Mutated RAS and constitutively activated Akt delineate distinct oncogenic pathways, which independently contribute to multiple myeloma cell survival

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We have recently shown that approximately half of primary multiple myeloma (MM) samples display constitutive Akt activity, which disposes them for sensitivity to Akt inhibition. The Akt pathway counts among the signaling conduits for oncogenic RAS and activating mutations of K- and N-RAS frequently occur in MM. We therefore analyzed the relation between RAS mutation and Akt dependency in biopsies and CD138-purified cells from MM patients (n = 65) and the function of oncogenic RAS for MM cell survival in a range of MM cell lines with differing RAS status. Whereas RAS mutations do not predict Akt dependency, oncogenic RAS retains an important role for MM cell survival. Knockdown of either K- or N-RAS strongly decreased the viability of MM cells that harbored the respective oncogenic isoform, whereas ablation of wild-type RAS isoforms had little or no effect. Silencing of oncogenic RAS did not affect the Akt pathway, again indicating lack of a direct link. Combined inhibition of RAS and Akt strongly enhanced MM cell death. These data suggest that oncogenic RAS and Akt may independently contribute to MM cell survival. Targeting of both pathways could provide an attractive therapeutic strategy for patients with oncogenic RAS and dysregulated Akt signaling. (Blood. 2011;117(6):1998-2004)

Introduction

Multiple myeloma (MM) is an incurable neoplasia of the terminally differentiated plasma cell and accounts for approximately 10% of all hematologic cancers.¹ Intrinsic genetic lesions as well as the bone marrow microenvironment contribute to the activation of proliferation and survival pathways, impairment of cell death mechanisms, and drug resistance.²⁻⁵ Two pathways commonly dysregulated in MM involve activation of the serine/threonine kinase Akt (Akt pathway) and of the guanine nucleotide exchange factor RAS (RAS/MAPK pathway). The Akt pathway has repeatedly been found to be important for MM cell survival,⁶,⁷ and we have recently shown that approximately 50% of primary MM samples display an Akt-dependent, phospho-Akt-positive phenotype.⁷ Little is known about the mechanisms that lead to constitutive Akt activation in MM, but suitable genetic lesions, such as deletion of PTEN,⁸⁻¹⁰ or activating mutations of PIK3CA,¹¹⁻¹³ appear to be relatively rare in this disease. Conversely, the reported prevalence of activating mutations of K- and N-RAS in MM ranges from approximately 30% to 50% of patient samples.¹²⁻¹⁷ The occurrence of RAS mutation appears independent of clinical stage,¹³,¹⁷ but oncogenic RAS is associated with disease progression, aggressive phenotype, and shorter survival.¹²,¹⁷⁻¹⁹ It is also implied in the transition to MM because RAS mutations are rarely found in monoclonal gammapathy of undetermined significance.¹³,¹⁶ However, analyses of the functional role of oncogenic RAS in MM are rare, mainly because of the entire lack of specific inhibitors. Prenylation blockers, such as farnesyltransferase inhibitors, had initially been developed to curb RAS signaling. Encouraging preclinical data, however, did not translate into clinical efficiency, and it has subsequently been shown that the observed effects were largely independent of oncogenic RAS.²⁰⁻²³ It has been reported that ectopic overexpression of oncogenic RAS induces MM cell proliferation²⁴ and lowers drug efficacy.²⁵ However, it is unknown whether endogenous oncogenic RAS still contributes to the malignant phenotype of clinically overt MM and whether it therefore constitutes a potential therapeutic target in its own right. Because oncogenic RAS is a potential activator of the PI3K/Akt signaling axis,²⁶⁻²⁹ we investigated the correlation between RAS mutation and presence of dependency on activated Akt in primary MM samples. We also used knockdown approaches in MM cell lines to assess the effects of oncogenic RAS depletion on MM cells. We show that isoform-specific knockdown of RAS reduces the viability of MM cells harboring the respective oncogenic K- or N-RAS mutation, whereas little or no effects are seen when RAS isoforms are lost against a wild-type background. Because RAS mutation was not predictive for sensitivity to Akt blockade and knockdown of oncogenic RAS did not affect Akt activation, constitutively activated RAS and Akt appear to contribute independently to MM cell survival. These observations might have direct implications for the development of targeted therapies in MM.

