Modulating cell-to-cell variability and sensitivity to death ligands by co-drugging

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Abstract
TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) holds promise as an anti-cancer therapeutic but efficiently induces apoptosis in only a subset of tumor cell lines. Moreover, even in clonal populations of responsive lines, only a fraction of cells dies in response to TRAIL and individual cells exhibit cell-to-cell variability in the timing of cell death. Fractional killing in these cell populations appears to arise not from genetic differences among cells but rather from differences in gene expression states, fluctuations in protein levels and the extent to which TRAIL-induced death or survival pathways become activated. In this study, we ask how cell-to-cell variability manifests in cell types with different sensitivities to TRAIL, as well as how it changes when cells are exposed to combinations of drugs. We show that individual cells that survive treatment with TRAIL can regenerate the sensitivity and death-time distribution of the parental population, demonstrating that fractional killing is a stable property of cell populations. We also show that cell-to-cell variability in the timing and probability of apoptosis in response to treatment can be tuned using combinations of drugs that together increase apoptotic sensitivity compared to treatment with one drug alone. In the case of TRAIL, modulation of cell-to-cell variability by co-drugging appears to involve a reduction in the threshold for mitochondrial outer membrane permeabilization.

Introduction

TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) is a member of the TNF family of death ligands that induces apoptosis via an extrinsic receptor-mediated cell death pathway (Ashkenazi 2008). TRAIL ligand and antibodies that function as receptor agonists are under investigation as anti-cancer drugs because of their ability to promote apoptosis in cancer cells while sparing normal tissue. However, many cancers are resistant to TRAIL-mediated apoptosis and others exhibit partial sensitivity, such that only a fraction of cells dies in response to treatment (Gonzalvez and Ashkenazi 2010). These and related factors have complicated the clinical development of TRAIL and TRAIL receptor agonists.

TRAIL induces apoptosis via binding to DR4/5 receptors on the surface of target cells (Gonzalvez and Ashkenazi 2010). Binding causes recruitment of death-inducing signaling complex (DISC) proteins to the intracellular tails of DR4/5 receptors and activation of initiator caspases-8/10 (Kischkel et al 1995, Martin et al 1998). In some cell types (Type I cells), cleavage of effector caspases-3/7 by caspase-8/10 is sufficient to trigger cell death, but most cells (Type II cells) require mitochondrial outer membrane permeabilization (MOMP) to undergo apoptosis (Barnhart et al 2003, Deng et al 2002, Sun et al 2002). MOMP is regulated by caspase-8/10 cleavage of Bid into tBid, followed by tBid translocation to the mitochondrial membrane where it activates pro-apoptotic Bcl-2 family proteins such as Bax/Bak (Eskes et al 2000).
When sufficient active Bax/Bak is present to overcome inhibition by resident anti-apoptotic Bcl-2 proteins, MOMP ensues, leading to release of Smac and cytochrome C into the cytosol (Li et al. 2002, Luo et al. 1998). Cytochrome C activates the caspase-9-containing apoptosome, while Smac displaces the inhibitor of apoptosis protein XIAP from caspase-3. These events result in a dramatic increase in effector caspase catalytic activity, ultimately leading to cleavage of the genome, proteome and consequent cell death (Deveraux et al. 1997, Riedl and Salvesen 2007).

Resistance to TRAIL is a natural feature of some cell types but may also be acquired following TRAIL treatment, and multiple mechanisms underlie resistance (Gonzalvez and Ashkenazi 2010, Johnstone et al. 2008). Mutation or downregulation of DR4/5 receptors or upregulation of DcR1/2 decoy receptors, which bind TRAIL but lack signaling domains, account for TRAIL resistance in some cases but are not broadly prognostic (Ashkenazi and Dixit 1999, Lee et al. 2001, MacFarlane et al. 2005). Changes in DISC signaling components, such as downregulation of caspase-8 or upregulation of the inhibitor protein c-FLIP, changes in the levels or activities of pro- or anti-apoptotic Bcl-2 family proteins, or changes in expression of IAP proteins such as XIAP can also cause resistance to TRAIL (Alldridge et al. 2011, Zhang and Fang 2005). Survival signaling pathways, such as those mediated by the NF-κB transcription factor or pro-survival kinases, are also implicated in resistance (Falschlehner et al. 2007). Finally, it has been shown that post-translational modification of DR4/5 receptors affecting clustering and subsequent recruitment of DISC proteins can determine whether cells respond to treatment with TRAIL and whether they subsequently activate death or survival pathways (Mazurek et al. 2011, Song et al. 2007, Wagner et al. 2007).

Using combinations of drugs is widely considered to be a promising strategy for overcoming resistance to TRAIL and increasing tumor cell killing (Hellwig and Rehm 2012, Johnstone et al. 2008). With the exception of cancers in which essential components of the extrinsic cell death pathway are mutated or silenced (resulting, for example, in inactivation of TRAIL receptors or caspase-8), co-drugging has been shown to increase the efficacy of TRAIL in cell lines, tumor models and patients (Ashkenazi and Herbst 2008). Sub-lethal doses of chemotherapy or ionizing radiation are known to sensitize resistant cancer cells to TRAIL-induced apoptosis via modulation of TRAIL receptors, DISC or mitochondrial components (Broaddus et al. 2005, Di Pietro et al. 2001, Ehrhardt et al. 2008, Galligan et al. 2005, Ganten et al. 2004, Keane et al. 1999, Morizot et al. 2010). Drugs that inhibit histone deacetylases or the proteasome (which modulate gene expression and protein stability, respectively) have also been shown to be effective in overcoming resistance to TRAIL in cell lines and tumor models (Bagci-Onder et al. 2012, Brooks et al. 2005, Butler et al. 2006, Frew et al. 2008, Inoue et al. 2004, Kabore et al. 2006, Vanoosten et al. 2005). Drugs that target pro-survival or mitogenic kinases such as the EGF receptor inhibitor gefitinib and the multikinase inhibitor sorafenib sensitize resistant cancer cells to TRAIL through a variety of mechanisms involving downregulation of the anti-apoptotic proteins Mcl-1, c-FLIP and members of the IAP family (Kim et al. 2008, Ricci et al. 2007, Rosato et al. 2007, Shrado et al. 2007). Smac mimetics and XIAP inhibitors directly promote caspase-3 activity in resistant cell types (Bockbrader et al. 2005, Karikari et al. 2007). Finally, specific inhibition of Bcl-2 and Bcl-2XL using the small molecule inhibitor ABT-263 (or its clinical version ABT-737) has shown promise in combination with TRAIL in certain contexts (Cristofanilon and Fulda 2012, Hetschko et al. 2007, Huang and Sinicrope 2008, Lickliter et al. 2007, Song et al. 2008).

