNF- α) can regulate proapoptotic and antiapoptotic signaling pathways through interaction with two receptors, TNF receptor 1 (TNF-R1) and TNF receptor 2 (TNF-R2) [1]. Following TNF- α triggering, TNF receptor is trimerized, and subsequently,

various signaling proteins were recruited to the receptor cytoplasmic domains [1-3]. Activation of TNF-R2 yields an antiapoptotic and proinflammatory cascade [4], while TNF-R1 trimerization results in apoptosis through the recruitment and activation of caspase-8 as blocked the activation of the anti-apoptotic and proinflammatory transcription factor, nuclear factor κ B (NF κ B) [4-6]. Furthermore, NF κ B target genes, including caspase inhibitors such as c-IAPs, XIAP, and c-FLIP; Bcl-2 family members; and other proteins like A20, counteract TNF- α -induced apoptosis [7, 8].

Human cancers are defined as an upregulated proliferation and a downregulated apoptosis. Among the proteins implicated in this dysregulation, Her-2 functions as a pivotal role in breast cancer cells [9-11]. Her-2 oncogene induces the expression of a transmembrane receptor protein structurally similar to epidermal growth factor receptor (EGFR). It was reported that Her-2 was over-expressed in approximately one-third of primary breast cancer, which was correlated with a poor prognosis and decreased overall or disease-free survival [9-11]. Her-2 over-expression is potential to enhance proliferation, metastasis, and prosurvival signals in breast cancer cell lines and to induce resistance to hormonal therapy [12, 13]. Moreover, anti-Her-2 monoantibody, trastuzumab has clinical activity either alone or combined with chemotherapy in Her-2-positive breast cancers [12]. Because association between Her-2 over-expression and NF κ B currently is elusive, we explored the effect of TNF- α treatment and/or NF κ B activation on Her-2 expression in MCF-7 breast adenocarcinoma cells. Our outcomes showed that TNF- α treatment led to intracellular Her-2 cleavage via a caspase-8-dependent signaling pathway in MCF-7 cells defective for NF κ B activation. Therefore, our results provided the evidence to refer Her-2 as a brand-new caspase-8 substrate and demonstrated that NF κ B can remarkably inhibit Her-2 degradation, that could be a novel mechanism for NF κ B antiapoptotic role.

Materials and Methods

Reagents

Human recombinant TNF- α and actinomycin D were purchased from Sigma (St. Louis, U.S.A). Recombinant caspase-8 and caspase-8 inhibitors were purchased from Calbiochem (La Jolla, CA).

Cell culture and transfection

MCF-7 breast adenocarcinoma cells (generously supplied by Dr. Chen Huang, Medical College of Xi'an Jiao Tong University, Xi'an, Shaanxi Province, P.R. China) were cultured and passaged in RPMI 1640 medium supplemented with 10% stripped fetal bovine serum, 1% L-glutamine (200 mM), penicillin (100 IU), and streptomycin (100 μ g/ml). For the stably transfected cell lines (pcDNA3.0 and I κ B α MT), culture medium was supplemented with G418 (500 μ g/ml, Roche). For DNA transfection, cells were plated at a concentration of 7×10^5 cells per 35-mm-diameter culture dish and transfected 24 h later with FuGENE (Sigma, U.S.A), according to the protocol provided by the manufacturer (Roche, Switzerland).

Protein extraction and Western blot analysis

Whole cell extracts were obtained by resuspending the PBS-washed cellular pellets in 1% SDS. Then, lysates were boiled for 10 min, and protein amounts were quantified with Micro BCA Protein Assay reagent (Pierce, Rockford, IL) using a BSA standard solution curve as the manufacturer's protocol. Protein extracts were separated on SDS-PAGE gels and blotted onto a polyvinylidenedifluoride (PVDF) membrane (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 first antibody, washed with Tris-buffered saline/Tween 20%, and incubated for 1 h with 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).

Fig. 1. Tumor necrosis factor α (TNF- α)-mediated Her-2 degradation in nuclear factor κ B (NF κ B)-defective MCF-7 cells. pcDNA3.0 or I κ B α MT MCF-7 cells were treated with TNF- α (100 units/ml) as indicated. 10 μ g of total protein extract was analyzed by Western blot with a specific anti-Her-2 COOH-terminal antibody. A nonspecific band (NS) was also shown as loading control.