Methods

Cell culture and primary MM cells

The human MM cell lines AMO-1, JJN-3, and U266 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). MM.1S


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cells were purchased from LGI Biolabs (ATCC-CRL-2974). Cell line INA-6 was a gift from Prof Martin Gramatzki (Kiel, Germany). MM cell culture conditions and acquisition of primary MM cells are described in detail by Stühmer et al. All cell cultures were regularly tested for mycoplasma. Bone marrow aspirates from myeloma patients were obtained after informed consent. Permission was granted by the Ethics Committee of the Medical Faculty of the University of Würzburg, Würzburg, Germany (reference no. 73/05).

**Application of drugs**

MM cells were seeded in 100 μL full medium in 96-well-plates. An equal amount of medium was added containing the respective drug in double concentration. Control incubations with dimethyl sulfoxide were always included. Akt-1,2 was obtained from Merck, and PD184352 was from Axon Medchem.

**Cell viability assay**

The fraction of apoptotic cells was identified by flow cytometry as described before. Briefly, cells were washed in phosphate-buffered saline, stained with propidium iodide and annexin V-fluorescein isothiocyanate or annexin V-allophycocyanin (Bender MedSystems), and analyzed using a FACSCalibur with CellQuest software Version 5.2 (BD Biosciences).

**RAS mutation analysis**

Primary MM samples and cell lines were screened for K- and N-RAS mutations at codons 12, 13 (both exon 2), and 61 (exon 3). A total of 10⁵ cells from freshly isolated (CD138⁺) selected patient samples or from cultured MM cell lines were washed twice with ice-cold phosphate-buffered saline and either genomic DNA (using digestion buffer, pH 8, containing 5M NaCl, 1M Tris, 0.5M ethylenediaminetetraacetic acid, 10% sodium dodecyl sulfate, 0.1 mg/mL proteinase K, followed by phenol-chloroform extraction) or total RNA (using the RNeasy Mini Kit, QIAGEN) were prepared. cDNA was synthesized using the RevertAid First-Strand cDNA Synthesis Kit (Fermentas). Polymerase chain reaction products were isolated from agarose gels and sequenced at LGC Genomics. The PCR was performed for 35 cycles, with 1 minute each for denaturation (94°C), annealing (61°C), and extension (72°C). Polymerase chain reaction products were isolated from agarose gels and sequenced at LGC Genomics. The following primers for amplification off of genomic DNA were used: 5'-ACCTTAATGTTGACATGTC-3' (K-RAS forward for exon 2), 5'-GACTGTGAATAACTCCAGCT-3' (K-RAS reverse for exon 3), 5'-ATTACTCTTAATGTCAGCT-3' (K-RAS reverse for exon 3), 5'-AGTACTGTAATGTCAGCT-3' (N-RAS forward for exon 2), 5'-TGATCCGACAAGTGAGAGAC-3' (N-RAS reverse for exon 2), 5'-CTTCTGGATTAAGCTGTC-3' (N-RAS forward for exon 3), and 5'-GATGTCCTTATACCTGTCAGT-3' (N-RAS reverse for exon 3). For amplification off of first-strand cDNA, the primers 5'-GCCGCTGTCGAAAT-GACTGAA-3' (K-RAS forward), 5'-CTGTCTGAAAGCTGTCAGCT-3' (K-RAS reverse), 5'-CTCTGCTAAAGCCATGAGG-3' (N-RAS forward), and 5'-AGTGTCACTTGCGTGAAGG-3' (N-RAS reverse) were used.

**Construction of shRNA and HA-tagged RAS expression vectors**

shRNA expression constructs were based on pSUPER.32 The following target sequences were used: 5'-GGTGGAGCCTGGCTTCTAG-3' (human K-RAS wild-type), 5'-GGTGGAGCCTGGCTTCTAG-3' (K-RAS as mutated in cell line MM.1S), 5'-GGTGGAGCCTGGCTTCTAG-3' (human N-RAS wild-type), 5'-GGTGGAGCCTGGCTTCTAG-3' (N-RAS as mutated in cell line INA-6), and 5'-ACGAGGGGATACATCAACAGC-3' (human Akt1). A pcDNA1.1-based expression vector for N-terminally triple-hemagglutinin (HA)-tagged human K-RAS was purchased from the University of Missouri-Rolla cDNA Resource Center (no. RASK20TN). The insert was excised by KpnI/XhoI digest and subcloned into pBluescript (SK) (yielding plasmid pBluescript-HA-K-RAS wt), from which the insert was transferred via KpnI/NcoI digest into a modified pCAGGS protein expression vector34 (pCAGGS-SE-HA-K-RAS wt). This construct produced considerably better expression levels of HA-tagged K-RAS in MM cells.

