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Inhibition of tissue transglutaminase sensitizes TRAIL-resistant lung cancer cells through upregulation of death receptor 5

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ABSTRACT

Tissue transglutaminase (TG2) is implicated in cellular processes such as apoptosis and cell migration. Its acyl transferase activity cross-links certain proteins, among them transcription factors were described. We show here that the TG2 inhibitor KCC009 reversed resistance to tumor necrosis factor-related apoptosis-inducing factor (TRAIL) in lung cancer cells. Sensitization required upregulation of death receptor 5 (DR5) but not of death receptor 4. Upregulation of DR5 involved the first intron of the DR5 gene albeit it was independent from p53 and nuclear factor kappa B. In conclusion, inhibition of tissue transglutaminase provides an interesting strategy for sensitization to TRAIL-induced apoptosis in p53-deficient lung cancer cells.
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1. Introduction

Lung cancer is the leading cause of cancer death in the United States among both, men and women. The projected number of new lung cancer cases in 2008 in the United States was 215 020, accounting for 15% of all new cancer cases and for 29% of all cancer deaths. In fact, more people die each year from lung cancer than from breast, colorectal, prostate, and ovarian malignancies combined [1].

A potential new anticancer drug provides the cytokine tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL). TRAIL is closely related to TNF-α and FasL, members of the tumor necrosis factor family. TRAIL induces apoptosis via interacting with death receptor 4 (DR4, TRAIL-R1) and death receptor 5 (DR5, TRAIL-R2) leading to the formation of the death-inducing signaling complex (DISC) with subsequent binding of caspase-8 (FLICE). Recruitment of caspase-8 to the DISC activates its proteolytic properties, which initiates a cascade of protease such as caspase-3, promoting the cleavage of death substrates and finally resulting in apoptosis [2]. Different studies have shown that TRAIL induce apoptosis only in tumor but not in normal cells [3,4] suggesting that TRAIL can be used as a new cancer-selective treatment option. One problem for the use of TRAIL as anticancer agent is provided by the fact that about half of tumor cells are resistant to TRAIL-induced apoptosis. Consequently, there is a need for strategies to sensitize cancer cells but not normal cells to TRAIL-mediated cell death.

In the present study we show that tissue transglutaminase (TG2, tTG) might provide an interesting target how to sensitize lung cancer cells to TRAIL-induced apoptosis. TG2 belongs to a family of transglutaminase proteins that cross-links and thereby stabilizes proteins by a Ca^{2+}-dependent formation of amide bounds between glutamine side chains and e-amino groups of lysine residues. In addition to the acyl transferase activity, TG2 has a GTP hydrolyzing activity which is Ca^{2+}-independent [5,6]. Expression of TG2 was shown to be elevated in various forms of cancer.

Abbreviations: TG2, tissue transglutaminase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DR4 (DR5), death receptor 4 (5); NF-κB, nuclear factor kappa B; siRNA, small interfering RNA

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Moreover, downregulation of TG2 expression level or inhibition of TG2 enzymatic activity reversed chemoresistance in cancer cells [7,8].

2. Materials and methods

2.1. Reagents and cells

Soluble, non-trimerized TRAIL was kindly provided by Genentech (South San Francisco, CA). KCC009 was a kind gift of Alvine Pharmaceuticals, Inc. (Palo Alto, CA). The human lung cancer cell lines A549, Calu1 and H1299 (American Type Culture Collection) were maintained as described before [9].

2.2. Apoptosis assays

Cell death was evaluated by the assessment of propidium iodide uptake as described before [9]. Caspase-3 and caspase-9 activities were determined essentially as described previously [10] using DEVD-amc and LEHD-afc as substrates.

2.3. Immunoblot analysis

Fifty micrograms of protein were separated by polyacrylamide gel electrophoresis. The following antibodies were used: PARP (clone C-2-10, Alexis), cleaved lamin A (Cell Signaling Technology, Inc.) transglutaminase II (clone CUB 7402, Lab Vision Corp.) and α-tubulin (Sigma).

