ras Oncogene Activation Does Not Induce Sensitivity to Natural Killer Cell–Mediated Lysis in Human Melanoma

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An important phenomenon in tumor immunology that has come under recent attention is the impact of oncogene activation in tumor cells on the sensitivity to lysis by immune effector cells. Several studies suggested that transfer of an activated *ras* oncogene into cultured rodent fibroblasts induces susceptibility to natural killer cell (NK)-mediated lysis. Experiments using human tumor cells, however, have produced conflicting data on the effect of *ras* activation in this respect. In studying the activation of the oncogene c-*myc*, which is often overexpressed in human melanoma, we have found that in cell lines expressing high levels of Myc protein, the sensitivity to lysis by NK cells was dramatically increased due to reduced expression of Human Leukocyte Antigen B locus products. Since the N-*ras* oncogene was found to be activated in 15% of human melanomas, we examined the possibility that in melanoma, in analogy to the murine systems, the mutated *ras* oncogene may influence NK susceptibility of human melanoma cells. Two N-*ras* genes harboring frequently found mutations were cloned into an expression vector. Transfection of the IGR39D melanoma cell line with wildtype and mutant N-*ras* constructs yielded several ras-expressing clones that were tested for NK sensitivity. Neither high expression of the wildtype N-ras protein, nor expression of two mutant proteins (N61-arg, N61-lys) was shown to result in enhanced NK-mediated lysis. We conclude that activation of *ras* oncogenes does not lead to the induction of an NK-sensitive phenotype in human melanoma cells.
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The acquisition of transforming properties is a dramatic event in the development of a tumor cell, reflected by a number of phenotypic changes. Such changes might elicit an immunologic response, resulting in generation of either tumor antigen-specific cytotoxic T lymphocytes (CTL), or modulation of tumor cell sensitivity to natural killer (NK) cells. The basis for recognition of target cells by NK cells is still unknown [1,2], but several studies have indicated that reduced levels of MHC class I may trigger lysis by NK cells [3–5].

NK cells may constitute a first line of defense against growing tumor cells [6], because there is no priming step required to activate their lytic activity. Furthermore, considering the effects of reduced MHC class I expression often found in tumor cells [7], NK cells may function as a defense system supplementary to CTL mediated lysis [8]. First identified as the transforming gene in the Harvey and Kirsten strains of murine sarcoma virus [9], the *ras* gene encodes a protein (p21), that constitutes a major link in the signal transduction pathways starting with triggering of tyrosine kinase receptors at the cell surface [10–13]. Activation of *ras* oncogenes is frequently found in varying types of human tumors, including melanoma [14,15]. In many cases, it is unclear whether mutant *ras* proteins are involved in the earlier stages of tumor initiation and growth or contribute to development of a fully malignant tumor phenotype. Several studies have addressed the question of whether *ras* activation may influence the NK sensitivity of tumor cells, and may thereby enhance their sensitivity to the patients' immune system. Rodent fibroblasts transformed by activated H- or K-*ras* showed an NK sensitive phenotype [16–19], a finding that has been extended to a human breast cancer cell line, which upon transfection of activated H-*ras* became highly susceptible to NK-mediated lysis [20]. On the other hand, it has also been reported that this phenomenon may be cell type specific [21–23], and therefore an effect of *ras* on NK sensitivity cannot always be anticipated. Previously, in a panel of human melanoma cell lines, we have found an association between mutation of endogenous *ras* and high NK sensitivity [24]. To establish if this relationship is causal, we set out to examine whether *ras* oncogene transfection into a human melanoma cell line would influence the lysis by NK cells. Two commonly detected N-*ras* mutations were generated and transfected into the NK resistant melanoma cell line IGR39D, that earlier was shown to be induced to NK-sensitive phenotype by transfection of the c-*myc* oncogene [25]. No enhanced lysis of N-*ras* transfected clones could be shown, indicating that in this human melanoma cell line N-*ras* mutation or overexpression does not induce elevated lytic susceptibility to NK cells.

