Combining radiotherapy with death ligands in cancer treatment: feasibility and molecular mechanisms
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TRAIL enhances efficacy of radiotherapy in a p53 mutant, Bcl-2 overexpressing lymphoid malignancy

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Background and purpose: Resistance to apoptosis is a contributing factor in the response to radiotherapy. Aim of this study was to evaluate whether TRAIL – in a soluble isoleucine zipped form – enhances the cytotoxic effect of irradiation on tumour cells with a blockade in the mitochondrial apoptosis route and/or a dysfunctional p53 pathway. Materials and methods: The p53 mutant human T acute lymphoblastic leukemia line Jurkat transduced with the Bcl-2 gene was used as model system in vitro and in a subcutaneous transplant setting in immunodeficient mice. Sensitivity to single and combined treatment was read out by apoptosis hallmarks and clonogenic survival in vitro, and by bioluminescence and palpation in vivo. Results: Jurkat cells overexpressing Bcl-2 did not undergo apoptosis after irradiation, but the combination with TRAIL synergistically induced apoptosis without breaking mitochondrial resistance. TRAIL also reduced clonogenic survival after irradiation. In vivo, radiotherapy or TRAIL alone delayed tumour outgrowth, but combination treatment had the most profound effect. Conclusions: Isoleucine zipped TRAIL can strongly enhance the efficacy of tumour therapy with ionising radiation in an unfavourable setting of p53 mutation and Bcl-2 overexpression.

Introduction

The anti-tumour effect of radiotherapy can be improved by combined modality treatment. Combination therapy with conventional anti-cancer drugs, such as cisplatin, is standard of care for an increasing number of tumour types [1]. However, a need exists for drugs that achieve higher cytotoxic efficacy, bypassing death resistance in tumour cells, while leaving normal tissues unaffected. The death receptor ligand TRAIL is an interesting candidate for combination with radiotherapy. Caspases are proteolytic enzymes that initiate and execute the apoptotic program. Death receptors can directly activate inducer Caspase-8 and/or -10 at their cytoplasmic tail [2]. Caspases-8/10 in turn activate effector caspases. All other apoptotic stimuli are reliant on the mitochondrial pathway for caspase activation, which is regulated by the Bcl-2 protein family. Apoptotic stimuli first activate certain BH3-only members of this family, which subsequently stimulate their relatives Bak and/or Bax to induce mitochondrial outer membrane permeability. Among the pro-apoptotic factors that are consequently released into the cytosol, Cytochrome c (Cyt c) acts as cofactor for Caspase-9 activation. Inhibitory Bcl-2 family members block the function of BH3-only proteins and Bak/Bax and thereby prevent apoptosis [3]. Death receptors target the mitochondrial pathway for caspase activation via the BH3-only protein Bid. They require the mitochondrial pathway to execute apoptosis in some cell types, but not in others, depending on the efficacy of Caspase-8 activation [4]. Ionising radiation (IR) induces DNA damage, which can activate the p53 pathway, p53 triggers cell cycle arrest to allow for DNA repair, but may also mediate apoptosis [5]. This proceeds via p53 targets such as BH3-only proteins Puma and Noxa [6, 7]. In absence of p53, IR can also induce apoptosis via Puma and Noxa [8, 9]. Both p53-dependent and -independent IR-induced apoptosis pathways are blocked by Bcl-2, indicating that they are reliant on the mitochondrial route [10]. In such a scenario, with IR operating via the mitochondria and TRAIL being able to bypass the mitochondrial pathway, it can be envisioned how combined treatment can increment the apoptotic response as compared to single treatment. Moreover, p53 can also upregulate TRAIL receptor-1 (TRAILR-1) and TRAILR-2 as well as Caspase-10 [11-13], which may bring about synergistic effects. TRAIL is a homotrimeric membrane-bound Tumour Necrosis Factor (TNF) family member [14]. For therapeutic purposes, it is produced in soluble form by truncation carboxy-terminal of its transmembrane segment. Multimerisation of trimers seems crucial to achieve an apoptotic effect on some cell types. This can be achieved by adding exogenous sequences such as a leucine zipper, but native TRAIL can also multimerise, depending on purification conditions [15]. In vitro, soluble human TRAIL in native [16] or leucine zipped version [17] killed a variety of tumour cell lines, many of them notoriously resistant to conventional anti-cancer drugs and IR. In contrast, normal cells appeared resistant to TRAIL. In mice, neither TRAIL preparation showed toxicity, while xenografted human tumours regressed [16, 17]. Native TRAIL was also non-toxic in cynomolgus monkeys [18]. This pioneering work made TRAIL an interesting lead for cancer therapy. In this study, we have investigated the therapeutic efficacy and potential toxicity of a novel form of recombinant soluble human TRAIL – isoleucine zipped (IZ)-TRAIL – in combination with IR on xenografted Jurkat cells overexpressing Bcl-2. Moreover, we have examined the apoptotic pathway that is employed upon combined treatment.