The importance of understanding drug sensitivity at the level of individual cells has been demonstrated in several recent studies (Cohen et al. 2008, Sharma et al. 2010) and this is also true for TRAIL (Bagci-Onder et al. 2012, Spencer et al. 2009). The dynamics of TRAIL-mediated apoptosis in single cells have been most extensively studied in HeLa lines using live-cell microscopy of cells expressing fluorescent reporters for MOMP and caspase activity (Albeck et al. 2008a, 2008b, Hellwig et al. 2008, Rehm et al. 2006). Timing of cell death following TRAIL treatment was shown to vary dramatically from one cell to the next, even within a single cell clone (Albeck et al. 2008a, Spencer et al. 2009). Within an individual cell, death was shown to be controlled by a ‘snap-action, variable-delay switch’ in which initiator caspases are slowly activated and effector caspases suddenly turned on only when MOMP is triggered and XIAP neutralized. The delay period is highly variable in length and its duration is determined by both the rate of initiator caspase activation and the threshold for MOMP, which is set by the levels of pro- and anti-apoptotic Bcl-2 proteins. However, once MOMP is triggered and effector caspases activated, cell death occurs at a rapid, relatively constant rate, in the order of minutes (Albeck et al. 2008a, Goldstein et al. 2000, Rehm et al. 2003). Variability in the timing of cell death is controlled in a non-genetic manner and appears to involve stochastic, naturally occurring differences in the concentrations of positive and negative regulators of apoptosis (Bhola and Simon 2009, Gaudet et al. 2012, Spencer et al. 2009). Recently, similar dynamics of caspase activation in single cells was demonstrated in glioblastoma cell lines exposed to TRAIL, and these dynamics were shown to be modulated by co-treatment with histone deacetylase inhibitors (Bagci-Onder et al. 2012). Thus, co-drugging appears to impact TRAIL sensitivity at the single-cell level but a detailed analysis of these effects has not been previously performed.

In this paper, we investigate how cell-to-cell variability in both timing of cell death and cell fate (death versus survival) manifests in cell lines having different intrinsic sensitivities to TRAIL, and we examine how cell-to-cell variability changes in response to therapeutic drugs individually and in combination. We first show how cell-to-cell variability affects the apparent sensitivity, at a population level, of a panel of cell types treated with TRAIL. Second, we show in multiple cell types that cells surviving treatment with TRAIL or other inducers of apoptosis regenerate the sensitivity of the original cell population within several days of outgrowth. This demonstrates that fractional killing is a stable, self-generating property of cell populations. Third, we examine the effects of co-drugging on cell-to-cell variability and show that variability is reduced when cells...
are sensitized to TRAIL using a variety of drugs. Finally, we demonstrate that modulating cell-to-cell variability contributes to sensitization to TRAIL-mediated apoptosis at least in part by lowering the threshold for MOMP.

Results

Net sensitivity to death ligands reflects variability in timing of cell death combined with heterogeneity in cell fate

To explore the relationship between cell-to-cell variability and sensitivities to death ligands, we treated a panel of cell lines with recombinant TRAIL (50 ng ml\(^{-1}\)) and monitored death times by live-cell microscopy using either the time of MOMP, as measured with an RFP-based reporter of mitochondrial outer membrane permeabilization (IMS-RP), or cellular morphology, which changes dramatically within minutes of MOMP (Albeck et al 2008a) (figure 1(a)). Cells that survived beyond a typical 16 h movie were considered to be ‘survivors’ since we observed that such cells were able to recover and successfully divide when TRAIL was removed. In agreement with previous reports, we found that primary endothelial cells (HUVEC) were almost completely resistant to TRAIL (90% surviving a 50 ng ml\(^{-1}\) treatment) and breast cancer cell lines (Hs578T and T47-D) were only slightly more sensitive, with 85% and 65% survivors, respectively. Colon cancer lines exhibited greater variability in response: HCT116 cells were relatively sensitive (10–15% survivors) and HT29 cells relatively resistant (80% survivors). Non-transformed but immortalized MCF10A mammary epithelial cells were also sensitive at this dose of TRAIL (10–15% survivors) (figure 1(b); (Ruhman et al 2009, Secchiero et al 2003, van Geelen et al 2003)). At a population level, sensitivity to TRAIL is a measure of heterogeneity in cell fate (death versus survival), with some cell lines exhibiting a more even distribution between the two fates, and thus more heterogeneity in cell fate, than others.

To explore the effects of variability in time of death on heterogeneity in fate, populations of MCF10A and T47-D cells were exposed to TRAIL at a range of doses and imaged for 18 h by live-cell microscopy. From the distribution of death times (figures 1(c)–(d)), the percentage of cells still alive at 2 h, 5 h or 18 h was determined and plotted as a set of time-resolved dose–response curves (figures 1(e)–(f)). Time was an important factor in determining sensitivity in the case of highly sensitive MCF10A cells (with the IC50 varying from 55 ng ml\(^{-1}\) at 2 h and 45 ng ml\(^{-1}\) at 5 h to 10 ng ml\(^{-1}\) at 18 h). In contrast, for T47-D cells, time was less important: T47-D cells either died within 6 h of TRAIL exposure or survived indefinitely. Thus, variability in the timing of cell death affects the dose-dependent sensitivity of some cell types to TRAIL more than others.

