Delineation of apoptotic genes for synergistic apoptosis of lexatumumab and anthracyclines in human renal cell carcinoma cells by polymerase chain reaction array

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Lexatumumab, a human agonistic monoclonal antibody against tumor necrosis factor (TNF)-related apoptosis-inducing ligand receptor-2 (TRAIL-R2), is a promising molecular-targeted therapeutic agent. Our past study indicated that low concentrations of doxorubicin sensitized renal cell carcinoma (RCC) cells to lexatumumab-mediated apoptosis. The present study was designed to examine the cellular and molecular effects of lexatumumab and anthracyclines in RCC cells. The treatment of human RCC cells with lexatumumab in combination with anthracyclines, epirubicin, and pirarubicin had a synergistic cytotoxicity. A marked synergistic apoptosis was induced by lexatumumab in combination with epirubicin or pirarubicin. Epirubicin and pirarubicin significantly increased the TRAIL-R2 expression at both the mRNA and the protein levels. The combination-induced cytotoxicity was significantly suppressed by the human recombinant DR5:Fc chimeric protein. To further explore the molecular mechanisms in this synergistic cytotoxicity with lexatumumab and anthracyclines, the changes in 84 apoptosis-related genes were evaluated by a quantitative polymerase chain reaction (PCR) array. Among these genes, 18 (*CD40LG*, *FASLG*, *LTA*, *TNSF7*, *FAS*, *BAG3*, *BAK1*, *BAX*, *BID*, *BIK*, *BCL10*, *caspase-1*, *caspase-5*, *caspase-6*, *caspase-10*, *TNF receptor-associated factor 1*, *PYCARD*, and *CIDEA*) were significantly upregulated and eight (*TNF receptor-associated factor 4*, *TNFRSF11B*, *TNF*, *BCL2*, *BCL2L1*, *BNIP3L*, *caspase-9*, and *DAPK1*) were downregulated at mRNA levels in RCC cells cotreated with lexatumumab and epirubicin. Furthermore, the upregulation of mRNA levels of *PYCARD* and *CIDEA* was confirmed using real-time reverse transcriptase-PCR analysis. The present study demonstrates that anthracylines sensitize RCC cells to lexatumumab-mediated apoptosis by inducing TRAIL-R2 expression, and the utility of PCR array to elucidate the mechanism of synergistic apoptosis. *Anti-Cancer Drugs* 23:445–454

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Introduction

Renal cell carcinoma (RCC) is the tenth leading cause of cancer mortality in Western countries, is resistant to conventional cancer treatments, and its development of distant metastases is high [1,2]. Although molecular-targeted drugs, such as sorafenib, sunitinib, or bevacizumab, have significantly increased survival for patients with advanced RCC, the response induced by these drugs is transient [3,4]. Development of novel and effective therapeutic strategies for metastatic RCC, therefore, is urgently needed.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is potentially an effective anticancer agent, because it selectively induces apoptosis in a variety of tumor cells [5,6]. TRAIL triggers apoptosis by binding to two death receptors: TRAIL-R1 and TRAIL-R2 [7–9]. The activation of these death receptors results in a signal transduction cascade that initiates both intrinsic and extrinsic apoptotic pathways [10]. TRAIL also binds to two other receptors, TRAIL-R3 and TRAIL-R4, which lack a functional cytoplasmic death domain (DD), and to a secreted TNF receptor homolog osteoprotegerin [7,11]. The last three receptors have been proposed to inhibit TRAIL-induced apoptosis. Thus, development of monoclonal agonistic antibodies (mAbs) to TRAIL-R1 and TRAIL-R2 may help avoid competitive interaction with decoy receptors [12].

Over the past 10 years, numerous mAbs to human TRAIL-R1 or TRAIL-R2 have been generated with antitumor activities *in vitro* and *in vivo* [13,14]. These agonistic antibodies work by activating TRAIL-mediated apoptotic pathways in a manner similar to TRAIL [15]. Mapatumumab (HGS-ETR1) and lexatumumab (HGS-STR2) are human agonistic mAbs specific to TRAIL-R1 and TRAIL-R2, respectively, and have been shown to reduce the viability of multiple types of tumor cells *in vitro* and inhibited tumor growth *in vivo* [16,17].

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Many studies, including ours, demonstrated that doxorubicin sensitized a variety of human cancer cells to TRAIL-R2-mediated apoptosis through the induction of TRAIL-R2 [15,18]. However, doxorubicin has been known to have serious cardiac toxicities, whereas its derivatives have fewer cardiac toxicities. From clinical viewpoints, therefore, it is imperative to clarify whether doxorubicin derivatives also sensitize TRAIL-R2-mediated apoptosis against RCC, which is highly resistant to systemic chemotherapy.

In this study, we investigated whether the cytotoxic effect of lexatumumab would be enhanced in combination with anthracyclines epirubicin or pirarubicin. Furthermore, we explored the molecular mechanisms that may be involved in the reversal of drug resistance.

Methods

Renal cell carcinoma cells

The human RCC ACHN cell line was purchased from the American Type Culture Collection (Rockville, Maryland, USA). The cells were cultured in Roswell Park Memorial Institute-1640 supplemented with 25 mmol/l of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal bovine serum at 37°C in a humidified 5% CO_2 atmosphere.

Primary RCC cells were separated from surgical specimens of three patients with untreated RCC, as described previously [19]. These patients had been diagnosed as having RCC of the clear-cell subtype by histological examination. Pathological stage and grade were consistent with 2000 WHO criteria as follows: patient 1, $T_1N_0M_0$, grade 1; patient 2, $T_1N_2M_0$, grade 2; and patient 3, $T_3N_0M_1$, grade 2.

