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Bleomycin Induced Sensitivity to TRAIL/Apo-2L-Mediated Apoptosis in Human Seminomatous Testicular Cancer Cells is Correlated with Upregulation of Death Receptors

Mujgan Timur[1], Aysegul Cort[1], Evrim Ozdemir[1], Sureyya Bilmen Sarikcioglu[2], Salih Sanlioglu[3], Ahter Dilsad Sanlioglu[3] and Tomris Ozben[1,#,*]

[1]*Akdeniz University Medical Faculty, Department of Biochemistry 07070 Antalya Turkey;* [2]*Akdeniz University Vocational School of Health Services, 07070 Antalya Turkey;* [3]*Akdeniz University Medical Faculty Department of Medical Biology and Genetics, 07070 Antalya Turkey*

Abstract: The most common solid tumor is testicular cancer among young men. Bleomycin is an antitumor antibiotic used for the therapy of testicular cancer. TRAIL is a proapoptotic cytokine that qualified as an apoptosis inducer in cancer cells. Killing cancer cells selectively *via* apoptosis induction is an encouraging therapeutic strategy in clinical settings. Combination of TRAIL with chemotherapeutics has been reported to enhance TRAIL-mediated apoptosis of different kinds of cancer cell lines. The molecular ground for sensitization of tumour cells to TRAIL by chemotherapeutics might involve upregulation of TRAIL-R1 (TR/1, DR4) and/or TRAIL-R2 (TR/2, DR5) receptors or activation of proapoptotic proteins including caspases. The curative potential of TRAIL to eradicate cancer cells selectively in testicular cancer has not been studied before. In this study, we investigated apoptotic effects of bleomycin, TRAIL, and their combined application in NTera-2 and NCCIT testicular cancer cell lines. We measured caspase 3 levels as an apoptosis indicator, and TRAIL receptor expressions using flow cytometry. Both NTera-2 and NCCIT cells were fairly resistant to TRAIL's apoptotic effect. Incubation of bleomycin alone caused a significant increase in caspase 3 activity in NCCIT. Combined incubation with bleomycin and TRAIL lead to elevated caspase 3 activity in Ntera-2. Exposure to 72 h of bleomycin increased TR/1, TR/2, and TR/3 cell-surface expressions in NTera-2. Elevation in TR/1 cell-surface expression was evident only at 24 h of bleomycin application in NCCIT. It can be concluded that TRAIL death receptor expressions in particular are increased in testicular cancer cells *via* bleomycin treatment, and TRAIL-induced apoptosis is initiated.

Keywords: Apoptosis, bleomycin, caspase-3, flow cytometry, testicular cancer, TRAIL.

[#]**Author's Profile:** Prof. Tomris Ozben is a full professor at the Dept. of Clinical Chemistry, Faculty of Medicine, Akdeniz University, Antalya, Turkey. She has over 150 publications; 8 book chapters; 3 books as editor. She is Editorial Board member of several Scientific Journals and invited speaker at more than 200 international conferences.

INTRODUCTION

Testicular cancer represents 1% of tumors seen in males [1, 2]. Bleomycin, etoposide and cisplatin (BEP) combination therapy is the most effective treatment in metastatic nonseminomatous tumors [3]. Bleomycin, which is a glycopeptide antibiotic with anticancer activity produced by the bacterium *Streptomyces verticillus,* was first discovered by Hamao Umezawa [4, 5]. Bleomycin triggers apoptosis in actively growing cells by inducing DNA strand breaks. DNA cleavage by bleomycin anticipates the presence of oxygen and ferrous ions [6]. Like many other anticancer drugs, bleomycin has many side effects with a potential to cause serious fatal complications [4].

"Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL, Apo-2 ligand), a member of Tumor Necrosis Factor (TNF) family, is a type II transmembrane protein" [7] whose extracellular domain can be released as a soluble cytokine [8, 9]. TRAIL displays an important regulatory performance in apoptosis by interacting with transmembrane receptors, mainly TRAIL-R1(TR/1)/DR4 and TRAIL-R2(TR/2)/DR5 [10]. Coupling of TRAIL to "TRAIL-R1/DR4" or "TRAIL-R2/DR5" cause receptor trimerization, and subsequent activation of the intracellular Fas-associated death domain (FADD). Receptor activation ultimately leads to caspase activation and apoptosis [11]. In addition to death receptors, TRAIL also binds to "decoy receptors 1 (TRAIL-R3(TR/3)/DcR1) and 2 (TRAIL-R4(TR/4)/DcR2)", which are transmembrane proteins with inactive or deficient intracellular caspase-activating death domains, thus unable to induce apoptosis [12]. Another target for TRAIL binding is the soluble osteoprotogerin (OPG) receptor, which is claimed to be involved in osteoclastogenesis [13]. The physiological role of decoy receptors is controversial [12, 14, 15]. It has been postulated that they modulate death receptor affinity to TRAIL by ligand-independent formation of death/decoy receptor complexes [16]. In addition to its apoptosis-inducing effects, TRAIL also has various non-apoptotic functions in normal tissues [17].