Survivor cells regenerate the original sensitivity profile

To investigate the origins of incomplete cell killing, we treated cells with TRAIL, collected survivors, allowed them to grow for several days and then exposed them to a second TRAIL challenge. To minimize the impact of genetic heterogeneity on these measurements, we examined early-passage cell populations obtained by single-cell cloning. First, we treated HeLa cells with 50 ng ml\(^{-1}\) TRAIL and followed them for 16 h by live-cell microscopy, at which point about 20% of the cells were still alive. We then collected these survivors and expanded them for approximately 1 week in the absence of TRAIL. When the survivor population was re-challenged with 50 ng ml\(^{-1}\) TRAIL and imaged for 16 h, we observed that once again about 20% of the cells survived (figure 2(a)). Moreover, not only the fraction of survivors, but also the shape of the time of death distribution was indistinguishable from that of the parental population: the mean, median and variances of the time of death distributions did not vary significantly between control and survivor cells (figure 2(b)). Regeneration of cell line-specific death-time distributions upon outgrowth was observed for multiple cell types with varying degrees of sensitivity to TRAIL (figure 2(c) and figure S1(a) available at stacks.iop.org/PhysBio/10/035002/mmedia). HeLa cells surviving treatment with staurosporine also re-established the sensitivity profile of the starting population within 2 weeks of outgrowth, showing that the emergence of a sensitive cell population from an initially resistant population is characteristic of both intrinsic and extrinsic apoptosis inducers (figure S1(b) available at stacks.iop.org/PhysBio/10/035002/mmedia). We have previously shown that with respect to the timing of TRAIL-mediated cell death, cell-to-cell variability is dominated by non-genetic factors including the unequal distribution of proteins between sister cells (Spencer et al 2009). In the current study, we extend these observations by showing that cell populations exist in a dynamic state of “equilibrium” between sensitive and resistant states that are unlikely to differ in genotype. Moreover, the shape of the death-time distribution for a cell population varies from one cell type to the next (and from one drug treatment to the next) but appears to be a stable property of that line, since it can be regenerated from individual surviving cells.

Co-drugging decreases variability and increases sensitivity

One approach to sensitizing resistant tumors to TRAIL is to co-treat with a second drug; many general and targeted chemotherapeutics have been shown to work in this way, at least in some cell lines (Ashkenazi and Herbst 2008, Holloway and Rehm 2012). To examine the effects of co-drugging on cell-to-cell variability in response to TRAIL, we treated HeLa cells expressing a reporter for MOMP (IMS-RP) (Albeck et al 2008a) with TRAIL alone or with TRAIL in combination with sorafenib, paclitaxel, gefitinib or ABT-737. We observed that all four drugs sensitized HeLa cells to TRAIL-induced death (Johnstone et al 2008), increasing the fraction killed at 16 h from ∼80% to 100% at 50 ng ml\(^{-1}\) TRAIL (as assayed by whether a cell had undergone MOMP by the 16 h timepoint) (figures 3(a)–(d)). In contrast, HeLa cells were relatively resistant to sorafenib and paclitaxel alone, and a significant fraction also survived exposure to gefitinib or ABT-737 alone. In all cases, the timing of cell death was altered by co-drugging, with a significant decrease in the mean and median death times (p < 0.001 for all combinations tested versus TRAIL).
Figure 1. Variability in timing of death and cell fate in different cell types treated with TRAIL. (a) Representative microscopy images (20×, N.A. = 0.75) depicting time of MOMP and the morphological changes used to score cell death in (b). Images are of HeLa cells expressing the MOMP reporter, and time (minutes) is indicated relative to time of MOMP (t = 0) for the cell indicated by an arrow. For this cell, morphological changes associated with death began occurring within approximately 9–12 min of MOMP. (b) Death-time distributions and percentage of surviving cells for primary human endothelial cells (HUVEC), MCF10A cells and four cancer cell lines (HT29, HCT116, Hs578T and T47-D) treated with TRAIL (50 ng ml\(^{-1}\)) and imaged at 3 min intervals for 16 h. The red bar at 16 h represents the percentage of cells surviving at the end of the 16 h movie (right side of the dotted line). At least 100 cells were analyzed for each cell type. (c)–(d) Death-time distributions and percentage of surviving cells for MCF10A and T47-D cells treated with a range of doses of TRAIL and imaged by live-cell microscopy at 5 min intervals for 18 h. The percentage of surviving cells at the end of the 18 h movie is plotted to the right of the dotted line. 100 cells were analyzed for each condition. (e)–(f) Dose–response curves calculated from the time of death distributions. The percentage of surviving cells at three timepoints (2 h, 5 h and 18 h) across a range of doses was calculated from the time of death distributions and plotted as a dose–response curve.

Variability in death time was also reduced in co-drugged cells compared to cells treated with a single agent. To quantify this effect we estimated the interquartile range (IQR) of death times for cells that underwent apoptosis (this
Figure 2. Survivor cells re-acquire sensitivity to TRAIL and regenerate the time of death distribution of the original cell population. (a) HeLa cell survivors of a 16 h TRAIL treatment (50 ng ml$^{-1}$) (red bar and oval, left-hand panel) were grown for 6 days in the absence of TRAIL and then re-treated with the same dose of TRAIL (right-hand panel). The cells were imaged by live-cell microscopy and time of death was scored based on morphological changes associated with cell death. Death-time distributions (blue bars) and the percentage of surviving cells at the 16 h timepoint (red bars, right side of dotted line) are plotted for both the primary treatment and the treatment of survivor cells. (b) Quantitation of the death-time distributions of primary treatment control (naive) and survivor cells from (a) monitored on the same day. Cells surviving at the 16 h timepoint are excluded from the analysis. Neither the mean (red crosses), median (lines through boxes) nor variances of the death times were significantly different ($p > 0.05$ for unpaired Welch’s $t$-test for distribution means, Mann–Whitney test for medians of non-Gaussian distributions, $F$ test for variances). At least 200 cells were analyzed for each sample. (c) Time of death distributions and percentage of surviving cells plotted for control (naive) and survivor MCF10A cells (6 days after the initial treatment), treated with 50 ng ml$^{-1}$ TRAIL for 16 h and imaged by live-cell microscopy.

is a more appropriate measure of variability than coefficient of variation because the death time histograms were not normally distributed). Cells treated with 50 ng ml$^{-1}$ TRAIL alone had an average IQR of 5.3 ± 0.9 h across all experiments, falling to 0.2–1.7 h in co-drugged cells (figures 3(e)–(h), and table 1). The effect was particularly striking for sorafenib which had little impact on its own but dramatically reduced variability in time of death when combined with TRAIL. We observed similar effects in other cell types such as MCF10A cells co-treated with TRAIL and gefitinib or sorafenib (figure S2 available at stacks.iop.org/PhysBio/10/035002/mmedia) and in U251 glioblastoma cells co-treated with TRAIL and the HDAC inhibitor MS-275 (Bagci-Onder et al 2012). Thus, co-drugging increases sensitivity to TRAIL at least in part through a reduction in death time variability among individual cells.