Results

IR-induced apoptosis in J16 cells is p53-independent

Functional analysis has shown that J16 cells contain
one wild-type and one mutant p53 allele [19]. To investigate whether the remaining functional p53 allele affected apoptotic responses to DNA damaging anti-cancer regimens, p53 was downregulated by RNA interference (RNAi) (Fig. 1a, insert). Cells were treated with different doses of IR (Fig. 1a), the topoisomerase inhibitor etoposide (Fig. 1b), or TRAIL (Fig. 1c). As examined by nuclear fragmentation, both magnitude and kinetics of apoptosis induced by IR and etoposide were unaffected by p53 downregulation. As expected, TRAIL-induced apoptosis was also not affected. We conclude that J16 cells undergo apoptosis in response to IR and etoposide in a p53-independent manner.

**Combined treatment does not break mitochondrial resistance**

From our results, we hypothesized that IR sensitises J16-Bcl-2 cells to TRAIL-induced apoptosis. The TRAIL signalling pathway in this case might bypass the mitochondria, or overrule inhibition by Bcl-2. To test whether mitochondrial resistance was broken upon combined treatment, we determined the extent of Cyt c release. Empty vector-transduced J16 cells were used as positive control. J16-Bcl-2 cells were first irradiated and 15 h later stimulated with TRAIL. In this setting, Cyt c release was minimal and not significantly greater than upon single treatment (Fig. 3a). In contrast, Caspase-3 activation by combined treatment was clearly more than additive at the same time point (Fig. 3b). We conclude that after irradiation of J16-Bcl-2 cells, TRAIL can more efficiently activate Caspase-3 independent of the mitochondria (Fig. 3c).

**TRAIL reduces clonogenic survival after IR**

To investigate combined effects of TRAIL and IR on cell survival, we also used long-term clonogenic assays that incorporate effects on cell cycle activity, apoptotic and non-apoptotic death [5]. Colonies formed in methylcellulose were counted at day 11 and survival curves were generated (Fig. 4). Clonogenicity in medium (42%) versus clonogenicity in presence of TRAIL only (3%) were both set at 100% for the

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**Figure 1.** Jurkat cells undergo apoptosis in a p53-independent manner. Dose response curves are shown for J16 after treatment for 16 or 24 h with (A) IR, (B) etoposide and (C) TRAIL. Inset in (A) shows p53 protein levels in J16 cells containing the p53 RNAi construct (+) or control vector (-). Actin served as loading control. Data are expressed as means of duplicate samples.

**Figure 2.** TRAIL synergizes with IR in J16 cells that overexpress Bcl-2.

(A) Vector transduced J16 cells (Control) and J16-Bcl-2 cells (Bcl-2) were treated with indicated doses of IR (Gy) and/or IZ-TRAIL (T, ng/ml). Data are expressed as means (+SD) of triplicate samples. (B) Isobolographic analysis for the indicated observed effects (‘e’) of IZ-TRAIL and IR in control and J16-Bcl-2 cells. The shaded area between the lines represents values of additivity. The dots indicate the actual combinations used to generate the observed effect on apoptosis at t = 48 h. Data points on the left of the area of additivity indicate synergy.
Efficacy of combined treatment with TRAIL and radiation

Survival curves, in which the effects of radiation were plotted. The steeper slope of the curve representing the combined treatment indicates that TRAIL reduced clonogenic survival of irradiated cells.