Concurrent application of TRAIL with chemotherapeutic agents in cancer cells has been demonstrated to enhance the efficacy of treatment, diminish the TRAIL resistance, and to increase apoptosis in many *in vitro* and *in vivo* studies, mainly through elevation of death receptor expression [18-20]. Although BEP combination therapy has a high cure rate in testicular cancer, this treatment approach has certain side effects that should be eliminated [4]. TRAIL is broadly tested for especially combination therapies in various types of cancer, mainly for creating a selective apoptotic effect on tumor cells without detectable side effects on normal cells. To our knowledge, the potential of bleomycin to increase the sensitivity of human testicular cancer cells to TRAIL has not been studied and reported before. Thus, we investigated caspase 3 activity as an indicator of apoptosis, and expression levels of death and decoy receptors in NTera-2 and NCCIT" testicular cancer cell lines, representing seminomatous and nonseminomatous germ cell tumors, respectively, that were incubated with bleomycin or TRAIL separately, or in combination.

MATERIALS & METHODS

Testicular Cancer Cell Lines

"NTera-2 and NCCIT" cell lines were kindly provided by Dr. Peter Andrews (The University of Sheffield, South Yorkshire, UK).

*Address correspondence to this author at the Akdeniz University Medical Faculty Department of Biochemistry 07070 Antalya Turkey; Tel: 90 242 2496895; Fax: 90 242 2274495; E-mail: ozben@akdeniz.edu.tr

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"NTera-2 and NCCIT" cells were matured to confluence in "Dulbecco's Modified Eagle Medium (DMEM)" and "Roswell Park Memorial Institute Medium (RPMI)", respectively. The incubator maintained the 37°C humidified atmosphere with 5% CO_2. RPMI and DMEM media were supplemented with 10% fetal bovine serum, "100 IU/ml pennicillin" and "10 µg/ml streptomycin "(Life Technologies-Invitrogen).

Cell Viability Assay

Colorimetric "MTT (3-(4,5) dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)" assay was used to measure the viability of cancer cells (EMD-Millipore). Different concentrations of bleomycin were used in incubations during 72 h, or with different doses of TRAIL for 24 h. The cell viability of the NTera-2 and NCCIT cells (control) incubated with no agent was arbitrarily set as 100% and the cell viability of the other groups was compared with the viability of the control cells. BioTek Spectrophotometer was set to 570 nm wavelength to measure the absorbances. "For calculation of the viable cell percentages, the mean absorbance values of the cells incubated with different agents were compared with the mean absorbance values of the control cells" [21].

TRAIL Receptor Analysis by Flow Cytometry

Flow cytometry was utilized to measure cell surface expressions of TRAIL receptors. Briefly, a total of $5x10^4$ testicular cancer cells were incubated with or without different agents for 24 h, 48 h, or 72 h. Then FITC-conjugated anti-TR/1, TR/2, TR/3, and TR/4 primary antibodies or control IgG (Mouse) antibodies were given to the cells and incubated for 30 min on ice in dark. Ice-chilled PBS was used to wash cells, and TRAIL receptor surface expressions were detected utilizing FACScan flow cytometer.

Caspase 3 Assay

Cells were incubated with one of these agents for 24 h: bleomycin, TRAIL, or bleomycin+TRAIL. Control cells were incubated with only media. Caspase 3 was measured by using a commercial kit (BioSource, #KHZ0022). This colorimetric protease assay is convenient for quantification of caspase 3 enzyme activity. "DEVD (synthetic tetrapeptide, the upstream amino acid sequence of the caspase 3 cleavage site) amino acid sequence, coupled with p-nitroanilide (pNA) is released upon substrate cleavage by caspase 3" [21]. Absorbance of pNA released is measured at 400-405 nm spectrophotometrically.

Statistical Analysis

Mean ± standard error (mean ± SE) was used to present all the data. SPSS-Windows version 10.0 (SPSS Inc.) was used to perform the statistical analysis. Significant difference was considered as $p<0.05$. We used Student's t test and ANOVA for comparison of categorical and continuous variables. The Mann-Whitney U test and Kruskal-Wallis tests were used for comparing the non-parametric variables.