Reagents

Lexatumumab was kindly provided by Human Genome Sciences (Rockville, Maryland, USA). Epirubicin and pirarubicin were obtained from Meiji Pharmaceutical and Kyowa Hakkou (Tokyo, Japan), respectively.

Cytotoxicity assay

A 100-μl suspension of 2×10^4 cells was added to each well of 96-well flat-bottom microtiter plates, and cultured at 37°C in a humidified 5% CO_2 atmosphere for 24 h. A 100-μl drug solution was distributed in the 96-well plates and each plate was incubated for an additional 24 h. Cytotoxicity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, as described previously [19,20].

Apoptosis assays

Apoptosis was determined in two ways. After incubation with lexatumumab and/or anthracyclines for 12 h or 24 h, floating and adherent cells were harvested. DNA was extracted from the prepared cells using the Apoptosis Ladder Detection Kit (MBL, Nagoya, Japan), as described previously [21]. Extracted DNA samples were resolved by electrophoresis on a 2% agarose gel and stained with ethidium bromide. DNA fragmentation was quantitatively evaluated using the Cell Death Detection enzyme-linked immunosorbent assay (ELISA) Kit (Roche, Penzberg, Germany) according to the manufacturer's instructions.

Flow cytometric analysis of tumor necrosis factor-related apoptosis-inducing ligand receptor-2

Cell-surface expression of TRAIL-R2 was determined using flow cytometry with EPICS XL [15]. In brief, RCC cells were seeded in 60-mm dishes at 5×10^5 cells per dish and cultured for 24 h. Cells were then treated with 0.1–10 μg/ml of epirubicin or pirarubicin for 6 – 24 h. After treatment, cells were harvested from the substrate using 0.05% trypsin and 0.02% EDTA, and washed twice in phosphate-buffered saline containing 0.2% fetal bovine serum and 0.01% NaN_3. The number of cells was counted, and 2×10^5 cells were incubated with PE-conjugated anti-TRAIL-R2 mAb (Genzyme Techne, Minneapolis, Minnesota, USA) at 4°C for 30 min, washed, and analyzed.

Caspase activity assay

The activities of caspase-8, caspase-9, caspase-6, and caspase-3 were measured by a quantitative colorimetric assay with caspase-8, caspase-9, caspase-6, and caspase-3 Colorimetric Protease Assay Kits (MBL, Nagoya, Japan), as described previously [22,23].

Polymerase chain reaction array analysis

The Human Apoptosis RT^{2}Profiler™ PCR Array (SuperArray, Frederick, Maryland, USA) was used following the supplier's protocol. The array profiles the expression of 84 genes involved in apoptosis. The genes are grouped into 12 categories according to their functional and structural features, including the B-cell lymphoma-2 (Bcl-2), caspase, TNF ligand, TNF receptor, the inhibitor of apoptosis proteins, TNF receptor-associated factor (TRAF), caspase recruitment domain (CARD), DD, death effector domain, cell death-inducing DFF45 effectors (CIDE) domain family members, and genes involved in the p53 and DNA damage response and anti-apoptosis pathways.

Cells were seeded in the 100-mm dishes at 1.5×10^6 cells per dish and cultured for 24 h. Cells were then treated with lexatumumab and/or anthracyclines for 12 or 24 h. After treatment, total RNA was extracted from the cells using the Qiagen RNeasy Kit (Qiagen, Valencia, California, USA). A260/A280 ratios and RNA gel electrophoresis were used to verify the high quality of the RNA. RNA was reverse transcribed and cDNA was prepared using the RT^2 PCR Array First Strand Kit (SuperArray), and cDNA was used in the array as per the supplier's protocol. The analysis of the obtained data was performed with the

Excel-based PCR Array Data Analysis template obtained from the SuperArray web site.

Real-time reverse transcription-PCR

The real-time reverse transcription (RT)-PCR was carried out using LightCycler Fast-Start DNA Master SYBR Green 1 (Roche Diagnostics, Mannheim, Germany). The forward and reverse primers were for TRAIL-R2 5′-GGGAG CCGCTCATGAGGAAGTTGG-3′ and 5′-GGCAAGTCTC TCTCCCAGCGTCTC-3′ [24], for PYCARD 5′-TCCAG CAGCCACTCAACG-3′ and 5′-GCACTTTATAGACCAG CA-3′ [25], and for CIDEA 5′-AAGAGGTCGGGAATAGC GAGAGTC-3′ and 5′-CTGCATCCCTATCCACACGTGA ACC-3′ [26]. The samples were preincubated at 95°C for 10 min, and amplified for 40–50 cycles using denaturation at 95°C for 10 s, annealing at 63°C to 65°C for 1–10 s, and extension at 72°C for 10 s. Quantitative analysis of the data was performed using LightCycler software version 3.5 (Roche Diagnostics). Standard curves for templates of TRAIL-R2 and glyceraldehyde-3-phosphate dehydrogenase were generated by serial dilution of the PCR products.

Western blot analysis

Western bolt analysis was carried out as described previously [18]. Forty micrograms of protein was loaded in each lane. Mouse anti-PYCARD mAb (Santa Cruz Biotechnology, Santa Cruz, California, USA), rabbit polyclonal Ab (Santa Cruz Biotechnology), and mouse anti-β-actin mAb (Abcom, Cambridge, UK) were used as primary Abs. Signals were detected using the ECL kit (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA).

Statistical analysis

All experiments were carried out three times, and the results were expressed as the mean ± SD. Statistical significance was determined using Student's *t*-test, with *P* value of less than 0.05 considered to be significant. Synergy was assessed by isobolographic analysis [27].