2.4. Determination of TRAIL receptor expression

Protein expression of DR4 and DR5 were determined as described before [9]. TRAIl receptor antibodies clones DR4-M271 and DR5-M413 were kindly supplied by Immunex Corp. (Seattle, WA)

2.5. Quantitative real-time RT-PCR

Total RNA was isolated using the GenElute™ kit (Sigma) and cDNA was synthesized. Assay-on-Demand probes for the TaqMan real-time PCR system for DR4 and DR5 and the internal housekeeping control-gene, *GAPDH*, were obtained from Applied Biosystems.

2.6. Transfection with siRNA

Cells were transfected with 1 μg small interfering RNA (siRNA) (Qiagen) by using 2 μl Dharmafect™ reagent (Dharmacon). All siRNAs were described before [7,11] 24 h after transfection, cells were stimulated for 48 h with 500 μM KCC009 and/or 100 ng/ml TRAIL.

2.7. Transfection with reporter plasmids and luciferase assay

Luciferase reporter plasmids (for details see [12–14]) were transfected using DharmafectDUO reagent (Dharmacon). Eight hours after transfection cells were treated with 500 μM KCC009 for 40 h before harvesting for luciferase assay. Firefly luciferase and Renilla luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega).

2.8. Statistical analysis

If not stated otherwise data were subjected to one- or two-way ANOVA using GraphPad Prism 4.03. Differences between experimental groups were determined by Bonferroni post-hoc test. Differences were considered statistically significant at P values <0.05.

3. Results

3.1. Inhibition of TG2 sensitizes lung cancer cells to TRAIL-induced apoptosis

We first analysed whether lung cancer cells can be sensitized to TRAIL-induced apoptosis by pharmacological inhibition of TG2 activity. We used KCC009 which was demonstrated to be a highly specific and irreversible inhibitor of TG2 acyl transferase activity [15]. Initial experiments showed cytotoxicity of KCC009 alone in concentrations exceeding 500 μM (data not shown). Therefore, 500 μM of KCC009 was used for further experiments. Although treatment of the three TRAIL-resistant lung cancer cell lines (Calu1, H1299 and A549) with KCC009 alone for 48 h was not toxic, the combination of KCC009 and TRAIL induced considerable cell death suggesting that TG2 acyl transferase activity served as an inhibitor of TRAIL-induced apoptosis (Fig. 1). To prove whether this effect was in fact related to TG2, we abolished the expression level of TG2 using TG2 specific siRNA followed by treatment with TRAIL.

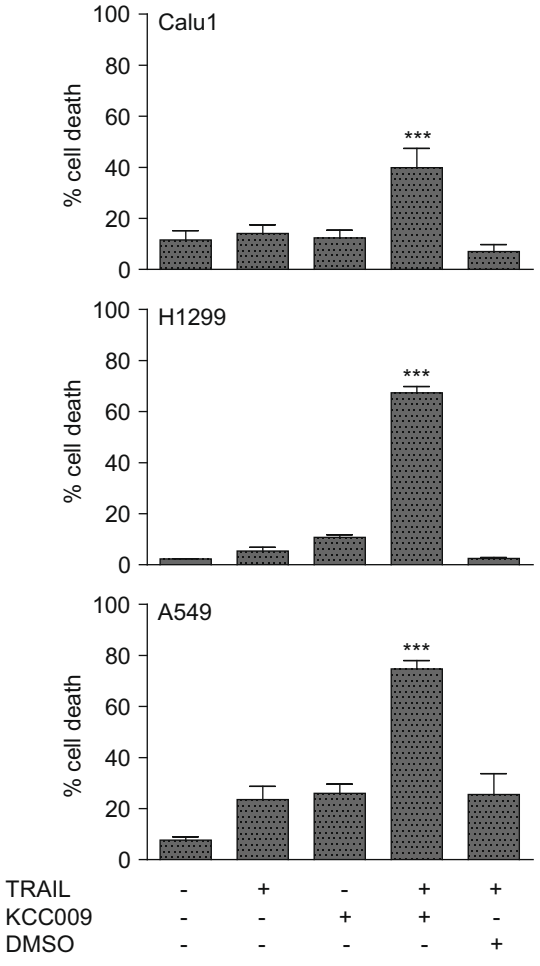

Fig. 1. Cell death in lung cancer cells induced by combined treatment with TRAIL and the TG2 inhibitor KCC009. Different lung cancer cell lines were treated with 100 ng/ml TRAIL and/or 500 μM KCC009 for 48 h. Cell death was assessed by PI staining followed by flow cytometry. Data represent the mean of at least three independent experiments in duplicates ± S.D. ***P < 0.001 vs. TRAIL.