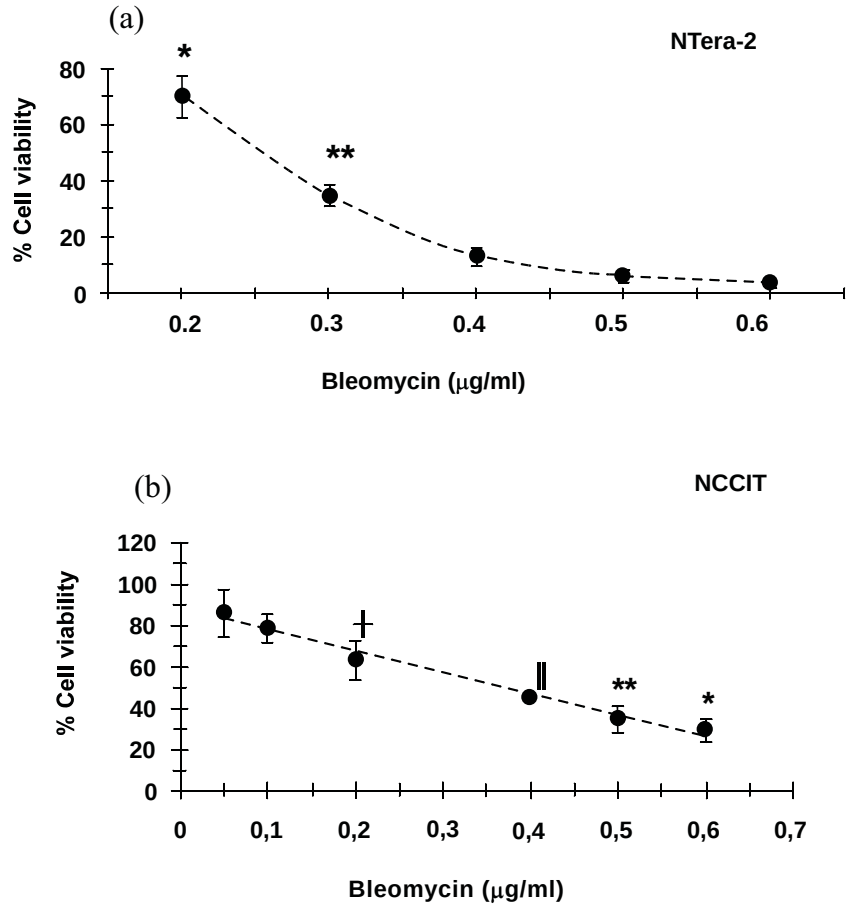

Fig. (1). Cytotoxicity of bleomycin in NTera-2 and NCCIT cell lines. Each data represents the mean ± SE of six independent experiments.
(a) NTera-2 cells were incubated with bleomycin (0.2-0.6 µg/ml) for 72 h.
*p<0.001 vs 0.3, 0.4, 0.5, 0.6; **p<0.001 vs 0.2, 0.4, 0.5, 0.6
(b) NCCIT cells were incubated with bleomycin (0.05-0.7 µg/ml) for 72 h.
*p<0.001 vs 0.05, 0.1, 0.2, 0.4; **p<0.001 vs 0.05, 0.1, 0.2; ‖ p<0.01 vs 0.05, 0.1, 0,2; ┼ p< 0.05 vs 0.05, 0.1

RESULTS

Bleomycin Sensitivity in NTera-2 and NCCIT Cells

Bleomycin's cytotoxic effect on testis cancer cells and half maximal lethal dose (LD_{50}) of bleomycin (the dose killing 50% of the cancer cells) were determined using MTT test by incubating NTera-2 cells with 0.2-0.6 μg/ml bleomycin, and NCCIT cells with 0.05-0.7 μg/ml bleomycin (Fig. **1**). LD_{50} dose of bleomycin was found as 0.25 μg/ml for NTera-2 cells, and 0.37 μg/ml for NCCIT cells. NCCIT cells were more resistant to bleomycin than NTera-2 cells. The optimum bleomycin dose to avoid high toxicity in combined applications of bleomycin and TRAIL was determined as 0.2 μg/ml for both NCCIT and NTera-2 cells. We incubated both NCCIT and NTera-2 cells with a bleomycin dose of 0.2 μg/ml (a dose lower than LD_{50} dose in order to keep more than 50% of both cells viable) in concurrent incubations with bleomycin and TRAIL.

TRAIL Sensitivity in NTera-2 and NCCIT Cells

We tested different doses of TRAIL (1, 10, and 100 ng/ml) to determine its cytotoxicity on testis cancer cell lines. These doses were similar to those used in the previous studies in different cancer cell types such as prostate and breast cancer cells. However, none of these three doses exerted any cytotoxic effect on NCCIT and NTera-2 cells. So, we applied higher doses such as as 1, 2, 3, 4 and 5 μg/ml. We found the TRAIL dose inhibiting 50% of NTera-2 cells as 5 μg/ml, and for NCCIT cells as 4 μg/ml (Fig. **2**). The TRAIL dose keeping 70% of the cells viable was 3 μg/ml for NCCIT cells, and 4 μg/ml for Ntera-2 cells. We incubated both NCCIT and NTera-2 cells with a bleomycin dose of 0.2 μg/ml, and with a TRAIL dose of 3 μg/ml for NCCIT cells and 4 μg/ml for Ntera-2 cells in concurrent incubations with bleomycin and TRAIL.

