H-ras Up-regulates Expression of BNIP3

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Abstract. *Background: Bcl-2/adenovirus E1B 19-kDa protein-interacting protein 3 (BNIP3) is a key regulator of cell death/autophagy and can act as an effector of a necrosis-like, atypical death program. It was implicated in execution of cell death induced by cluster of differentiation 47 (CD47). Despite the postulated role of BNIP3 in the regulation of survival of cancer cells, the influence of oncogenic transformation on BNIP3 expression is unclear. Materials and Methods: The influence of oncogenic transformation on expression of BNIP3 was studied using H-ras-transformed cells. The consequences of BNIP3 expression for sensitivity to CD47-mediated cell death were assessed using tetrazolium salt-based assay. Results: Here, the enforced and endogenous expression of Ras coincided with the up-regulation of BNIP3 across a wide spectrum of cancer cells, providing the first experimental evidence that BNIP3 is a regulatory target of H-Ras. This indicated that merely the introduction of a single oncogene may result in the up-regulation of BNIP3. The consequences of CD47 ligation strongly depended on the BNIP3 presence, which in turn correlated with Ras expression. Interestingly, the indirect effect of that phenomenon was the selective sensitivity of Ras-transformed cells to CD47-mediated cell death.*

Bcl-2/adenovirus E1B 19-kDa protein-interacting protein 3 (BNIP3) is classified as a member of Bcl-2 homology domain 3 (BH3)-only subfamily of the Bcl-2 protein family that regulate programmed cell death (1-3). BH3-only proteins are thought to be predominantly the mediators of proapoptotic signals of various origin, due to the activity of their BH3 domain. Although there are numerous similarities between BNIP3 and other proteins that belong to this group, *i.e.* the capacity to interact with antiapoptotic Bcl-2 proteins, BNIP3 is undoubtedly distinct in regard to its activity and regulatory mechanisms. Beyond its ability to induce apoptosis, BNIP3 can act as an effector of a necrosis-like, atypical death program (4), through its transmembrane domain (1). It is also a key regulator of cell death/autophagy caused by oxygen deprivation (5). On the other hand, BNIP3 possesses antiapoptotic activities, *i.e.* after heart ischemia/reperfusion injury, BNIP3 induces autophagy of the miocardiocytes, suppressing their apoptotic death (6). Interestingly, BNIP3 was also shown to act as the transcription repressor of apoptosis-inducing factor (AIF) gene in astrocytes and glioma cells, preventing causeless death induction in normal cells, but also conferring chemoresistance to cancer cells (7).

The control of *BNIP3* gene expression has been extensively studied and was shown to be regulated mainly by hypoxia and hypoxia-inducible factor 1 (HIF-1) in cooperation with nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB), E2F1 and also retinoblastoma protein (8-11). On the other hand, much less is known about the expression control of the *BNIP3* gene in normoxic conditions. During normoxia, in macrophages, nitric oxide (NO) can stimulate BNIP3 expression, whereas in other cells (hepatocytes), NO suppresses the transcription of *BNIP3* gene (12).

One of the well known aspects of the proapoptotic properties of BNIP3 is its participation in the activation-induced cell death of lymphocytes (13, 14), macrophages (15), and fibroblasts under mechanical stress (16). These activities were attributed to the interaction of BNIP3 with cluster of differentiation 47 (CD47), a ubiquitously expressed receptor of thrombospondin 1 (TSP-1), a potent angiogenesis inhibitor). This interaction can be mimicked using 4N1K, a decapeptide derived from the *C*-terminal domain of TSP-1 (17). Upon lymphocyte activation, BNIP3 accumulates in cells. CD47 ligation by CD47 agonists (antibodies, TSP-1 or 4N1K) triggers BNIP3 migration to mitochondria and the induction of the atypical death pathway (13, 14, 18, 19).

