Preclinical and Clinical Estimates of the Basal Apoptotic Rate of a Cancer Predict the Amount of Apoptosis Induced by Subsequent Proapoptotic Stimuli

Lian Zhang¹, Brian D. Kavanagh², Andrew M. Thorburn¹, and D. Ross Camidge³

Abstract

Purpose: We hypothesized that the basal apoptotic rate (BAR) of a cancer would predict sensitivity to subsequent proapoptotic stimuli. To explore this, preclinical and clinical BAR assays were developed measuring cumulative apoptotic events through ELISAs for soluble caspase-cleaved cytokeratin 18 (M30) normalized to either cell number increase or total tumor volume, respectively.

Experimental Design: The BARs of A549, HCC44, and SW1573 non–small cell lung carcinoma cell lines were measured following different pro/antiapoptotic manipulations. In isogenic wild-type and stable knockdown (KD) series, pretreatment BARs were correlated with response to proapoptotic stimuli and compared with established apoptosis assays. Pretreatment and posttreatment serum was available from stereotactic body radiation therapy patients.

Results: Caspase inhibition and p53 KDs reduced the BAR, whereas serum deprivation, XIAP, or Bcl2 KDs increased the BAR. The nontreated BAR rank ordering of the XIAP series recapitulated that with terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling and caspase-3/7 activity assays, and predicted each line's sensitivity to TRAIL or irradiation. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling, however, underestimated basal apoptosis during increased apoptotic stress, and caspase-3/7 activity detected minimal death in the media. P53 KDs with lower nontreated BARs were less sensitive to TRAIL and cisplatinum than wild-type. Stereotactic body radiation therapy increased serum M30 values, and the pretreatment clinical BAR strongly correlated with fold change in M30 on treatment (r = 0.93).

Conclusions: M30-based BAR assays reflect apoptosis accurately and are more amenable to clinical application than existing apoptosis assays. The pretreatment BAR correlates with cell and/or tumor sensitivity to extrinsic and intrinsic apoptotic pathway stimulation. Prospective clinical exploration is warranted.

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Translational Relevance

The proapoptotic and antiapoptotic signaling balance determines the proximity of a cell to the apoptotic threshold. Cancers closer to this threshold may have a higher basal apoptotic rate (BAR) and a greater propensity to commit apoptosis in response to a proapoptotic stimulus. Using specific normalizations of the M30 Apoptosense ELISA (which can assess cumulative cell death in culture medium or serum) based on viable cell number or total tumor volume, we have estimated the BAR of cancer cell lines and clinical cancers, respectively. The M30 BAR correlates with apoptotic outcomes following different proapoptotic stimuli, including radiation treatment in patients. This assay may now be explored prospectively as a predictive marker of clinical radiation sensitivity and as a specific pharmacodynamic marker, looking for changes in the BAR to predict benefit from the direct apoptosis-manipulating agents currently being explored within clinical trials.

hypothesize that the distance a cell (or a cell population, on average) is from this threshold determines how easy or difficult it will be to kill the cell following an additional uniform proapoptotic stimulus (Fig. 1A). Second, we hypothesize that, given the natural variation in proapoptotic and antiapoptotic stimuli occurring in the cellular microenvironment as a result of, for example, growth factor, nutrient, and oxygen level fluctuations, a cancer with a closer proximity to the apoptotic threshold will have a higher basal apoptotic rate (BAR), which provides a surrogate of the cancer's overall apoptotic threshold proximity (Fig. 1B).

Although clinically some correlation has been reported between the histologically derived apoptotic index of cancers and subsequent response to radiation/chemoradiotherapy in tumors accessible for pretreatment and post-treatment biopsies/resections, findings have not been consistent (8–11). Beyond the problems of intratumoral heterogeneity when biopsying, histopathologic estimates of apoptosis and in vitro methods based on detecting cells in the process of apoptosis at any one time point may also significantly underestimate the true BAR. This is because cells undergoing apoptosis are unlikely to remain available to be counted but, rather, they will rapidly dismantle themselves, undergo phagocytosis, and disappear from view.