Combined effects of radiation and IZ-TRAIL on tumour growth in vivo

The effects of combined treatment in vitro prompted us to explore the clinical potential of this treatment in a xenograft setting. Immunodeficient mice were inoculated s.c. with J16-Bcl-2-Luc cells and treated with IR, IZ-TRAIL or with the combination. Caliper measurements showed that in control mice tumours grew steadily and reached the DEP of 12 mm at 27 (±3) days (Fig. 5a). Tumours of mice that were treated with 10 Gy local irradiation or IZ-TRAIL alone showed initial regression, followed by regrowth. They reached the DEP after 53 (±3) and 64 (±2) days, respectively (Fig. 5a and b), indicating a significant tumour growth delay as compared to controls ($p < 0.01$ and $p < 0.001$). Combined treatment with IR and IZ-TRAIL also resulted in initial tumour regression, but in this case no regrowth of the tumour was measurable by caliper up to 70 days after treatment.

The kinetics of tumour cell growth was also evaluated with bioluminescence imaging (illustrated in Fig. 5d). Tumour regression in the treatment groups was clearly revealed with this method. Mice that received single treatment with either IR or IZ-TRAIL showed a modest and transient (1–2 weeks) decrease in bioluminescence (Fig. 5c). In mice treated with the combination, bioluminescence was dramatically reduced to background level in between 2 and 4 weeks post-treatment (Fig. 5c and d). A slow increase in signal was seen in later weeks (Fig. 5c).

No normal tissue toxicity in vivo

Changes in body weight were used as an index of general toxicity (Fig. 6a). Body weights steadily increased in all groups. To examine potential liver toxicity, serum AST and ALT levels were determined on Day 11 and Day 25 (Fig. 6b and c). No significant changes in AST and ALT levels were observed in the different treatment groups as compared to buffer-injected controls. Thus, both general and organspecific toxicity were minimal.

Discussion

Evasion of apoptosis is a hallmark of cancer and may be an important factor in resistance to conventional anticancer treatment. This has been convincingly
demonstrated in a lymphoid malignancy where loss of the tumour suppressor p53 and/or overexpression of Bcl-2 promoted resistance to chemo- or radiotherapy in vivo [21]. In this study, we tested whether systemic treatment with a highly agonistic recombinant form of death ligand TRAIL [22] could improve the outcome of radiotherapy in a p53 mutant, Bcl-2 overexpressing lymphoid tumour model.

TRAIL is a very interesting candidate for combination treatment because of its proven capacity to induce apoptosis in a great variety of tumour tissues and its lack of normal tissue toxicity in preclinical models. Moreover, in many studies with human tumour lines in vitro, cell death was enhanced when TRAIL was combined with radiation. This has been demonstrated for haematopoietic cell types [23-25], but also for solid tumour types, such as breast [26, 27], lung [27] and colon carcinoma [28]. Combined effects have also been shown upon xenografting of human tumour cells in mice, in case of breast [26, 29] and prostate cancer [30]. Recombinant soluble TRAIL has also been combined with conventional DNA damaging chemotherapeutics in xenograft settings. Like for radiation, dramatic regressions and cures were only seen after combined treatments.

TRAIL has no cytostatic or genotoxic effects, but acts by inducing apoptotic cell death. Whereas wild-type Jurkat cells were sensitive to single treatments, cells overexpressing Bcl-2 resisted apoptosis to radiation or TRAIL. However, the combination resulted in a synergistic apoptotic response, as shown by isobolographic analysis. This is consistent with observations by Belka et al. [23] who used another form of recombinant TRAIL. In the Jurkat cell model, synergistic responses did not require a mitochondrial contribution, as Cyt c release was blocked but Caspase-3 was activated. In other cell types, the mechanism of synergy may be distinct. For instance, in prostate carcinoma cells, synergy between TRAIL and radiation depended on Bax proficiency, indicating an essential role of the mitochondrial route [31]. The mechanism of synergy in Jurkat cells is under investigation, but does not appear to rely on upregulation of the TRAIL receptor or other pro-apoptotic components (results not shown).