Considering the fact that BNIP3 is involved in multiple cell death pathways, it is not surprising that its role in cancer cells

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Key Words: Ras, BNIP3, CD47, cancer, programmed cell death, 4N1K.

is highly intricate and controversial. On the one hand, there are numerous studies showing the silencing of *BNIP3* gene expression in some colorectal (20, 21), gastric (20) and pancreatic carcinomas (22, 23), as well as in acute lymphocytic leukemias (24), where the loss of its expression was correlated with shorter survival. On the other hand, *BNIP3* was shown to be up-regulated in non-small cell lung cancer (25), follicular lymphomas (26), ductal carcinomas (27) and colorectal adenocarcinomas, where its elevated levels were correlated with high-grade and invasive disease (28).

Elevated levels of BNIP3 in cancer cells are usually attributed to the appearance of hypoxic regions widespread in solid tumors. This indicates HIF-1 as a key regulator of *BNIP3* transcription. Interestingly, in ductal carcinomas and cervical cancer, association between hypoxia or the expression of hypoxia-inducible factor 1, alpha subunit (HIF-1α) and BNIP3 levels was not found (27, 29). This suggests the presence of an additional cancer-related mechanism of regulation of BNIP3 expression. Despite the studies showing correlations between BNIP3 expression and progression of some cancer types, no study has linked alterations of BNIP3 expression with the introduction of a single oncogene. The present study focuses on the impact of the presence of mutated *Ras* oncogene, one of the most common alterations associated with human malignancy (30) on the expression of BNIP3 in normoxic conditions and its consequences for susceptibility to CD47 induced cell death.

Materials and Methods

Cells and reagents. The immortalized, non-tumorigenic, polyclonal mouse dermal fibroblastic cell line MDFB6 was derived from dermal explants of C57Bl/6 mice. These cells and their stably transformed isogenic polyclonal variant B6ras harboring a mutant *H-ras* oncogene have been described elsewhere in detail (31). The cells were cultured in Dulbecco modified Eagle's medium (HyClone; Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen Corp., Carlsbad, CA, USA). The human cancer cell lines were obtained from Polish Academy of Sciences Cell Collection. The A549 (carcinomic human alveolar basal epithelial cell), A498 (kidney carcinoma), PC3 (hormone-independent prostate cancer), and MCF7 (invasive breast ductal carcinoma) were cultured in a 1:1 mixture of OptiMEM (Gibco) and RPMI (Institute Immunology and Experimental Therapy, Wroclaw, Poland) supplemented with 5% FBS (PAA Laboratories GmbH, Pasching, Austria). The LnCap (hormone-dependent prostate cancer) and HL60 (human promyelocytic leukemia) cells were grown in Iscove medium (Gibco) with 10% FBS (Gibco). All the cultures were supplemented with antibiotic and antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA). The culture plates and flasks were purchased from Nunc (Thermo Fisher Scientific Inc.). The 4N1K peptide was purchased from Lipopharm (Gdansk, Poland). The primary antibodies used in the study included rabbit anti-BNIP3 (2 μg/ml; Abcam Inc., Cambridge, MA, USA) and goat anti-actin (1 μg/ml; Dako, Glostrup, Denmark) antibodies.

Determination of CD47 expression. CD47 expression was determined by flow cytometry using anti-CD47 (0.5 μg/ml, 15 min, 4˚C; MIAP301; Santa Cruz Biotechnology, Heidelberg, Germany) and anti-human CD47 (0.5 μg/ml, 15 min, 4˚C; B6H12; Abcam) antibodies. This was followed by incubation with the appropriate FITC-conjugated secondary antibodies (1/1000, 15 min, 4˚C; Dako).

Western blotting. The cells were lysed in a buffer (20 mM Tris-HCl, 100 mM NaCl, 0.5% NP-40) supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich). The proteins were resolved by SDS-PAGE (12% gel) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Billerica, MA, USA). The membranes were blocked and incubated with the indicated primary antibodies followed by detection with the secondary horseradish peroxidase (HRP)-conjugated antibodies (1/4000; Dako) and an ECL (Enzymatic Chemiluminescence) blotting detection system (Pierce, Thermo Fisher Scientific Inc., Rockford, IL USA).