Results

Synergistic cytotoxicity of lexatumumab and anthracyclines against RCC cells

We examined whether the treatment of ACHN cells with lexatumumab in combination with anthracyclines would result in a synergistic cytotoxic activity. The treatment of ACHN cells with lexatumumab (1–100 ng/ml) in combination with subtoxic concentrations of epirubicin or pirarubicin (0.1–10 μg/ml) for 24 h resulted in a significant potentiation of cytotoxicity, and a synergistic cytotoxic effect was achieved, although each agent alone had little cytotoxicity (Fig. 1). This synergy was specific for lexatumumab, because epirubicin or pirarubicin in combination with a control IgG had no synergistic effect (data not shown).

There was a marked decrease in cell numbers and apoptotic bodies were also observed under the phase-contrast microscope when lexatumumab and epirubicin/pirarubicin were used in combination, although each agent induced cytotoxicity in a few cells (data not shown).

The synergy was not selective for this RCC cell line, as primary RCC cells derived from three patients were also sensitized to lexatumumab-induced cytotoxicity in the presence of epirubicin or pirarubicin (Fig. 2).

Together, these findings clearly demonstrate that the treatment of the RCC cell line or primary RCC cells with a combination of lexatumumab and anthracyclines results in the potentiation of cytotoxicity.

Sensitization of renal cell carcinoma cells to lexatumumab-induced cytotoxicity by anthracyclines

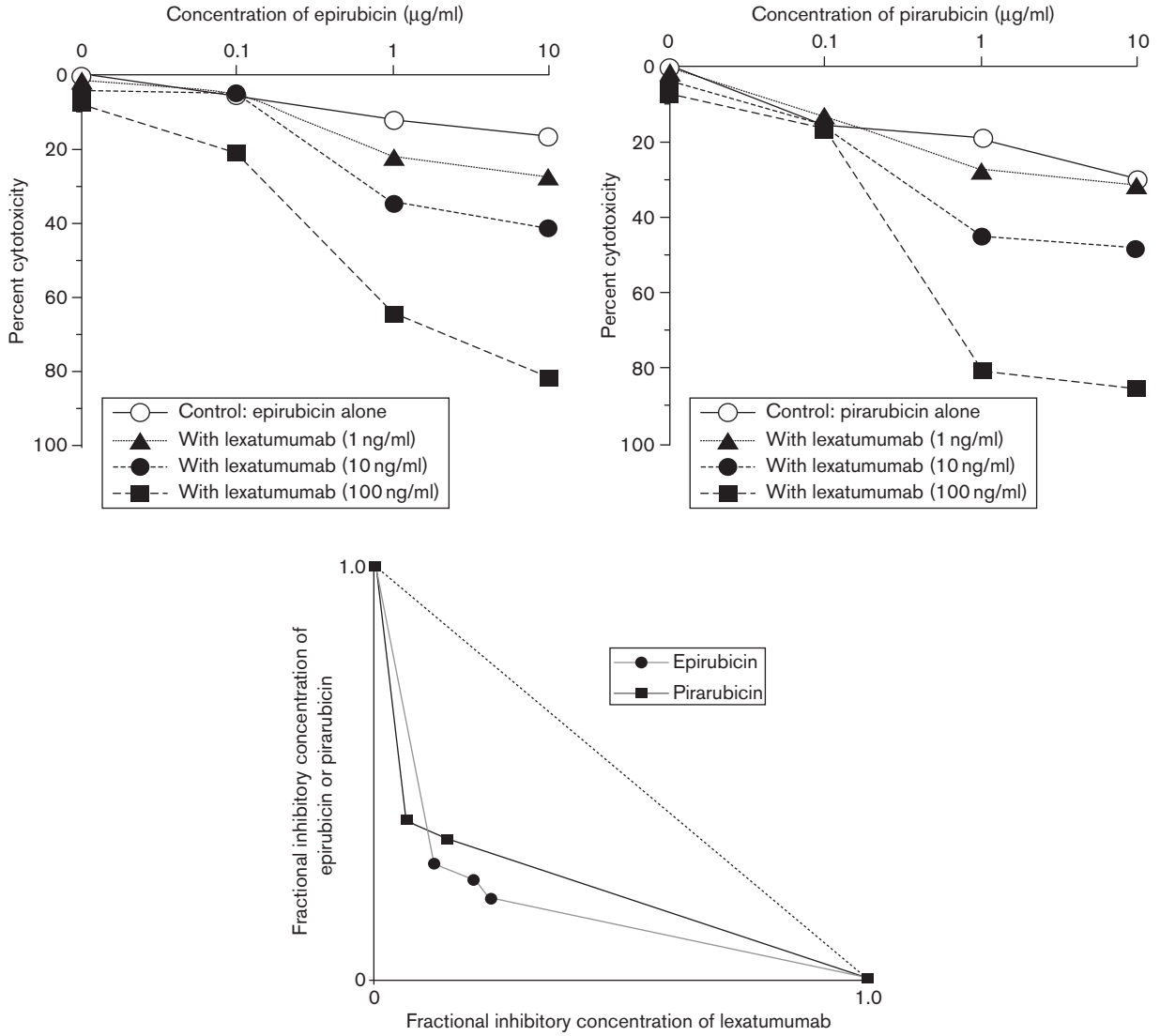
To explore the underlying mechanisms in this synergistic cytotoxicity with lexatumumab and anthracyclines, the effect of sequential treatment with lexatumumab and epirubicin/pirarubicin was examined next. Pretreatment of ACHN cells with epirubicin or pirarubicin (10 μg/ml) for 8 h, followed by treatment with lexatumumab (100 ng/ml) for 16 h, induced more cytotoxicity than either reverse treatment or simultaneous treatment using these agents (Fig. 3). These findings indicate that anthracyclines sensitize RCC cells to lexatumumab-induced cytotoxity and provide a foundation to optimize the administration of these drugs for application in the clinical setting.