To generate HA-tagged N- and H-RAS expression vectors, the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) was used to introduce an in-frame BstI1071 restriction site between the triple-HA-tag and the K-RAS coding sequences in pBluescript-HA-K-RAS wt. First-strand cDNA generated from U266 cells was used to amplify N- and H-RAS genes with primers 5’-AGTCGTATATCGAGCAACTCTGGTGG-3’ (N-RAS forward), 5’-AGTCTCGAGTTTACATCCACACATAGC-3’ (N-RAS reverse), 5’-AGTCGTATATCGAGCAACTCTGGTGG-3’ (H-RAS forward), and 5’-AGTCCTCGAGTTTACATCCACACATAGC-3’ (H-RAS reverse). The primers introduced BstI1071 and XhoI restriction sites (boldface type) to immediately flank the 5′- and 3′-ends of the coding regions, and they lead to deletion of the native start codons. The genes were cloned to replace K-RAS in pBluescript-HA-K-RAS wt and again transferred to pCAGGS/SE.

Point mutated forms of K-RAS (representing the mutation in MM.1S cells) and N-RAS (as mutated in INA-6 cells; supplemental Table 1B, available on the Blood web site; see the Supplemental Materials link at the top of the online article) were generated with the Quik Change II kit in the respective pBluescript constructs described above, and subcloned into pCAGGS/SE.

**Transfection of MM cells by electroporation**

AMO-1, INA-6, JJN-3, MM.1S, and U266 cells were washed and resuspended in fresh, pure RPMI 1640 medium at a density of up to 1.5 × 10⁶ cells/500 μL. Transient transfection of shRNA and protein expression vectors was performed at 950 μL and 280 V (310 V for MM.1S cells; Gene Pulser 2, Bio-Rad; in 4-mm cuvettes, PoqLab). AMO-1, JJN-3, MM.1S, and U266 cells were cotransfected with an expression plasmid for enhanced green fluorescent protein (pcDNA3.1-EGFP), INA-6 cells were cotransfected with an expression plasmid for truncated CD4.31 Cells were kept for one day in full RPMI 1640 medium with 10% fetal bovine serum, and purification of strongly transfected cells was achieved either by CD4 microbead selection31 or by cell sorting of EGFP+ cells (MoFlo; Beckman Coulter). For electroporation of control cells, shRNA-expressing pSUPER constructs were substituted for the same amount of empty pSUPER vector. For Western analyses, cells were harvested 2 days after electroporation. After one wash in ice-cold phosphate-buffered saline, cell pellets were snap frozen in liquid nitrogen and stored at −80°C until further use.

**Western analysis**

Western blotting was performed essentially as described before.35 Briefly, cell pellets were dissolved in 20 μL of lysis buffer, protein concentrations determined, and equal amounts mixed with Laemmli buffer run on sodium dodecyl sulfate 10%-polyacrylamide gels before blotting on nitrocellulose membranes. The anti-HA-tag antibody (ab9110) was purchased from Abcam, anti-K-RAS (sc-30, lot A2309) and anti-N-RAS antibodies (sc-31, lot K1709) were from Santa Cruz Biotechnology. Antibodies detecting pan-Akt (no. 9272), phospho-Akt (Ser473, no. 4058), phospho-Akt (Thr308, no. 2965), ERK1/2 (no. 9102), phospho-ERK1/2 (no. 9101), phospho-FOXO1/3a (no. 9464), and phospho-GSK-3β (no. 9356) were purchased from Cell Signaling Technology. Antiβ-actin antibody (A5316) was available on the Blood Web site; see the Supplemental Materials link at the top of the online article. (no. 9356) was purchased from Sigma-Aldrich, anti-tubulin (no. 03568) was from Biozol. Secondary antibodies specific for rabbit (no. 111-036-045), mouse (no. 115036-003), or rat (no. 112036-062) were from Jackson ImmunoResearch Laboratories.