![Figure 4. TRAIL reduces clonogenic survival after IR. J16-Bcl-2 cells were treated with indicated doses of IR in presence or absence of 10 ng/ml TRAIL and cultured in duplicate in methylcellulose. Data are expressed as mean values of two independent observations. Data were fitted according to a second order polynomial model.](image)

![Figure 5. Inhibition of tumour growth in vivo by IR and/or IZ-TRAIL.](image)
Clonogenic assays are more adequate to assess cell survival after radiation than apoptosis assays, since they also incorporate the effects of cell cycle arrest and non-apoptotic death [5]. In the clonogenic assays, treatment with TRAIL alone reduced plating efficiency from 42% to 3%. The numbers of cells seeded in the combined treatment settings were adjusted to compensate for this. Clearly, the cytotoxic effect of the combined treatment is predominantly due to apoptosis. However, the survival curve also revealed an apparent radiosensitising effect of TRAIL.

The combined effects of IR and TRAIL on the p53 mutant, Bcl-2 overexpressing Jurkat tumour were subsequently evaluated in mice. Tumour cells were implanted in Matrigel, which prevented systemic spread and promoted tumour take. The recipient RAG2-/-; IL2Rc -/- mice have no lymphocytes or natural killer cells and therefore accepted this human xenograft. The subcutaneous localisation allowed for local irradiation and monitoring of tumour growth by palpation. In response to a single dose of 10 Gy, tumours displayed a significant growth delay. TRAIL alone also significantly delayed tumour growth. This can be explained by the fact that high doses of TRAIL and prolonged treatment can induce apoptosis via the mitochondrion-independent pathway (Fig. 3c). When radiation was combined with TRAIL, tumours regressed dramatically. Whereas tumours reached the end-point size around day 60 upon single treatments, tumours treated by combined modality showed no regrowth as determined by palpation. Bioluminescence was reduced to background levels for a period of 2 weeks, followed by a slow increase. Bioluminescence monitors the presence of metabolically active cells that can convert Luciferin and gives therefore not only quantitative, but also qualitative information on tumour status. At the DEP, tumours that were 12 mm according to calliper measurement did not show the same level of bioluminescence. This may be attributed to differences in tumour composition between treatment groups, in particular the presence of necrotic cells and/or scar tissue.

In addition to treatment efficacy, normal tissue toxicity determines the success of a novel treatment modality. Although body weight and liver enzymes did not show significant treatment-induced alterations, these data should be interpreted with caution, because we have found that TRAIL shows a certain degree of species-specificity (results not shown). Clearly, potential normal tissue toxicity deserves attention in future studies.

In summary, combined treatment with radiation and IZ-TRAIL in an unfavourable setting of p53 mutation and Bcl-2 overexpression, synergistically induced apoptosis and led to effective tumour regression in vivo. These results support the development of this combination treatment for future clinical application.

Materials and methods

Reagents

IZ-TRAIL was produced as described [22] and kept frozen in storage buffer (20 mM Tris–HCl, pH 8, 0.5 M arginine–HCl, 100 mM NaCl and 0.02% Tween 20). Soluble human FLAG-tagged TRAIL and enhancer were obtained from Alexis (Lausen, Switzerland) and etoposide from Sigma. Anti-Cyt c monoclonal antibody (mAb) (clone 6H2.B4) was from BD Biosciences (San Jose, CA), anti-p53 mAb (clone DO-1) from Santa Cruz Biotechnology (Santa Cruz, CA), anti-actin mAb (clone C4) from Chemicon International (Temecula, CA) and rabbit anti-active Caspase-3 from BD Biosciences. AlexaFluor 633-conjugated goat anti-mouse Ig and Alexa-Fluor 647-conjugated goat anti-rabbit Ig were from Molecular Probes (Leiden, The Netherlands). Horseradish peroxidase-conjugated rabbit anti-mouse Ig was from DAKO A/S (Glostrup, Denmark) and the enhanced chemoluminescence kit from Amersham Biosciences.