We have developed a more representative and practical method for assessing the BAR using specific normalizations of the M30 Apoptosense ELISA analysis to estimate cumulative cell death both in vitro and in vivo. M30 is an antibody directed against a neoepitope produced during the caspase-mediated cleavage of cytokeratin 18, a type I intermediate filament common in single layer and glandular epithelial cells (12). In addition to being used for immunohistochemical detection of apoptotic cells in solid tissues, the cleaved fragment detected by M30 antibody is both soluble and stable, offering the potential to look at completed apoptosis events in cell culture media or blood. The M30 Apoptosense assay has been used to show preclinical and clinical pharmacodynamic increases in apoptosis following both novel and traditional anticancer drug treatments (13–15). However, appropriate normalization of the M30 assay to explore a cancer's BAR and its predictive significance have not been described before. We chose to predominantly explore non–small cell lung cancer (NSCLC) as an example of a major cancer health problem (16). The limited effectiveness of many of the established treatments in lung cancer patients, particularly with increasing lines of therapy in advanced disease, suggests resistance to apoptosis is common in this population (16). Here, we describe how using the M30 Apoptosense assay on unchanged culture media normalized to viable cell number increase over time was used to assess the BAR of NSCLC cells in vitro. We then extended our observations to a clinical setting in which a clinically derived BAR was assessed by normalizing M30 reactivity in cancer patients' serum to the baseline total tumor burden determined by volumetric computed tomography scanning. The validation and potential predictive significance of these BAR assays in NSCLC cell lines undergoing different proapoptotic stimuli and in patients undergoing stereotactic body radiation therapy (SBRT) are reported.

Materials and Methods

Reagents

Reagents were RPMI 1640 growth medium (HyClone) supplemented with 10% fetal bovine serum (HyClone). Unless otherwise noted, chemicals and reagents were obtained from Sigma. z-VAD-fmk was from Alexis Biochemicals. M30 Apoptosense ELISA kits (PEVIVA) were obtained from DiaPhama. Human recombinant TRAIL was purchased from R&D Systems. Lexitumumab was generously given by Human Genome Sciences.

Cell line and cell cultures

All the lung cancer cell lines were the gift of Barb Helfrich at the University of Colorado, Aurora, CO, and cultured in RPMI 1640 supplemented with 10% FCS, 1% sodium pyruvate, 2.0 mg/mL sodium bicarbonate, and 10 mmol/L glucose at 37°C with 5% CO₂ unless specified otherwise. Cell lines were DNA profiled using polymorphic short tandem repeat markers (Identifiler Plus PCR Amplification kit, Applied Biosystems). A549 originally from the American Type Culture Collection was matched by profiling in May 2006 and again in December 2009. HCC44 obtained from John D. Minna and Adi Gazdar (University of Texas Southwestern, Dallas, TX) was matched by profiling in December 2007. SW1573 was obtained from H Broxterman (Amsterdam) but has not yet been profiled at the University of Colorado.

In vitro BAR assay

Unless specified otherwise, the in vitro BAR assay was done as follows: 80,000 cells were seeded per plate and...
grown in the presence of 10% FCS without changing media until cells reached confluence (all cells in contact, and the entire surface of the plate was covered). Replicate plates were sacrificed daily to collect the media and to count the cell number. The media were subjected to the M30 Apoptosense ELISA assay according to the manufacturer's instruction. Time points at approximately equivalent viable cell numbers before confluence were compared, with M30 values normalized to the corresponding exact cell number at the time of harvesting.
MTS cell viability assay
Cells were plated at a density of 4,000 cells per well in a 96-well plate in triplicate wells. Cells were treated with the indicated concentrations of drugs for 24 to 48 hours. At the end of the experiment, the cells were treated with 20 μL of MTS, inner salt (Promega) and incubated at 37°C until optimal color changes occur. The absorbance values at wavelength of 490 nm were measured with a Bio-Rad Benchmark Plus Microplate Spectrophotometer (Bio-Rad Laboratories).

Clonogenic survival assay
Five hundred cells were plated in six-well plates in triplicate manner, followed by human recombinant TRAIL or lexatumumab the next day. After treating with TRAIL or lexatumumab for 18 to 24 hours, the drugs were removed by washing cells with prewarmed PBS, and fresh media were added. Following 7 to 10 days of incubation, colonies were fixed with a fixer (10% acetic acid, 10% methanol, 80% diH2O) and stained with crystal violet (0.4% crystal violet, 20% ethanol). After the images were scanned, the colonies were solubilized with 30% acetic acid, and the absorbance was read at a wavelength of 540 nm.

Caspase-3/7 activity assay
Cells were plated in a 96-well black-walled plate at a density of 4,000 cells per well in a triplicate manner, followed by the indicated concentrations of TRAIL treatment for 4, 8, or 24 hours. One hour before the end of the experiment, Caspase-Glo 3/7 reagent (Promega) was added to each well according to the manufacturer's instructions. After 1 hour of incubation in the dark, the caspase-3/7 activities of the samples were measured using a Veritas microplate luminometer (Turner BioSystems).