**Immunofluorescent histochemical stainings of bone marrow biopsies**

To analyze the expression of phospho-Akt (Ser473) and of phospho-Akt (Thr308) in MM cells, immunofluorescent stainings were performed for either antigen in combination with detection of CD138 in formalin-fixed, paraffin-embedded bone marrow biopsies heavily infiltrated with MM cells. For both the CD138/phospho-Akt (Ser473) and the CD138/phospho-Akt (Thr308) double stainings, the primary antibodies were derived from different species. The following antibodies were used: anti-CD138 (M7728,
We have previously shown that the phospho-Akt status in MM cells is largely predictive of cell death induction through inhibition of Akt, and that for primary MM samples approximately 50% displayed a phospho-Akt-positive/Akt inhibition-sensitive phenotype. Because a similar number has been reported for activating mutation status does not predict dependency on Akt in MM. Collectively, these data suggest that oncogenic RAS is not a primary driver of intrinsic Akt activity in MM.

Validation of isoform-specific shRNA expression constructs against K- and N-RAS

Because of the lack of suitable inhibitors, it remains unclear whether oncogenic RAS retains a functional role in MM. We therefore decided to establish an isoform-specific knockdown approach (ie, to investigate the loss-of-function consequences in these gain-of-function phenotypes). Genetic analyses of 5 MM cell lines suitable for transfection by electroporation showed that AMO-1 and U266 cells are RAS wild-type, INA-6, and JJN-3 cells display an activating N-RAS mutation, and MM.1S cells harbor oncogenic K-RAS (supplemental Table 1B and supplemental Figure 1; see legend to supplemental Figure 1 for comments on discrepancies in the published RAS status for some of these cell lines). Because of the high sequence homology of RAS isoforms at protein as well as at DNA level, and to assess potential selectivity for wild-type and mutant RAS alleles, it was mandatory to control the specificity of the reagents. Complicating matters, isoform-specific RAS antibodies can display some cross-reactivity (supplemental Figure 2), and RAS proteins may be present in different levels in different MM cells. We therefore cotransfected expression vectors for HA-tagged wild-type and mutant RAS proteins to assess the suitability of the allele- and isoform-specific shRNA expression vectors. Both the K-RAS and the N-RAS-specific knockdown constructs led to strong and selective depletion of their respective target (Figure 2A). However, the constructs did not distinguish between the wild-type and mutant alleles of either the K-RAS (Figure 2B) or N-RAS isoform (data not shown), showing that the single-base differences between target sequences were insufficient to confer effective selectivity. The constitutively active K- and N-RAS proteins are therefore of necessity always depleted in concert with their respective wild-type twin.

Silencing of oncogenic RAS induces MM cell death

To assess the dependency of MM cells on oncogenic RAS, we transfected each of the 5 MM cell lines (AMO-1, INA-6, JJN-3, MM.1S, and U266) with K- or N-RAS knockdown constructs and measured cell viability over the course of 5 days in culture. Whereas the 2 RAS wild-type cell lines (AMO-1, U266) remained resistant (Figure 1A). Comparing the distribution of the survival rates between the RAS wild-type and RAS mutated groups, we found no statistical correlation (P = .05, Student t test; Figure 1A). Considering these results with immunohistochemical detection of phosphorylated Akt (Ser473 and/or Thr308) in the matching bone marrow biopsies, however, largely confirmed our previous observations in that, of the 19 MM samples for which both pharmacologic and immunohistochemical data were available, the large majority of phospho-Akt-positive samples was sensitive to Akt inhibition, whereas phospho-Akt-negative samples were mostly resistant (P = .005, Student t test; Figure 1B). For the correlation between phospho-Akt positivity in biopsies and the presence of oncogenic RAS in the cognate MM samples, we found that mutant RAS was present in 16 of 34 (47%) phospho-Akt-positive cases, as well as in 8 of 25 (32%) phospho-Akt-negative samples (Figure 1C). These numbers do not imply a statistically significant correlation between the presence of oncogenic RAS and phospho-Akt status (P = .29, Fisher exact test), although with the qualification that at n = 59 the statistical power for these conclusions is still limited. Collectively, these data suggest that oncogenic RAS is not a primary driver of intrinsic Akt activity in MM.