Cells and stimulation

The J16 clone was derived from the human T-acute lymphoblastic leukemia cell line Jurkat by limiting dilution [32]. J16 cells stably overexpressing Bcl-2 (J16-Bcl-2) were generated by retroviral transduction [20]. J16 cells and derivatives were cultured in Iscove’s modified Dulbecco’s medium (IMDM) with 8% foetal bovine serum (FBS). For apoptosis assays, J16 cells were stimulated with the indicated doses of TRAIL, etoposide, or IR in IMDM with 5% FBS. After addition of stimulus, cells were incubated for the indicated time periods at 37°C, 5% CO2. Irradiation of cells was performed using a 137Cs source.
and national guidelines and approved by the Experimental Animal Committee of The Netherlands Cancer Institute. For tumour cell inoculation, 5-week-old mice were anaesthetised with ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally (i.p.). Mice were injected subcutaneously (s.c.) in the midline of the lower back with 10^6 J16-Bcl-2-Luc cells, which had been resuspended in PBS and mixed with Matrigel™ Matrix Growth Factor Reduced (BD Biosciences) (1:1) in a final volume of 500 µl. Tumour size was measured by calliper and represented as mean diameter (length + width + height)/3. Treatment started when tumours reached a mean diameter of 8 mm (Day 0). Treatments with 4–6 mice per group consisted of: (1) 10 daily i.p. injections with 500 µl storage buffer (control); (2) 10 Gy local irradiation followed by 10 daily i.p. injections with storage buffer; (3) 10 daily i.p. injections with 500 µg IZ-TRAIL; (4) 10 Gy followed by 10 daily i.p. injections with 125 µg IZ-TRAIL. IZ-TRAIL injections were started on the same day as irradiation. Irradiation was performed in lead-shielding jigs, minimizing irradiation of normal tissue, with 250 kV X-rays, operating at 12 mA and filtered with 0.6 mm Cu. The dose rate at the position of the tumour was 2.35 Gy/min and mice were rotated through 180° halfway through each irradiation in order to maximize dose uniformity. A mean tumour diameter of 12 mm was set as designated end-point (DEP).

Bioluminescence

Tumour growth and response to treatment were monitored non-invasively using bioluminescence imaging. Bioluminescence was measured once or twice weekly using the IVIS 200 Imaging System (Xenogen Corporation, Alameda CA). An aqueous solution of D-Luciferin (150 mg/kg body weight; Xenogen) was injected i.p. Subsequently, mice were anaesthetised with isoflurane and placed in a lighttight imaging chamber. Eighteen minutes after injection, photons emitted by Luciferase-expressing tumour cells were acquired using Living Image® 4.02 for Windows (Graph Pad Software, San Diego, CA). Isobolographic analysis [23] was performed to determine synergy between IR- and TRAIL-induced apoptosis.

Normal tissue toxicity

To investigate normal tissue toxicity, mice were weighed five times a week. In addition, levels of aspartate- and alanine aminotransferase (AST and ALT) were determined in serum two days after the last i.p. injection (Day 11) and two weeks later (Day 25). AST and ALT levels were determined with enzyme-specific kits in a Hitachi 917 analyser (Roche Diagnostics) at the Department of Clinical Chemistry of The Netherlands Cancer Institute.

Statistics

Differences between treatment groups were analysed with paired Student’s t-tests and considered significant when \( p < 0.05 \). Analyses were performed using GraphPad Prism version 4.02 for Windows (Graph Pad Software, San Diego, CA). Isobolographic analysis [23] was performed to determine synergy between IR- and TRAIL-induced apoptosis.

References

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