In situ terminal deoxynucleotidyltransferase-mediated dUTP biotin nick-end-labeling assay
Eighty thousand cells were plated in 24-well plates in triplicate wells. The next day, the cells were treated with TRAIL (50 ng/mL) for 4, 8, or 24 hours. Apoptosis was measured by using the TiterTACS In situ apoptosis detection kit (R&D Systems) according to the manufacturer's instructions except that the amount of reagents used in every step was doubled.

Small hairpin RNA transfection
The XIAP, BCL2 small hairpin RNA (shRNA) lentiviral constructs were obtained from Thermo Scientific Open Biosystems. The shRNA lentiviral constructs were cotransfected with packaging plasmid psPAX2 and envelope plasmid pMD2.G into 293T cells. Two days posttransfection, the virus was harvested and filtered through a 0.45-µm filter and aliquoted for infection or storage at −80°C. A549 and SW1573 cells were infected with XIAP or BCL2 shRNA lentivirus and selected with puromycin (2 µg/mL). The p53 shRNA constructs (sc-29435-sh) were purchased from Santa Cruz Biotechnology. A549 Cells were transfected with p53 shRNA plasmids using DharmaFect 1 (Dharmacon) according to the manufacturer's instructions, followed by puromycin (1.5-3 µg/mL) selection for 2 to 3 weeks to establish stable transfectants.

Immunoblotting analysis
Cell lysate preparation, electrophoresis, membrane transferring, signal detection, and film developing were conducted as previously described [17]. The primary antibodies used were as follows: anti-XIAP (#2045) and anti-BCL2 (#2872) were from Cell Signaling Technology. Anti-p53 (sc-126) antibody was from Santa Cruz Biotechnology.

SBRT-treated patient samples
Quantitative serum M30 assays were done on serum from patients treated on a prospective Institutional Review Board–approved protocol. Eligibility for this protocol included the planned delivery of SBRT (minimum dose, 10 Gy per fraction for three fractions) to one or more lung or liver primary or metastatic lesion(s) from a solid malignancy. From the entire cohort enrolled on the protocol, patients eligible for the BAR analysis met the following criteria: (a) epithelial tumors expected to contain cytokinin 18 positive cells, (b) SBRT given to all known sites of gross disease, (c) no concurrent use of systemic agents with known effects on cellular apoptosis (e.g., systemic

Fig. 1. Proximity to the apoptotic threshold may influence propensity to apoptosis in response to a proapoptotic stimulus. A, schematic representation of how cancer cells may avoid apoptosis (passage beyond the apoptotic threshold) in response to a therapeutic insult. Black bars, i and ii, differing proximities to the apoptotic threshold. In Cell 1, the prodeath signaling from insult A1 is sufficient to induce completed apoptosis. In Cell 2, the prodeath drive from insult A is reduced (A2), for example through increased repair of drug-induced damage, and given the same resting proximity to the apoptotic threshold, the cell remains alive. In Cell 3, the prodeath drive from insult A remains the same as in Cell 1 (A1), but the cell does not die because the composite of all its other prodeath and antideath signaling has shifted its resting position away from the apoptotic threshold (ii), and the drive from insult A1 is now insufficient to reach the threshold. Other insults with stronger prodeath drive (B1) may still kill Cell 3. NB-specific molecular factors could influence either the prodeath drive resulting from an insult (the downward arrows), the proximity of the cell to the threshold (the black bars), or both, as the resting proximity is a composite of prodeath and antideath signaling from many different cellular stressors that may or may not share death-related signaling pathways with the therapeutic stressors. B, schematic representation of how, given the normal fluctuations in prodeath and prosurvival stimuli that cells continually experience even under standard growth conditions, proximity to the apoptotic threshold may be estimated by measuring the BAR. The distance of the cell relative to the ground is represented as a balance between proapoptotic “gravity” (size of the balloon’s basket) and antiapoptotic “lifto” (size of the balloon). Contact with the ground represents commitment to cell death. The population of cells closer to the apoptotic threshold (the dotted balloons and the dotted fluctuation line) will reach the apoptotic threshold more often given the same fluctuation of pro/antiapoptotic stimuli manifesting as a higher BAR compared with the other (solid) population of cells. C, schematic representation of the in vitro BAR assay looking at cumulative products of caspase-mediated cell death in the unchanged culture media of growing cells.
steroid anti-inflammatory agents). Serum collection times were pretreatment and immediately after the final fraction of SBRT. Absolute M30 reactivity increases in the patients’ sera pre- and post-SBRT were compared using two-tailed paired t testing. The correlation between the pre-SBRT clinically determined BAR [baseline M30 divided by the total gross tumor volume (GTV)] and fold change in M30 reactivity from pre-SBRT to immediately post-SBRT was determined by calculating the product-moment correlation coefficient, r. Typically, the GTV was expanded 5 to 10 mm in all directions to generate the planning target volume.