Blocking protein synthesis decreases mean death time and variability in death time, while stabilizing protein levels reverses this effect

Sub-lethal concentrations of the protein synthesis inhibitor cycloheximide (CHX) are well known to sensitize many types of cells to TRAIL-induced apoptosis (Albeck et al 2008a, Kang et al 2003). This effect is dramatic in HeLa cells which
Figure 3. Co-drugging sensitizes cells to TRAIL-mediated apoptosis and reduces variability in timing of cell death. (a)–(d) Time of death distributions and percentage of surviving HeLa cells treated with TRAIL alone (50 ng ml$^{-1}$ in (a), (c), (d); 10 ng ml$^{-1}$ in (b)), with TRAIL (50 ng ml$^{-1}$) + sorafenib (10 μM; (a)); TRAIL (10 ng ml$^{-1}$) + paclitaxel (300 nM; (b)); TRAIL (50 ng ml$^{-1}$) + gefitinib (10 μM; (c)); TRAIL (50 ng ml$^{-1}$) + ABT-737 (10 μM; (d)), or with each drug alone, monitored for 16 h by live-cell microscopy. For paclitaxel, cells were pre-treated with the drug for 24 h to arrest them in mitosis prior to TRAIL addition at the start of the movie (in the continued presence of paclitaxel). Cells surviving at the end of the movie are plotted to the right of the dotted line. (e)–(h) Quantitation of death times for dying cells only. Box plots represent the spread of the data; the box spans the IQR, with the median indicated as a line through the box; bars span the range of the data, from the minimum to the maximum death time. 50–100 cells were analyzed for each condition.

exhibit both dose-responsive cell killing by TRAIL and dose-responsive sensitization to TRAIL with CHX (figure 4(a)). Several explanations have been suggested for this sensitizing effect, including a reduction in the production of pro-survival factors, particularly those that are short-lived or induced by TRAIL treatment, or activation of a JNK-mediated stress response (Clohessy et al 2006, Kang et al 2003, Kreuz et al 2001, Sah et al 2003, Wajant et al 2000). To study the effects of CHX on variability in death time, HeLa cells were exposed to TRAIL (50 ng ml$^{-1}$) with or without the addition of CHX (2.5 μg ml$^{-1}$). Under these conditions, mean death time fell significantly ($p < 0.0001$ (Albeck et al 2008a))
Cycloheximide reduces mean death time and variability in death times, and proteasome inhibition partially reverses this process. 

(a) Dose–response curves of HeLa cells treated with increasing doses of TRAIL ± increasing doses of CHX for 4 h, monitored for cell death by flow cytometry using an antibody that recognizes cleaved PARP. (b) Time of death distributions and percentage of surviving HeLa cells treated with TRAIL (50 ng ml\(^{-1}\); yellow), TRAIL + CHX (2.5 \(\mu\)g ml\(^{-1}\); blue) or TRAIL + CHX + MG-132 (10 \(\mu\)M; red) for 16 h and imaged by live-cell microscopy. Per cent of cells surviving at the end of the movie is plotted to the right of the dotted line. Shown is a representative of three independent experiments that yielded similar results. (c) Quantitation of the spread of the death times for cells pre-treated with CHX for 2 h prior to treatment with TRAIL + CHX, compared to co-treatment with TRAIL + CHX or treatment with TRAIL alone. (d) Quantitation of the spread of the death times in (b). Median, IQR and range of death times for each treatment appear in the box and whisker plots as described for figure 3. 50–100 cells were analyzed per condition.

and IQR decreased from \(~4.1\) h for TRAIL alone to \(1.3\) h for TRAIL + CHX (these curves passed a normality test, allowing us to estimate a CV of 40\% for TRAIL versus 26\% for TRAIL + CHX; figures 4(b)–(c); table 1). The effect of CHX was enhanced when cells were pre-treated with CHX for 2 h prior to addition of TRAIL (IQR for TRAIL + pre-treatment with CHX was \(~0.2\) h; figure 4(c) and table 1). Since CHX acts rapidly to block potential TRAIL-induced protein synthesis, this implies that CHX is altering the basal state of the apoptotic machinery.

We reasoned that if degradation of short-lived anti-apoptotic proteins were important for the observed sensitization by CHX, then stabilization of these proteins using a proteasome inhibitor might counteract this effect. We found that this was the case: when HeLa cells were treated with TRAIL in combination with both 2.5 \(\mu\)g ml\(^{-1}\) CHX and 10 \(\mu\)M of the proteasome inhibitor MG-132, median death time increased \((p = 0.0002)\) and variability was also increased (IQR = 1.3 h for TRAIL + CHX versus 2.1 h for TRAIL + CHX + MG-132; figures 4(b), (d) and table 1). The shift induced by MG-132 did not fully recreate the death profile observed with TRAIL alone; this is consistent with the fact that MG-132 by itself sensitizes cells to TRAIL-induced apoptosis, due to the multiple targets of this inhibitor (Laussmann et al 2011, Sayers and Murphy 2006). A similar proteasome inhibitor-mediated delay in TRAIL+CHX-induced cell death has been observed by others (Laussmann et al 2012) and attributed to stabilization of the anti-apoptotic DISC protein FLIP. We conclude that degradation of short-lived anti-apoptotic proteins has a significant effect on the timing and probability of TRAIL-mediated cell death and that variability in death times can be modulated simply by altering the balance between protein synthesis and degradation. Since CHX and MG-132 affect the synthesis and degradation of many apoptotic regulators, including Mcl-1, FLIP and XIAP (Clohessy et al 2006, Kreuz et al 2001, Lane et al 2006), further work is required to determine precisely which proteins play the most significant roles in different cell types.