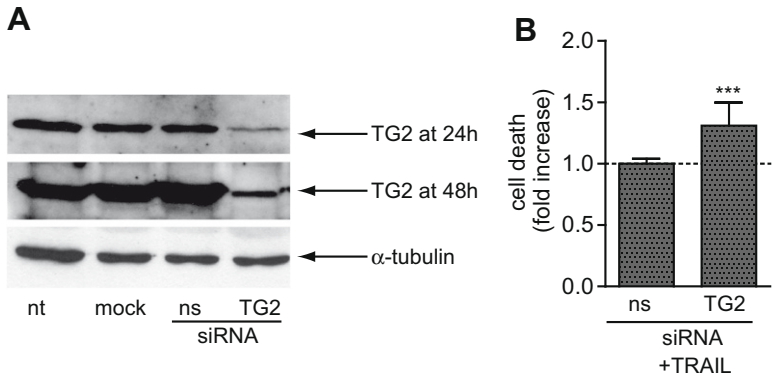

Fig. 2. Silencing of TG2 by siRNA lead to a significant increase in TRAIL-mediated cell death. Calu1 cells were transfected with TG2 specific siRNA or non-silencing control siRNA. (A) siRNA-mediated knockdown of TG2 was confirmed by Western blot analysis 24 or 48 h after transfection. (B) Transfected cells were treated with 100 ng/ml TRAIL; and cell death was assessed 48 h after stimulation by PI staining followed by flow cytometry. Mean ± S.D. of four independent experiments in duplicates. ***$P < 0.001$, *t*-test.

Subsequently, cells with reduced expression of TG2 were rendered more susceptible to TRAIL-induced apoptosis (Fig. 2A and B).

3.2. Cell death induced by combined treatment with KCC009 and TRAIL reveals typical hallmarks of apoptosis

We further analysed apoptosis-related events induced by the combination of the TG2 inhibitor KCC009 and TRAIL. While KCC009 and TRAIL alone were not able to trigger caspase-3 activity, the combination of both substances induced remarkable caspase-3 activation (Fig. 3A). Similar results were demonstrated for caspase-9 (Fig. 3B) suggesting an involvement of the mitochondrial type II apoptotic pathway. In addition, cleavage of the so called death substrates lamin A and PARP were found in cells treated with the combination of KCC009 and TRAIL but not in cells treated with either agent alone (Fig. 3C).

3.3. KCC009 increases cell surface expression and mRNA of DR5, but not of DR4

Next we asked what molecular mechanism might be responsible for KCC009-mediated sensitization to TRAIL-induced apoptosis. Therefore, we measured changes in DR4 and DR5 expression after treatment with KCC009. For DR4, no changes in expression neither at the protein nor at the mRNA level were determined. In contrast to DR4, cell surface expression of DR5 was markedly increased in cells treated with KCC009. This effect was due to an augmentation of DR5 transcription demonstrated by higher mRNA level (Fig. 4A and B). However, despite lung cancer cells were also sensitized to TRAIL-induced apoptosis by siRNA-mediated knockdown of TG2, we were not able to observe increased DR5 expression in these cells (data not shown).

3.4. KCC009-mediated sensitization to TRAIL-induced apoptosis depends on upregulation of DR5

Given higher expression levels of DR5 in KCC009 treated lung cancer cells; we next determined whether this effect was essential for sensitization to TRAIL-induced apoptosis. Therefore we blocked receptor upregulation using siRNA against DR4 and DR5. Subsequently, cells were treated with KCC009 and TRAIL followed by the measurement of cell death. As expected, inhibition of DR4 upregulation demonstrated no influence on cell death induced by the combination of KCC009 and TRAIL. Instead, blockage of DR5 upregulation significantly diminished the extent of apoptosis proposing that upregulation of DR5 plays an essential role in KCC009-mediated sensitization to TRAIL-induced apoptosis (Fig. 5).