Basal Receptor Levels

Basal TRAIL death receptor TR/2 and decoy receptor TR/4 expressions were at substantial levels, while the basal TR/1 death receptor and TR/3 decoy receptor expressions were very low in NTera-2 cells. The receptor with the highest expression was TR/4, which was followed by TR/2 receptor expression in NTera-2 cells (Fig (**3a**)). Similarly, the basal TR/2 and TR/4 receptors were synthesized distinctly, while TR/1 and TR/3 expressions were quite low in NCCIT cells. TR/2 was the receptor with the highest expression in this cell line, which was followed by the TR/4 receptor (Fig. (**3b**)).

Effects of Bleomycin and TRAIL on TRAIL Receptor Levels

The TRAIL receptor composition in NCCIT and NTera-2 cells were determined following 24 h, 48 h, and 72 h of bleomycin incubation. TR/1, TR/2 and TR/3 expression levels increased in NTera-2 cells at 72 hours of bleomycin incubation (Fig. **4**). NTera-2 cells TR/1, TR/2, TR/3 and TR/4 receptor levels were similar to control cell receptor levels after incubation with TRAIL for 24, 48, 72 hours.

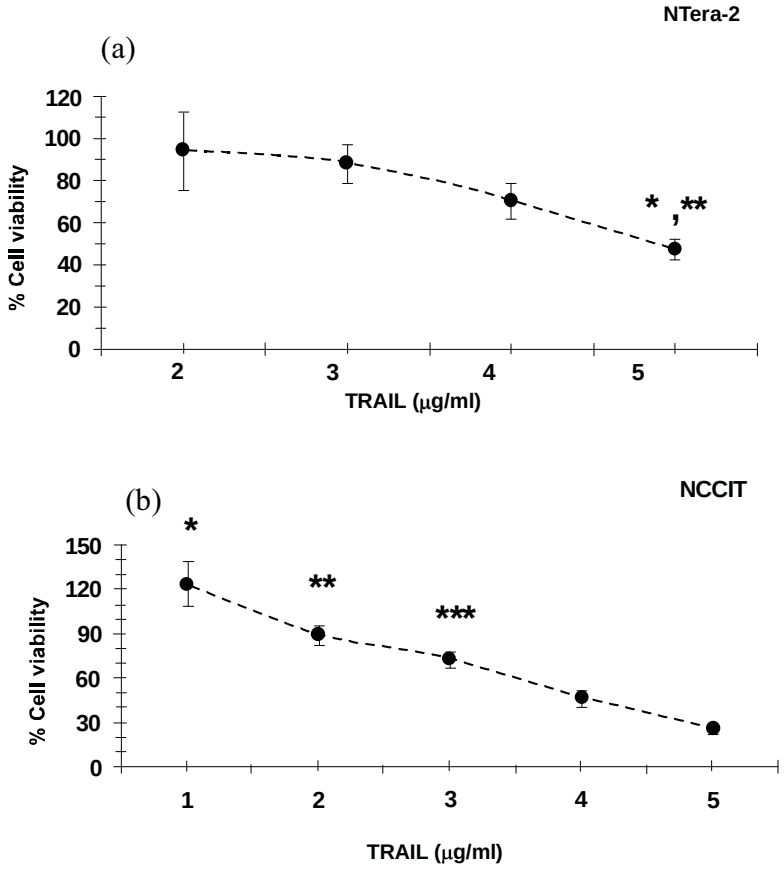

Fig. (2). Cytotoxicity of TRAIL in NTera-2 and NCCIT cell lines. Each data represents the mean ± SE of six independent experiments.
(a) NTera-2 cells were incubated with TRAIL (2-5 μg/ml) for 24 h.
*p<0.001 vs 2 μg/ml; **p=0.002 vs 3 μg/ml
(b) NCCIT cells were incubated with TRAIL (1-5 μg/ml) for 24 h.
*p<0.01 vs 2, 3, 4, 5 μg/ml; **p<0.01 vs 1, 4, 5 μg/ml; ***p<0.01 vs 5 μg/ml