Quantitative polymerase chain reaction (real-time PCR). The total RNA was extracted from the cells using a Total RNA Minipreps Super Kit (Bio Basic, Ontario, Canada) followed by DNase I (Fermentas, Ontario, Canada) treatment. The RNA was reverse-transcribed with oligo(dT) primers (Sigma-Aldrich) using SuperScript III Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA, USA). Quantitative PCR was performed with a 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using reagents from Applied Biosystems. The expression levels of *BNIP3* were evaluated using the primer/probe set Mm00833810_g1 (Applied Biosystems). Expression of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous control (Applied Biosystems).

MTS assay. Cell viability was determined using the MTS Assay (Promega Corp., Madison, WI, USA) as recommended by the supplier. Briefly, 5000 cells/well were cultured in a 96-well plate for 72 h in the presence of the indicated concentrations of 4N1K peptide, or other agents as indicated. The results are presented as percentages of the control samples±standard deviation (SD); *p*-values were calculated using unpaired Student's *t*-test.

Suppression of BNIP3 expression. Target-validated *BNIP3*-directed small interfering RNA oligonucleotides (siRNA) were transfected into the cells using an Accell Delivery System (Dharmacon, Inc., Chicago, IL, USA). Briefly, the cells were transfected with 1 μM Accell siRNA per well diluted in Accell Delivery Media supplemented with 1% serum and incubated for 72 h prior to treatment. The down-regulation of *BNIP3* expression by specific siRNA and not by control oligonucleotides, was validated by Western blotting.

Ras activity assay. Ras pull-down assay was performed using an EZ-Detect™ Ras Activation Kit (Pierce) according to the manufacturer's instructions. Briefly, 10^6 cells were lysed in 0.5 ml of the lysis buffer and equivalents of 0.5 mg of total protein were incubated with the GST-protein binding domain (PBD) fusion protein (GST-Raf1-PBD) and loaded into preconditioned columns. The active Ras was affinity-purified by binding to glutathione and eluted from the glutathione resin using SDS-PAGE sample loading buffer. The eluate was resolved using SDS-PAGE and probed with Ras-specific antibody.

