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Tissue-specific expression of an anti-*ras* ribozyme inhibits proliferation of human malignant melanoma cells

Yukinori Ohta+, Hiroshi Kijima, Tsukasa Ohkawa, Mohammed Kashani-Sabet[1] and Kevin J. Scanlon*

Biochemical Pharmacology, Department of Medical Oncology, City of Hope National Medical Center, Duarte, CA 91010, USA and [1]Department of Dermatology, University of California at San Francisco, San Francisco, CA 94143, USA

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ABSTRACT

In this study, we have compared the efficacy of a tissue-specific promoter (tyrosinase promoter) with a viral promoter to express anti-*ras* ribozyme RNA in human melanoma cells. The retroviral vector containing the tyrosinase promoter was superior in its ability to suppress the human melanoma phenotype *in vitro* as characterized by changes in growth, melanin synthesis, morphology and H-*ras* gene expression. These data support the use of tissue-specific expression of anti-oncogene ribozymes as a rational therapeutic strategy in human cancers.

INTRODUCTION

A variety of genetic abnormalities, such as mutation or overexpression of cellular oncogenes, have been implicated in the pathogenesis of neoplastic diseases (1). These cancer-related genetic alterations have attracted considerable attention as targets for gene therapy of cancer. In recent years, many efforts have been made to reverse the malignant phenotype of cancer cells by using gene modulators such as antisense DNA or RNA, catalytic RNA (ribozyme) and triplex DNA (2,3).

For successful gene therapy of cancer, it would be desirable to introduce recombinant genes directly into tumor cells *in vivo*. This requires the use of an effective and safe DNA vector. Much of the preclinical study of delivery systems has focused on viral (such as retroviruses and adenoviruses) and non-viral liposome-mediated methods which can effectively transfer 'therapeutic' DNA into the cell effectively (4,5). Although no technique currently exists for the reliable transduction of 100% of cells *in vivo*, most approaches entail non-specific delivery and expression in both normal and cancer tissue. Assuming that the target gene for therapeutic manipulation has biological importance, transfer and expression of gene modulators should be restricted to cancer cells without affecting normal cells.

One such approach concerns the use of hammerhead ribozymes, which represent newly developed *trans*-acting agents for the modulation of gene expression (3). We have investigated the utility of ribozymes against *ras* genes to suppress the malignant phenotype in relevant human model systems. In fact, the ribozyme against activated H-*ras* codon 12 has been shown to discriminate between the activated oncogene and its normal counterpart both *in vitro* (6) and in transformed cells (7) as well as to suppress human tumor growth in several murine model systems (8–10). In this manner, the anti-*ras* ribozyme can act as a tumor-specific therapeutic agent.

Another important component of this strategy involves restricting expression of the transgene to the target organ. In melanoma, this has been achieved using the tissue-specific tyrosinase promoter (11,12). Tyrosinase, one of the key enzymes in melanogenesis (i.e., the process of melanin production seen in normal melanocytes and melanoma cells), is synthesized almost exclusively in the melanocytic system (13). Melanoma cell-specific expression has been obtained with as little as 0.27 kb of the 5′ sequences of the tyrosinase gene, indicating the presence of *cis*-regulatory elements important for expression of this gene in melanoma (14).

In this study, we have attempted to evaluate the therapeutic potential of a ribozyme against the activated H-*ras* oncogene (*ras*Rz) driven by the tyrosinase promoter in FEM human melanoma cells, containing a heterozygous codon 12 mutation H-*ras*. The rationale for targeting *ras* genes in melanoma stems in part from studies demonstrating *ras* gene mutations in 45% of melanomas beyond Clark's level II (15) and in part from studies revealing that activated *ras* genes upregulate cytokines thought to be involved in melanoma cell proliferation (16). We have shown that expression of the *ras*Rz under the transcriptional control of the 5′ flanking sequence of the tyrosinase gene leads to suppression of the growth potential of human melanoma cells.