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MATERIALS AND METHODS

Cell Culture The human melanoma cell line IGR39D and its c-*myc*-transfectant (referred to as IGR-myc, also clone 3) have been described elsewhere [26,27]. Cell lines and *ras* transfectants were cultured in DMEM supplemented with 8% fetal bovine serum (FBS). The NK-sensitive promyelocytic cell line K562 was grown in DMEM + 10% FBS. All cell lines were checked for mycoplasma infection and found to be negative.

Plasmids and Mutagenesis The wildtype human genomic N-*ras* clone pBNras as well as the normal N-*ras* cDNA clone pcN1 have been described before [28,29]. For mutagenesis the N-*ras* cDNA was subcloned from pcN1

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by *Ava*II digestion and ligation into the *Sma*I site of pBSK+ (Stratagene, La Jolla, CA). This vector was designated pBCR1 and used for *in vitro* mutagenesis. For *in vitro* mutagenesis the following primers were used. The M13 primer 5′-GTAAAACGACGGCCAGT-3′ and reverse primer 5′-CAGGAAACAGCTATGAC-3′ hybridize to pBSK+ vector sequences. For the generation of the 61-arg (61R) mutation the sense primer 5′-TACAGCTGGACGAGAAGAGTACA-3′ and anti-sense primer 5′-TGTACTCTTCTCGTCCAGCTGTA-3′ were utilized. Similarly, for the 61-lys (61K) mutation the sense 5′-TACAGCTGGAAAAGAAGAGTACA-3′ and anti-sense 5′-TGTACTCTTCTTTTCCAGCTGTA-3′ primers were synthesized. Polymerase chain reaction (PCR) based mutagenesis was done according to the general method as described by Higuchi *et al* [30]. In a first round of amplification a 5′ and a 3′ fragment of the N-*ras* cDNA were generated in separate reactions, using a primer hybridizing to pBCR1 vector sequences in combination with a primer containing a point-mutation as the indicated codon. Both 5′ and 3′ fragments were purified and mixed for a second round of amplification with the vector binding primers only, resulting in a full-length N-*ras* cDNA containing the indicated point-mutation. After digestion with *Sac*I and *Kpn*I the cDNA was cloned into pBSK+. The integrity of the cDNA sequences was verified by PCR-SSCP analysis [31] and sequencing. The *Sac*I/*Kpn*I fragment was subcloned into the *Bam*HI site of the expression vector pCMV neo [32]. Finally, the expression vectors were tested in a focus forming transformation assay using Rat-2 fibroblast cells.

Transfection and Selection Transfection of IGR39D was performed using the calcium-phosphate method [33], with two 10 μg of the N-*ras* vector, mixed with 0.5 to 1 μg of pSV2neo, when appropriate. Transfected cells were selected in medium containing 600 μg/ml of G418 (Gibco BRL, Paisley, Scotland). Transfectants were screened for genomic integration of the N-*ras* cDNA by PCR.

Western Analysis Whole cell extracts were prepared using 0.14 M NaCl, 0.2 M triethanolamine, 0.2% Na-deoxycholate, and 0.5% Nonidet P-40, supplemented with 1 mM phenylmethylsulphonyl fluoride and 0.2 mg/ml aprotonin. Extracts were quick frozen twice and centrifuged to remove debris. Fifty micrograms of total protein was separated on sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to PVDF membranes (Tropix, Bedford, MA). Immunodetection was performed using the rat monoclonal antibody Y13-259 [34] at 1:400 dilution. Second antibody incubations were carried out using goat–anti-rat conjugated to Alkaline Phosphatase (1:2500, Promega, Madison, WI) and the chemiluminescent substrate CPSD (Tropix) according to manufacturers' instructions. Membranes were exposed to Fungi RX X-Ray film for 30 seconds.

Cytotoxicity Assays Cytotoxicity assays were carried out as described before [25] in a standard 4-h chromium release assay, using as effectors peripheral blood mononuclear cells freshly isolated from a healthy donor.

