Sensitization to Apoptosis Underlies $Kras^{D12}$ -Dependent Oncolysis of Murine C26 Colorectal Carcinoma Cells by Reovirus T3D

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Reovirus T3D is an oncolytic agent that preferentially targets tumor cells expressing an activated *Ras* oncogene. *Ras* signaling interferes with the cellular stress response that inhibits translation of reovirus RNAs. Murine C26 colorectal carcinoma cells express a mutant $Kras^{D12}$ gene. Reovirus T3D efficiently kills C26 cells, but not C26 cells in which the $Kras^{D12}$ mRNA is stably repressed by expression of $Kras^{D12}$ -directed short-hairpin RNAs. Surprisingly, neither reovirus T3D protein synthesis nor T3D virus yields were suppressed by deletion of $Kras^{D12}$. Rather, reovirus-induced tumor cell apoptosis was completely abrogated as a result of *Kras* knockdown. We conclude that sensitization of C26 tumor cells to reovirus-induced apoptosis underlies the *Ras* dependency of reovirus T3D oncolysis.

Reovirus T3D is a double-stranded RNA-containing virus belonging to the *Reoviridae* family. Fibroblasts transformed by an activated *Hras* oncogene are highly sensitive to reovirus oncolysis (7, 17, 23). In untransformed cells, viral RNAs stimulate a cellular defense mechanism by activation of double-stranded RNA-dependent protein kinase (PKR) (23). Activated PKR prevents the translation of transcripts by inactivation of translation initiation factor 2 α (eIF2 α) through phosphorylation on ser51 (6, 10). *Ras* signaling interferes with this defense mechanism by inhibiting virus-induced PKR activation, thereby allowing reovirus replication (23). Reovirus infections are nonpathogenic in immunocompetent adults, which makes this virus an interesting candidate for exploitation as an oncolytic agent (13–15, 19, 23).

C26 is an aggressive colorectal cancer cell line that contains constitutively activated *Kras* due to an activating point mutation in codon 12 (G12D) (22). The *Nras* and *Hras* genes in this cell line do not contain activating mutations. We previously established cell lines in which the endogenous $Kras^{D12}$ allele is stably suppressed by mutant-specific RNA interference by using a lentivirus vector (C26-*Kras*KD). As a control, we established cell lines transduced with the empty lentivirus pLL3.7 vector (C26-pLL). Efficient and specific knockdown of *Kras* was demonstrated by Western blot analysis for *Kras* and, as control, *Nras* (22). These cell lines were used to analyze the effect of *Kras* on C26 sensitivity to reovirus-induced oncolysis. Tumor cells (5,000/well) were plated in a 96-well plate and immediately infected with reovirus T3D (25 PFU/cell). Tumor cell viability was then analyzed for 6 consecutive days with

standard 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assays. After an initial growth phase (1 to 3 days), reovirus-infected C26-pLL cells rapidly lost viability, resulting in near-complete cell death by day 6 (Fig. 1A). In contrast, C26-*Kras*KD cells were completely refractory to reovirus-induced cell death (Fig. 1A). Evaluation of cellular morphology by light microscopy clearly showed the cytopathic effect of reovirus T3D on C26-pLL cells, but not on C26-*Kras*KD cells (Fig. 1B).

We expected that C26-*Kras*KD cells were refractory to reovirus T3D infection due to inhibition of viral protein synthesis and replication (23). To test this, 5×10^4 C26-pLL and C26-*Kras*KD cells/well were plated in a 24-well plate and infected with reovirus T3D (25 PFU/cell) or treated with a vehicle control and labeled for 4 h with [³⁵S]methionine at 5 days postinfection (dpi). Surprisingly, the production of reovirus proteins could be clearly demonstrated in both C26-pLL and C26-*Kras*KD cells (Fig. 2A). However, cellular protein synthesis was detectable only in C26-*Kras*KD cells and had been completely shut off in C26-pLL cells (Fig. 2A). PKR-mediated inhibition of cellular protein synthesis is part of the immediate integrated stress response by which cells respond to virus infections, oxidative stress, endoplasmic reticulum stress, or amino acid deprivation (11, 23). We have not been able to detect an inhibition of cellular or viral protein synthesis in C26 cells or in C26-*Kras*KD cells at 4, 8, 24, and 48 h postinfection by performing 1-h [³⁵S]methionine-labeling experiments (not shown). Rather, inhibition of cellular protein synthesis in reovirus-infected C26-pLL cells is evident only at 5 and 6 dpi, when primarily viral RNAs are transcribed (Fig. 2A). Total abrogation of protein synthesis (both viral and cellular), reflecting massive cell death, usually occurs 1 to 2 days later. Our study is in apparent contradiction with published studies showing that ectopic expression of exogenous $Hras^{V12}$ in fibroblasts

