Suppression of the Malignant Phenotype in Human Pancreatic Cancer Cells by the Overexpression of Manganese Superoxide Dismutase

Christine Weydert, Benjamin Roling, Jingru Liu, Marilyn M. Hinkhouse, Justine M. Ritchie, Larry W. Oberley, and Joseph J. Cullen

Departments of Surgery (B. R., J. J. C.), Radiation Oncology (C. W., J. L., L. W. O., J. J. C.), and the Holden Comprehensive Cancer Center (J. M. R., L. W. O., J. J. C.), University of Iowa College of Medicine, Iowa City, Iowa 52242, and Veterans Affairs Medical Center (M. M. H., J. J. C.), Iowa City, Iowa 52242

Abstract

Cells contain a large number of antioxidants to prevent or repair the damage caused by reactive oxygen species. One component of the antioxidant system, manganese superoxide dismutase (MnSOD), is localized in the mitochondria, and the levels of this protein have been previously shown to inversely correlate with pancreatic cancer cell growth. The aim of the present study was to determine whether MnSOD overexpression could suppress the in vitro and in vivo malignant phenotype of a human pancreatic cancer cell line. Tumor cell behavior was determined in the pancreatic cancer cell line MIA PaCa-2 by examining cell growth, plating efficiency, and anchorage-independent growth in soft agar. MnSOD was overexpressed in the pancreatic cancer cell line MIA PaCa-2 by infection with an adenovirus-MnSOD construct. Cells were also injected s.c. in nude mice and tumor volume was calculated. Single and multiple direct injections of the adenoviral MnSOD construct (10^8 plaque-forming units) were delivered to the tumor. Increases in MnSOD immunoreactivity and activity were seen after transduction with the adenovirus-MnSOD construct. Increasing MnSOD levels correlated with increased doubling time. Cell growth, plating efficiency, and growth in soft agar decreased with increasing amounts of the adenovirus MnSOD construct. Tumors grew slower and survival was increased in nude mice injected with the adenoviral MnSOD construct compared with the parental cell line, whereas multiple injections of the adenoviral MnSOD construct further inhibited tumor cell growth and extended survival. These results suggest that MnSOD may be a tumor suppressor gene in human pancreatic cancer. Delivery of the MnSOD gene may prove beneficial for suppression of pancreatic cancer growth.

Introduction

Pancreatic adenocarcinoma is now the fourth leading cause of cancer death in the United States with an overall 5-year survival rate of less than 5% (1). Even after curative resection, the 5-year survival rates achieved at specialized centers are less than 20%, and the majority of patients die of metastatic cancer recurrence (2). Other adjuvant treatments such as radiation therapy and chemotherapy have not improved long-term survival after resection. Thus, novel treatment strategies directed against this devastating malignancy are greatly needed.

Many solid tumors have low levels of antioxidant enzymes (3, 4). Cells contain a large number of antioxidants to prevent or repair the damage caused by reactive oxygen species. There are three major types of primary intracellular antioxidant enzymes in mammalian cells: SOD, catalase, and peroxidase, of which glutathione peroxidase is the most prominent. The SODs convert $O_2^-$ into $H_2O_2$, whereas the catalases and peroxidases convert $H_2O_2$ into water. In this way, two toxic species, $O_2^-$ and $H_2O_2$, are converted to water. These antioxidant enzymatic functions are thought to be necessary for life in all oxygen-metabolizing cells (5). An important feature of these enzymes is that they are highly compartmentalized. In general, MnSOD is localized in the mitochondria, CuZnSOD is localized in the cytoplasm, catalase is localized in peroxisomes and cytoplasm, and glutathione peroxidase is localized in many subcellular compartments.

As with most other solid tumors, pancreatic cancer has been demonstrated to have low levels of antioxidant enzymes (6). Immunohistochemistry demonstrated that MnSOD, CuZnSOD, catalase, and glutathione peroxidase are decreased in human pancreatic ductal carcinoma specimens when compared with normal human pancreas. Similar findings are seen in primary pancreatic cancer cell lines, including the pancreatic cancer cell line MIA PaCa-2, which has decreased levels of MnSOD immunoreactivity and enzyme activity when compared with normal human pancreas (7). This study also demonstrated that cell-doubling time was most rapid in the cell lines with the lowest levels of MnSOD. There was no correlation between cell growth and the levels of other antioxidant enzymes including CuZnSOD, catalase,

Received 11/13/02; revised 2/5/03; accepted 2/12/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by NIH Grants DK 60618 and CA 66081 and by the Medical Research Service, Department of Veterans Affairs.

2 To whom requests for reprints should be addressed, at 4605 JCP, University of Iowa Hospitals and Clinics, Iowa City, IA 52242. Phone: (319) 353-8297; Fax: (319) 356-8378; E-mail: joseph-cullen@uiowa.edu.

3 The abbreviations used are: SOD, superoxide dismutase; MnSOD, manganese-containing SOD; CuZnSOD, copper- and zinc-containing SOD; MOI, multiplicity/multiplicities of infectivity; PFU, plaque-forming unit(s); AdgbII, adenovirus containing no added DNA.
or glutathione peroxidase; however, MnSOD activity and immunoreactive protein correlated with pancreatic tumor cell doubling time. These findings suggest that MnSOD may play a role in the growth of pancreatic cancer in vitro, and that MnSOD may be effective in tumor growth suppression in this devastating disease.