Tumor necrosis factor-related apoptosis-inducing ligand receptor-2-dependent synergistic apoptosis of lexatumumab and anthracyclines

Cytotoxic drugs have been reported to increase the expression of TRAIL-R2 in cancer cells [18,28]. We used flow cytometry to determine whether this mechanism could account for the sensitization of RCC cells to lexatumumab-induced apoptosis by anthracyclines. Treatment of ACHN cells with 1 μg/ml of epirubicin or pirarubicin for 24 h increased the expression of TRAIL-R2 (Fig. 4a). The upregulation was also seen when the epirubicin or the pirarubicin treatment was shortened from 24 to 6 h (data not shown). Real-time PCR analysis showed that epirubicin significantly increased mRNA levels of TRAIL-R2 in a time-dependent manner. The upregulation of TRAIL-R2 mRNA levels was also obtained after pirarubicin treatment (Fig. 4b).

To clarify the significance of upregulation of TRAIL-R2 by anthracyclines in synergy, the effect of a human recombinant DR5:Fc chimeric protein was examined. The synergistic cytotoxicity of lexatumumab and epirubicin/pirarubicin against ACHN cells was significantly inhibited by DR5:Fc chimeric protein (Fig. 4c). The inhibitory effect of DR5:Fc chimeric protein was also observed when its concentration was increased from 10 to 20 μg/ml (data not shown).

Fig. 1



Synergistic cytotoxicity of lexatumumab and anthracyclines in ACHN cells. Cells were treated for 24 h with lexatumumab alone, epirubicin alone, pirarubicin alone, or a combination of lexatumumab with epirubicin or pirarubicin at the indicated concentrations. Cytotoxicity was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and synergy was assessed by isobolographic analysis, respectively. Data represent means from three independent experiments.

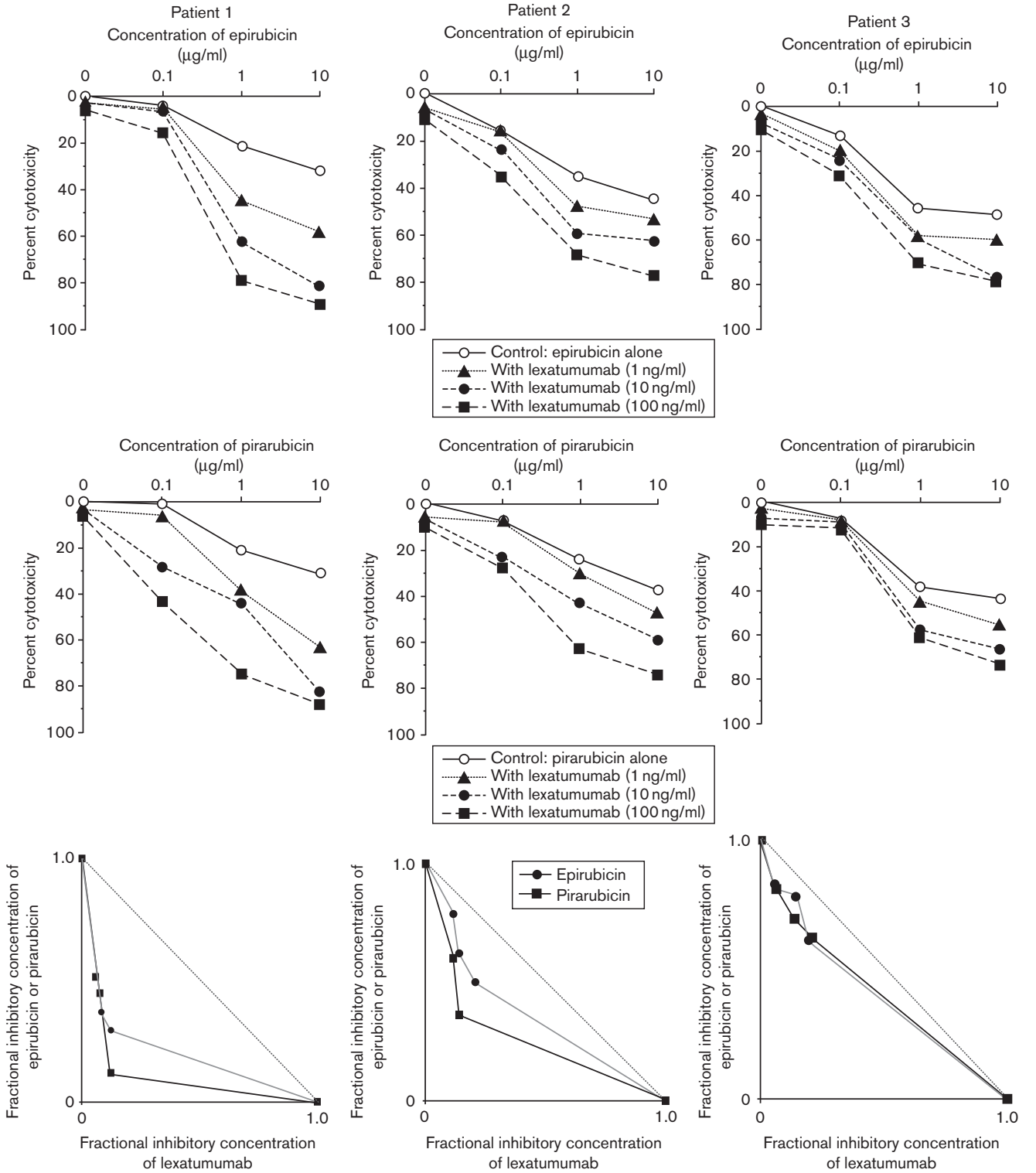
These results indicate that synergistic cytotoxicity and apoptosis of lexatumumab and anthracyclines is TRAIL-R2 dependent.