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FIG. 1. KrasD12 knockdown sensitizes C26 colorectal carcinoma cells to reovirus T3D. (A) Cell viability of C26 and C26-KrasKD cells was measured on 6 consecutive days following infection with reovirus T3D by using the MTT assay. (B) Light microscopy images of C26 and C26-KrasKD cells infected with reovirus T3D at 5 dpi.

promotes reovirus protein synthesis (14, 20, 23). A possible explanation for this discrepancy is that the strength and selection of signaling pathways which are activated by endogenous versus overexpressed exogenous Ras genes may be different. Indeed, the expression level of $Kras^{V12}$ is a highly critical parameter in determining cellular responses to Ras expression (1). In this respect, it is worth mentioning that our results show for the first time that the knockdown of an endogenous *Kras* oncogene abrogates cellular sensitivity to reovirus T3D.

Recently, it was reported that the facilitation of reovirus replication by overexpressed $Hras^{V12}$ in fibroblasts depends on the activity of p38 mitogen-activated protein kinase (20). However, several studies show that cancer cell lines harboring an endogenous mutant K-Ras allele display very low to undetectable levels of constitutive p38 activity (2, 21, 25). Furthermore, p38 activity in human colorectal cancer cells was not affected by $Kras^{D13}$ deletion or by $Hras^{V12}$ overexpression (2). Therefore, differential signaling to p38 by overexpressed $Hras^{V12}$ in fibroblasts and by endogenous $Kras^{D12}$ in tumor cells may explain the differential effects of these *Ras* genes on reovirus replication.

Next, we assessed whether *Kras* knockdown affects reovirus T3D propagation in C26 cells. To this end, we infected C26-pLL and C26-KrasKD cells with reovirus T3D (25 PFU/cell) and analyzed reovirus protein synthesis, as well as the number of infectious particles produced by both cell types over time. We made use of a polyclonal antibody raised against UV-inactivated reovirus T3D in rabbits. This antibody primarily recognizes the major structural μ_1 protein, with an apparent

molecular mass of 76 kDa. Figure 2B shows that both C26-pLL and C26-KrasKD cells synthesize μ_1 following infection with T3D. In addition, infectious particles were assayed both in the medium and in freeze-thawed lysates from cell populations. To determine the amount of reovirus released by the cells, the medium was carefully harvested from the cultures. The cell debris was pelleted by low-speed centrifugation (2 min, $250 \times g$, room temperature). The reovirus in the supernatant was quantified by plaque assays on 911 cells. To determine the quantity of cell-associated reovirus, the medium was carefully removed. Subsequently, the remaining adherent cells were detached in 100 μ l fresh medium by tapping the dish and by triturating using a small-volume pipette. The cell suspension was removed from the dish and added to the pellet fraction obtained after centrifugation of the conditioned medium. The cells were resuspended and lysed by three cycles of freeze-thawing. Subsequently, the lysate was added to a new tube and spun at $1,600 \times g$ for 10 min. The amount of infectious T3D particles in the supernatant was then assessed by plaque assays on 911 cells. Figure 2C shows that reovirus T3D is efficiently propagated in and released from C26-pLL, as well as C26-KrasKD, cells. In fact, virus propagation was more pronounced in the T3D-resistant C26-KrasKD cells (Fig. 2B and C). Taken together, the results show that C26-KrasKD cells can support reovirus T3D replication and release without overt cytopathic effects.