Serologic Reagents and FACS Analysis Cell surface expression of HLA class I and adhesion molecules was determined using the following antibodies: W6/32 (Sera Lab, Crawley Down, Sussex, UK), specific for a monomorphic epitope of HLA-A,-B and -C; 4E [35], recognizing all HLA-B and -C alleles, in addition to those of the HLA-Aw19 crossreactive group; F10.2 (a generous gift of A. Bloem, Dept. of Immunology, Utrecht, the Netherlands), specifically staining ICAM-1; TS2/9, recognizing LFA-3 [36]. Cells were trypsinized, washed, and suspended in PBS, containing 0.5% (w/v) BSA and 0.02% NaN_3 (PBA). After a 30-min incubation at 4° C with first antibody, cells were washed three times with PBA and incubated for another 30 min with fluorescein-conjugated goat-anti-mouse antibody (Nordic Immunological Laboratories, Tilburg, the Netherlands). Finally cells were washed again and analyzed using a FACScan fluorocytometer (Becton Dickinson, Mountainview, CA).

RESULTS

Generation of N-*ras* Transfectants Using a panel of 14 human melanoma cell lines, we have previously found that the presence of mutated N-*ras* alleles correlated with high sensitivity to NK-mediated lysis [24]. To investigate whether this augmentation was actually caused by expression of mutant Ras proteins, a cell line with low sensitivity to NK lysis, IGR39D, was chosen for transfection experiments.

The 61-arg (N61R) and 61-lys (N61K) mutations are frequently found in varying types of tumors [15], including melanoma (our unpublished observations). These two mutations were introduced into the cDNA clone of human N-*ras*, using PCR mutagenesis. The mutant cDNA constructs were placed under the control of the CMV immediate early promoter/enhancer to induce high expression in melanoma cell lines (see below). We also tested the transforming ability of these CMV-driven *ras* plasmids in a Rat-2 fibroblast focus assay and found them to be at least as potent in transformation as the EJ H-*ras* (H-*ras* gene carrying a 12-val mutation) oncogene (data not shown). The pBNras plasmid, containing the wildtype genomic clone of human N-*ras* with its own promoter region, and the mutant N-*ras* constructs were transfected into IGR39D. The wildtype genomic N-*ras* gene was transfected to test whether a mere overexpression of p21 would already exert an effect on the NK phenotype of these melanoma cells. The resulting clones were tested for expression of the exogenous $p21^{N\text{-}ras}$ by Western blotting. A cell extract from the wildtype H-*ras* overexpressing cell line Hras13 [37] was included as a control **(Fig 1*A*)**. The parental cells and control clones IGR-neo1 and -neo2 showed low basal levels of endogenous wildtype p21 **(Table I, Fig 1*A*)**. Several clones expressing wildtype and mutant N-*ras* protein were obtained after transfection. Because the frequency of G418 resistant colonies expressing the mutant p21 was found to be very low, a prescreening procedure was divised, using PCR amplification of exons 1 and 2 to check for integration of the transfected cDNA. In five out of eight wildtype clones we found raised p21 levels, whereas in a similar experiment only six out of 61 clones transfected with mutant *ras* yielded detectable p21 expression. All clones listed in **Table I** were found to have integrated exogenous *ras* (data not shown). Clones IGR-Q1, -Q2 and -Q4 express high levels of the exogenous wildtype N-*ras*, whereas clone IGR-Q3 harbored integrated copies of a wildtype N-*ras* cDNA, but did not show evidence of protein expression **(Fig 1*A,B*)**. The mutant cDNA constructs resulted in a number of clones showing varying p21 levels, IGR-R1 and -R2 having a fair expression of 61-arg N-*ras*, whereas IGR-K1 produced a very high amount of 61-lys p21. The IRG-K2 transfectant was positive for integration, but no detectable mutant p21 could be seen in these cells **(Fig 1*B*)**.