MATERIALS AND METHODS

Cells and transfections

Human melanoma FEM, FEMX-1 and LOX cells were obtained from Dr Oystein Fodstad (Oslo, Norway) and have been previously described (17). The FEM cells harbor a H-*ras* codon 12 mutation, in which the normally glycine-encoding GGC sequence is converted

* To whom correspondence should be addressed

+Present address: Department of Dermatology, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagamihara, Kanagawa 228, Japan

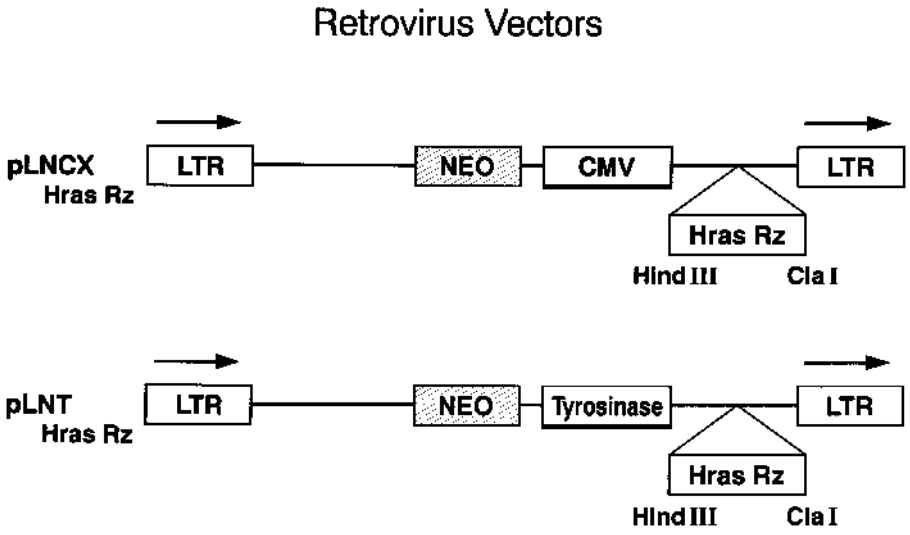

Figure 1. Structure and sequence of the two retroviruses containing the H-*ras* ribozyme. The CMV promoter (pLNC) and the tyrosinase promoter-driven (pLNT) retroviral constructs containing the H-*ras* ribozyme were sequenced by the dideoxynucleotide method (data not shown).

to GUC, encoding valine (18). BUX and PDJ are non-small cell lung carcinoma cells that were obtained from Dr Fodstad. Cells were grown as a monolayer with RPMI 1640 medium and supplemented with 10% fetal bovine serum. The pCAT control vector, containing a SV40 promoter, was obtained from Promega (Madison, Wl). The pCAT-Tyr-NT1 was obtained from Dr G. Schütz (Heidelberg, Germany). Cellular extracts were prepared after 24 h and assayed for CAT activity as described previously (14). Thymidine uptake was used to determine the rate of [^{3}H]dThd incorporation into trichloroacetic acid-precipitable material. Colony formation in soft agar was performed as previously described (18). For determination of relative melanin content, 5×10^6 cells were collected and dissolved in 1 ml of 1 N KOH and measured by O.D. at 492 nm (18).

Log-phase growing cells were transfected by electroporation according to a previously published method (19). The cells were then selected in growth medium containing 500 µg/ml geneticin (G418, Gibco) for 3–6 weeks. Individual G418-resistant colonies were picked, grown and screened for expression of the ribozyme by reverse-transcription (RT)-PCR (20).