Results

The BAR of a cancer cell line can be measured in vitro using a normalized M30 ELISA assay applied to unchanged culture media.

To assess the BAR of a cancer cell line in vitro, we applied the M30 ELISA assay to unchanged culture media

to determine the cumulative caspase-related cell death that occurred during the time it took for the cell line to increase its viable cell number from a fixed seeding of cells (n) to a second, larger, viable cell number (N). Cells were grown to confluence, but media were taken to calculate the BAR at a time before confluence was reached. Growth times were adjusted to allow approximately the same number of viable cells to be present and then normalized to the exact number on harvesting. The experimental design is illustrated in the schematic diagram in Fig. 1C.

When A549 NSCLC cells (adenocarcinoma) were grown under optimal growth conditions, M30 reactivity was detectable in the culture media. The addition of 25 μmol/L of the pan-caspase inhibitor z-VAD-fmk to the media resulted in a faster growth rate of the cells, such that equivalent cell numbers were achieved ~1 day earlier (Fig. 2A) and decreased M30 reactivity normalized to cell number before approaching confluence compared with nontreated

![Fig. 2. The BAR of a cell line changes in response to antisurvival and/or proapoptotic stimuli from the extracellular microenvironment. The pan-caspase inhibitor z-VAD-fmk increased the growth rate (A) and reduced the BAR (B) of A549 cells measured before confluence by the method depicted in Fig. 1C. Z-VAD-fmk was added to the medium (final concentration, 25 μmol/L) the day after the cells were seeded and remained in the medium until the cells were harvested. The graphs represent typical experiments repeated at least once. Points, mean from three replicates; bars, SD. In contrast, serum starvation produces the opposite results. C, growth curves of HCC44 cells in the presence of 10%, 2%, or 0.5% serum concentration. D, growth times were adjusted to reach approximately similar numbers of viable cells before confluence, and M30 reactivity normalized to exact cell number on harvesting to determine the BAR of the cell lines under the different growth conditions. Columns, mean from three replicates after normalization to the corresponding cell number; bars, SD. *** P values were obtained using Student’s t test of comparing the BAR in 10% versus the BAR in 2% or 0.5% serum concentration. *** P < 0.0001.](https://cancerres.aacrjournals.org/content/16/17/4482/F2.large.jpg)
cells (Fig. 2B). There was a dramatic increase in the BAR as confluence was approached presumably due to the increased stress of cell crowding and nutrient depletion. Consequently, in all subsequent assays, the BAR was calculated based on media taken during the early exponential phase of growth (approximately 650-850,000 cells), adjusting the growth time as necessary to achieve these levels, normalized to the exact cell number at harvesting.

The BAR of a cell line changes in response to antisurvival and/or proapoptotic stimuli from the extracellular microenvironment

Serum deprivation has been shown to trigger the intrinsic apoptotic pathway (18). Following the observation relating to the potential effects of cell density as a microenvironmental stressor on the BAR of A549 cells (Fig. 2A and B) we tested whether the BAR assay could reflect changes in the proapoptotic/antisurvival stimuli caused by serum deprivation. The BARs of two NCSLC cell lines, HCC44 (adenocarcinoma) and SW1573 (squamous carcinoma), were determined in the presence of 10%, 2%, or 0.5% fetal bovine serum. In contrast to the effects of z-VAD-fmk, reduction of serum concentration resulted in slower growth rate in HCC44 (Fig. 2C) and SW1573 cells (data not shown), in conjunction with an increase in the BAR of both cell lines (Fig. 2D).