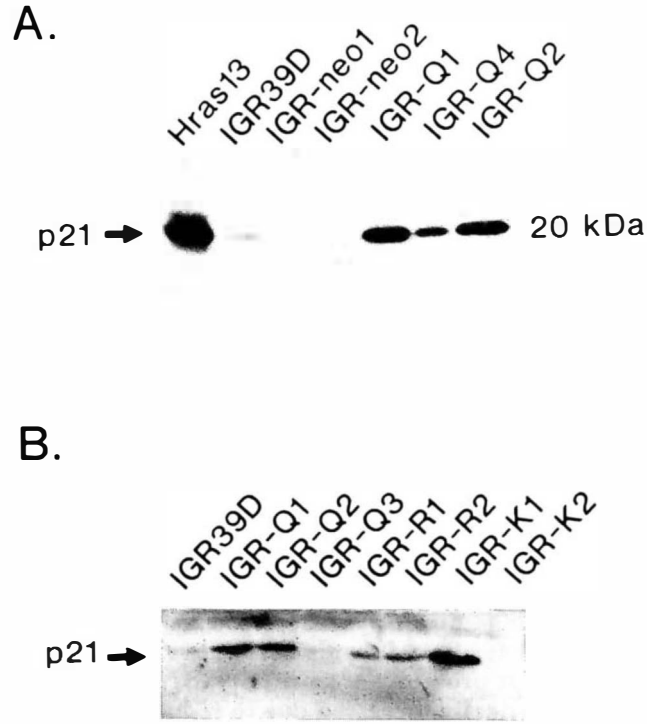

Figure 1. N-*ras* p21 expression in IGR39D and its derivatives. N-*ras* p21 protein expression levels of the IGR39D cell line and transfectants were assayed by Western blotting. *A, lane 1* control Hras13 extract, *lane 2,* IGR39D, *lane 3,* IGR-neo1, *lane 4,* IGR-neo2, *lanes 5–7,* IGR-Q1, -Q4, and -Q2 as indicated. *B, lane 1,* IGR39D, *lanes 2–8,* IGR-Q1, -Q2, -Q3, -R1, -R2, K1, -K2 as indicated.

Table I. P21*ras* Expression Levels of Transfectants[a]

Clone	Transfected with	N-*ras* Protein Expression[b]
IGR39D	—	—
IGR-myc	*neo* + c-*myc*	—
IGR-neo1	neo	—
IGR-Q1	*neo* + wt N-*ras*	++
IGR-Q2	*neo* + wt N-*ras*	++
IGR-Q3	*neo* + wt N-*ras*	±
IGR-R1	*neo* + 61R N-*ras*	+
IGR-R2	*neo* + 61R N-*ras*	+
IGR-K1	*neo* + 61K N-*ras*	++
IGR-K2	*neo* + 61K N-*ras*	—

[a] IGR39D clones carry wildtype (IGR-Q1, -Q2, -Q3) or mutant 61-arg (IGR-R1, -R2) or 61-lys (IGR-K1, -K2) N-*ras* constructs. IGR-myc, *c-myc* overexpressing clone; IGR-neol, a control *neo* transfectant.

[b] p21 levels are indicated by — (no expression), + (moderate), or ++ (high). One clones showed either low or no expression (±) in different cell extracts.

NK Sensitivity of Mutant *ras* Expressing Clones The influence of exogenous p21 expression in the IGR39D cell line on lysis by NK cells was evaluated, using freshly isolated peripheral blood mononuclear cells (PBMC) from a healthy donor as effector cells. The K562 promyelocytic cell line was used as a positive control for NK activity in the PBMC isolate. IGR-myc was taken as a positive control for the induction of NK sensitivity in the IGR39D cell line. c-*myc* transfection of this line was previously shown to induce strongly enhanced NK sensitivity [25]. In the present experiments, IGR-myc was two to four times more susceptible to NK cells as compared to the parental cell line **(Figs 2,3)**.

Two clones overexpressing wildtype N-Ras (IGR-Q1 and -Q2) showed little, if any, alteration of NK sensitivity **(Fig 2*B,C*)**, also when compared to control *neo* transfectants, of which a representative clone is shown in **Fig 2*A*** (IGR-neo). Clone IGR-Q3, which had integrated *ras* DNA but did not produce p21, was equally well lysed by NK cells. These findings indicate that mere overexpression of (wildtype) p21 does not induce NK susceptibility. Two clones expressing comparable amounts of the 61-arg (R) N-Ras protein, were obtained from two independent transfection experiments (IGR-R1 and -R2). NK lysis of these transfectants was similar to that of the parental cell line and the IGR-neo control **(Fig 3*A,B*)**. We observed the same effect when two 61-lys (K) mutants were tested. The IGR-K1 clone, expressing rather high levels of the activated protein even shows a slight decrease in NK lysis **(Fig 3*C*)**. On the other hand, clone IGR-K2, in which the 61K protein is not detectable seems to be more sensitive than the parental IGR39D cells **(Fig 3*D*)**. These fluctuations therefore seem to be a consequence of clonal variation, rather than of direct effects of activated *ras*. A third 61K clone producing similar amounts of p21 as the 61R mutants, was equally sensitive to NK mediated lysis as the parental and the *neo*-transfected cells (data not shown).