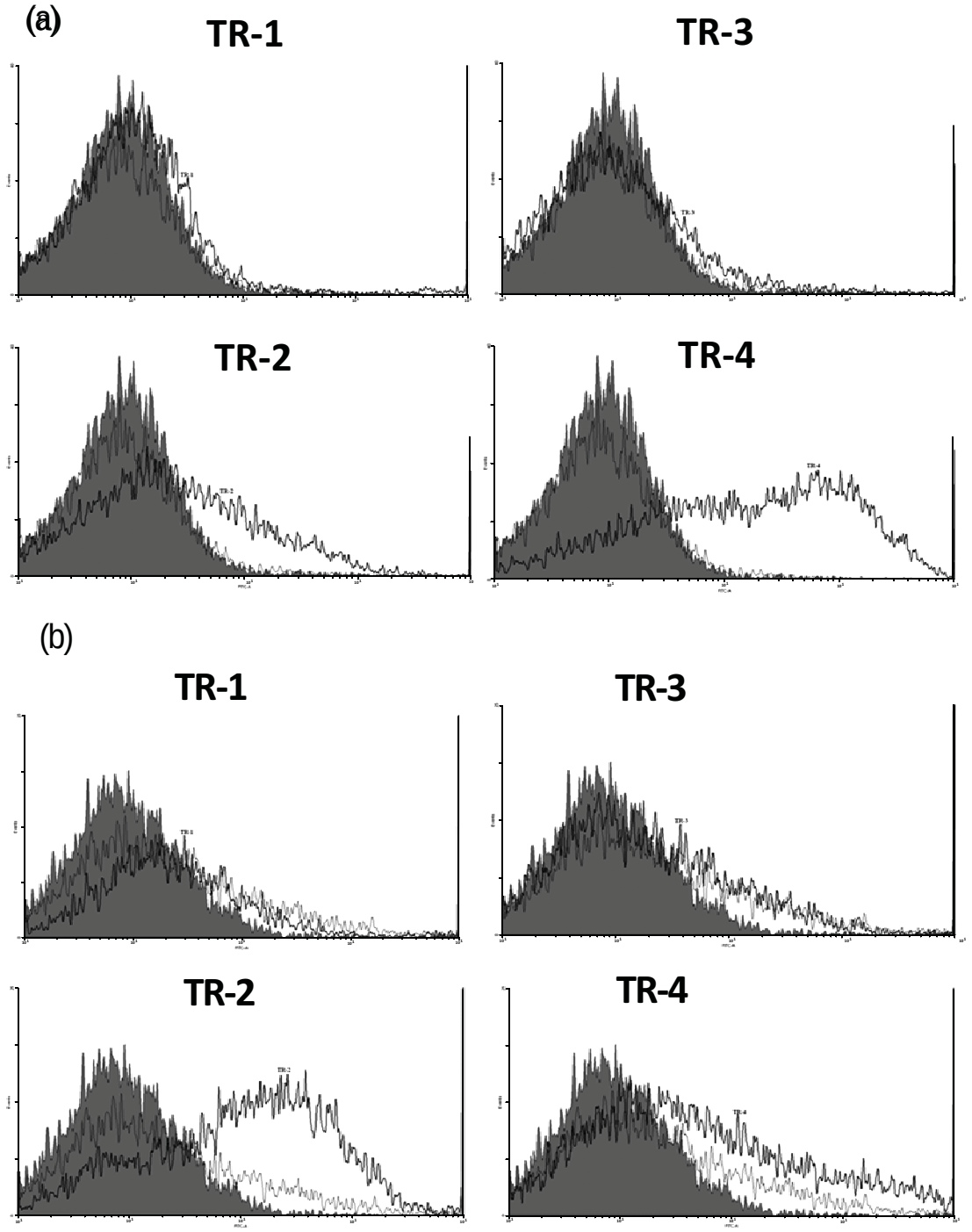

Fig. (3). (a) The basal expression levels of TR/1, TR/2, TR/3 and TR/4 receptors in NTera-2 cells. Filled line, unstained control; thin line, isotype control; thick line, TR/1, TR/2, TR/3 or TR/4 (b) The basal expression levels of TR/1, TR/2, TR/3 and TR/4 receptors in NCCIT cells. Filled line, unstained control; thin line, isotype control; thick line, TR/1, TR/2, TR/3 or TR/4

A significant increase in TR/1 expression level compared to the basal receptor level was detected in NCCIT cells only after incubation with bleomycin for 24 h. There was no increase in TR/1 expression level after 48 and 72 hours of bleomycin incubation (Fig. **5**). TR/2, TR/3 and TR/4 receptor levels in NCCIT cells were similar to the control cell receptor levels after incubation with bleomycin for 24, 48 and 72 hours. Incubation with TRAIL for 24, 48, 72 hours did not cause any alteration in the TR/1, TR/2, TR/3 and TR/4 receptor levels in NCCIT cells compared to the levels in NCCIT cells not incubated with TRAIL.