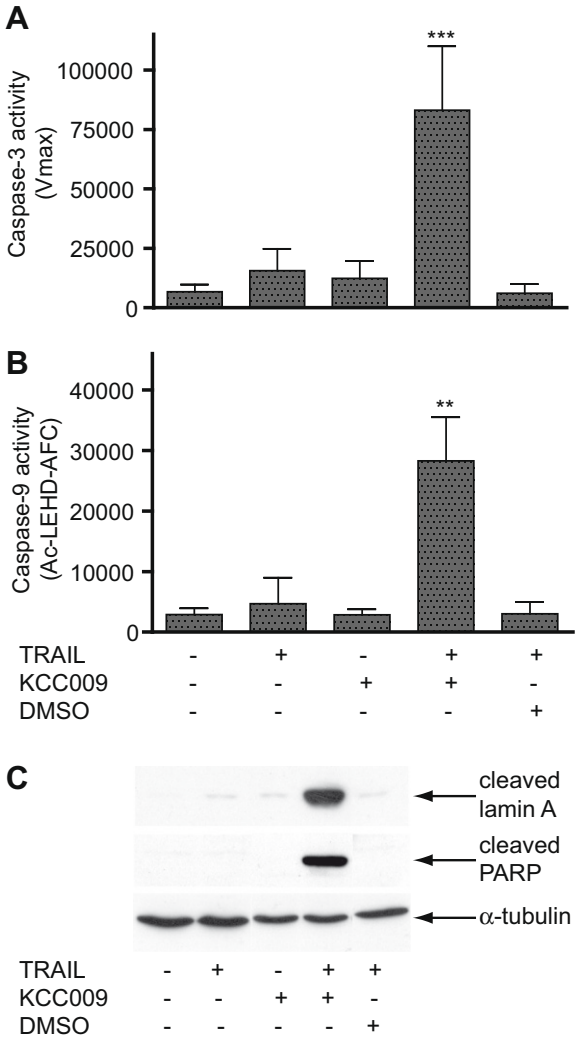

Fig. 3. Activation of caspases in lung cancer cells treated with the combination of TRAIL and KCC009. Calu1 cells were treated for 24 h with TRAIL in the presence or absence of KCC009 and subsequently harvested in lysis buffer. Caspase-3 and -9 activities were measured by determination of (A) hydrolysis of DEVD-amc and (B) cleavage of LEHD-afc, respectively. Data of three independent experiments are expressed as mean ± S.D. **$P < 0.01$ and ***$P < 0.001$ vs. TRAIL. (C) Cleavage of the caspase substrates lamin A and PARP were determined by Western blot analysis.