Synthetic oligonucleotides

Synthetic oligodeoxynucleotides were prepared as previously described (8). The sequences for primers used in this study were as follows:

(i) primers for the PCR assay:

5′-AGG CTG AGA GTA TTT GAT GT-3′
5′-CAG GTG GGG TCT TTC ATT CC-3′

(ii) probe for ribozyme detection:

5′-CTC ACG GGA CTC ATC AGG-3′

(iii) oligonucleotides for cloning the ribozyme:

5′-AGC TTC ACA CCC TGA TGA GTC CGT GAG GAC GAA ACG GCG CCA T-3′

5′-CGA TGG CGC CGT TTC GTC CTC ACG GAC TCA TCA GGG TGT GA-3′

(iv) primers for detecting ribozyme gene expression by PCR:

pLNCX*ras*Rz: 5′-GAG ACG CCA TCC ACG CTG TT-3′ and
5′-CAG GTG GGG TCT TTC ATT CC-3′

pLNT*ras*Rz: 5′-AGG CTG AGA GTA TTT GAT GT-3′ and
5′-CAG GTG GGG TCT TTC ATT CC-3′

Construction of plasmids

All recombinant DNA techniques were carried out as described (8,18). The retrovirus vector in which the ribozyme was driven by the cytomegalovirus (CMV) promoter was constructed as follows. The retroviral vector pLNCX (obtained from Dr A. D. Miller) was digested by *Hin*dIII and *Cla*I downstream of the neomycin(Neo)-resistance gene and the CMV promoter (21). The *ras*Rz, prepared from two synthetic oligodeoxynucleotides with flanking restriction sites, was then subcloned into the linearized vector. The resulting retroviral vector with the CMV promoter was designated pLNCX*ras*Rz (Fig. 1). The retroviral ribozyme-expressing vector with the murine tyrosinase promoter was constructed using pLNCX*ras*Rz. The CMV promoter was removed from the pLNCX*ras*Rz plasmid by digestion at *Bam*HI and *Hin*dIII and partially filled-in with the Klenow fragment. The ribozyme sequence was still included in this vector. The insert containing the 0.27 kb murine tyrosinase promoter was taken from pTYRCAT6 with *Xho*I and *Sal*I and was further subcloned into the linearized vector, pLNCX*ras*Rz, without the CMV promoter (14). The resulting retroviral vector with the murine tyrosinase promoter was designated pLNT*ras*Rz (Fig. 1).

Detection of gene/ribozyme expression

RT-PCR followed that of a commercially available protocol (GeneAmp, Perkin-Elmer-Cetus) to detect ribozyme expression using aforementioned primers and probe as previously described (20). Briefly, 100 ng of total RNA from each cell line was subjected to 25 cycles of RT-PCR. An identical amount of PCR product from each cell line was used for electrophoresis. The probe used to detect *ras*Rz expression, *ras*P-4, is encoded by the following sequence complementary to the conserved catalytic core: 5′-CTG ACG GAC TCA TCA GG-3′ (8). RNA was

size-fractionated and transferred to a nylon membrane (Hybond-N, Amersham). Hybridizations were carried out with probes radiolabeled by the random primer method as described (8,18). Total RNA and poly(A) mRNA were prepared by the guanidium isothiocyanate method (22).

RESULTS AND DISCUSSION

In order to demonstrate tissue specificity, the murine tyrosinase promoter and the chloramphenicol acetyltransferase (CAT) expression system were utilized. The tyrosinase promoter, containing 0.27 kb of the 5′ flanking sequences of the mouse tyrosinase gene, has been determined to regulate expression of the tyrosinase gene in the presence of *cis*-regulatory elements in human melanoma cells (14). A mouse tyrosinase promoter was used because of its availability when the experiments were initiated and was shown to be active in human cells that synthesized melanin. We assumed that melanin either from a mouse source or a human source would activate the tyrosinase promoter. Two CAT gene plasmids were generated where the expression was directed either by the SV40 promoter (denoted pCAT-SV40) or by the tyrosinase promoter (denoted pCAT-Tyr-NT). Transfection of each vector into FEM cells and FEMX-1 cells (a derivative of FEM cells grown in nude mice) revealed strong CAT expression (Table 1). Furthermore, transfection of the pCAT-SV40 vector into a number of other tumor cell lines resulted in CAT gene expression. However, when the pCAT-Tyr-NT plasmid was transfected into the LOX human amelanotic cell line, little expression was observed. And there was minimal expression in the human non-small cell lung carcinoma cell lines BUX and PJD (Table 1). These results indicate that the tyrosinase promoter was selectively active in melanin-producing cells.

