Activated N-Ras Contributes to the Chemoresistance of Human Melanoma in Severe Combined Immunodeficiency (SCID) Mice by Blocking Apoptosis

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Abstract

Activation of the N-ras gene by point mutations occurs in about 15% of all human melanomas. Using recently established melanoma severe combined immunodeficiency-human mouse xenotransplantation models, here we further investigate the biological significance of these mutations. We demonstrate that activated N-ras significantly contributes to the chemoresistance of human melanoma both in vitro and in vivo by blocking apoptosis. Overexpression of wild-type N-ras had no such effects. With antisense oligonucleotides and farnesyltransferase inhibitors, tools capable of blocking Ras function on the therapeutic horizon, our observation that activated N-ras is not a bystander but a factor worth targeting to improve therapeutic outcome in melanoma gains additional importance.

Introduction

Malignant melanoma, an example for poor response to a variety of treatment strategies including chemotherapy, expresses activated N-ras in about 15% of all cases (1). Two recently described SCID-human mouse xenotransplantation models for human melanoma overexpressing activated N-ras genes (2) provided a tool to study the role ras genes play in human melanoma and to shed some light on their biological function. Mutated N-ras genes had been shown to influence the growth characteristics of human melanoma both in vitro and in vivo in SCID mice without altering its metastatic potential (1), an observation consistent with recent clinical findings (2). Based on reports in experimental nonmelanoma systems (3—8), we hypothesized that activated ras genes may also play a role in the chemoresistance of human melanoma. In the present study, we test this concept.

Materials and Methods

Melanoma Cells and Cell Culture. The human melanoma cell lines 518A2 and IGR39D, both carrying wild-type N-, H-, and K-ras genes, were transfected and characterized as described earlier (2). The mutated N-ras transfectants 518-L1 (61-Leu/L1) and IGR-K1 (61-Lys/K) and the neomycin control transfectants (518-neo and IGR-neo) of these parental cell lines were cultured in DMEM (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 8% FCS (Life Technologies, Inc.) in a humidified 5% CO2, 95% ambient air atmosphere at 37°C. IGR39D cells transfected with wild-type N-ras (61-Glu/Qu) were used as an additional control (IGR-Q1). No wild-type N-ras 518A2 transfectants could be generated despite great efforts, and such clones are consequently not available for comparison.

Influence of Cisplatin on In Vitro Cell Growth. For the comparison of in vitro growth with and without cisplatin (Ebewe, Unterach, Austria), ras-transfected and vector control melanoma cells were plated at a density of 4 × 104 cells/well in 96-well microtiter plates (0.1 ml/well, 4 wells/group). At 24 h after plating, the cells were rinsed with medium, and fresh medium or fresh medium supplemented with cisplatin at final concentrations of 1 μM, 10 μM, 100 μM, and 1000 μM was added for 4 h. After an incubation period of 72 h, the number of viable cells was determined by MTS assay (Promega, Madison, WI).

Western Blot Analysis. Western blotting for p21 was performed by TUNEL staining (Ref. 9; Boehringer Mannheim, Mannheim, Germany).

Statistical Analysis. Statistical significance of differences in tumor weight between treated and untreated animals in the respective groups (N-ras-transfectants versus controls) and between cell numbers of the various groups in vitro were determined using the Mann-Whitney U test. P values of <0.05 were considered to be of statistical significance.

Results

Influence of Activated N-ras on the Resistance of Human Melanoma Cells to Cisplatin in Vitro. We demonstrate that mutated N-ras in the tested human melanoma cells contributes significantly (P < 0.002) to chemoresistance in a liquid culture system in vitro (Fig. 1). As shown in Fig. 1A, in vitro cell growth of chemotherapy-treated IGR-neo cells reached 93.2% (± SD = 4.0%), 78.6% (± SD = 3.2%), 60.0% (± SD = 2.3%), and 12.2% (± SD = 4.1%) of the saline-treated control after exposure to cisplatin at 1 μM, 10 μM, 100 μM, and 1000 μM, respectively. Transfection with wild-type N-ras (IGR-Q1) led to very similar results (92.5%, ± SD = 3.9%; 75.7%, ± SD = 4.5%; 63.3%, ± SD = 9.0%; and 3.2%, ± SD = 0.8%, respectively; P, not significant). However,
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genes on the chemoresistance of human melanoma in vivo using SCID-hu xenotransplantation models (Fig. 2).

As shown in Fig. 2A, the mean tumor weight of animals injected s.c. with IGR-neo was 2.69 g (± SD = 0.73 g). Cisplatin treatment rendered 3 of 7 animals injected with IGR-neo without detectable tumors [mean tumor weight, 0.08 g (± SD = 0.13 g; P < 0.002] and reduced the mean tumor weight by 92.9% (± SD = 4.8%) compared to the untreated animals. SCID mice carrying cells overexpressing wild-type N-ras (IGR-Ql) reached a mean tumor weight of 1.93 g (± SD = 0.47 g). Notably, all animals (8 of 8) injected with IGR-Ql cells were found to be free of detectable tumors at 14 weeks (P < 0.001; reduction in mean tumor weight by 100%). Mice injected s.c. with IGR-K1 cells developed melanomas of a mean tumor weight of 4.87 g (± SD = 0.86 g). Cisplatin treatment in this group did not render any animals (8 of 8) without detectable tumors and reduced the mean tumor weight to 2.20 g (± SD = 0.81 g; P < 0.01), which reflects a reduction in mean tumor weight by 54.8% ± SD = 3.7%.