When LAK cells (lymphokine activated killer cells PBMC stimulated for 5–7 d *in vitro* with interleukin-2) were used as effectors, a slightly decreased sensitivity was observed for clones IGR-Q1, -Q2, and -R1, but not for the other clones described here (results not shown). The NK and LAK data were similar with PBMC isolated from one other (healthy) individual. These experiments altogether show that *ras* transfected melanoma cells are not more prone to NK-mediated cytolysis.

Cell Surface Expression of HLA Class I and Adhesion Molecules To investigate whether possible effects of *ras* transfection on accessory molecules involved in determining NK susceptibility might have obscured or counteracted an effect of *ras* on NK sensitivity, we analyzed HLA class I, ICAM-1 and LFA-3 expression of these transfectants. FACS analysis of HLA class I and HLA-B,-C locus products showed downregulated expression on the *c-myc* transfected, NK-sensitive clone IGR-myc, as compared to the parental IGR39D cells **(Table II)**. To a lesser degree, the same observation was done for IGR-R2, but this was not accompanied by

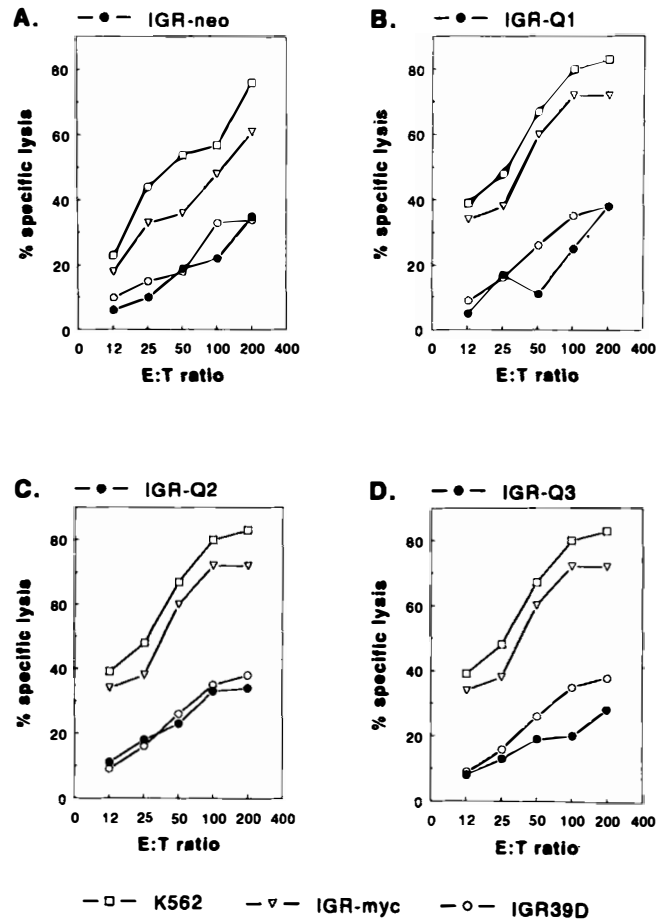

Figure 2. NK susceptibility of wildtype N-*ras* transfectants. Peripheral blood mononuclear cells were freshly isolated from a healthy donor, and used as NK effector cells in a standard 4-h chromium release assay. The K562 cell line was included as a control for the lytic activity of the effectors. *A* shows lysis of IGR-neo, in addition to the lysis of parental IGR39D, its c-myc transfectant IGR-myc, and the control cell line K562. In *B*, *C* and *D*, the NK sensitivity is indicated by three clones expressing elevated levels of wildtype N-*ras*, IGR-Q1,-Q2 and -Q3, respectively. *Open squares* represent the lysis of K562; *open circles*, IGR39D; *open triangles*, IGR-myc; *closed circles*, the indicated transfected clone.

enhanced NK sensitivity. Expression of ICAM-1 was found to be variable on several clones including the *neo*-transfectants, hinting to clonal polymorphism for this marker. No consistent differences, however, were found with the NK sensitivity data. Apparently, in the clones showing less ICAM-1 expression, the down modulation was not strong enough to abolish NK resistance. LFA-3 expression was unchanged on all transfectants.