The antibodies were used for Western blot analysis: rabbit polyclonal anti-Her-2 (ab-2428), mouse monoclonal anti-actin (ab-3280), rabbit polyclonal anti-XIAP (ab-21278), rabbit polyclonal anti-c-FLIP (ab-8421), rabbit polyclonal anti-c-IAP-1 (ab-2399), and anti-c-IAP-2 (ab-23423) were obtained from Abcam (U.S.A.).

Real-time quantitative PCR

Total RNA was extracted using RNeasy columns from Qiagen (Valencia, Spain) according to the manufacturer's recommendations. After DNase treatment, RNAs were eluted and quantified using a spectrophotometer. 1.0 μ g of RNA was reverse transcribed using the first-strand cDNA synthesis kit for reverse transcription-PCR (Roche). The quantitative PCR reaction system involved 2.0 μ l of 20 $\times$ diluted cDNAs, 2.0 μ l of 10 $\times$ SYBR Green PCR mix buffer, 1.6 μ l of 25 mM MgCl₂ and 7.0 μ M of each primer. The number of cycles was selected to allow linear amplification of the cDNAs. For quantitative PCR, GAPDH was selected as control. Quantification was performed with the Light Cycler PCR (Eppendorf). 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 MCF-7 cells and I κ B α 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 (Sigma, U.S.A.). Subsequently, the fluorescence of generated cleaved product was assessed using a spectrofluorometer (380 nm and 460 nm). The experimental procedure followed the manufacturer's recommendations.

FLIP antisense experiments

c-FLIP shRNA, 5'-ACTTGTCCCTGCTCCTTGAA-3'; control shRNA, 5'-ATCACGTATCGTCGCTTCTC-3' bearing phosphorothioate linkages were delivered into cells by FuGENE (Roche, U.S.A.) at a final concentration of 10 μ M for 8 h before TNF- α treatment.

Results

TNF- α -induced Her-2 degradation in NF κ B-defective cells

To investigate the effects of TNF- α and NF κ B on Her-2 expression, we used MCF-7 breast adenocarcinoma cells stably transfected with either an empty vector pcDNA3.0 (control cells) or a vector encoding an NF κ B repressor, mutated I κ B α at the sites of serines 32 and 36 (I κ B α MT or MT cells), that inhibited NF κ B activity and in turn led to enhanced apoptotic response to TNF- α . Both cell lines were stimulated with TNF- α for 24 h. Then, Her-2 expression was investigated by Western blot. An obvious decrease in Her-2 expression was detectable in MCF-7 cells with the mutated form of I κ B α (Fig. 1). To further explore the transcriptional influence on decreased Her-2 protein, MCF-7 cells with pcDNA 3.0 or I κ B α MT was stimulated with TNF- α for 24 h. Then, the transcriptional level of Her-2 was tested by quantitative real-time PCR using Her-2-specific primers and GAPDH gene as control. No significant differences

Fig. 2. Her-2 mRNA synthesis and stability. A, pcDNA3.0 or I κ B α MT MCF-7 cells were stimulated by tumor necrosis factor α (TNF- α , 100 units/ml) as indicated. Quantitative real-time PCR was performed using Her-2-specific oligonucleotides and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control. B, control or MT cells were treated with actinomycin D (5 μ g/ml) as indicated. Quantitative real-time PCR was performed as in 2A.

Fig. 3. Her-2 protein stability. I κ B α MT cells were incubated with or without Phenylmethylsulfonyl fluoride (PMSF, 10 μ g/ml) for 1 h before tumor necrosis factor α (TNF- α , 100 units/ml) time course stimulation. 10 μ g of total cellular extract was subjected to anti-Her-2 Western blot analysis.

in Her-2 mRNA level were observed after both cell lines were stimulated by TNF- α (Fig. 2A). Subsequently, control and I κ B α MT MCF-7 cells were preincubated with actinomycin D for 1 h to block RNA neosynthesis before a TNF- α stimulation. The degradation of Her-2 mRNA was identical in both cell lines (Fig. 2B). These outcomes showed that TNF- α -mediated Her-2 downregulation was not the consequence of decreased RNA transcription.