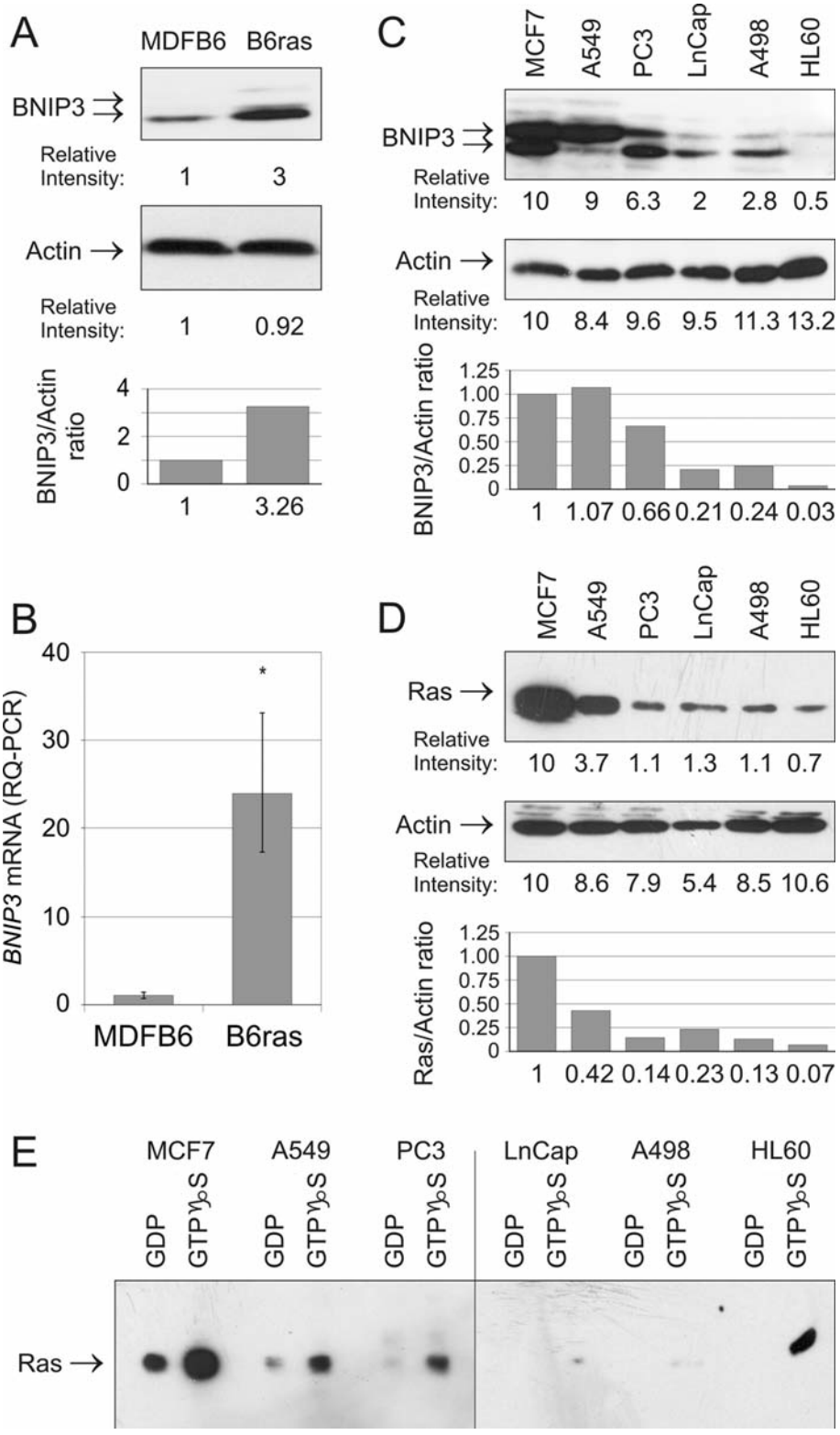

Figure 1. *Expression of BNIP3 and activated Ras. BNIP3 expression in ras-transformed cells (B6ras) compared with their isogenic, non-transformed counterparts (MDFB6) detected at the protein (Western blot) (A) and mRNA (average and ranges of real-time-PCR) (B) levels. BNIP3 (C) and Ras (D) expression shown by Western blot in a panel of human cancer cells (E). Activation of Ras measured by GTP ras activation assay. Western blots were quantified by densitometry and normalized to the signal for β-actin. A representative result of four independent repeats is displayed (A, C-E). *p<0.01; n=6.*