Statistical analysis

A 2-tailed Student t test was applied to perform statistical analysis of cell viability assays. For categorical data, Fisher exact test was used. Results were considered significant at P < .05. Calculations were performed with IBM SPSS Statistics 18.

Results

RAS mutation status does not predict dependency on Akt in primary MM

We have previously shown that the phospho-Akt status in MM cells is largely predictive of cell death induction through inhibition of Akt, and that for primary MM samples approximately 50% displayed a phospho-Akt-positive/Akt inhibition-sensitive phenotype. Because a similar number has been reported for activating mutation status.
virtually unaffected by isoform-specific RAS depletion (Figure 3) or by combined knockdown of K- and N-RAS (supplemental Figure 3), the shRNA expression vectors led to decreased viability specifically in those MM cells that harbor the respective oncogenic RAS isoform (Figure 3). Thus, knockdown of N-RAS entailed significant cell death specifically in N-RAS mutated INA-6 (29% viability relative to empty vector treated controls) and JJN-3 (46% viability) cells. Conversely, MM.1S cells, which harbor oncogenic
levels of Akt substrates GSK-3β and FOXO1/3α were not affected by K-RAS knockdown in MM.1S cells, either (Figure 4). Because oncogenic RAS and Akt may therefore represent components of 2 independent survival pathways, we tested the effects of simultaneous inhibition of these proteins. An shRNA expression vector against Akt1 (shown to be effectively inducing cell death in MM.1S cells) or the small molecule Akt1&2 inhibitor Akti-1,2 were combined with the K-RAS knockdown vector (Figure 5). The K-RAS mutant MM.1S cells, which are dependent on both K-RAS and Akt, displayed significantly enhanced levels of cell death when these proteins were down-regulated together (Figure 5 left). This effect was also very prominent when K-RAS knockdown was complemented with pharmacologic inhibition of Akt (Figure 5 middle). Contrarily, the viability of RAS wild-type AMO-1 cells, which neither depend on RAS nor display constitutive activity of Akt, was unaffected by combined knockdown of K-RAS and Akt1 (Figure 5 right).

Inhibition of MEK/MAPK alone does not recapitulate the effects of RAS knockdown

The observation that knockdown of oncogenic RAS in MM.1S and JJN-3 cells strongly decreased the levels of phospho-ERK1/2 led us to examine to what extent oncogenic RAS might exert its prosurvival effect via the MAPK pathway. Treatment of MM cells with 10 μM of the MEK inhibitor PD184352 did not lead to significant decreases in viability, even though at this concentration the phosphorylation of the MEK substrates ERK1 and ERK2 is completely and permanently blocked (supplemental Figure 5). The prosurvival effect of constitutively active RAS in MM cells is therefore not primarily mediated via the MEK/ERK module, again underpinning that RAS represents a potentially useful therapeutic target in its own right.

Discussion

Both the RAS/MAPK and the PI3K/Akt pathway have garnered considerable attention for their presumed role in the pathogenesis and potential suitability for therapeutic intervention in MM.36-38 We have recently shown that Akt contributes to tumor cell survival in approximately 50% of primary MM cases and that sensitivity to Akt inhibition is predicted by the presence of constitutive Akt