Reovirus T3D causes cell death by inducing apoptosis (4). As Ras may control apoptosis signaling either positively or negatively (9), we hypothesized that resistance to reovirus-

FIG. 2. *Kras*^{D12} knockdown does not affect reovirus protein synthesis or virus propagation. (A) C26 and C26-*Kras*KD cells were infected with reovirus T3D and labeled with [³⁵S]methionine for 4 h at 5 dpi. Total cell extracts were analyzed by gel electrophoresis and subsequent autoradiography. (B) C26 and C26-*Kras*KD cells were infected with reovirus T3D. Cell extracts were prepared on the indicated days postinfection and tested for the presence of the reovirus μ₁ protein by Western blot analysis. Each bar on the right indicates the position of the 75-kDa marker. (C) C26 and C26-*Kras*KD cells were infected with reovirus T3D. Medium samples (sup) were subsequently analyzed at 4, 5, 6, and 7 dpi. The cells were counted, and cell extracts were prepared by freeze-thawing. The number of infectious virus particles (PFU) in both fractions was determined by using 911 cells (12).

induced apoptosis may underlie the differential sensitivity of C26-pLL and C26-*Kras*KD cells to reovirus-induced cell death. To test this, C26-pLL and C26-*Kras*KD cells were exposed to control vehicle or reovirus T3D (25 PFU/cell) and after 5 days the induction of apoptosis was analyzed by FACScan analysis of propidium iodide-stained cells, as well as by indirect immunofluorescence and Western analysis for activated caspase 3. The fraction of apoptotic C26-pLL cells (with sub-G1 DNA content) increased to 31% at 5 dpi. In contrast, reovirus T3D infection had no effect on the fraction of C26-*Kras*KD cells with sub-G1 DNA content (Fig. 3A, right side). In addition, immunofluorescence analyses showed that 18.2% of C26-pLL cells were positive for activated caspase 3, whereas only 2.6% of C26-*Kras*KD cells were positive for activated caspase 3 (Fig. 3A, left side [C92-605; BD Biosciences PharMingen], and B). Furthermore, Western blot analysis showed that activated caspase 3 was readily detected in lysates of C26-pLL cells but

not in lysates of C26-*Kras*KD cells (Fig. 3C). Genomic DNA is cleaved during apoptosis, yielding single- and double-stranded DNA breaks with free 3'-OH termini that can be labeled with the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) reaction. Figure 3B shows that at 5 days following infection with reovirus T3D, 21% of the C26-pLL cells were TUNEL positive whereas C26-*Kras*KD cells remained negative (in situ cell death detection kit; Roche Applied Science). Baculovirus protein p35 inhibits virus-induced apoptosis (5). To determine the contribution of reovirus-induced apoptosis to the cytopathic effect on C26 cells, we transduced C26 cells with a lentivirus vector carrying the p35 coding region, resulting in cell line C26-p35. C26-p35 and, as a control, C26-pLL cells were subsequently infected with reovirus T3D (25 PFU/cell), and 5 days after infection cell viability was assessed by MTT assays. Whereas reovirus T3D infection reduced the viability of C26-pLL cells by 52%, the viability of p35-expressing C26 cells was unaffected (Fig. 3D). Reovirus T3D-induced apoptosis requires virus binding to sialic acid via the σ1 protein (8). Type 1 Lang (T1L) reovirus does not bind to sialic acid and does not induce apoptosis (24). A reassortant virus strain carrying the T3D S1 gene in a T1L background (T3SA+) has gained the potential to bind sialic acid and to induce apoptosis (8). In contrast, a reassortant T1L virus carrying a mutated (L204P) T3D S1 gene (T3SA-) fails to do so (8). We compared T3SA+ and T3SA- for the abilities (i) to induce oncolysis and (ii) to replicate in C26 cells. We found that T3SA+ induced oncolysis with kinetics similar to that induced by T3D (Fig. 3E). The apoptosis-defective T3SA- mutant, however, did not induce oncolysis of C26-pLL cells (Fig. 3E). Importantly, the amounts of reovirus μ₁ protein produced at the end of the experiment (8 dpi) were similar for both T3SA+ and T3SA-, indicating comparable virus production (Fig. 3E). C26-*Kras*KD cells were completely resistant to either T3SA+ or T3SA-, as they are to T3D (data not shown and Fig. 1A).