The BAR of a cancer cell line can be manipulated either toward or away from the apoptotic threshold by genetically changing molecules affecting different aspects of the apoptotic balance within the cell. A, BARs of the WT and the isogenic XIAP KDs measured by the M30-based BAR assay. ShRNA XIAP KDs confirmed by Western analysis in A549 and SW1573 cells (inset). B, BARs of the WT and the isogenic BCL2 KDs measured by the M30-based BAR assay. C, ShRNA p53 KDs confirmed by Western analysis in A549 cells in response to doxorubicin. D, BARs of the WT and the isogenic p53 KD lines measured by the M30-based BAR assay. The M30 values presented are means ± SD from three replicates after normalization to the corresponding cell number. *P values were obtained using Student’s t test of comparing the BAR in WT versus the KDs. *, \( P < 0.01 \); **, \( P < 0.001 \); ***, \( P < 0.0001 \).
blotting for the expressed protein (Fig. 3A). Knocking down XIAP resulted in increased BARs compared with the isogenic wild-type (WT) lines (Fig. 3A). Stable KDs of Bcl2 similarly increased the BAR of A549 compared with the isogenic WT line (Fig. 3B).

To manipulate the pro/antiapoptotic balance in the opposite direction, we then knocked down p53 as an example of a damage sensing (i.e., proapoptotic) stimulatory molecule. Stabilization of p53 protein in response to doxorubicin treatment in the p53 KDs (p53-1, p53-2) was both delayed and reduced in magnitude compared with the A549 WT line, indicating efficient KD by the shRNA (Fig. 3C). Consistent with our central hypothesis, knocking down p53 (reducing proapoptotic signaling) reduced the BAR of the cells compared with the isogenic A549 WT line (Fig. 3D).

Comparison of the M30-based cumulative cell death BAR assay with terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling and caspase-3/7 activity-based apoptotic assays

To further validate our M30-based BAR assay, we compared it to both terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) and caspase-3/7 activity assays in our A549 WT and XIAP KD cell lines. The rank ordering of the apoptotic rates of the three lines measured by in situ TUNEL staining of fixed cultured cells correlated well with that determined by our M30-based BAR assay (compare Figs. 3A and 4A). However, when the cells were challenged with 50 ng/mL TRAIL for 8 hours, the apoptotic rates measured by TUNEL decreased in WT and XIAP KDs compared with no treatment, with a greater reduction in the XIAP KD lines (Fig. 4A). In contrast, when the apoptotic rates were measured using the M30-based BAR assay, TRAIL treatment increased the apoptotic rates in all the cell lines with the greatest effects being in the XIAP KDs and in those with the highest nontreated BAR (Fig. 4B). These data suggest that the ability to measure completed apoptotic events as is provided by the M30-based BAR assay provides a more accurate measure of the total amount of apoptosis that has taken place compared with the “snapshot” provided by TUNEL staining.

We next explored increasing concentrations of TRAIL for 8 hours, followed by caspase-3/7 activity determination in the culture wells (media and cells combined; Fig. 4C). Unlike with TUNEL, caspase activity revealed
identical sensitivity rankings across the WT and XIAP KD lines to the M30-based rankings even at the highest concentrations of TRAIL. To explore how much might be due to caspase activity in the media, we compared caspase-3/7 activity in the media with activity in the cell pellet. The results showed that almost all caspase-3/7 activity was restricted to the cell pellet and not the media (Fig. 4D), thus offering less potential for translational application to body fluids than the M30-based assay (see below).

The M30-based BAR predicts for apoptotic sensitivity in response to extrinsic apoptotic pathway stimulation in vitro

To further address the question whether the BAR can predict treatment outcome from extrinsic apoptotic pathway stimulation, MTS assays were done to measure cell viability after 48 hours of TRAIL treatment across a range of concentrations. XIAP KD sensitized A549 and SW1573 cells to TRAIL treatment (Fig. 5A). More importantly, the sensitivity rank ordering by MTS assay in the WT and XIAP KDs in A549 and SW1573 cells correlated well with their nontreated BAR ranking (Figs. 3A and 5A). This correlation also applied to the monoclonal antibody lexatumumab, which recognizes and stimulates Death Receptor 5 (data not shown). To determine whether the BAR predicts for long-term survival and proliferation capability after treatment, clonogenic survival assays were conducted with or without TRAIL/lexatumumab treatment. Again, the cell lines with the highest BAR in their isogenic series correlated with the poorest survival rate on TRAIL or lexatumumab treatment (Fig. 5B and C). On the other hand, pushing cells away from the apoptotic threshold, reducing their BAR by knocking down p53, made the cells more resistant to the TRAIL treatment compared with the WT parent line in clonogenic survival assays (Fig. 5D).