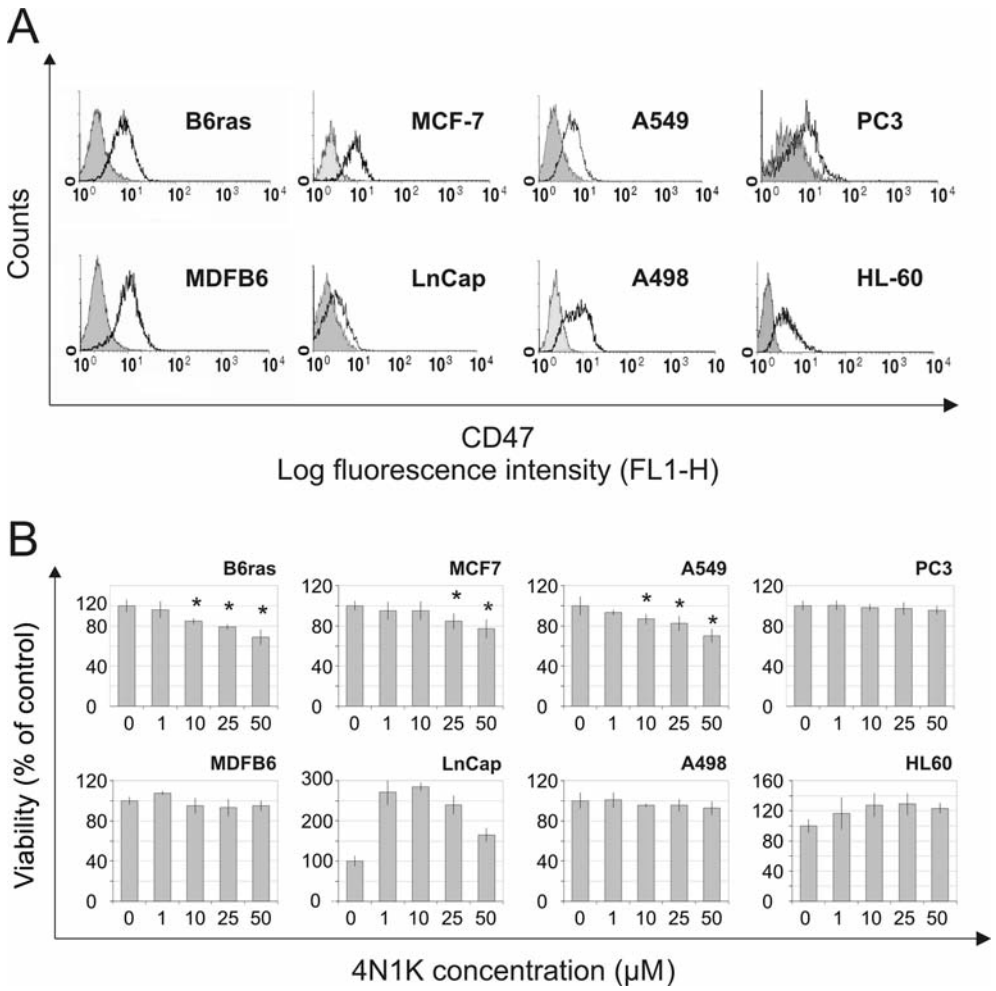

Figure 2. *Sensitivity of human tumor cell lines to CD47 ligation. A: Representative expression of the CD47 receptor on the cell surfaces, assayed by flow cytometry. The filled histograms represent control staining with the FITC-conjugated secondary antibody only (no primary). Isotypic control antibody resulted in identical staining (data not shown). B: Cell viability measured by MTS assay of cultured mouse dermal fibroblasts (MDFB6 and B6ras) and human tumor cells in the presence of 4N1K peptide (anti-CD47). Percentages of the MTS signal in peptide-treated cultures relative to untreated control. Average±SD of at least three independent experiments. *p<0.02; 6<n<12.*

Results

Expression of BNIP3 and Ras. BNIP3 was markedly up-regulated in normoxic conditions by the introduction of the *H-ras* oncogene. The expression of protein (Figure 1A) as well as mRNA (Figure 1B) was a few-fold up-regulated in the *H-ras*-transformed B6ras cells compared with their normal counterparts. To extend these observations, a panel of human cancer cell lines was also screened for the expression and the activation (GTP-loading) of Ras protein as well as for their *BNIP3* status. While this screening revealed considerable variation in the levels of Ras (Figure 1D), the Ras level positively correlated with the expression of BNIP3 (Figure 1C) and with GTP loading (Figure 1E), with exception of low Ras expressing PC3 cell line, which showed the significant activation of Ras. The highest levels of BNIP3 were found in the cells with a particularly high Ras expression (*e.g.* MCF7 and A549) and lower in those in which less Ras protein was detectable (*e.g.* LnCap). Collectively, these observations suggested that both enforced (Figure 1AB) and endogenous (Figure 1C) expressions of the activated Ras coincided with the up-regulation of *BNIP3* across a wide spectrum of cancer cell types (Figure D).