Table 1. CAT gene expression in mammalian cells

Cell line	pCAT-SV40[a]	pCAT-Tyr[a]
FEM	2.23	0.84
FEMX-1	2.39	1.07
LOX	4.63	0.02
BUX	4.37	0.03
PDJ	1.55	0.01

Activity for a melanoma cell-specific tyrosinase gene promoter (14) was determined in human melanin and non-melanin producing cell lines. Two melanoma cell lines were melanin positive (FEM and FEMX-1). One melanoma cell line was melanin negative (LOX) and two human non-small cell lung carcinoma cell lines were melanin negative (BUX and PDJ). The plasmids were transfected into the different cell lines by electroporation and assayed for CAT activity as described in the Methods section.

[a]The specific activity was pmol/min/mg of protein. These results are the average of three experiments. There was $<10\%$ standard deviation for the activity.

In order to test the efficacy of the tyrosinase promoter in driving ribozyme expression in FEM cells, retroviral plasmids were utilized. Figure 1 depicts the schematic representation of ribozyme-expressing retroviral vectors used in this study. Ribozyme expression was regulated by the CMV promoter and mouse tyrosinase promoter in pLNCX*ras*Rz and pLNT*ras*Rz, respectively. FEM human melanoma cells were transfected with either pLNCX*ras*Rz or pLNT*ras*Rz by electroporation. After G418 treatment for 3–6 weeks, several neomycin-resistant clones were selected based on a range of altered morphology. Two representative clones from each group (designated FEM pLNCX*ras*Rz-1, *ras*Rz-2 and FEM pLNT*ras*Rz-1, *ras*Rz-2, respectively) were utilized for subsequent analysis. RT-PCR was performed and ribozyme RNA detected in all *ras*Rz-transfected clones; however, relatively higher amounts of ribozyme RNA transcripts were observed in pLNT*ras*Rz transfectants when compared to that in pLNCX*ras*Rz clones (Fig. 2). We have previously demonstrated that *ras*Rz generated inside cells retains the capacity to cleave *ras* transcripts *in vitro* (8,9). Moreover, the *ras*Rz has been shown to be superior to antisense *ras* sequences in inhibiting target gene expression (8–10).

Expression of H-*ras* RNA, the target for the *ras*Rz, was downregulated in *ras*Rz-expressing clones compared with that of parent FEM cells and control FEM cells transfected with pLNCX only. In FEM cells with pLNT*ras*Rz, the expression of H-*ras* was significantly decreased, whereas pLNCX*ras*Rz transfectants were shown to express almost the same level as parental or vector-only cells. In addition, FEM cells with pLNT*ras*Rz (clone 2), with the strongest ribozyme expression, revealed the lowest level of target H-*ras* mRNA (Fig. 2), suggesting an inverse correlation between ribozyme expression and H-*ras* transcript levels.

In vitro growth characteristics were determined and are summarized in Table 2. The pLNCX*ras*Rz clone resembled the control clone as characterized by several growth characteristics. The pLNT*ras*Rz transfectants were shown to have ~4.0–4.7 times longer doubling time in clones 1 and 2, respectively, than that of the control clone. Thymidine uptake studies demonstrated approximately a 50% reduction in DNA synthesis in FEM pLNT*ras*Rz when compared with parent FEM cells. Colony formation in soft agar, or capacity for anchorage-independent growth, was virtually abolished in pLNT*ras*Rz transfectants. Thus, the *ras*Rz driven by the tyrosinase promoter made a stronger impact on the cell growth of FEM human melanoma cells than that regulated by the CMV promoter. In order to guard against clonal variability, four transfected clones demonstrating a range of ribozyme expression were pooled and the studies repeated. The results reveal the consistently greater growth inhibition in FEM pLNT*ras*Rz cells than in FEM pLNCX*ras*Rz cells (Table 2). The pLNT only vector and a pLNT reverse *ras*Rz vector containing the tyrosinase promoter in the opposite orientation had similar results to the control and the pLNCX only vector (Table 2).