The M30-based BAR predicts for apoptotic sensitivity in response to intrinsic apoptotic pathway stimulation in vitro and in patients undergoing SBRT

Having shown the role of the BAR in determining outcomes from extrinsic apoptotic pathway–directed stimuli, we tested its relevance to intrinsic apoptotic pathway stimulation. The lower nontreated BAR in p53 KD lines was associated with less M30 induction following growth in the presence of 1 μmol/L cisplatinum (Fig. 6A). Although the rank ordering of the BARs in the presence of cisplatinum in the p53 KD series did not recapitulate their nontreated BAR rank ordering exactly, it did parallel their phenotypic sensitivity to cisplatinum in clonogenic survival assays (Fig. 6B).

We also tested radiation exposure in vitro as a way of manipulating the intrinsic apoptotic pathway. Two grays of radiation clearly increased the postexposure BARs across WT, XIAP (Fig. 6C), and p53 (data not shown) KD cell lines compared with no treatment, indicating radiation-induced caspase activation in these cells. Similar to what we had observed before, lines with higher nontreated BARs (XIAP KDs) manifested higher elevations in their postexposure BAR following radiation treatment, whereas lower nontreated BARs (p53 KDs) manifested lower elevations in their postexposure BARs.

To test if these measurements apply during patient treatment, we analyzed serum samples from patients with primary or metastatic cancer collected before and on the last day of a course of SBRT to all known sites of disease (liver and/or lung). The clinical characteristics of the SBRT patients (n = 13) are shown in Table 1. The median prescription dose to the planning target volume was 60 Gy in three fractions (range, 36-60), administered in a total of 3 to 7 days. Consistent with our in vitro work, absolute M30 reactivity increased in the 13 patients' sera pre- and post-SBRT. The baseline M30 levels [mean ± SEM, U/L] were 145 ± 14 and increased to 232 ± 34 immediately post-SBRT (P < 0.01 by paired t test). Plotting the M30 ratio of post- to pre-SBRT against the clinically determined BAR [pre-SBRT M30 divided by the total tumor volume (all sites) in mm³] showed that this clinically determined BAR strongly correlated with the apparent amount of apoptosis induced by the treatment (correlation coefficient of 0.93; Fig. 6D).

Discussion

Multiple proapoptotic and antiapoptotic signals interact to determine whether, at any particular moment, a cell will irrevocably commit to undergo apoptosis. The commitment to die is likely to result from sufficient imbalance in pro apoptotic and antiapoptotic signaling, or what may be viewed as transition past a hypothetical apoptotic threshold. We hypothesized that the theoretical distance a cell, or a cell population on average, is from this threshold will determine how easy it will be for a subsequent proapoptotic stimulus to push the cell past this threshold (Fig. 1A). Moreover, we have hypothesized that this theoretical distance may be estimated by measuring the BAR of a cell population (Fig. 1B). Using the Apoptosense M30 ELISA to quantify cumulative apoptotic events in the unchanged culture media, it is clear that a significant amount of caspase-dependent apoptosis occurs during normal growth in vitro (Fig. 2A and B). The effect of the pan-caspase inhibitor z-VAD-fmk on both the time to reach confluence and M30 reactivity suggests that the BAR directly influences the perceived "growth rate" of a cancer, increases in the BAR reducing the total viable cell number, just as increases in the division rate would increase the viable cell number.

Many factors affect the BAR. In addition to caspase inhibitors reducing the BAR, serum deprivation increases the BAR (Fig. 2D). This is consistent with our primary hypothesis. Specifically, cells grown in reduced serum concentrations are likely to be closer to the apoptotic threshold and have a higher tendency to spontaneously undergo apoptosis as shown by an increase in the cumulative caspase-mediated cell death products detectable...
Fig. 5. The M30-based BAR predicts for sensitivity to extrinsic apoptotic pathway stimulation. A, A549, SW1573, and their isogenic XIAP KD cells were treated with the indicated concentrations of TRAIL for 48 h; the cell viability rates were determined by MTS assay. B, C, and D, Clonogenic survival assays done with 500 cells treated with TRAIL (25 ng/mL) or lexatumumab (50 ng/mL) for 48 h. The stained colonies were solubilized with 30% acetic acid; the absorbances were read at 500 nm and normalized to the no treatment ones. Points, mean from three replicates; bars, SD. P values were obtained using Student’s t test of comparing the survival rates of WT versus KD in response to TRAIL or lexatumumab. #, P < 0.05; **, P < 0.001; ***, P < 0.0001. In addition, there was a significant difference between the KDs in each series (P < 0.01 in XIAP KDs in A549 and SW1573 background and P < 0.05 in p53 KDs), suggesting that the BAR predicts for survival on TRAIL and lexatumumab treatment.
in the media. Again, as a direct consequence of their elevated apoptotic rate, plus or minus any additional effect of serum deprivation on the actual division rate of the cells, in the presence of serum deprivation, the cell lines take longer to increase their viable cell number (Fig. 2C). Genetic manipulation of different proapoptotic and antiapoptotic signaling molecules also moved the BAR of lung cancer cell lines in vitro in the expected directions. XIAP and Bcl2 KDs increased the BAR, whereas p53 KDs reduced the BAR (Fig. 3).