Activation of caspases and induction of synergistic apoptosis by lexatumumab and anthracyclines

We analyzed caspase-8, caspase-9, caspase-6, and caspase-3 activities in ACHN cells by a quantitative colorimetric assay. Epirubicin/pirarubicin and lexatumumab combination significantly activated caspase-8, caspase-9, caspase-6, and caspase-3 (Fig. 5a). In contrast, neither lexatumumab alone nor epirubicin/pirarubicin-alone activates caspase-8, caspase-9, caspase-6, or caspase-3.

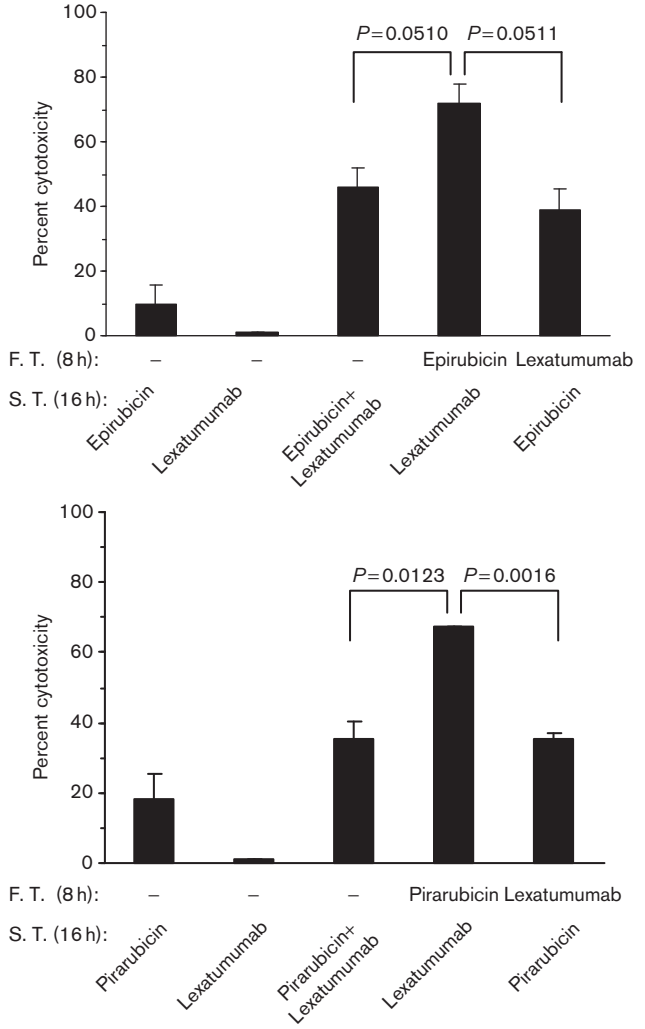
To assess whether the enhanced cytotoxity of lexatumumab and anthracyclines combination was mediated by apoptosis, ACHN cells were monitored both by DNA ladder and by ELISA assays. Epirubicin, pirarubicin, or lexatumumab did not significantly cause DNA fragmentation. Obvious DNA fragmentation, however, was observed when the cells were incubated with lexatumumab in combination with epirubicin or pirarubicin (Fig. 5b). The synergistic apoptosis of cells treated with lexatumumab and anthracyclines was confirmed using a quantitative apoptosis-specific ELISA kit (Fig. 5c). These results indicate that the synergistic cytotoxicity was achieved by inducing apoptosis.

Fig. 2



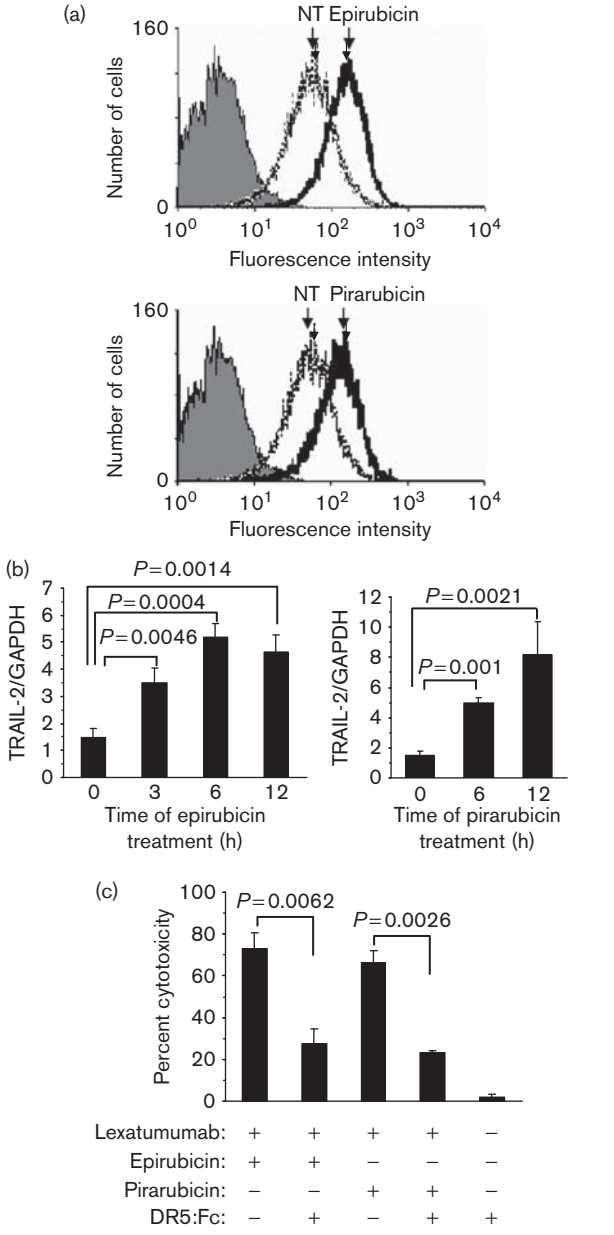
Synergistic cytotoxicity of lexatumumab and anthracyclines in primary renal cell carcinoma cells derived from three patients. Cells were treated for 24 h with lexatumumab alone, epirubicin alone, pirarubicin alone, or a combination of lexatumumab with epirubicin or pirarubicin at the indicated concentrations. Cytotoxicity was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and synergy was assessed by isobolographic analysis, respectively. Data represent means from three independent experiments.