Sensitizing agents decrease the threshold for cell death

Previous work in our lab demonstrated that variability in factors upstream of MOMP has the greatest contribution to variability in timing of cell death; these factors can be divided...
Figure 5. Bcl-2 inhibition lowers the threshold for cell death. (a) Schematic illustrating sample IC-RP trajectories for individual HeLa cells (each indicated by a different color) treated with TRAIL (50 ng ml\(^{-1}\)). Arrows indicate levels of IC-RP activity (threshold, y-axis) at the time of MOMP (x-axis) for two individual cells. (b) Box plots indicating the MOMP threshold (height of the IC-RP FRET trajectory at the time of MOMP) for a population of 50–100 HeLa cells treated with the indicated doses of TRAIL and ABT-737. Initiator caspase FRET trajectories for individual cells were normalized to the minimum and maximum intensity for each trajectory, and the threshold was calculated as the normalized intensity at the time of MOMP. Box plots span the entire range of the data; means are plotted as crosses. (c) IC-RP trajectories of representative individual HeLa cells treated with TRAIL (50 ng ml\(^{-1}\)), ABT-737 (1 \(\mu\)M) or TRAIL + ABT-737 and imaged for 16 h by live-cell microscopy. The relatively flat trajectories in cells treated with ABT-737 alone (middle panel) indicate baseline activity of the reporter under conditions in which extrinsic cell death is not activated and pre-MOMP initiator caspase activity is absent. (d)–(e) Dot plots showing the MOMP threshold plotted relative to time of MOMP for individual cells treated with the indicated doses of TRAIL and ABT-737. Thresholds for ABT-737 treatment alone are plotted to indicate baseline activity of the reporter.
Table 1. Death time statistics for cells treated with TRAIL in combination with a second agent

| Treatment | Median (h) | IQR (h) | Range (h) | CV |
|-----------|------------|---------|-----------|----|
| TRAIL     | 1.5        | 0.5     | 6.0       | 111%|
| TRAIL+Sorafenib | 2.9  | 1.3     | 6.4       | 69% |
| Sorafenib | 3.6        | 2.2     | 7.6       | 74% |
| TRAIL+CHX | 4.3        | 2.1     | 6.3       | 40% |
| TRAIL+ Pre-treat | 8.5  | 0.2     | 1.5       | 40% |

Storage of data to the box plots and time of death distributions in figures 3-4. All data were generated in HeLa cells. Data are median death time (hours) for dying cells (excluding surviving cells), followed by the IQR and range of the spread of the data and the coefficient of variation (CV).

Discussion

Extensive literature exists on the sensitivity of cells to death ligands such as TRAIL and FasL individually and in combination with various anti-cancer drugs. The vast majority of these papers involve endpoint, population-average measures of apoptosis in combination with detailed biochemical or genetic analysis of a few apoptotic regulators. In this paper, we ask whether dynamic, single-cell assays such as those used to develop mathematical models of receptor-mediated apoptosis in genetically diverse tumor models with and without drug-co-drugs. Our focus on assaying key reactions that control extrinsic apoptosis in individual cells provides a window into the physiologic impact of cell death and sets the stage for physicochemical modeling of combination therapy. By examining diverse cell lines that cover a range from TRAIL-sensitive to relatively resistant we observe a diversity of death-time distributions. In all lines examined, we find that cells can switch between states of sensitivity and resistance over a time period of several days and that fractional killing is a stable property of specific cell types. Cells from a sensitive line that survive an initial TRAIL challenge give rise, within several days of outgrowth, to a population of cells with the same degree of sensitivity and distribution of death times; the same is true of relatively resistant lines. Variability in the time at which single cells

anti-apoptotic proteins (Callus et al. 2008, Davids et al. 2012, Letai 2008) and thus might be expected to alter the threshold in particular. As a positive control for this hypothesis, we first measured the MOMP threshold in cells treated with TRAIL and ABT-737, a Bcl-2/Bcl-XL inhibitor that blocks binding of Bcl-2/Bcl-XL to pro-apoptotic Bcl-2 proteins, as compared to treatment with TRAIL alone. When HeLa cells expressing IC-RP and the MOMP reporter were treated with TRAIL plus ABT-737 and analyzed by live-cell microscopy, we observed a decrease in the MOMP threshold that was dose dependent for ABT-737 (figures 5(b)–(c)). Treatment with ABT-737 alone did not lead to a significant increase in pre-MOMP initiator caspase activity over time, even in cells that died (as would be expected for a drug that does not activate extrinsic cell death; figure 5(c), middle panel); thus, the threshold for ABT-737 alone is plotted only to indicate the baseline activity of the reporter (figures 5(d)–(e)). An overall decrease in the threshold was observed when ABT-737 was combined with TRAIL compared to treatment with TRAIL alone, even at matched MOMP times (figures 5(d)–(e)).