Effect of BNIP3 on cancer cell death induced by CD47 ligation. First the expression of CD47 on the surface of mouse dermal fibroblasts and human cancer cell lines was examined. The MDFB6, B6ras, MCF7, A549, A498 and HL60 cells were highly CD47 positive, while both prostate cancer cell lines PC3 and LnCap exhibited noticeably lower surface expression

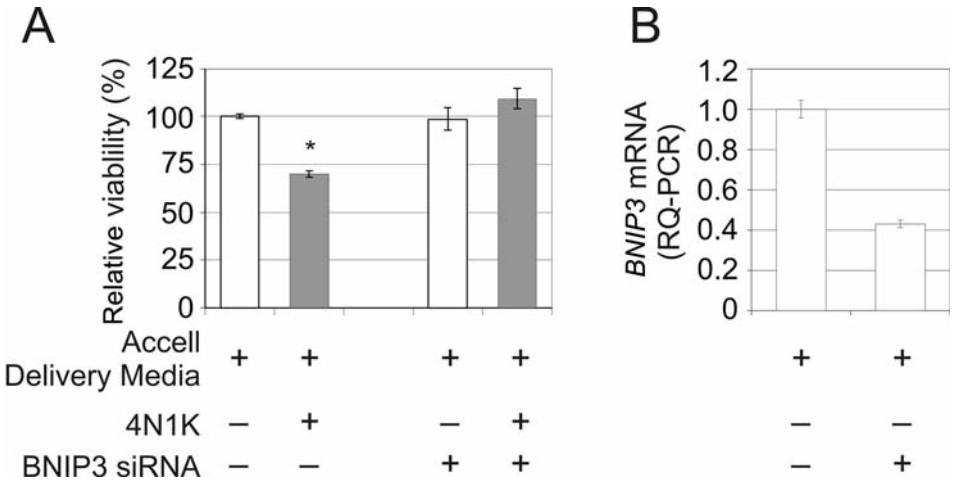

Figure 3. *Effect of BNIP3 on CD47-mediated cytotoxicity in A549 cancer cells harboring oncogenic ras. A: BNIP3 mRNA down-regulation by a mixture of BNIP3-directed siRNA oligonucleotides. Average and ranges of real-time-PCR. B: Cell viability with or without siRNA-mediated down-regulation of BNIP3 expression and/or 4N1K peptide (anti CD47) MTS assay; average±SD; *p<0.02; n=4.*

of this receptor (Figure 2A). These various human and rodent cells were subsequently cultured in the presence of the agonistic anti-CD47 peptide (4N1K) and assayed for changes in cellular viability by the MTS assay (Figure 2B). The viability of the immortalized MDFB6 fibroblasts expressing high levels of CD47, but low levels of BNIP3 remained unaffected in the presence of TSP-1 derived 4N1K peptide, which in contrast caused the death of BNIP3-expressing B6ras cells. Similar determinants of the response to the CD47-directed peptide were apparent amongst the human cancer cell lines (Figure 2B) 4N1K induced a dose-dependent loss of cell viability within a wide range of concentrations (1-50 μM), as detected by the MTS assay, in cells co-expressing high levels BNIP3 and CD47. In particular the MCF7 and A549 cells were the most sensitive to this treatment. On the other hand, cell death was mitigated in the cells expressing low levels of CD47 (PC3) or BNIP3 (A498). In the case of the HL60 cells, which are nearly negative for BNIP3, 4N1K treatment induced a mild increase in proliferation. Moreover, in the LnCap cells, low expressers of BNIP3 and CD47, growth was stimulated with low concentrations of 4N1K, but this effect was partially reversed with increasing doses of this peptide (Figure 2B).

Effect of BNIP3 on induction of CD47-mediated cell death. Exposure of A549 cells, which express high levels of activated Ras (Figure 1CE), BNIP3 (Figure 1D) and CD47 (Figure 2A), and are highly sensitive to 4N1K peptide (Figure 2B) to siRNA oligonucleotides which selectively down-regulate human *BNIP3*, as expected, selectively down-regulated the levels of protein (by approximately 50%, not shown) and mRNA (Figure 3B) and markedly diminished the loss of the viability observed upon treatment of these cells with 4N1K peptide (Figure 3A). In contrast, neither the transfection media, nor a cocktail of unspecific siRNA oligonucleotides (not shown) exerted any discernable effect in these assays.