Her-2 cleavage by TNF- α -activated caspase-8 in NF κ B-defective cells

In the light of the outcomes mentioned above, we postulated that downregulated Her-2 in response to TNF- α treatment might be a consequence of protease activation. To test such a hypothesis, we preincubated cells with protease inhibitors and tested their ability to counteract TNF- α -mediated Her-2 decay. Phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor) did not prevent Her-2 decay in response to TNF- α in the cells overexpressing the mutated I κ B α (Fig. 3). Because it was reported that Her-2 may be a caspase substrate, it was of interests to investigate this signaling pathway in our study. It was observed that endogenous caspase-8 activation following TNF- α addition in the I κ B α MT cells, which was perfectly paralleled with Her-2 decay (Figs. 4A and B). Moreover, a specific caspase-8 inhibitor, z-IETD-fmk, was used to block TNF- α -induced Her-2 degradation in MCF-7 cells with I κ B α MT (Fig. 5). Her-2 cleavage presented and was inhibited by the specific caspase-8 inhibitor. Taken together, these data indicated that caspase-8 was responsible for the TNF- α -induced Her-2 degradation in the NF κ B-deficient cells.

Fig. 4. Caspase-8 cleavage and activation in response to tumor necrosis factor α (TNF- α). A, pcDNA3.0 MCF-7 cells and I κ B α MT MCF-7 cells were stimulated with TNF- α . Proteolysis of caspase-8 in total cellular extract (10 μ g) was assessed by Western blot analysis. Caspase-8 proform was indicated by an arrow, and asterisks showed the cleaved fragments. B, pcDNA3.0 and I κ B α MT MCF-7 cells were stimulated with TNF- α as indicated. Cellular extracts were incubated with a fluorogenic caspase-8 substrate. The fluorescence of generated cleaved product was assessed with a spectrofluorometer.

Fig. 5. Her-2 cleavage by caspase-8. I κ B α MT MCF-7 cells were stimulated with tumor necrosis factor α (TNF- α , 100 units/ml) as indicated in the absence (left) or presence (right) of the caspase-8 inhibitor (20 μ M). Western blot analysis was carried out with 10- μ g total extract. Proteins were tested with anti-Her-2, and anti-actin antibodies as indicated.

Inhibition of caspase-8-mediated Her-2 cleavage by NF κ B-regulated c-FLIP

Our report showed that caspase-8-induced Her-2 decay only occurred in the MCF-7 cells with the inhibition of NF κ B activation or synthesis. Furthermore, we identified NF κ B-regulated genes in control and MT cells treated with TNF- α after total cellular lysates were analyzed by Western blot analysis revealed with the specific antibodies. Accordingly, c-IAP-1, c-IAP-2, and XIAP were identical in the TNF- α -treated control or I κ B α MT MCF-7 cells. However, c-FLIP-L was increased remarkably in the TNF- α -stimulated control cells as compared with MT cells (Fig. 6). Because c-FLIP-L is a well-known caspase-8 inhibitor and an NF κ B target gene [14, 15]. Therefore, we further investigated whether modulation of c-FLIP-L affected Her-2 cleavage. Specific shRNA targeting c-flip mRNA efficiently attenuated c-FLIP-L expression in MCF-7 control cells, whereas control shRNA did not (Fig. 7). The inhibition of c-FLIP-L expression in control MCF-7 cells was associated with TNF- α -induced Her-2 degradation (Fig. 7). In addition, the opposite experiment was performed by re-introducing c-FLIP-L exogenous transient expression in MT cells. Ectopic c-FLIP-L expression partially inhibited TNF- α -mediated Her-2 cleavage (Fig. 8).

Fig. 6. Expression of nuclear factor κ B (NF κ B)-regulated genes. pcDNA3.0 and I κ B α MT MCF-7 cells were stimulated with tumor necrosis factor α (TNF- α , 100 units/ml) for 24 h. Total extracts (10 μ g) were analyzed by Western blot with anti-c-IAP-1, anti-c-IAP-2, anti-XIAP, anti-c-FLIP-L, and anti-actin antibodies, respectively.

Fig. 7. Increased Her-2 cleavage by inhibition of c-FLIP. pcDNA3.0 MCF-7 cells were transfected with c-FLIP-L shRNA or control shRNA. After stable transfection, cells were untreated or were stimulated with tumor necrosis factor α (TNF- α , 100 units/ml) for 24 h. Total extracts (10 μ g) were evaluated by Western blot with anti-Her-2, anti-c-FLIP-L, and anti-actin antibodies, respectively.

