Brief Definitive Report

TRANSFECTION OF A RAT CELL LINE WITH THE v-Ki-ras ONCOGENE IS ASSOCIATED WITH ENHANCED SUSCEPTIBILITY TO NATURAL KILLER CELL LYSIS

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Natural killer (NK) cells have been proposed as a first level of defense against the initiation and spread of tumors in vivo (1). Recent studies with syngeneic mice have directly correlated levels of NK activity with resistance to tumor growth (reviewed in 2). However, most of these investigations have used long-passaged transplantable tumor cell lines rather than newly transformed cells. We had predicted that, if NK cells played a true surveillance role, host cells should become susceptible to NK-mediated cytotoxicity during transformation to the malignant phenotype. The present study demonstrates that NK-resistant rat fibroblasts transformed with the v-Ki-ras oncogene become anchorage independent, grow as tumors in nude mice, and acquire NK sensitivity. These results indicate that NK cells can recognize and kill newly transformed cells and that conceivably they play an early role in host surveillance against neoplasia.

Materials and Methods

Cells. Rat-1, a cell line derived from Fisher rat F2408 fibroblasts (3), was transfected and injected into NIH-II nu/nu mice (2×10^6 cells per mouse, subcutaneously). Large growing tumors were explanted in vitro after 3 wk and reestablished as cell lines. Injection of nontransfected rat-1 cells resulted in a barely palpable, benign nodule, which was also established as a cell line and used as a control in most experiments. The NK clones LH 49 (cytolytic) and L250-A9 (less cytolytic) were gifts of Dr. C. Brooks (4).

Transfection Protocol. Rat-1 was transfected with the entire 7.0 kilobase (kb) v-Ki-ras oncogene ligated into the Eco RI site of pBR322 (5). The transfection procedure has been previously described (6). Transfected cells were selected by colony formation in 1% methyl cellulose at 3–6 wk (7).

Cytotoxicity Assays. Target cells labeled with ^{51}Cr were analyzed in 6-h assays with a variety of effectors (see Table II) as previously described (1).

Southern Blot Analysis of Transfectants. DNA (15 μg) was digested with Eco RI, blotted (8), and hybridized to a 0.6 kb Kpn I fragment of v-Ki-ras corresponding to the long-terminal repeat sequence of the oncogene. After washing under high stringency, autoradiograms were exposed overnight.

Results and Discussion

Cytotoxicity assays performed on transformed and untransformed lines demonstrated that transfection of the v-Ki-ras oncogene enhanced NK-mediated

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killing. As shown in Fig. 1, the transformed cells were much more sensitive ($P < 0.01$) than the rat-1 parent to lysis by nylon wool–passed spleen cells from poly(I)C-boosted CBA/J mice. This difference was >20-fold in terms of lytic units calculated at 10% lysis. Since the parent cells are essentially resistant to NK lysis, they required $>10^6$ lymphocytes to even approach this level of killing. The difference was even more striking when the LH49 NK clone was used as an effector.

Transformed cells injected subcutaneously into nude mice grew as tumors, and were subsequently reestablished in culture. Cytotoxicity assays performed on tumor cells passaged through nude mice consistently showed that they were also highly NK sensitive. As summarized in Table I, data from seven replicate experiments demonstrate that the newly transformed and tumor-derived Ki-ras-A cells are 26–58-fold more NK sensitive than rat-1, and 6–12-fold less sensitive than the standard NK-sensitive tumor, YAC 1.2. Both the parental rat-1 cells and a line established from a benign, barely palpable nodule at the site of injection of untransfected rat-1 cells were NK resistant (<1 lytic unit per 10^6). Two additional, independently derived rat-1 transformants (Ki-ras-B and -C) were also converted to NK sensitivity after transfection with v-Ki-ras (Table I)

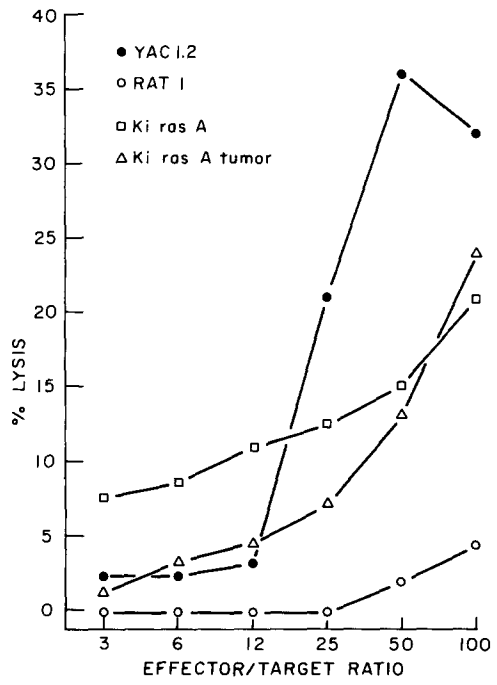

FIGURE 1. Nontransformed rat-1 cells (○), cells from the v-Ki-ras-transformed cell lines grown in vitro (□) or in vivo (△), and YAC 1.2 lymphoma cells (●) were labeled with ^{51}Cr and tested in a 6-h cytotoxicity assay against nylon wool–passed spleen cells from six CBA/J mice injected 24 h previously with 100 μg (1 U) of polyinosinic-polycytidylic acid [poly(I)C] per mouse. Values represent the mean percent lysis in triplicate wells. Values for the percent lysis of K-ras A transfectants and K-ras A tumor–derived cells were compared with the parent rat-1 line using Student's t test. Lysis values for both transformed cell lines were statistically different from rat-1 for all effector/target ratios >12:1, with $P < 0.01$. Standard errors for replicate experiments are indicated in Table I. This experiment was repeated seven times with similar results.