Next, we monitored initiator caspase activity and MOMP in HeLa cells treated with TRAIL plus a variety of other anti-cancer drugs. Sorafenib in combination with TRAIL was particularly interesting since it did not kill HeLa cells on its own even at the highest dose tested (10 μM for 16 h). Co-treatment with sorafenib led to a dose-dependent decrease in the MOMP threshold as compared to TRAIL alone (figure 6(a)). Combinations of TRAIL (50 ng ml⁻¹) plus gefitinib (10 μM) and TRAIL (50 ng ml⁻¹) plus paclitaxel (300 nM, with a 24 h paclitaxel pre-treatment) also reduced the threshold for MOMP compared with TRAIL treatment alone (figures 6(b)–(c)); thresholds for gefitinib and paclitaxel alone are plotted to indicate baseline activity of the reporter, which exhibited little or no change in response to either drug alone; figures 6(d)–(e)). Thus, sorafenib, gefitinib and paclitaxel reduced the threshold for TRAIL-induced MOMP, presumably by affecting the levels or states of pro- and anti-apoptotic mitochondrial proteins. Treatment of cells with TRAIL + CHX also reduced the threshold compared with TRAIL treatment alone, demonstrating that inhibition of protein synthesis—and a consequent reduction in the levels of short-lived anti-apoptotic mitochondrial proteins—contributes to a reduction in the MOMP threshold (CHX alone did not kill any cells and is excluded from the plot; figure 6(f)). Thus, co-drugging with the agents tested led to more rapid and less variable death times than death by TRAIL alone at least in part by lowering the threshold for MOMP.
Figure 6. Co-drugging lowers the threshold for cell death. (a) Box plots indicating the MOMP threshold for a population of 50–100 HeLa cells treated with the indicated doses of TRAIL and sorafenib and analyzed as in figure 5. Boxes span the 10th–90th percentile of the data; means, plotted as crosses, are significantly different (p < 0.05). (b) Box plots indicating the MOMP threshold for HeLa cells treated with TRAIL (50 ng ml\(^{-1}\)), gefitinib (10 \(\mu\)M) or TRAIL + gefitinib for 16 h. (c) Box plots indicating the MOMP threshold for HeLa cells treated with TRAIL (50 ng ml\(^{-1}\)) alone or pre-treated with paclitaxel (300 nM) for 24 h and then treated with TRAIL + paclitaxel or paclitaxel alone for an additional 16 h. (d) Representative FRET trajectories (colored lines, left panel) and MOMP thresholds (colored dots, right panel) for individual HeLa cells treated with TRAIL (50 ng ml\(^{-1}\); blue lines and dots), gefitinib (10 \(\mu\)M; pink lines and dots); or TRAIL + gefitinib (green lines and dots) for 16 h. (e) Representative FRET trajectories (colored lines, left panel) and MOMP thresholds (colored dots, right panel) for individual HeLa cells treated with TRAIL (50 ng ml\(^{-1}\); blue lines and dots), paclitaxel (300 nM; pink lines and dots); or TRAIL + paclitaxel (green lines and dots) for 16 h. Paclitaxel-treated cells were pre-treated with paclitaxel for 24 h. (f) Box plots indicating the MOMP threshold for HeLa cells treated with TRAIL (50 ng ml\(^{-1}\)) with or without CHX (2.5 \(\mu\)g ml\(^{-1}\)) for 16 h; CHX alone did not kill any cells and is excluded from the plot. In (b), (c) and (f), box plots span the entire range of the data.
in a population die is likely to be determined in a non-genetic fashion (Spencer et al 2009), whereas the shape of the death-time distribution appears to be a stable property of a particular cell line undergoing treatment with a particular dose of TRAIL. Anti-cancer drugs that increase sensitivity to TRAIL modulate the timing of cell death, at least in part by lowering the threshold for MOMP.

We and others have previously demonstrated that individual HeLa cells in a clonal population exposed to death ligands such as TRAIL and FasL differ markedly in time at which they die and that even at high ligand doses, a fraction of cells survives (Albeck et al 2008a, Neumann et al 2010, Spencer et al 2009). Fractional killing has also been observed following treatment of cells with agents that activate intrinsic cell death pathways (Cohen et al 2008, Gascoigne and Taylor 2008) and we find that it is associated with variability in time of death that is qualitatively similar to that observed with TRAIL (figure 3 and figure S1(b) available at stacks.iop.org/PhysBio/10/035002/mmedia). However, the connection between variability in time of death and fractional killing has not been previously examined in detail for either extrinsic or intrinsic apoptosis. In population-based studies, it is conventional to measure the sensitivity of cells to a pro-death ligand based on fixed-time, end-point measurement of the ligand concentration necessary to kill 50% of the cells in the initial population (the IC50). When only a subset of cells dies at the highest ligand doses, it is more informative to report the maximum fractional killing (E_{max}) and the ligand concentration at which half of this level of killing is observed (EC50 (Barretina et al 2012)). Particularly in the case of fractionally sensitive cells, it is thus important to distinguish between IC50 and EC50 at multiple timepoints when evaluating sensitivity to apoptosis.

We observe that even among sensitive cell lines the shape of the death-time distribution (sharp and narrow versus wide and evenly spread) is remarkably variable from one cell line to the next. The same is true for resistant lines, although in all cases the death-time distribution is a stable and characteristic feature of that line. For example, both T47-D and HCT116 cells exhibited narrowly distributed and early death-time distributions but T47-D cells were significantly more resistant than HCT116 cells (with a total of 35% of T47-D cells versus 85% of HCT116 dying at a 50 ng ml^{-1} TRAIL treatment over 16 h). In contrast, TRAIL-resistant Hs578T and HT29 cells exhibited broad death-time distributions in which a few cells died sporadically throughout a 16 h time-lapse experiment. The role of dose in shaping the death-time distribution was dependent on cell type: dose had a large effect on the shape of the death-time distribution in MCF10A cells but had a much smaller effect on the shape of the death-time distribution in T47-D cells.

The clear separation in time between the T47-D, HCT116 and MCF10A cells that died soon after exposure to TRAIL (within ∼4–6 h at 50 ng ml^{-1} TRAIL) and those that were resistant to prolonged ligand exposure (12–16 h) is suggestive of two distinct subpopulations. However, in multiple cell lines tested, and in multiple clones derived from the same parental culture, cells that survived treatment with TRAIL were not permanently resistant. Within several days of outgrowth surviving cells had regenerated the same fractional sensitivity and death-time distribution as the starting population. Conventional acquired resistance to pro-apoptotic ligands can be observed following long term or repeated exposure (Lane et al 2006, Li et al 2011) and this resistance is stable and genetically determined. In contrast, the resistance observed in our experiments is transient and likely to involve non-genetic factors such as epigenetic switching, differences in cell cycle state, stochastic fluctuations in the levels of pro- and anti-apoptotic proteins and differential induction of survival signaling (Brock et al 2009, Cohen et al 2008, Neumann et al 2010, Sharma et al 2010, Spencer et al 2009). In principle, these processes could interact on different timescales to influence the initial efficacy of a pro-apoptotic drug and the outgrowth of resistant populations. Regeneration of fractional sensitivity is reminiscent of bacterial persisters responding to antibiotic therapy (Balaban et al 2004) and has also previously been demonstrated in cancer cells undergoing drug treatments (Sharma et al 2010). Our results additionally raise the important question of how death-time distributions and E_{max} are independently and stably encoded in different cell types.