[Diagrams and tables related to the text are not transcribed here, as they are not available in the text format.]
phosphorylation. Because genetic lesions within the Akt pathway are relatively rare in MM, we decided to investigate whether oncogenic RAS is a main driver of intrinsic Akt activity and to analyze its role for MM cell viability. Oncogenic RAS is a potential upstream activator of the Akt pathway, and activating mutations in the K- and N-RAS genes are generally found in up to 50% of MM cases. Our study, based on the correlation of the RAS mutation status of primary MM samples with sensitivity to pharmacologic Akt inhibition and with the phosphorylation status of Akt in corresponding bone marrow biopsies, again denoted the presence of phosphorylated Akt as indicator for Akt dependency. However, it also showed that even if RAS-mutated MM samples tended to express phosphorylated Akt more frequently, no correlation between Akt dependency and the presence of oncogenic RAS existed. Accordingly, knockdown of oncogenic RAS in cell lines MM.1S and JJN-3 failed to attenuate the intrinsic phosphorylation of Akt or of Akt substrates, such as GSK-3β or FOXO1/3a, also indicating that the presence of oncogenic RAS is not required to sustain endogenous Akt activity. This is in contrast to studies in RAS wild-type ANBL-6 MM cells, where stable overexpression of oncogenic RAS entailed increased levels of Akt phosphorylation. However, our results are in agreement with loss-of-function analyses in solid tumor entities where oncogenic RAS was not found to be a primary driver for constitutive Akt activation. Notwithstanding its statistical lack of significance, a larger number of RAS mutant primary MM samples did also show constitutive activation of Akt. We would therefore, not rule out that oncogenic RAS may well support Akt activity in a subset of MM cases.

The oncogenic RAS isoforms therefore constitute potential therapeutic targets in their own right. Although RAS is currently not drugable, the development of strategies to block oncogenic RAS function is warranted and highly desirable for novel treatment approaches, which would be of immediate relevance for a well-defined patient subgroup.

Figure 5. Combined knockdown of oncogenic RAS and Akt enhances cell death in MM.1S cells. Simultaneous depletion of oncogenic K-RAS and attenuation of Akt activity, either by shRNA-mediated knockdown (left) or by pharmacologic inhibition of Akt1 and 2 with Akti-1,2 (middle), significantly enhanced cell death in K-RAS mutated MM.1S cells. The concentrations chosen for the expression vectors for K-RAS shRNA (15 μg/mL) and Akt1 shRNA (10 μg/mL) and for Akti-1,2 (2.5 μM) were such that an approximately medium size effect on the viability after 5 days in culture could be expected. RAS wild-type AMO-1 cells, which are insensitive to Akt inhibition, were not significantly affected by either single or combined knockdown of K-RAS and Akt1 (right).

Our study suggests that in MM oncogenic RAS mutation and Akt dependency are not 2 sides of the same medal: neither does oncogenic RAS necessarily activate Akt nor does constitutive activation of Akt depend on the presence of oncogenic RAS. This indicates that intrinsically active RAS and Akt may delineate distinct oncogenic pathways, which independently contribute to MM cell survival. Such a notion is supported by our observation that depletion of K-RAS, when combined with Akt blockade (via knockdown of Akt1 or via pharmacologic inhibition of Akt), led to enhanced cell death in the K-RAS mutant/Akt-dependent cell line MM.1S but was without effect in RAS wild-type/Akt-independent AMO-1 cells.

Oncogenic RAS is known to sustain activity of the RAS/MAPK pathway, and in our hands, too, knockdown of oncogenic RAS led to a strong decrease in the level of phosphorylated ERK1/2 in MM.1S and JJN-3 cells. Nevertheless, although sustained blockade of this pathway with suitable concentrations of MEK inhibitor PD184352 abrogated ERK1/2 phosphorylation, it had little or no effect on the survival of MM cells regardless of their RAS mutation status. To that end, we have previously shown that knockdown of ERK1&2 did not impair survival of N-RAS mutated INA-6 cells. This indicates that the prosurvival effects of oncogenic RAS in MM are unlikely to be exclusively transmitted via the MEK/ERK module. Pharmacologic intervention at the level of MEK is thus not a substitute for direct RAS blockade. Oncogenic K-RAS has recently been shown to be associated with the presence of activated nuclear factor-kB signaling in various human carcinoma cell lines. However, comparison of the RAS mutation status of the MM cell lines represented here with their ranking for nuclear factor-kB activity (nuclear factor-kB transcriptional signature index) recently published by Demchenko et al. does not suggest a clear pattern. RAS mutant cell lines appear equally well represented among those ranking highest (eg, MM.1 and JJN-3) or lowest (eg INA-6, NCI-H929) in the index.

In conclusion, our study shows, for the first time, that oncogenic RAS isoforms sustain the survival of MM cells and that this effect is unlikely to be mediated via either the Akt or the MAPK pathway. The oncogenic RAS isoforms therefore constitute potential therapeutic targets in their own right.

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