Other in vitro methods for determining apoptosis already exist. Yet, many of these methods rely on quantifying apoptotic events at a particular time point. However, as apoptosis results in cell destruction, methods based on assaying apoptotic cells/bodies represent only a snapshot of the true extent of apoptosis and therefore have the potential to significantly underestimate the true apoptotic rate. These limitations are reduced with the M30-ELISA, which looks at completed/cumulative apoptotic events. In line with this, although TUNEL rank ordering of our different XIAP KDs correlated with the M30 BAR rank ordering under standard growth conditions, TUNEL readings actually decreased in the presence of death receptor stimulation (Fig. 4A). When apoptosis occurs in large amounts, cells are lost for subsequent quantitation, thereby evading detection by the TUNEL assay but still being detectable by the M30-based BAR assay applied to the culture media. Assessing TUNEL at 4 and 24 hours, in addition to 8 hours, post-TRAIL exposure made no difference to the results, suggesting that apoptosis under these conditions was rapid and, as expected based on recent single cell studies examining cell death (19), occurred at different times for different cells within the population (data not shown). Caspase-3/7 activity tracked the M30 assay more robustly under standard and prodeath conditions, but offers little potential for translational extension (Fig. 4C and D) because the active caspase is found primarily in the cell remnants rather than released into the media or body fluids.

Data shown in Figs. 4–6 support our hypothesis that apoptotic threshold proximity, estimated through M30-based BAR assays in vitro, correlates with propensity to undergo apoptosis following an additional proapoptotic stimulus.

**Fig. 6.** The M30-based BAR predicts for apoptosis induction following intrinsic apoptotic pathway stimulation in vitro and in patients undergoing SBRT. A, A549 and the p53 KD cell lines p53-1 and p53-2 had their M30-based BAR determined following exposure to 1 μmol/L cisplatinum or no treatment (NT). The morning after cells were seeded, a final concentration of 1 μmol/L freshly made cisplatinum in DMSO, or the same amount of DMSO only for the no treatment control, were added to the cells. At comparable viable cell numbers, preconfluence cells and the media were collected for the M30 assay. B, the same cell lines and treatments as in A were explored for phenotypic correlates through clonogenic survival assays. C, A549 and the XIAP KD cell line X-2, SW1573, and the XIAP KD cell line X-4 had their M30-based BAR determined following 2 Gy of radiation or no treatment. The radiation was delivered through a RS2000 irradiator; the morning after the cells were plated. The BAR values presented in the graphs are means ± SD from three replicates of the M30 values after normalization to the corresponding cell number. D, a clinically determined BAR was calculated by normalizing the baseline M30 value to the GTV (determined by volumetric computed tomography scanning used in the radiation planning). The correlation between the fold change in M30 reactivity on radiation treatment (post-SBRT/pre-SBRT) and the clinical BAR is shown. P values were obtained using Student’s t test of comparing the BAR (A and C) or survival rate (B) of WT versus KDs in response to cisplatinum or radiation. *, P < 0.01; ***, P < 0.0001; #, P < 0.05.
Changes in M30 reactivity within the BAR assay on treatment increased significantly more among those cell lines with higher pretreatment BARs and vice versa, and it is clear from Figs. 4–6 that this reflects much more than a simple additive phenomenon. For example, the pretreatment BARs in the XIAP KD series in adenocarcinoma and in squamous carcinoma NSCLC cell lines predict, not only changes in the M30-based BAR on treatment, but also phenotypic cell death determined through both MTS and clonogenic assays in response to death receptor stimulation. Similar outcome correlations in the XIAP series were apparent for M30 induction postirradiation (Fig. 6C). From Fig. 3A, although the M30-based BAR correlated well with outcomes, the rank ordering of XIAP protein reduction by the different KDs did not fully match with their different BARs. These data support the idea that the absolute protein level of a molecule involved in the apoptotic pathway (which continues to be explored as a potential predictive biomarker) may poorly correlate with the cells sensitivity or insensitivity to apoptosis, as, instead, cell fate decision making is actually determined by the overall balance of multiple different signals interacting (as is integrated together in the M30-BAR assay readout).