Fig. 8. Inhibition of Her-2 cleavage by c-FLIP expression. I κ B α MT MCF-7 cells were transfected with pcDNA3.0 or c-FLIP-L expression vectors. After stable transfection, cells were stimulated with TNF- α (100 units/ml) as indicated. Western blot analysis was performed with total extracts (10 μ g) using anti-Her-2, anti-c-FLIP-L, and anti-actin antibodies.

Discussion

The apoptosis depends on the activity of caspases family, a growing family of aspartyl-specific cysteine proteases that are essential for the execution of apoptotic cascade by the cleavage of broad-spectrum substrates, including DNA repair molecule like PARP, focal

adhesion kinase, oncoproteins such as Bcl-2, and signaling proteins [16-19]. Because caspases are responsible for the cell apoptotic cascade, identification of their cellular substrates is essential and provides new insights into downstream events involved in apoptosis signaling. Our outcomes clearly defined Her-2 as a caspase-8 substrate. It was of interest that Her-2 molecule cleavage by activated caspase-8 occurred after TNF- α treatment, implying that this cleavage might be involved in proapoptotic signaling progression.

It was previously demonstrated that TNF- α treatment led to a decrease in Her-2 mRNA synthesis and expression level of Her-2 protein [20, 21]. We did not observe any effects of TNF- α on Her-2 transcription level. It was likely that these discrepancies resulted from the differences in the cell lines. However, it was the first time that we reported a caspase-dependent cleavage of Her-2 following TNF- α treatment.

In breast cancer cells, it was proven that oncoprotein Her-2 was able to promote proliferative, metastatic, and prosurvival events and correlated with a poor prognosis [9, 22]. Furthermore, Her-2 expression induced a resistance to chemoradiotherapy, and its inhibition by a specific monoclonal antibody, trastuzumab, yields the predominant clinical activity either alone or in combination with chemotherapy in the Her-2-positive breast cancers [23-26]. Moreover, it has been uncovered that Her-2 over-expression could induce resistance to TNF- α stimulation and that trastuzumab can restore the cytotoxic response [20, 21]. Our results raised a hypothesis that caspase-8-mediated Her-2 cleavage following TNF- α treatment could take part in the cell apoptosis. Wild-type Her-2 over-expression by the transfection of the plasmid bearing wild-type Her-2 is potential to significantly inhibit TNF- α -induced cell death, and a survival disadvantage was not displayed following the over-expression of an uncleavable Her-2, demonstrating that Her-2 cleavage leads to apoptosis.

Her-2-related signalings counteract cell apoptosis by inducing antiapoptotic pathways, such as Bcl-2 and Bcl-XL upregulation or by activating the Akt/NF κ B prosurvival cascade [27-29]. NF κ B is an extensively defined antiapoptotic factor, whose nuclear DNA binding is potently and rapidly induced by TNF- α treatment in almost all of the cancer cell lines [30, 31]. Constitutive NF κ B activation has been observed in a wide variety of cancers and associated with a resistance to apoptosis owing to its target genes encoding for antiapoptotic molecules [30, 31]. In our study, TNF- α stimulation led to NF κ B activation, and subsequent expression of one of its target genes, c-FLIP. c-FLIP is extensively defined as a caspase-8 inhibitor and therefore counteracts Her-2 cleavage triggered by TNF- α stimulation. c-FLIP-mediated cleavage inhibition did not occur when NF κ B activity was inhibited by I κ B α MT expression, demonstrating that it required the integrity of the NF κ B pathway. We demonstrated that Her-2 cleavage might participate in TNF- α -induced apoptosis, indicating that NF κ B-dependent blockage of Her-2 cleavage was a novel mechanism for NF κ B antiapoptotic role.

In conclusion, we provide evidence, for the first time, that caspase-8 cleaves Her-2 protein in response to TNF- α stimulation, and we propose an original model in which NF κ B exerts a new antiapoptotic function through c-FLIP-induced expression and subsequent inhibition of TNF- α -triggered cleavage of Her-2. Therefore, this antiapoptotic mechanism underlines the opportunity to evaluate the NF κ B activation status and the Her-2 expression level in breast cancer cells and opens new insights to develop combined anticancer therapies for Her-2-overexpressing breast cancers. Because NF κ B inhibitors currently are being evaluated as anticancer therapeutic agents, our results indicate a putative novel activity for these agents. Antibodies against Her-2 and NF κ B inhibitors may be combined with chemotherapy or biological modifiers for the management of Her-2-overexpressing cancers.

Grant Support

Supported by national natural science foundation of China (NNSFC, No.30740022 and 30951128).

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