Active Caspase 3 Levels

The most essential caspase in the apoptotic process is caspase 3, and it is activated by initiator caspases such as caspase 8, 9, 10 through extrinsic (death ligand) and intrinsic (mitochondrial) pathways. It mediates endonuclease activation, chromatin condensation and membrane blebbing during apoptosis [22, 23]. We measured active caspase 3 levels in NCCIT and Ntera-2 cells after incubation with either bleomycin, TRAIL or their combination in comparison to the level in the control (Fig. **6**). Although incubation with bleomycin or

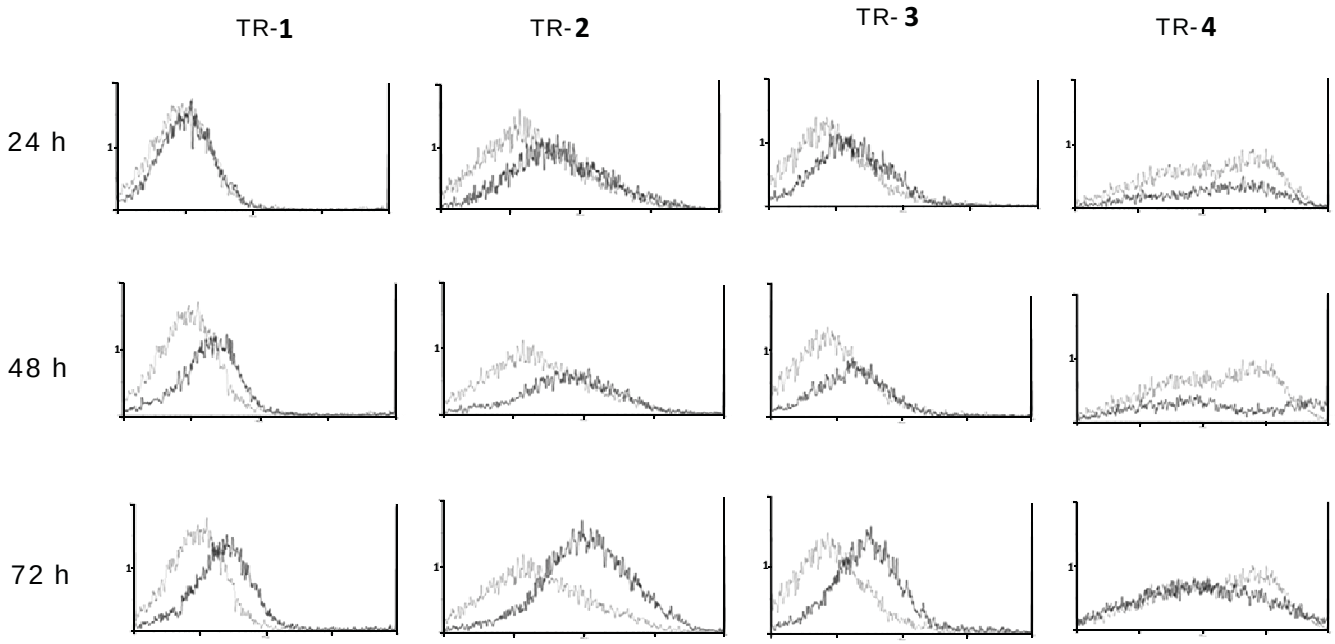

Fig. (4). TR/1, TR/2, TR/3 and TR/4 receptors in NTera-2 cells after incubation with bleomycin for 24, 48, and 72 hours. Thin line, untreated control; thick line, TR/1, TR/2, TR/3 or TR/4.