Fig. 3



Effect of the sequence of treatment with lexatumumab and anthracyclines. ACHN cells were preincubated for 8 h with medium only, 100 ng/ml of lexatumumab, 10 μg/ml of epirubicin or pirarubicin, washed three times with medium, and exposed to 100 ng/ml of lexatumumab and/or 10 μg/ml of epirubicin or pirarubicin for 16 h. Cytotoxicity was measured using a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. FT, first treatment; ST, second treatment.

Fig. 4



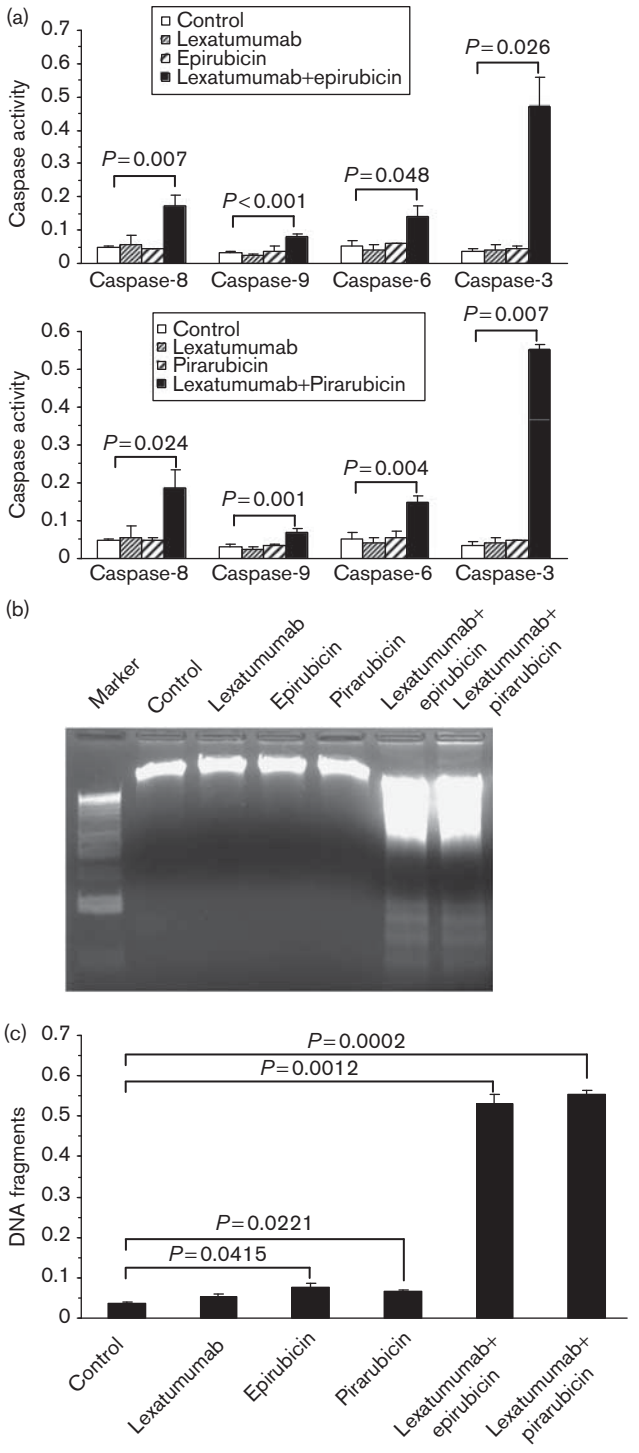
Tumor necrosis factor (TNF)-related apoptosis-inducing ligand receptor-2 (TRAIL-R2)-dependent synergistic cytotoxicity of lexatumumab and anthracyclines. (a): ACHN cells were treated for 24 h with medium only or 1 μg/ml of epirubicin or pirarubicin. Cells were harvested, incubated with PE-conjugated anti-TRAIL-R2 mAb for 30 min at 4°C, and the protein levels of TRAIL-R2 were analyzed using flow cytometry. Gray areas represent IgG1 isotype staining. The thin histogram indicates TRAIL-R2 staining, whereas the thick histogram indicates TRAIL-R2 staining after epirubicin or pirarubicin treatment. (b): Total RNA was extracted with TRIzol reagent and used for cDNA synthesis. Cells were treated with 1 μg/ml of epirubicin for 3–12 h. The mRNA levels of TRAIL-R2 were determined using real-time reverse transcriptase-PCR. (c) Cells were treated with 100 ng/ml of lexatumumab plus 10 μg/ml of epirubicin or pirarubicin in the absence or presence of 10 μg/ml of DR5:Fc chimeric proteins for 24 h. Cytotoxicity was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Regulation of apoptosis-regulatory genes by lexatumumab and anthracyclines

Among 84 genes involved in apoptosis, 18 (*CD40LG*, *FASLG*, *LTA*, *TNSF7*, *FAS*, *BAG3*, *BAK1*, *BAX*, *BID*, *BIK*, *BCL10*, *CASP1*, *CASP5*, *CASP6*, *CASP10*, *APAF1*, *PYCARD*, and *CIDEA*) were found to be consistently upregulated more than 2.0-fold and eight (*TRAF4*, *TNFRSF11B*, *TNF*, *BCL2*, *BCL2L1*, *BNIP3L*, *CASP9*, and *DAPK1*) were downregulated more than 2.0-fold in the ACHN cells cotreated with lexatumumab and epirubicin (Table 1).