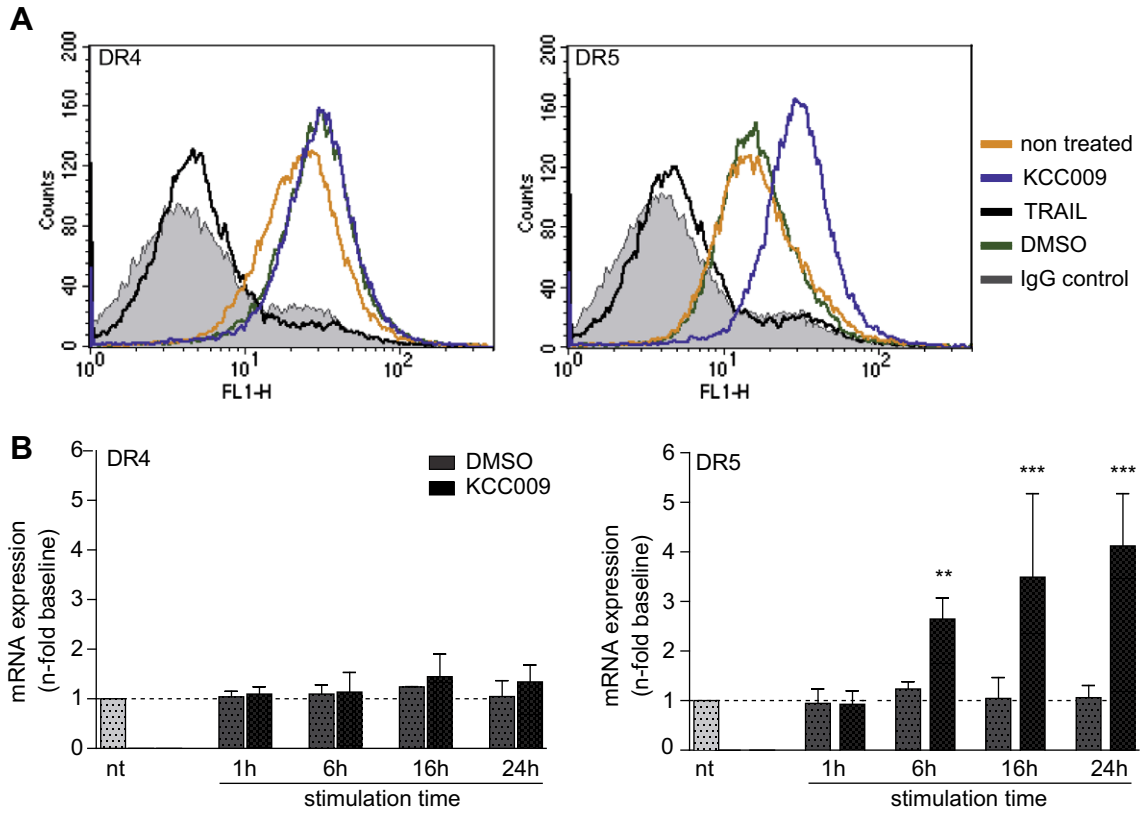

Fig. 4. KCC009 increases expression level of DR5 but not of DR4. (A) Calu1 cells were treated for 24 h with 500 μM KCC009, immunostained and then analyzed for TRAIL receptor expression. Representative data of three independent experiments. (B) Cells were treated with 500 μM KCC009 for the indicated time points; DR4 and DR5 mRNA expression was detected by quantitative RT-PCR. Mean ± S.D. of three independent experiments in duplicates. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. non-treated (nt) cells.

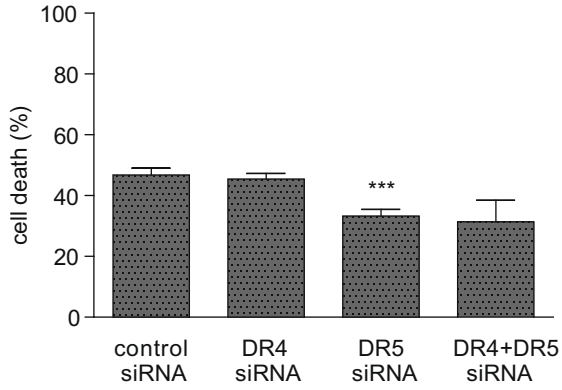

Fig. 5. Elevated DR5 expression accounts for TRAIL-sensitizing effects in lung cancer cells. KCC009-mediated upregulation of DR5 was selectively blocked using siRNA followed by stimulation with TRAIL and KCC009 for 48 h. Mean ± S.D. of three independent experiments in duplicates. $^{***}P < 0.001$ vs. control siRNA.

3.5. KCC009-mediated upregulation of DR5 requires the first intron of the DR5 genomic promoter region

To further evaluate the mechanism how KCC009 upregulates the expression of DR5 we expressed luciferase reporter plasmids with deletion mutants of the DR5 promoter as well as constructs containing the full length promoter plus part of the first intronic region of the DR5 gene. Construction of these plasmids has been described before [12–14]. Surprisingly, none of the plasmids containing the full length promoter region (pDR5 PF) as well as 5′-deletions mutants of this construct demonstrated increased luciferase activity after stimulation with KCC009. Only the two plasmids containing the full length DR5 promoter region and parts of the first intron (pDR5/MluI and pDR5/mtNF-κB) showed an impressive activation of luciferase activity (Fig. 6). Since the only difference between pDR5/MluI and pDR5/mtNF-κB is a mutated