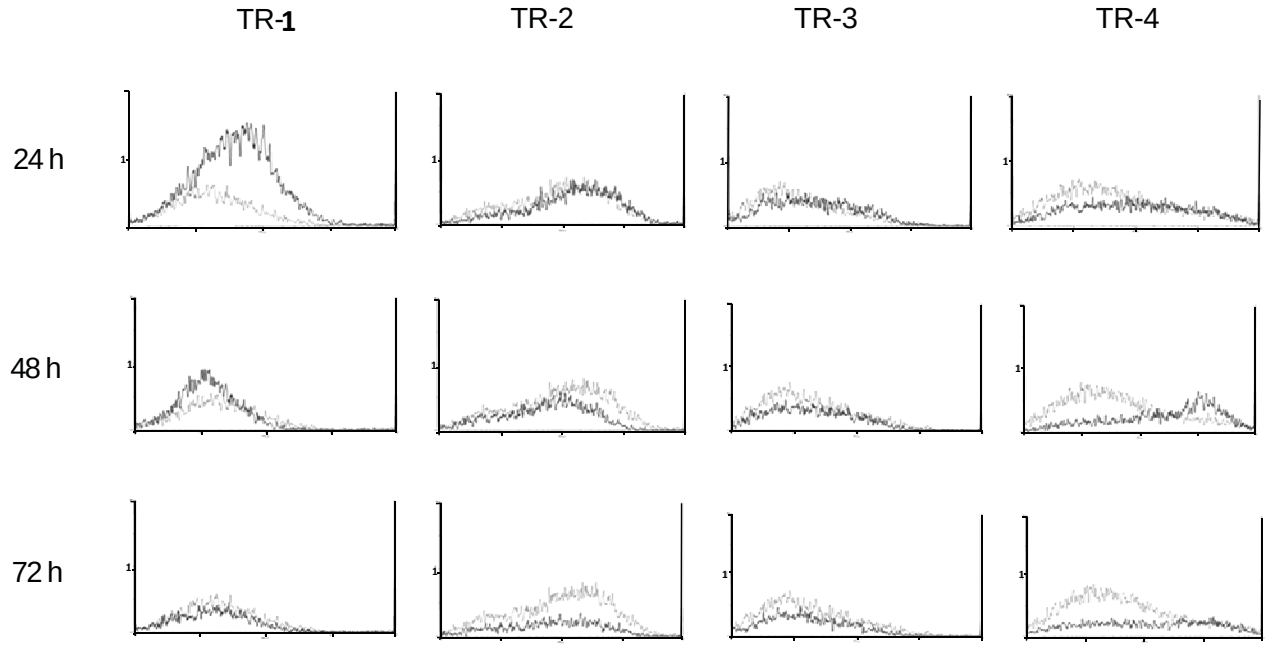

Fig. (5). TR/1, TR/2, TR/3 and TR/4 receptors in NCCIT cells after incubation with bleomycin for 24, 48 and 72 hours. Thin line, untreated control; thick line, TR/1, TR/2, TR/3 or TR/4.

TRAIL increased active caspase 3 levels in NTera-2 cells, the rise was not statistically significant. In contrast to NTera-2 cells, incubation with bleomycin for 24 hours significantly increased caspase 3 levels in the NCCIT cells. No significant increase was evident after TRAIL incubation in both cell lines. In contrast, active caspase 3 levels in NTera-2 cells increased significantly after TRAIL and bleomycin combined incubation compared to NTera-2 testicular cancer cells incubated with either TRAIL or bleomycin solely. There was no change in the caspase 3 activation level in NCCIT cells incubated with TRAIL and bleomycin combination.

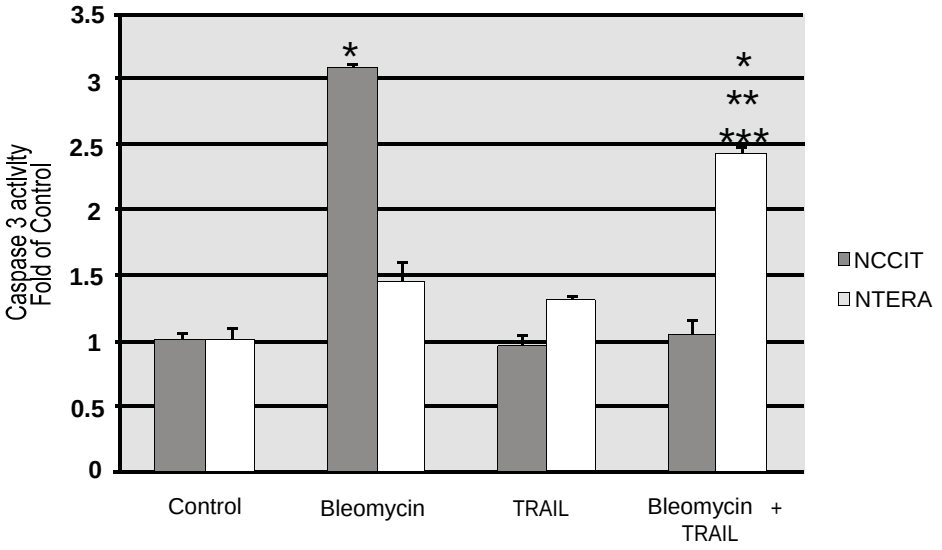

Fig. (6). Caspase 3 activation in NTera-2 and NCCIT cells incubated with either bleomycin, TRAIL, or bleomycin+TRAIL. *p<0.01 vs control; **p<0.05 vs bleomycin; ***p<0.01 vs TRAIL.

DISCUSSION

As in other cancer types, specificity and therapeutic effects of the current treatment methods in testis cell cancers need to be improved. Although bleomycin, etoposide, and cisplatin (BEP) combined therapy is successful in treating testicular cancer, both chemotherapy and radiotherapy may lead to undesired side effects such as serious lung damage and adverse effects on spermatogenesis and sperm chromatin quality [4, 24]. Thus, further investigation is required to develop new therapeutic approaches for highly effective lesser doses of chemotherapeutic applications, and safer and more targeted treatments with minimized side effects. TRAIL is the most widely studied molecule in cancer treatment regimens. The fact that TRAIL can induce cancer cell apoptosis without affecting physiologically normal cells, and its p53-independent mechanism of action makes TRAIL a suitable candidate for new therapeutic approaches, including combinatory treatments with known chemotherapeutics [25]. We used NTera-2 cells as a representative of seminomatous germ cell tumors, and NCCIT cells to represent nonseminomatous germ cell tumors, to test the efficiency of separate and combined applications of bleomycin and soluble TRAIL ligand.