Taken together, our results demonstrate an alternative mechanism for the selective sensitivity of tumor cells carrying a *Kras* oncogene to reovirus T3D: sensitization to reovirus-induced apoptosis.

There has been an increasing awareness that Ras, depending on the cell type and context, can induce either pro- or anti-apoptotic signaling (9). Our results clearly show that the presence of *Kras*^{D12} facilitates the induction of apoptosis by reovirus T3D. Reovirus-induced apoptosis in human embryonic kidney 293T cells and in HeLa cells is mediated by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (3). Furthermore, overexpression of *Hras*^{V12} sensitizes normal human fibroblasts and human embryonic kidney cells to TRAIL-induced apoptosis (18). Therefore, Ras-dependent sensitization to TRAIL may underlie the differential susceptibility of C26 and C26-*Kras*KD cells to reovirus-induced apoptosis. However, treatment of C26-pLL and C26-*Kras*KD cells with recombinant TRAIL (up to 500 ng/ml, 24 h) did not induce apoptosis in either cell line, whereas HCT 116 cells were efficiently killed (Fig. 4). The TRAIL batch used was highly active, as it induced HCT-116 apoptosis already at 100 ng/ml (not shown). Although TRAIL did not induce apoptosis in C26 or C26-*Kras*KD cells, it caused a marked increase in the percentage of G1 DNA in both C26 cells (from 40 to 58%) and

FIG. 3. *Kras*^{D12} knockdown prevents apoptosis induction by reovirus. (A) C26-pLL and C26-KrasKD cells were infected with reovirus T3D (25 PFU/cell), and at 5 dpi they were analyzed by anti-active caspase 3 immunofluorescence (A [left side] and B), by FACScan analysis of propidium iodide-stained cells (A [right side]), by the TUNEL reaction (B), or by Western blot analysis for activated caspase 3 (Casp 3a) (C). (D) Baculovirus p35-expressing cells and control cells were infected with reovirus T3D (25 PFU/cell), and cell viability was determined at 5 dpi by the standard MTT assay. (E) C26-pLL cells were infected with reovirus T3SA⁻ and T3SA⁺ (25 PFU/cell), and cell viability was measured at the indicated time points by using the MTT assay. Synthesis of the viral μ_1 protein was analyzed by Western blotting at 8 dpi as a measurement of virus production.

C26-KrasKD cells (from 52 to 68%). Recently, it was found that TRAIL reduced the proliferation of human T-cell lines, possibly by suppressing cdk4 levels (16). It is unknown whether this is a general phenomenon in TRAIL-resistant tumor cells. In conclusion, the Ras-dependent susceptibility of C26 cells to reovirus-induced apoptosis is not accompanied by susceptibility to TRAIL. This suggests that TRAIL is not the only critical factor in reovirus-induced apoptosis, at least in the C26 cells studied here.

Whether an activated Ras pathway sensitizes transformed

cells to reovirus oncolysis by interfering with cellular antiviral stress signaling or by facilitating apoptosis may be cell type dependent. It is even conceivable that these pathways act in concert to allow tumor-specific oncolysis, although this was not observed in the present study. Taken together, our results support the feasibility of using reovirus T3D as an oncolytic agent for tumor cells carrying mutant *Kras*. Nonetheless, the mechanism underlying oncolytic selectivity may involve Ras-dependent sensitization of tumor cells to reovirus-induced apoptosis, rather than to facilitation of reovirus replication. This

FIG. 4. Resistance to TRAIL-induced apoptosis. Exponentially growing C26-pLL, C26-KrasKD, and HCT116 cells were treated with recombinant TRAIL (500 ng/ml) for 24 h. All detached and adherent cells were collected and processed for FACSscan analysis of DNA content by using propidium iodide. WinMDI software was used to determine the percentage of apoptotic cells displaying sub-G1 DNA content. Values represent means of triplicates.

may have ramifications for the design of combination therapies involving reovirus T3D.

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