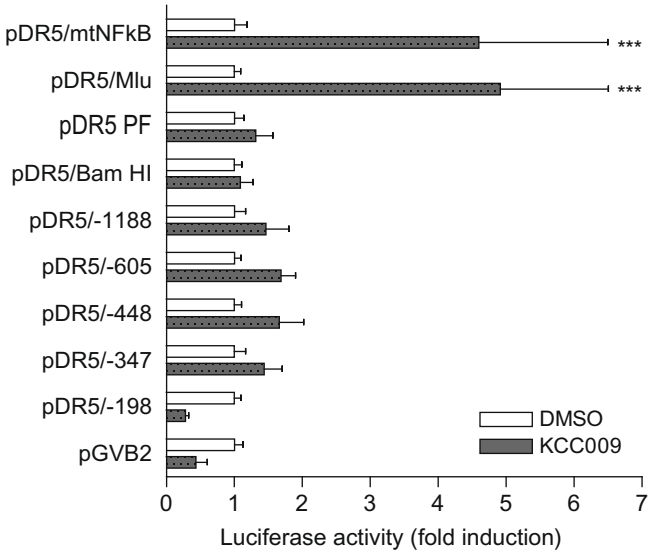

Fig. 6. KCC009-mediated activation of DR5 genomic promoter region. H1299 cells were transfected with luciferase reporter plasmids containing 5′-deletion mutants of human DR5 promoter as well as constructs including the DR5 promoter plus the first intronic region. After 8 h cells were washed and treated with solvent or 500 μM KCC009 for 40 h before harvesting for luciferase assay.

nuclear factor kappa B (NF-κB) binding site within the first intron it suggests that NF-κB does not critically contribute to the enhanced expression of DR5 induced by KCC009.

4. Discussion

The data presented here suggest that application of the TG2 inhibitor KCC009 in combination with TRAIL provides a new strategy to sensitize lung cancer cells to TRAIL-induced apoptosis. The pivotal question was on how inhibition of TG2 activity reverses resistance to TRAIL. In glioblastoma the involvement of protein kinase B (Akt) in KCC009-mediated reversal of chemoresistance was demonstrated [16]. However, in ovarian cancer protein kinase B was excluded to be responsible for KCC009-induced sensitization to chemotherapy; instead NF-κB was shown to be involved [17]. Our data obtained in lung cancer cells propose a new mechanism how KCC009 reverses resistance to apoptosis. It is known that TG2 catalyzes cross-linking of certain intracellular proteins. Hence it might conceivable that in non-treated lung cancer cells transcriptions factors responsible for regulation of DR5 expression are inactive through TG2-mediated cross-linking. In turn, treatment of lung cancer cells with the TG2 inhibitor KCC009 prevented cross-linking and subsequently, DR5 transcription was activated. TG2-initiated cross-linking and subsequent inactivation of transcription factors has been shown before for Sp1 in liver cells [18].

Which transcription factors are involved in KCC009-mediated upregulation of DR5 remains unsolved yet. Our data demonstrated the requirement for the first intronic region of the DR5 gene. The need of the first intron and their p53 and NF-κB binding sites in DR5 upregulation was shown before for the TRAIL-sensitizing agents etoposide and the proteasome inhibitor MG132 [13,19]. In our experimental setup mutation in the NF-κB binding site did not abrogate KCC009-induced promoter activity suggesting that NF-κB is not critically involved DR5 upregulation. Furthermore, we were able to exclude p53 to play a role in DR5 upregulation since H1299 cells are null-mutants of p53 [20] but were sensitized by KCC009 nonetheless. Of note, in H1299 cells the p53-dependent proteasome inhibitor MG132 was unable to induce DR5 expression and consequently did not sensitize to TRAIL-induced apoptosis [19]. Since lung cancer cells are most frequently mutated in p53 [21], TRAIL-sensitizing agents acting independently from p53 as shown for KCC009 might provide an important advantage for this type of cancer.

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