Although TRAIL is known to have a selectively apoptotic effect on cancer cells, but not on normal cells, some cancer cells appear resistant to TRAIL's apoptotic effects. This resistance to TRAIL in normal cells and some cancer cell types may result from differential expression rates of TRAIL death and decoy receptors on the cell surface, and/or from high levels of intracellular antiapoptotic molecules [26]. The receptors themselves can also trigger anti-apoptotic systems inside the cells. High expression levels of cell surface decoy receptors TR/3 and TR/4 are particularly crucial in TRAIL resistance [27]. We observed that NTera-2 and NCCIT cells were resistant to TRAIL's apoptotic effect as indicated by higher LD_{50} doses compared to other cancer cells (5 µg/ml and 4 µg/ml, respectively). On the other hand, McKee *et al.*, reported a three-fold increase in baseline apoptosis in rat testicular germ cells obtained from primary testis explants, following addition of only 0.5 µg/ml TRAIL [28]. In search for a clue for mechanism of resistance, we analysed TRAIL receptor levels expressed on the cell surface. While basal TRAIL death receptor TR/2 and decoy receptor TR/4 expressions were in substantially high levels, TR/1 and TR/3 expressions were negligible in both testis cancer cell lines. The higher rate of TR/4 expression in both cell lines might be responsible for TRAIL resistance, since high expression of decoy receptors, rather than low expression of death receptors are known to be more effective in TRAIL resistance.

Chemotherapeutic agents are known to enhance the sensitivity of tumor cells to TRAIL *via* increasing expression of TR/1 and/or TR/2 death receptors [29-31]. In a study by Seitz *et al.*, bleomycin was reported to increase apoptosis through intrinsic and extrinsic pathways in hepatocellular carcinoma. It induced caspases 2, 3, 5, 6, 8, and 9, while upregulated CD95, TNF-R1, TR/1 and TR/2 in Hep3B cells. Inhibition of TR/1 and TR/2 by specific blockers significantly decreased apoptosis induced by DNA-damaging agents [32]. On the other hand, chemotherapeutic agents enhanced TRAIL sensitivity of cancer cells without affecting the expression levels of the related cell surface receptors [33, 34]. In another study, no increase in TR/1 and TR/2 receptor levels were reported following bleomycin application compared to the control groups in the lymphoma cell line U937. Inhibition of TRAIL receptors did not interfere with bleomycin-induced apoptosis suggesting presence of a TRAIL receptor-independent mechanism for the apoptotic action of bleomycin [35]. Two other studies by the same researchers revealed that bleomycin-induced apoptosis occurred through caspase pathway, yet was independent of TRAIL receptors [36, 37].

In our study, we investigated whether basal receptor expression levels of TRAIL receptors were altered in NCCIT and NTera-2 cell lines following incubation with either bleomycin, TRAIL, or their combination. We observed an increase in TR/1, TR/2 and TR/3 receptor levels in NTera-2 cell line incubated with bleomycin for 24, 48 and 72 hours, but an increase in TR/1 receptor level only at 24 h in NCCIT cell line. No change in TRAIL receptor expression levels was observed in either cell line following incubation with TRAIL. Bleomycin's increasing effect on TR/1 and TR/2 receptor levels in Ntera-2 cells is in accordance with increased sensitivity of this cell line to TRAIL-mediated apoptosis following the combined application. TR/2 is the major death receptor reported to increase by the effects of various chemoterapeutics. Although TR/1 and caspase 3 levels were increased following bleomycin treatment in NCCIT cells, application of TRAIL and bleomycin together did not increase apoptosis in NCCIT cells as indicated by the low caspase 3 levels. In fact, caspase 3 activation was repressed by the combined application of the two agents in NCCIT cells. While it is hard to

suggest a particular mechanism for this observation, this result might be due to the activation of intracellular anti-apoptotic mechanisms by the combined treatment in NCCIT cells. It is well known that the potential of chemotherapeutics to improve TRAIL sensitivity may vary in different cell types, as the characteristics of the cancer cell type obviously play a very important role in the success of an anti-cancer therapeutic regimen. Accordingly, in contrast to NCCIT cells, application of TRAIL and bleomycin together increased caspase 3 activity in NTera-2 cell line.

CONCLUSION

We conclude that the application of TRAIL ligand with bleomycin in testis cell cancers, has a potential to increase the therapeutic effect at least in a subset of testis tumors. While further *in vitro* and *in vivo* research is required, the combination of TRAIL and bleomycin may be evaluated as a new therapeutical strategy that may improve life standard and quality of testis cancer patients, *via* utilizing the selective apoptotic effect of TRAIL on tumor cells, and minimizing side effects.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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The content is solely the responsibility of the authors. T. Ozben designed the study. M. Timur, AD. Sanlıoglu and A. Cort wrote the manuscript and designed the experiments. S. Sanlioglu contributed important reagents. E. Ozdenir, M. Timur, A. Cort, and SB. Sarıkcıoglu performed the experiments. This work has been supported by Akdeniz University and TUBITAK [COST-CM0603-15(107S291)].

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