We observed that treating cells with both TRAIL and a second pro-apoptotic drug reduces the mean and variability in death times and increases the maximum fraction of cells killed. To begin to dissect these effects we treated cells with the protein synthesis inhibitor CHX and the proteasome inhibitor MG-132, whose effects on protein levels are understood in broad terms. Treating cells with sub-lethal concentrations of CHX is known to reduce the levels of potent but short-lived anti-apoptotic proteins such as Mcl-1, c-FLIP and XIAP and thus to sensitize cells to death ligands (Clohessy et al 2006, Kreuz et al 2001, Lane et al 2006). Under some conditions, proteasome inhibitors can also sensitize cells to TRAIL-induced apoptosis via inhibition of NF-κB-mediated pro-survival signaling or caspase-3 degradation (Albeck et al 2008a, Lane et al 2006), stabilization of TRAIL receptors or tBid (Breitschopf et al 2000, Johnon et al 2003, Kabore et al 2006), or alteration of the DISC (Laussmann et al 2011, Sayers et al 2003). We observed that co-treating cells with TRAIL and sub-lethal doses of CHX reduced variability in death times and that further addition of MG-132 reversed this, at least partially (under these conditions, virtually all cells were ultimately sensitive to 16 h of TRAIL exposure). Antagonism between CHX and proteasome inhibition has also been observed using the clinical-grade proteasome inhibitor bortezomib (Laussmann et al 2012). These data strongly suggest that short-lived anti-apoptotic proteins play a significant role in the variability of death times. Cell-to-cell variability in the levels of proteins is often ascribed to stochastic fluctuation rates of transcription and protein synthesis; inhibition of these processes is thus expected to decrease variability while blocking protein degradation would in principle reverse this effect and maintain variability as we observe.

In TRAIL-treated cells, the timing of cell death is determined by the rate of accumulation of tBid (a pro-apoptotic Bcl-2 family member generated by caspase-8/10-mediated
cleavage of Bid) and the threshold level of tBid needed to trigger MOMP. In the case of TRAIL, this threshold is set primarily by the levels of anti-apoptotic Bcl-2 family proteins (or by the ratio of these proteins with pro-apoptotic Bcl-2 family members). It is logical to assume that when the threshold is lower, less time is needed for cleaved and active tBid molecules to accumulate to a level that exceeds the threshold and triggers cell death. To demonstrate that this is indeed the case we treated cells with TRAIL and ABT-737, a small molecule that inhibits Bcl-2 and Bcl-XL (Huang and Sinicrope 2008, Oltersdorf et al. 2005). As expected, we observed a dose-dependent reduction in the MOMP threshold, causing cells to die earlier. A variety of other anti-cancer drugs have been shown to synergize with TRAIL in cell killing and the proposed mechanisms are diverse: p53-dependent or -independent transcriptional upregulation of TRAIL receptors or caspase-8 (Di Pietro et al. 2001, Ehrhardt et al. 2008, Sheikh et al. 1998, Wu et al. 1997), downregulation of anti-apoptotic pathway components such as c-FLIP and Mcl-1 (Galligan et al. 2005, Kim et al. 2008, Morizot et al. 2010, Stagni et al. 2010) or transcriptional upregulation of pro-apoptotic BH3-only proteins such as Bim or Puma (Letai 2008). It is not yet possible to measure the MOMP threshold for inducers of intrinsic apoptosis such as gefitinib, sorafenib or paclitaxel in single cells (although BH3-profiling makes it possible in cell populations (Deng et al. 2007)), in large part because we do not know which pro-apoptotic regulators play the role that tBid does in extrinsic apoptosis. However, by measuring the caspase-8/10 threshold needed to trigger MOMP in cells treated with both TRAIL and an intrinsic apoptosis inducer, it is possible to infer the activities of factors that set the threshold for intrinsic apoptosis as the reduction in threshold relative to TRAIL alone. By analogy with mitochondrial priming, this reduction in threshold could involve either the inhibition of anti-apoptotic proteins such as Mcl-1 or the production of pro-apoptotic proteins such as Bim (Certo et al. 2006). We observed that when TRAIL was combined with sorafenib, gefitinib or paclitaxel the threshold for MOMP was significantly lower than for TRAIL alone. In the large body of literature on sorafenib, gefitinib and paclitaxel as a common element is their effect on Mcl-1, a protein expected to regulate the MOMP threshold directly (Chen et al. 2010, Henson et al. 2003, Meng et al. 2007, Ricci et al. 2007, Rosato et al. 2007, Sanchez-Perez et al. 2009, Wertz et al. 2011)

The second factor expected to change the timing of TRAIL-induced cell death is the rate of IC-RP cleavage. When we used a phenomenological rate equation to fit for caspase-8/10 activity in pre-MOMP cells (Spencer et al. 2009), we observed that earlier death times were associated with faster rates of IC-RP cleavage in both TRAIL-treated and co-drugged HeLa cells expressing the IC-RP reporter. Previous studies showed that in cells treated with TRAIL plus CHX, the rate of IC-RP cleavage for an individual cell was more predictive of time of death than the threshold (Spencer et al. 2009). In our current analysis, we observed that the shapes of the IC-RP trajectories were altered in co-drugged cells compared to cells treated with TRAIL alone (complicating efforts to apply a uniform function to compare rates among the different conditions), but we did not find consistent evidence demonstrating that increases in rate were a major determinant for the sensitizing effects we observed with co-drugging. Nevertheless, given that gefitinib, sorafenib and paclitaxel have been suggested to alter not only the levels of mitochondrial proteins but also the levels of DISC proteins and DR4/5 receptors (Nimmanapalli et al. 2001, Rosato et al. 2007, Singh et al. 2003), this question warrants further analysis.