Similar findings in the second tumor panel (Fig. 2B) support the general nature of our observation that activated N-ras is a factor contributing to the chemoresistance of human melanoma. The mean tumor weight of animals injected s.c. with 518-neo was 2.32 g (± SD = 0.24 g). Cisplatin treatment reduced the mean tumor weight by 55.3% (± SD = 17.8%) to 1.06 g (± SD = 0.45 g); P < 0.001. Notably, again mice injected with human melanoma cells transfected transfection with activated N-ras (IGR-K1) rendered melanoma cells more chemoresistant, leading to in vitro cell growth of 95.4% (± SD = 2.1%), 89.3% (± SD = 2.7%), 86.5% (± SD = 3.3%), and 56.6% (± SD = 4.6%), respectively, when compared to saline-treated controls (P < 0.002).

This observation could be confirmed with the transfectants of a second melanoma cell line (518A2). This cell panel lacks the additional wild-type control. As shown in Fig. 1B, in vitro cell growth of chemotherapy-treated 518-neo cells was found to be 83.7% (± SD = 5.7%), 77.2% (± SD = 2.6%), 48.5% (± SD = 10.2%), and 23.5% (± SD = 5.8%) of the saline-treated control after exposure to 1 μM, 10 μM, 100 μM, and 1000 μM of cisplatin, respectively. Notably, transfection with activated N-ras (518-L1) enhanced chemoresistance. Cell growth measured as relative absorbance in the colorimetric MTS assay reached 97.0% (± SD = 3.7%), 91.5% (± SD = 6.6%), 74.7% (± SD = 8.7%), and 52.2% (± SD = 4.6%) of the saline controls, respectively (P < 0.002).

Influence of Activated N-ras on the Cisplatin Resistance of Human Melanoma Grown in SCID Mice. Based on our in vitro findings, we set out to determine the influence of activated N-ras
with activated N-ras (518-L1) developed melanomas less sensitive to treatment with cisplatin. Animals injected s.c. with 518-L1 cells grew melanomas of a mean tumor weight of 4.02 g (± SD = 0.41 g). Cisplatin treatment reduced the mean tumor weight in the 518-L1 group by only 10.9% (± SD = 12.0%) to 3.59 g (± SD = 0.49 g; P, not significant). Ras expression was also stable in vivo in all systems investigated (data not shown).

Influence of Cisplatin on Apoptosis of Human Melanomas with or without Activated N-ras. In search of the mechanism behind the clear differences in chemoresistance of melanomas carrying activated N-ras genes compared to their controls and due to recent evidence that cisplatin exerts its action through the induction of apoptosis (10), we examined sections of the generated tumors for the presence of apoptotic cells using immunohistochemical methods (TUNEL DNA assay; Ref. 9).

In both cell panels, activated N-ras protects the human melanomas grown in SCID mice from naturally occurring apoptosis and even more so from cisplatin-induced apoptosis. Representative examples are shown in Fig. 3. Cisplatin treatment enhances apoptosis, leading to an about 5-fold higher number of apoptotic cells in 518-neo tumors (Fig. 3B) compared to untreated 518-neo tumors (Fig. 3A). In the larger 518-L1 tumors expressing activated N-ras genes, only single apoptotic cells could be detected (Fig. 3C). Cisplatin treatment (Fig. 3D) also caused an increase in apoptosis (2-fold when compared to untreated 518-L1 tumors), however, the total number of apoptotic cells remained low.

Discussion

Fifteen percent of all melanoma patients carry a tumor with activated ras genes. The mutations responsible for these activations are mainly located in codon 61 of the human N-ras gene (1). Until recently (2), there was no system available that allowed studies of the impact of activated N-ras genes on drug resistance, certainly one of the key issues of melanoma therapy. With novel ways to influence ras gene expression and ways to block Ras function on the horizon (11–15), the question of whether activated Ras in human melanoma is a bystander or a factor worth targeting to improve therapeutic outcome gains momentum.

In the present study, we report that activated N-ras clearly contributes to the resistance of human melanoma to cisplatin both in vitro and in two SCID-hu mouse xenotransplantation models. Overexpression of wild-type N-ras genes, available in one of the two melanoma model systems, failed to cause the chemoprotective effects seen with activated N-ras. Cisplatin, a chemotherapeutic agent used in experimental melanoma therapy (16–19), rather than the more widely used dacarbazine was the drug of choice in our study because it is active both in vitro and in vivo without metabolic activation and because the apoptosis-inducing mechanism of action of cisplatin is well described (10). We found a less pronounced increase in apoptosis and clearly fewer apoptotic cells after cisplatin treatment in the generated melanomas carrying activated N-ras oncogenes compared to melanomas grown in mice injected with the controls. These observations suggest
that activated N-ras may be able to contribute to the chemoresistance of human melanoma by blocking apoptosis.

Our finding that activated N-ras contributes to the chemoresistance of human melanoma may be of considerable relevance. Antisense oligonucleotides, ribozymes, or farnesyltransferase inhibitors are promising approaches for altering ras gene expression or Ras function (11–15) and may be important in the quest to overcome drug resistance in melanoma patients whose tumors carry activated N-ras genes.

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