Interestingly, *ras*Rz transfectants, especially the pLNT*ras*Rz clones, revealed a dendritic morphological alteration (Fig. 2). This can be interpreted as a sign of accelerated differentiation (18). Moreover, melanin synthesis was enhanced as a consequence of *ras*Rz action. Tyrosinase is one of the key enzymes in melanogenesis. Studies using expression assays with CAT-containing vectors have indicated that the 0.27 kb region upstream of the tyrosinase gene is sufficient to yield cell-specific expression in murine melanoma cell lines (14). In this study, the *ras*Rz was actively expressed in FEM cells transfected with pLNT*ras*Rz. Our results indicate that this tyrosinase promoter region can be a possible tissue-specific promoter for ribozyme expression in melanoma cells. In contrast, our results also indicated that the pLNCX*ras*Rz plasmid was far less effective as a negative regulator of the cell growth of FEM human melanoma cells. This retrovirus vector construct is essentially the same as that originally reported (21) and used in several gene-transfer studies. At present, we have no evidence to determine why this vector was not as effective in the FEM cells. It is possible that the efficacy of this expression vector may be dependent on the cell type utilized.

Table 2. Cell growth and melanin content of FEM human melanoma cells transfected with *ras*Rz in a pLN retroviral vector containing either the CMV promoter (pLNCX), the tyrosinase promoter (pLNT) or the tyrosinase promoter (pLN reverse T*ras* Rz) in the reverse orientation

Cell lines[a]	Doubling time (h)	[^{3}H] Thymidine uptake[b] (%)	Colonies in soft agar[c] serum: 1%/20%	Melanin content[d] (%)
FEM	18.2	100	84/168	100
FEM pLNCX only	19.6	91	80/142	115
FEM pLNCX*ras*Rz-1	21.1	80	76/152	111
FEM pLNCX*ras*Rz-2	20.2	81	90/148	116
FEM pLNCX*ras*Rz-P[e]	21.5	82	81/161	121
FEM pLNT*ras*Rz-1	78.0	43	8/24	415
FEM pLNT*ras*Rz-2	92.0	42	0/12	498
FEM pLNT*ras*Rz-P[e]	88.5	39	0/15	513
FEM pLN (reverse-T)*ras*Rz	19.0	85	80/155	111

[a]FEM, FEM human melanoma cells (parent); FEMpLNCX, FEM cells with pLNCX vector only; FEMpLNCX*ras*Rz-1,2, FEM cells with pLNCX*ras*Rz clone-1, -2; FEMpLNT*ras*Rz-1, -2, FEM cells with pLNT*ras*Rz clone-1,2.
[b]To determine the rate of [^{3}H] thymidine uptake in acid-soluble material, cells were grown for 48 h in medium and were pulsed for 1 h with [^{3}H] thymidine, washed, acid-precipitated and counted. The FEM pLNCX (100%) represented 3.29 fmol/mg DNA/h. These results represent the mean of three separate experiments.
[c]Viable cells (5×10^3)were grown in 0.3% agar with 1% or 20% fetal bovine serum. Thirteen days after seeding, colonies ≥ 250 µm in diameter were counted. The data are presented as the mean of triplicate plates.
[d]An equal number of cells was harvested and lysed in 1 N KOH. The hydrolysate was centrifuged at 12 000 *g* and the O.D. at 492 nm was determined. Melanin content (100%) in control FEM pLNCX represented 12.1 µg/10^6 cells.
[e]P denotes pooled clones.