To confirm the results of PCR array, we further performed quantitative real-time RT-PCR analysis. The upregula-

Fig. 5



Activation of caspases and induction of apoptosis by lexatumumab and anthracylines. ACHN cells were treated with 1 μg/ml of epirubicin alone, 1 μg/ml of pirarubicin alone, or in combination with 100 ng/ml of lexatumumab for 12 h. (a): Activities of caspase-8, caspase-9, caspase-6, and caspase-3 were measured by a quantitative colorimetric assay. (b): DNA was extracted and separated with by electrophoresis in 2% agarose gels and stained with ethidium bromide. (c) Quantification of DNA fragmentation was assessed with the apoptosis-specific enzyme-linked immunosorbent assay kit. Results are derived three different experiments.

Table 1 List of genes upregulated or downregulated greater than 2-fold in treated cells with lexatumumab and epirubicin[a]

Gene symbol	Gene	Mean fold change
Groups 1, 2, and 6: TNF ligand, TNFR, and TRAF families		
CD40LG	CD40 ligand (TNF superfamily, member 5)	3.75 ↑
FASLG	Fas ligand (TNF superfamily, member 6)	2.15 ↑
LTA	Lymphotoxin-α (TNF superfamily, member 1)	2.10 ↑
TNFSF7	TNF ligand superfamily, member 7	3.36 ↑
FAS	TNF receptor superfamily, member 6	7.20 ↑
TRAF4	TNF receptor-associated factor 4	2.91 ↓
TNFRSF11B	TNF receptor superfamily, member 11b	3.22 ↓
TNF	TNF superfamily, member 2	3.42 ↓
Group 3: Bcl-2 family		
BAG3	BCL2-associated athanogene 3	3.70 ↑
BAK1	BCL2-antagonist/killer 1	6.21 ↑
BAX	BCL2-associated X protein	2.36 ↑
BID	BH3 interacting domain death agonist	3.86 ↑
BIK	BCL2-interacting killer (apoptosis inducing)	2.37 ↑
BCL10	B-cell CLL/lymphoma 10	2.47 ↑
BCL2	B-cell CLL/lymphoma 2	2.38 ↓
BCL2L1	BCL2-like 1	3.08 ↓
BNIP3L	BCL2/adenovius E1B 19 kDa interacting protein 3-like	2.46 ↓
Groups 4 and 7: caspase and CARD families		
CASP1	Caspase 1, apoptosis-related cystein protease	3.28 ↑
CASP5	Caspase 5, apoptosis-related cystein protease	2.15 ↑
CASP6	Caspase 6, apoptosis-related cystein protease	3.35 ↑
CASP10	Caspase 10, apoptosis-related cystein protease	4.89 ↑
CASP9	Caspase 9, apoptosis-related cystein protease	2.98 ↓
APAF1	Apoptotic protease activating factor 1	4.75 ↑
PYCARD	PYD and CARD domain containing	4.54 ↑
Groups 8 and 10: DD and CIDE families		
DAPK1	DNA fragmentation factor, 45 kDa, α polypeptide	2.94 ↓
CIDEA	Cell death-inducing DFFA-like efector a	2.15 ↑

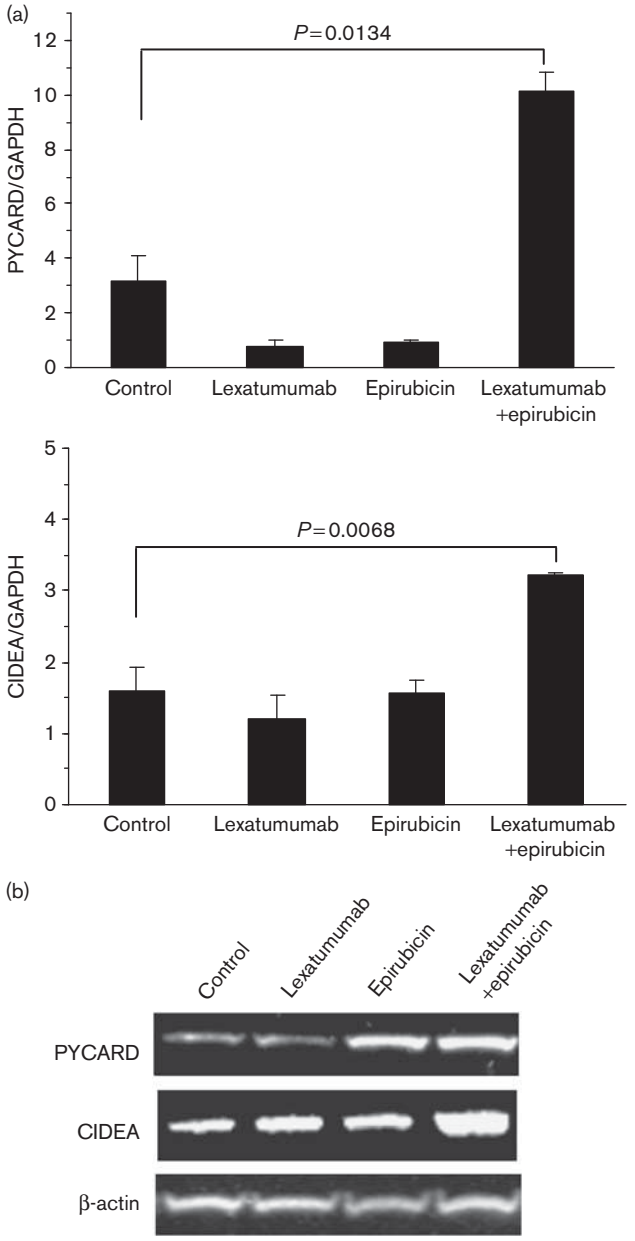
[a]ACHN cells were treated with 100 ng/ml of lexatumumab in combination with 1 μg/ml of epirubicin for 12 h. The gene expression was assessed by PCR array assay. The results are expressed as the mean ± SD of three different experiments. CLL, chronic lymphocytic leukemia; DD, death domain; TNF, tumor necrosis factor.
↓ means downregulation; ↑means upregulation.