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TABLE I

NK Sensitivity in a Panel of Transfected Targets and Nontransfected Controls

Target cells	No. of experiments	Cytolysis	
		LU/10^6	Pecent lysis (100:1 E/T)
YAC 1.2	7	332 ± 122	42 ± 2
Rat-1	5	<0.5 ± 0.2	3 ± 2
Nodule	6	1 ± 0.9	7 ± 2
K-ras A	7	26 ± 12	23 ± 10
K-ras A tumor	7	58 ± 20	32 ± 6
K-ras B	2	6 ± 1	20 ± 4
K-ras B tumor	1	10	17
K-ras C	2	8 ± 2	18 ± 2
K-ras C tumor	4	11 ± 3	21 ± 5

Fresh spleen cells from CBA/J mice boosted 24 h previously with 100 μg of poly (I)C were titrated in serial dilutions at effector/target (E/T) ratios of between 200:1 and 3:1. Lytic units (LU) were calculated from titration curves; and 1 LU is defined as the number of lymphocytes required to lyse 10% of ^{51}Cr-labeled targets in a 6-h assay; the values shown represent LU per 10^6 effector lymphocytes. Percent lysis is indicated at the 100:1 effector/target ratio. Data represent the mean and standard error of replicate experiments.

TABLE II

Characterization of the NK Effector Cell

Strain	Assay time	Poly (I)C	Spleen fractionation	Treatment	Cytolysis			
					YAC		Ki-ras A tumor	
					Lysis*	LU/10^6‡	Lysis	LU/10^6
	h				%		%	
CBA/J	6	−	None	None	33	ND	11	8
	6	+§	None	None	42	ND	26	50
	10	−	Nylon wool‖	Anti-Lyt-1.1, 2.1¶	80	160	55	45
	10	+	Nylon wool	Anti-Lyt-1.1, 2.1	82	250	52	50
	10	+	Nylon wool	Anti-Lyt-1.1, 2.1, asialo GM_1**	63	125	28	25
BALB/c	10	+	Nylon wool	Anti-Lyt-1.1, 2.1	74	100	35	41
	10	+	Nylon wool	Anti-Lyt-1.1, 2.1, NK-1.2**	44	34	1	<1
	10	+	Nylon wool	Anti-Lyt-1.1, 2.1, NK-2.1	47	31	2	<1
DBA/2	4	+	Nylon wool	None	17	18	12	10
Wistar-Firth rat	4	+	Nylon wool	None	17	25	33	115
C57BL/6	6	−	—	NK clone LH 49	21	500	41	1000
	6	−	—	NK clone L250-A9	7	62	9	110

* Percent cytolysis of ^{51}Cr-labeled target cells at a 100:1 effector/target ratio.

‡ A lytic unit (LU) was defined as the number of lymphocytes required to lyse 10% of targets. The value shown represent LU per 10^6 effector lymphocytes.

§ Mice were boosted intraperitoneally with 100 μg (1 U) of poly(I)C 24 h before assay.

‖ Spleen cells were pooled from 4 to 18 animals per group and passed over nylon wool columns.

¶ 10^7 Spleen cells/ml were treated 1 h at 4°C with a 1:20 dilution of monocolonal anti-Lyt-1.1 mixed with 1:20 anti-Lyt-2.1 (Cedarlane Laboratories), followed by washing and a 1-h incubation at 37°C with 1:10 rabbit complement preabsorbed with mouse tissues.

** After anti-Lyt-1.1, 2.1 and complement treatment, spleen cells were treated with 1:100 rabbit anti-asialo GM_1 sera or 1:80 mouse anti-NK-1.2 or -2.1 sera followed by 1:10 rabbit complement.

These experiments were repeated 3 times with similar results.

whereas cells transfected with v-fes remained NK resistant (data not shown).

The characteristics of effector cells that caused cytolysis of transfected targets are summarized in Table II. Although normal, unfractionated CBA/J mouse spleen cells killed the Ki-ras tumor, boosting of the mice with poly(I)C resulted

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in a sixfold enhancement of the effect. The poly(I)C augmentation was less apparent when using nylon wool column–passed spleen cells depleted of Lyt-1$^+$ and Lyt-2$^+$ effectors. In addition to being Lyt-1$^-$,2$^-$ phenotype, the antitumor effector cells were partially depleted by treatment with anti–asialo GM_1, and totally eliminated by treatment with anti-NK-1.2 or NK-2.1. Experiments with cloned NK cell lines of varying cytolytic potential, kindly provided by C. Brooks (4), demonstrated that the clones lysed YAC-1.2 and the transfected tumor line equally, but had negligible effects on the rat-1 parent. The phenotype of the effector cell that mediates cytolysis of rat-1 transformants is Lyt-1$^-$,2$^-$, NK-1.2$^+$, NK-2.1$^+$, asialo GM_1 positive, nylon wool nonadherent, and poly(I)C boostable. Therefore, the effect is most likely mediated by NK cells.