In conclusion, we have shown that variability in the time and probability of TRAIL-mediated cell death varies from one cell type to the next and is a stable property of a particular cell type. Co-drugging cells with TRAIL and a second anti-cancer drug has a significant impact on cell death time, at least partly by lowering the threshold for MOMP. Non-genetic variation among cells may impact the extent of synergism observed when drugs are combined (Fitzgerald et al. 2006), and the degree of synergy may in turn influence cell-to-cell variability in death times. Future studies in cell types exhibiting a range of fractional sensitivities could in principle shed light on these effects and aid in the design of more potent combination therapies.

Materials and methods

Cell culture and generation of stable cell lines

HeLa cells expressing IC-RP and the mitochondrial reporter IMS-RP were generated as described (Albeck et al. 2008a) and cultured in DMEM containing 10% FBS, 5 mM l-glutamine and 1% penicillin/streptomycin (Gibco). MCF10A and HCT116 cells expressing IMS-RP were generated using a retrovirus produced by co-transfection of 293T cells (ATCC) with pCL-ampho and pBabe-IMS-RP and were cultured as described (Aldridge et al. 2011, Deb Nath et al. 2003). HT29 (ATCC) were cultured in McCoy’s 5a medium; T47-D were grown in RPMI, and Hs578T cells were grown in DMEM, supplemented with 10% FBS, 2 mM l-glutamine and 1% penicillin/streptomycin (Neve et al. 2006). HUVEC (primary human endothelial cells) were maintained in M200 medium containing low-serum growth supplement (Cascade Biologics). Single-cell clones were generated by serial dilution followed by expansion and testing for TRAIL sensitivity that was either similar to or enhanced compared to that of the parental cell line.

Cell treatments

SuperKiller TRAIL was purchased from Alexis Biochemicals; other reagents include ABT-737 (Selleck Chemicals), gefitinib and sorafenib (LC Laboratories), paclitaxel (Sigma Aldrich), cycloheximide (Sigma Aldrich), MG-132 (Calbiochem) and staurosporine (EMD Biosciences).

Measurement of cell death by flow cytometry

Following treatment, floating dead cells were combined with trypanized adherent cells, fixed with 4% paraformaldehyde, permeabilized in methanol and labeled with Alexa Fluor 488-conjugated anti-cleaved PARP (cPARP) antibodies (Becton Dickinson) as described (Albeck et al. 2008a). Flow cytometry
Live-cell microscopy

Cells were seeded in glass-bottom chamber slides (Nunc) and imaged at 3–5 min intervals for 16–18 h as described (Albeck et al 2008a). For HUVEC, HS578T and T47-D cells, chamber slides were pre-coated with 50 μg ml⁻¹ fibronectin (Invitrogen) to facilitate cell adhesion. Cells were imaged in a 37 °C humidified chamber either in CO₂-independent media (Invitrogen) supplemented with 1% FBS, 2 mM l-glutamine, and 1% penicillin–streptomycin, or in ~5% CO₂ in a phenol-free medium containing full growth supplements. Unless otherwise indicated, treatments were performed 15–30 min prior to the start of the movie. Time of death was monitored as the time of MOMP in cells expressing the IMS-RP reporter (Albeck et al 2008a) or by morphological changes associated with apoptosis. Cells were imaged either on a Deltavision microscope (Applied Precision) at 10–20× magnification or on a Nikon TE2000E (Nikon Imaging Center, Harvard Medical School).

Survivor re-challenge experiment

Cells seeded in glass-bottom chamber slides (Nunc) were treated with 50 ng ml⁻¹ TRAIL and imaged at 3 min intervals for 16 h; survivors were collected by trypsinization, replated and recovered for 6 days in the absence of TRAIL. Survivor cells were then replated into chamber slides, re-treated with 50 ng ml⁻¹ TRAIL, and re-imaged for 16 h under identical conditions, alongside control cells that had not been previously exposed to TRAIL. Alternatively, cells were treated for 7–24 h with 50 ng ml⁻¹ TRAIL in 10 cm dishes; surviving cells were collected by trypsinization, replated into 10 cm dishes, and allowed to recover for 6–7 days in the absence of TRAIL. Survivor cells were then seeded alongside control (naïve) cells into glass-bottom chamber slides, treated with 50 ng ml⁻¹ TRAIL, and imaged for 16 h by live cell microscopy. For the staurosporine survivor re-challenge experiment, HeLa cells growing in 6-well dishes were treated for 16 h with 2 μM staurosporine; surviving cells were collected by trypsinization, replated and allowed to recover for 2 weeks in the absence of staurosporine prior to re-challenge with 2 μM staurosporine and live-cell imaging.

Image analysis and threshold calculations

Death times for 50–200 cells were monitored by visual inspection of IMS-RP translocation (MOMP) or by morphological changes in cells not expressing the IMS-RP reporter. The percentages of cells dying within 1 h intervals, as well as the fraction surviving at the end of the movie, were calculated and plotted. Analysis of IC-RP reporter activity was performed as described (Albeck et al 2008a). Briefly, Image J was used to compute the ratio of CFP:YFP fluorescence in single cells over time. Trajectories of this ratio were plotted for individual cells following subtraction of an average trajectory for an untreated photobleaching control. For threshold calculations, initiator caspase FRET trajectories for individual cells were normalized to the minimum and maximum intensity for each trajectory; the threshold was defined as the normalized intensity at the time of MOMP.

Statistics

Statistics, curve-fitting (for dose–response curves) and IC50 calculations were performed using GraphPad Prism Statistical Software. Time of death histograms were tested for normality using the D’Agostino Pearson test. Interquartile range (IQR) and coefficient of variation (CV) were reported as measures of variability. Mean and median death times were compared using the Mann–Whitney test for non-Gaussian distributions or using an unpaired Welch’s t-test for the death-time distribution values. An F test was used to compare variances determined from Gaussian approximations. The mean threshold values were compared across treatments using an unpaired t-test for the distributions of threshold values, or using ANOVA. Significant differences were reported for p < 0.05.

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