Our previous study (18) indicated that one consequence of *ras*Rz action was upregulation of melanin synthesis in human melanoma cells (23). Tyrosinase activity is well-correlated to melanin synthesis (14). It is therefore possible that upregulation of tyrosinase promoter activity (leading to enhanced melanin synthesis) as a consequence of *ras* ribozyme action led to a positive feedback loop resulting in increased ribozyme expression. And the tyrosinase promoter did act in a tissue-specific manner as it was found to be inactive in the amelanotic cell lines tested. In this manner, we have designed a tumor-specific therapeutic agent whose expression is restricted to the cell of interest. Taken together, our system using a ribozyme targeting the activated *ras* gene and the tyrosinase promoter may create an effective tumor-specific and tissue-specific therapeutic gene. These concepts need to be further explored in preclinical (*in vivo*) studies.

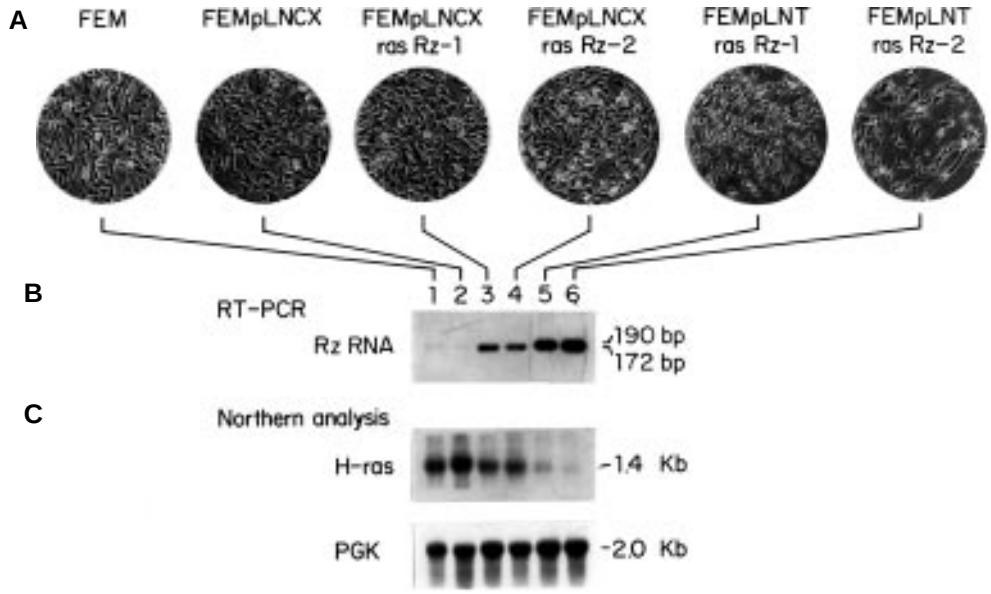

Figure 2. Morphology and gene expression in FEM transformants. (**A**) Morphology of FEM ribozyme transfected cells. Photographs of FEM cell morphology were taken at a magnification of 100×. Lane 1, FEM cells; lane 2, FEM pLNCX vector only; lanes 3 and 4, FEM pLNCX*ras*Rz clones 1 and 2; lanes 5 and 6, FEM pLNT*ras*Rz clones 1 and 2. (**B**) Semi-quantitative RT-PCR was performed on 100 ng of total RNA from each cell line (1–6). An identical amount of sample and probe was used to conduct each experiment. (**C**) Northern blotting of 2 µg RNA for each lane was used for detecting H-*ras* RNA and a control gene, phosphoglycerate kinase RNA (PGK). The experiments depicted in lanes 1–6 were conducted simultaneously and the samples handled in an identical manner. kb, kilobases; bp, base pair.

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