Discussion

The increased expression of *BNIP3* at both mRNA and protein levels was highly correlated with the enforced or endogenous expression of oncogenic Ras. This indicated that merely the introduction of a single oncogene (in this case *H-ras*) may result in the up-regulation of *BNIP3*, even in normoxic conditions. It clearly indicated the presence of a novel, hypoxia-independent, but Ras-dependent mode of regulation of BNIP3 expression. This could serve as the explanation for the phenomenon of elevated BNIP3 expression observed in ductal carcinoma (27) and cervical cancer, where no correlation with hypoxia and HIF1α was found (29).

The mechanisms by which *BNIP3* is up-regulated by activated Ras remain unclear. Some studies have suggested that protein kinase A may play a role in BNIP3-mediated cell death (19, 32). Notably, BNIP3 can associate with CD47, which protects it from proteasomal degradation. This results in the retention of the protein in the cell (13). Hence mechanisms that would lead to further up-regulation of *BNIP3* in CD47-expressing cells could potentially lead to the accumulation of this protein at high levels, a process that could ultimately precipitate programmed cell death in the presence of CD47 ligands, of which TSP-1 is a known and interesting example. Here, the induction of programmed cell death in murine and human cancer cells co-expressing high levels of activated Ras, CD47 and BNIP3 was documented when such cells were exposed to a CD47-binding TSP-1-derived peptide (4N1K). A

low expression of this molecule resulted in resistance to CD47-mediated cell death. Additionally, even 50% decrease in BNIP3 expression in (CD47- and Ras-expressing) A549 cells diminished its sensitivity to 4N1K. In such instances, Ras-dependent up-regulation of *BNIP3* leads to selective sensitivity of *H-ras* transformed cells to CD47-directed agents.

The increasing appreciation of the multifaceted involvement of oncogenic pathways in the progression of human malignancies has raised considerable interest in developing targeted anticancer therapies (33). It is recognized that while activated oncogenes, including mutant *Ras*, may impart a selective growth advantage on the affected cancer cells (34), notably through cell proliferation (35), survival (36) and angiogenesis (37), this is achieved at the cost of certain vulnerabilities. This process is commonly described as 'oncogene addiction' (38). For example, Ras-driven cancer cells rely on the continuous presence of this 'oncogene addiction' to form tumors *in vivo* (39, 40). This is believed to be, at least in part, a consequence of the Ras-dependent stimulation of angiogenesis (31, 37), and down-regulation of TSP-1 is believed to be among the key events (41, 42). In this sense, the present studies reveal a novel aspect of Ras-driven tumorigenesis, namely an increased sensitivity to CD47-specific ligands. We suggest that this property may open new therapeutic opportunities, especially in combination with other agents. In the present study, the minimal requirement for the induction of selective cancer cell demise through the ligation of CD47 consisted of the simultaneous expression of this receptor (a Ras-independent event) and the up-regulation of *BNIP3* (Ras-dependent). The cytotoxic activity of the 4N1K peptide differed among the human cancer cell lines studied, which may explain the diversity of anticancer responses to this peptide reported in the literature (13, 15, 16, 43-47).

The control of BNIP3 by activated Ras is worthy of further study, as similar regulatory influences could be found in association with other transforming events acting upstream (receptor tyrosine kinases) and downstream of Ras (mitogen-activated protein kinases, phosphatidylinositol 3-kinases) and could broaden the possibility of exploiting *BNIP3* up-regulation as a tumor-sensitizing mechanism. Overall, the co-expression of Ras, BNIP3 and CD47 could have both therapeutic and prognostic significance. The possibility of using CD47-directed agents in therapy for in a subset of patients with tumors that co-express CD47 and BNIP3 along with other agents represents an intriguing research direction for future studies.

Acknowledgements

We are most grateful to Ewa Ziolo and Pawel Skiba for their technical assistance.

This work was supported by grant no. N301 104 31/3087 and 1243/B/P01/2007/33 from the Polish State Committee for Scientific Research and a grant from the Canadian Cancer Society Research Institute to JR, who also holds the Jack Cole Chair in Pediatric Hematology/Oncology.

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Received April 12, 2011
Revised June 14, 2011
Accepted June 15, 2011