DISCUSSION

Immune Surveillance Against Induction and Growth of Tumor Cells The immune surveillance theory predicts that a major defense mechanism against tumor cells is constituted by the cellular immune system [8]. It has been established that CTL responses can occur in patients with various forms of human cancer including melanoma. For melanoma, tumor specific peptides that elicit a CTL response have been characterized [38]. In mice, a CTL response against activated *ras* protein epitopes can be instigated [39,40]. Also, a specific reaction of NK cells against tumor cells that express activated *ras*, has been observed for human tumors [20]. This last observation however, seems to be dependent on the cell type that is used [22]. We have previously found that transfection of *c-myc* into human melanoma cell lines resulted in a reduction of HLA expression, especially of B locus antigens [26], leading to

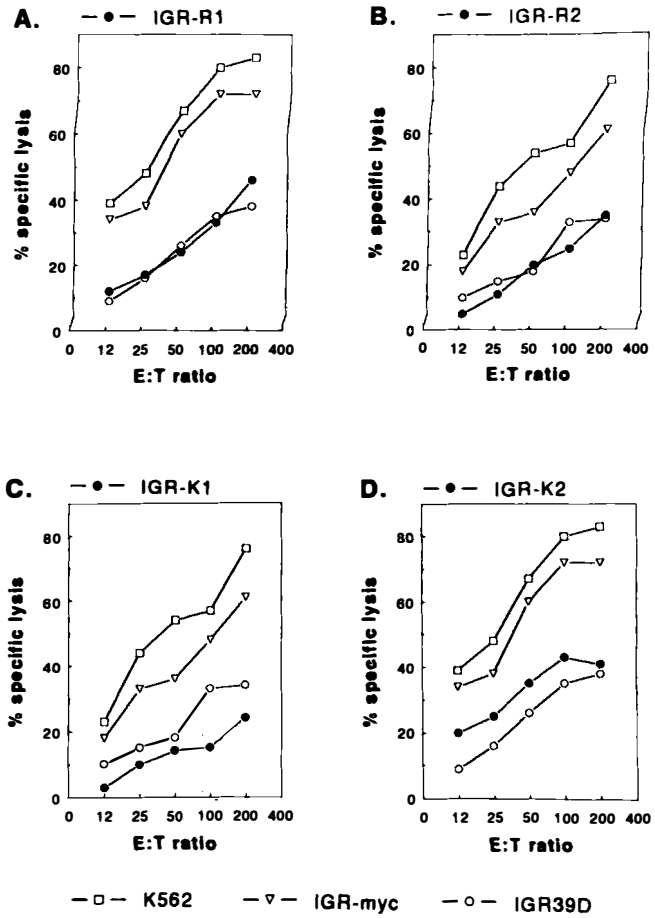

Figure 3. NK susceptibility of mutant N-ras transfectants. The IGR39D clones transfected with mutant N-*ras* as indicated in **Table I**, were tested for their sensitivity to NK mediated lysis. *A* shows the lysis of the 61R clone IGR-R1, *B, C,* and *D* show results obtained with clones IGR-R2, -K1, and -K2 respectively. *Open squares* represent the lysis of K562; *open circles,* IGR39D; *open triangles,* IGR-myc; marked by *closed circles* are the four mutant N-*ras* clones.

elevated lytic susceptibility to NK cells [25]. This implies that oncogene activation, as in the case of *c-myc*, can be involved in the regulation of phenotypical aspects important for immune recognition [41]. In a number of human melanoma cell lines high NK sensitivity was found to be associated with the presence of a mutated N-*ras* allele [24]. Here, we explored the possibility that *ras* activation may, directly or through some other cell component, induce sensitivity to NK-mediated lysis in human melanoma cells, similar to what has been reported for murine cells. The well-described cell line IGR39D was transfected with several wildtype and mutant plasmid constructs.