The nature of the presumptive target structures on the transformants was investigated using cold-target competition studies to ascertain if determinants were different from those expressed on YAC 1.2. As shown in Table III, unlabeled Ki-ras A tumor cells competed with NK cells for lysis of ^{51}Cr-labeled YAC. The degree of inhibition was comparable to that observed between labeled and unlabeled YAC. Similar results were obtained in the reciprocal experiment in which the Ki-ras A tumor cells were labeled and killing was reduced by competition with either unlabeled Ki-ras-A tumor cells or unlabeled YAC 1.2. Nontransformed cells did not compete in either system.

Transfection was verified by Southern blot analysis of genomic DNA hybridized to a 0.6 kb segment from the original v-Ki-ras-transfected gene (Fig. 2). This fragment, which corresponds to the viral long-terminal repeats (LTR) in the oncogene, hybridized strongly to DNA from the transfected cells but not control lines, under the conditions descibed above.

These results suggest that transformation of NK-resistant fibroblast lines with specific oncogenes such as v-Ki-ras converts the cells to an NK-sensitive, anchorage-independent, malignant phenotype. Tumor progression is likely related to a loss of NK sensitivity since studies of rat fibroblast cell lines transformed with adenovirus 2 have revealed an inverse correlation between NK susceptibility and

TABLE III

Competitive Inhibition by YAC and Ki-ras Transfectants

Labeled target	Unlabeled competitor	Competitors ($\times 10^3$) required for 50% inhibition of lysis
YAC 1.2	YAC 1.2	8
	K-ras A tumor	4
	Rat-1	>64
	Nodule	>64
K-ras A tumor	YAC 1.2	4
	K-ras A tumor	4
	Rat-1	>64
	Nodule	>64

YAC 1.2 and nude mouse–passaged, v-Ki-ras A–transfected, rat-1 tumor cells were labeled with ^{51}Cr and tested in a 6-h cytotoxicity assay against nylon wool–passed spleen cells from six CBA/J mice injected 24 h previously with 100 μg (1 U) of poly(I)C acid. Varying numbers of unlabeled target cells were added to a constant number of ^{51}Cr-labeled targets (2×10^3/well) at an effector/target ratio of 100:1. The mean percent lysis values from triplicate wells were plotted and the number of competitors causing 50% inhibition of cytolysis was calculated by interpolation. This experiment was repeated five times with similar results.

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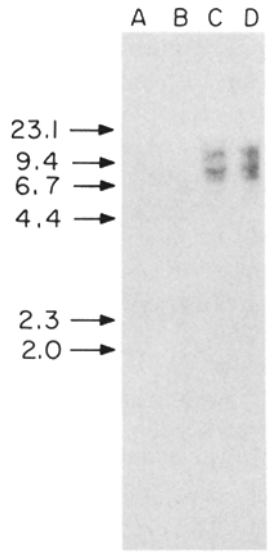

FIGURE 2. Southern blot of v-Ki-ras A transfectants. DNA was extracted from transfected and untransfected cell lines and equal amounts were digested with Eco RI, electrophoresed on an agarose gel, and transferred to Gene Screen Plus by Southern blotting. Blots were probed with a 0.6 kb fragment of the transfected gene and autoradiographed. Tracks correspond to equal amounts of DNA from (*A*) rat-1; (*B*) benign nodule at the site of injection of rat-1 into nude mice; (*C*) v-Ki-ras A transfectants; (*D*) tumor from nude mice injected with v-Ki-ras A transfectants. Size markers in kilobasepairs are shown on the left.

tumorigenesis in vivo (9, 10). In a similar fashion, the extended growth and selection of methylcholanthrene-treated fibroblasts in vivo resulted in the emergence of NK-resistant variants (11). More extensive studies are currently in progress in our system to correlate the level of oncogene expression with the degree of malignancy and relative NK sensitivity.

Summary

Transfection of the v-Ki-ras oncogene into rat-1 fibroblasts resulted in the establishment of cell lines that were transformed, tumorigenic, and sensitive to lysis by natural killer (NK) cells. Characterization of effectors indicated that the killing was not related to Lyt-1^+ or Lyt-2^+ cells (T cells) but was associated with cells bearing NK markers (asialo GM_1, NK-1.2^+, and NK-2.1^+). Transfected targets were also killed by cloned NK lines. The transformation determinants on rat-1 transfectants cross-competed with YAC 1.2 lymphoma cells, suggesting a common target structure on these two diverse cell types. The results indicate that the NK surveillance system can recognize and kill cells newly transformed by a member of the ras oncogene family.

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