Supporting the idea that multiple different factors affecting the BAR all influence the propensity of cells to complete apoptosis, the BARs of the p53 KDs, compared with their WT lines, also correlated with M30 and phenotypic outcomes to death receptor stimulation, cisplatinum, and irradiation (Figs. 5 and 6). In our model, most biomarkers predictive of treatment outcome in cancer patients that have been explored to date will have primarily focused only on factors determining the initial proapoptotic drive (the size of the downward arrows in Fig. 1A) and not how far the cell has left to move toward the apoptotic threshold to induce apoptosis. Thus, the ability to translate our in vitro findings into the clinic represents a novel direction in biomarker development. Specifically, we suggest that apoptotic threshold proximity, assessed through a clinically determined BAR, has the potential to be explored as an independent variable determining treatment outcome. Previous reports correlating the apoptotic index in cancer biopsies with subsequent response to radiation/chemoradiotherapy in tumors have been inconsistent (8–10). However, our clinically determined BAR assay (baseline M30 readout in blood normalized to total tumor volume determined by volumetric computed tomography scanning) seems to show very strong correlation with apoptosis induction following SBRT across a range of different cancers including NSCLC (Table 1; Fig. 6D). Although in vivo/clinical use of the M30 assay has been described before as a marker of cell death following standard or experimental anticancer treatments (15, 20, 21), its appropriate normalization to estimate a true clinical BAR represents a new development. Prospective validation of the clinically determined BAR with clinical end points of efficacy or toxicity from SBRT and with other therapies is now required.

Because the basis for the M30 assay is the detection of caspase-cleaved cytokeratin 18, in all of our in vitro work, we controlled for any differences in cytokeratin 18 expression between cell lines by conducting our analyses in isogenic systems. Of note, although there were some differences in cytokeratin 18 expression between different cell lines, none of our KDs or interventions directly affected cytokeratin 18 expression (data not shown). In our clinical assay, we explored fold change in M30 as our end point, both to create a nonconfounded variable with the BAR estimate and to compensate for any variation in cytokeratin 18 that may exist. Clinically, we do not know the extent of cytokeratin 18 differences between tumors. Yet the degree of correlation between the clinically determined BAR and the fold change in M30 following SBRT suggests either that within our series, cytokeratin 18 variation is not significant, or not significant enough to bias the result in the presence of a strong proapoptotic stimulus such as SBRT. Possibly confounding the clinical observations following SBRT is the likelihood that in addition to tumor cell apoptosis, there may also be a contribution to the serum levels of M30 by endothelial cell apoptosis, a phenomenon supported by direct preclinical and indirect clinical observations (22, 23).

One additional potential application of the assays we describe here relates to their use as a pharmacodynamic measure of the dependence of cancer on particular signaling molecules. Many direct manipulators of apoptosis are now being explored within oncology clinical trials, and preidentification of patients likely to derive the most benefit from each class of agent represents a major parallel research effort (24). Given our data on the feasibility of measuring a clinically determined BAR, it may be possible to use our assays to look for changes in the clinically determined BAR following short exposure to such agents, allowing the patient to act as their own control. In theory, those who were most dependent

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**Table 1. Clinical characteristics of analyzed patients treated with SBRT**

<table>
<thead>
<tr>
<th>Site treated</th>
<th>n</th>
<th>Male/female</th>
<th>Median age, y (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td>8:5</td>
<td>63 (30-82)</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Site of primary cancer</th>
<th>n</th>
<th>Male/female</th>
<th>Median age, y (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other*</td>
<td>8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Histology</th>
<th>n</th>
<th>Male/female</th>
<th>Median age, y (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other*</td>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Median GTV, cc (range)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>26 (4-40)</td>
<td>13</td>
</tr>
</tbody>
</table>

*One case each: ovary, bile duct, penis, maxillary sinus, tongue, esophagus, kidney, liver.
†Other includes NSCLC, not otherwise specified, or large cell neuroendocrine tumor.
on XIAP or Bcl2, for example, might then manifest the greatest change in their BAR on exposure to an inhibitor of apoptosis protein or Bcl2 inhibitor, allowing the assay to help identify those who will derive the most benefit from such drugs alone, or in subsequent combinations, in the future.

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References

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Preclinical and Clinical Estimates of the Basal Apoptotic Rate of a Cancer Predict the Amount of Apoptosis Induced by Subsequent Proapoptotic Stimuli

Lian Zhang, Brian D. Kavanagh, Andrew M. Thorburn, et al.


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