Generation of Mutant ras Expressing Melanoma Cells The isolation of four clones overexpressing wildtype N-Ras and four transfectants with varying expression of mutant p21 is described in this paper. In our experience, obtaining cells with the correct integration and expression of mutated N-*ras* gene constructs after transfection is a rather infrequently occurring event, especially when compared to transfections using the wildtype genomic fragments. We do not have an explanation for this phenomenon, but a suggestion may be that expression of activated p21 has a profound effect on the survival of recipient cells. It is well known, that activating point mutations in the Ras protein enhance the proliferative capacity of a tumor cell in early development. This may not, however, be the case in already malignant tumor cells, or cells adapted to culture conditions. Aberrant expression of the Ras protein in these cells may lead to cell death, because it is known that unscheduled entry into mitosis, as for instance induced by expression of a mutant Ras, may trigger the process of apoptosis. The clones with mutated *ras* that were isolated and studied here, are apparently resistant to *ras*-induced apoptosis. In these clones the expression of several molecules involved in recognition and lysis by NK cells and CTL such as HLA class I and adhesion molecules was found not to be changed grossly, as compared to the parental cell line. Analogous to the NK sensitive clone, IGR-myc, but not to the same extent, the ras tranfectant IGR-R2 showed decreased HLA-B,-C expression, in this case not followed by NK susceptibility. This may suggest a threshold level *of HLA class I is necessary to protect against lysis by NK cells.* IGR-R2 may then still express enough HLA-B and -C locus products for protection to NK mediated lysis, as opposed to IGR-myc.

Activation of ras Does Not Determine NK Sensitivity The IGR39D clones transfected with mutant and wildtype N-*ras*, were found to possess an unaltered susceptibility to NK-mediated lysis. If *ras* activation is lethal for these m lanoma cells (see above), then surviving colonies as used here for determining NK sensitivity must have undergone additional changes to survive. These changes potentially could mask a regular effect of mutated *ras* on NK susceptibility, which would weaken our conclusion that *ras* activation by mutation is not linked to determining the NK phenotype. On the other hand, excessive production of wildtype p21 in some experimental systems induces identical effects as compared to the mutant analogue [42], and in a number of human tumors it has been shown that overexpression of wildtype *ras* is associated with poor prognosis [43–46]. Therefore, our clones with overexpressed wildtype p21 may be considered as representative for *ras*-activated cells. These clones could be easily obtained, and possessed unaltered NK susceptibility. This corroborated our conclusion that *ras* activation is not involved in modulation of NK sensitivity in melanoma.

Activated ras Involved In Early Steps of Metastasis? In a recent study using a colon carcinoma cell line, it was shown that activated *ras* induces NK resistance, concomitant with the induction of a more differentiated phenotype of the tumor cells [22]. It has been speculated that *ras* activation or amplification might be an important step preceding the acquisition of metastasizing capacity [44,47]. In view of this, it is understandable that activation of the p21 protein induces resistance to NK cells, because it facilitates the successful entry into the circulation and colony-formation at distant sites in the body. We could not demonstrate prominant NK resistance following N-*ras* transfer, but several of the tested clones were slightly less susceptible to NK or LAK lysis. It will be interesting to see whether metastasizing capacity in immunocompetent mice is enhanced in these particular *ras* transfectants.

Table II. Cell Surface Expression of HLA Class I and Adhesion Molecules on IGR39D and Its Transfectants[a]

Clone	HLA class I	HLA-B,-C	ICAM-1	LFA-3
IGR39D	+++	+++	++/+	++
IGR-myc	++	+	+	++
IGR-neo1	+++	+++	+	++
IGR-Q1	+++	+++	+	++
IGR-Q2	+++	+++	++	++
IGR-Q3	+++	+++	++	++
IGR-R1	+++	+++	+	++
IGR-R2	+++	++	++	+
IGR-K1	+++	+++	++	++
IGR-K2	+++	+++	++	++

[a] Parental, control, and *ras* transfected cells were tested for expression of HLA class I (W6/32 antibody), HLA-B,-C (4E), ICAM-1 (F10.2), and LFA-3 (TS2/9) by FACS analysis. Expression levels are denoted as strongly positive (+++), positive (++), or weakly positive (+).

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