tion of PYCARD and CIDEA mRNA levels was obtained when ACHN cells were treated with lexatumumab in combination with epirubicin, although they were not with each agent alone (Fig. 6a). Western blot analysis showed that lexatumumab in combination with epirubicin increased PYCARD and CIDEA protein levels (Fig. 6b).

Discussion

RCC is one of the most resistant malignancies to conventional cancer therapies. No effective systemic therapy presently exists for patients with metastatic RCC, although several molecular-targeted agents to slow RCC growth are currently being used with some success [29]. Development of novel and effective therapeutic strategies for metastatic RCC is urgently needed. The present study demonstrates that (a) lexatumumab and anthracyclines, epirubicin and pirarubicin, had a synergistic effect on human RCC cells, which were resistant to each agent used alone; (b) the synergistic cytotoxicity of lexatumumab and anthracyclines was achieved by induction of apoptosis in a TRAIL-R2-dependent manner; and (c) the change in

Fig. 6



Upregulation of PYCARD and CIDEA expressions by lexatumumab and epirubicin. ACHN cells were treated with 100 ng/ml of lexatumumab and 1 μg/ml of epirubicin for 12 h. (a): Total RNA was extracted with TRIzol reagent and used for cDNA synthesis. The mRNA levels of PYCARD and CIDEA were determined using real-time reverse transcriptase-PCR analysis. (b): The expression of PYCARD and CIDEA was monitored by western blot analysis. β-actin was used as a loading control. Data represent means from three independent experiments.

other genes related to apoptosis by lexatumumab and anthracyclines might play a role in the regulation of these cellular events. These findings suggest that combination treatment using lexatumumab and anthracyclines is promising from a clinical perspective.

Several studies have shown that combination treatment with agonistic anti-TRAIL-R2 mAbs and chemotherapeutic agents had a synergistic apoptotic effect in some tumor cell lines, such as lymphoma, breast cancer, colorectal cancer, cervical cancer, and malignant mesotheloma [30–34]. However, the mechanisms by which chemotherapeutic agents enhance the TRAIL-R2-mediated apoptosis are not understood completely. The present study shows that both epirubicin and pirarubicin significantly upregulated TRAIL-R2 expression in RCC cells. Furthermore, the synergistic cytotoxicity of lexatumumab and anthracyclines was significantly inhibited by the DR5:Fc chimeric protein, which competes for binding to TRAIL-R2. These findings indicate that lexatumumab and anthracyclines synergistically induce apoptosis in RCC cells in a TRAIL-R2-dependent manner.

In this study, we found that some genes from CARD and CIDE families, not well characterized in RCC, also showed multifold increases in the RCC cells cotreated with lexatumumab and epirubicin, except for common apoptosis-regulatory genes including the TNF ligand, TNFR, TRAF, BcL-2, caspase, and DD gene families. PYCARD, one of the CARD family members, and CIDEA were increased multifold in the treated RCC cells. The CARD family is required for interactions between caspases and other proteins, allowing activation of the caspase family of proteins and induction of apoptosis [35]. The CIDE family also plays an important role in the prevention of tumorigenesis by helping induce apoptosis [36]. Rajandram *et al.* [37] recently demonstrated that TRAF, CARD, and CIDE gene families were upregulated in RCC cells treated with radiation plus interferon-α using a real-time PCR array method. Although further functional analysis is necessary, these results suggest that PYCARD and CIDEA gene families may be involved in the synergistic apoptosis of lexatumumab and anthracyclines in RCC cells.

A phase I clinical trial using lexatumumab to treat patients with advanced cancers was recently completed [38,39]. Although lexatumumab treatment was relatively nontoxic, only 32.4% of patients with lexatumumab achieved stable disease. Therefore, development of ways to optimize the effect of lexatumumab, particularly through combination with chemotherapy agents, is warranted. Epirubicin and pirarubicin are commonly used chemotherapeutic drugs for many solid cancers and demonstrate fewer cardiac toxicities than other anthracyclines. Importantly, the present results indicate that lexatumumab and epirubicin/pirarubicin have a synergistic cytotoxicity in RCC cells at subtoxic concentrations of epirubicin or pirarubicin. Pretreatment with epirubicin or pirarubicin further enhanced the synergistic effect of combination with lexatumumab. These results suggest that combining epirubicin or

pirarubicin with lexatumumab using an optimized dosing schedule may greatly enhance the efficacy of lexatumumab for the treatment of RCC.

Conclusion

We have demonstrated that subtoxic concentrations of anthracyclines enhanced lexatumumab-induced apoptosis and cytotoxicity against RCC cells in a TRAIL-R2-dependent manner. We also demonstrated by PCR array that *PYCARD* and *CIEDA* genes were significantly upregulated in the RCC cells treated with lexatumumab in combination with epirubicin. These results suggest that treatment of RCC using anthracyclines combined with lexatumumab is promising as a potential clinical application, and the utility of PCR array to elucidate the mechanism of synergistic apoptosis of lexatumumab and anthracyclines.

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Conflict of interests

There are no conflicts of interest.

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