To further define the role of MnSOD in the growth of pancreatic cancer, we investigated the effect of enforced expression of MnSOD on both the in vitro and in vivo growth of the pancreatic cancer cell line MIA PaCa-2. The aims of our study were to determine whether transgene-expressed MnSOD alters our study were to determine whether transgene-expressed MnSOD on both the pancreatic cancer, we investigated the effect of enforced expression of MnSOD on both the in vitro and in vivo growth of the pancreatic cancer cell line MIA PaCa-2. The aims of our study were to determine whether transgene-expressed MnSOD alters in vitro tumor cell behavior, including cell growth, plating efficiency, and anchorage-independent growth in soft agar. Additionally, we wanted to determine whether delivering the MnSOD gene directly to xenografts by injection would affect in vivo tumor growth and survival. Our results show that MnSOD overexpression has a profound effect on pancreatic cancer cell growth.

Materials and Methods

Cell Culture. MIA PaCa-2 cells were purchased from American Type Culture Collection (Manassas, VA) and are human primary pancreatic adenocarcinoma cells derived from tumor tissue of the pancreas obtained from a 65-year-old male. The cell cultures were maintained at 37°C in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum and 2.5% horse serum.

Adenovirus Gene Transfer. The adenovirus constructs used were replication-defective, E1- and partial E3 deleted recombinant adenovirus (8). Inserted into the E1 region of the adenovirus genome was either the human MnSOD gene or the LacZ reporter gene, both of which are driven by a cytomegalovirus promoter. The adenovirus constructs were obtained from the University of Iowa Gene Transfer Vector Core.

Approximately 10^6 MIA PaCa-2 cells were plated in 10 ml of complete media in a 90-cm² plastic dish and allowed to attach for 24 h. Cells were then washed three times in serum- and antibiotic-free medium. The adenovirus-MnSOD construct, suspended in 3% sucrose, was then applied to cells suspended in 4 ml of serum- and antibiotic-free medium at 0, 10, 25, 50, 100, and 200 MOI. Control cells were treated with 100 MOI of the adenovirus-LacZ construct. Cells were incubated with the adenovirus constructs for 24 h. Media was then replaced with 4 ml of complete media for an additional 24 h before cells were harvested.

Cell Homogenization and Protein Determination. Cells were washed three times in PBS (pH 7.0), scraped from the dishes using a rubber scraper, and then collected in phosphate buffer (pH 7.8). This was followed by sonic disruption on ice for 30 s in 10-s bursts using a VibraCell sonicator (Sonics and Materials Inc., Danbury, CT) at 100% power. Protein concentration was determined using the Bio-Rad Bradford dye-binding protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

Western Analysis. Immunoreactive protein corresponding to MnSOD was identified and quantitated from total cell protein by the specific reaction of the immobilized protein with its antibody. Total protein was electrophoresed in a 12.5% SDS-polyacrylamide running gel and a 5% stacking gel. Gels were stained for protein loading with Coomassie Blue staining to determine equal loading. The proteins were then electrotransferred to nitrocellulose sheets. After blocking in 20% fetal bovine serum for 1 h, the sheets were washed and then treated with antiserum to MnSOD (1:1000) for 1 h. Polyclonal rabbit-antihuman antibody to MnSOD has been prepared and characterized in our laboratory (9). This antibody has been shown to react with the appropriate protein in a variety of species, including hamster and human (9, 10). The blot was incubated with horseradish peroxidase-conjugated goat-antirabbit (Sigma) IgG (1:10,000) for 1 h at room temperature. The washed blot was then treated with enhanced chemiluminescence Western blot detection solution (Amersham Life Science, Buckinghamshire, United Kingdom) and exposed to X-ray film. Western blots were performed in duplicate.

SOD Activity Assay. SOD activity was measured using an indirect competition assay between SOD and an indicator molecule, nitroblue tetrazolium. This was performed in the crude homogenate according to the method of Spitz and Oberley (11). Sodium cyanide (5 mM) inhibits CuZnSOD; therefore, activity measured in the crude homogenate in the presence of sodium cyanide indicates only MnSOD activity. Specific activity was reported as units per mg protein.

Antioxidant Enzyme Activity Gels. In this technique, nondissociating slab gels were run essentially by the method of Davis (12) with ammonium persulfate used as the initiator in the running gel (12.5%) and riboflavin-light in the stacking gel (5%). Once run, the gels were stained for SOD activity by the method of Beauchamp and Fridovich (13). CuZnSOD and MnSOD were differentiated by the presence of sodium cyanide in the staining solution, which inhibits CuZnSOD. The protein concentration was measured by the method of Lowry et al. (14). All of the antioxidant enzyme activity gels were performed in duplicate.

Cell Growth. Cells (1 × 10⁴) were plated in triplicate in 1.5 ml of complete media in 24-well plates. Cells were trypsinized and then counted on alternate days for 2 weeks using a hemocytometer. Cell population doubling time in hours (DT) was determined using the following equation:

\[
DT(\text{hours}) = \frac{0.693 (t - t_o)}{\ln(N_f/N_o)}
\]

where \(t_o\) = time at which exponential growth began, \(t\) = time in hours, \(N_f\) = cell number at time \(t\), and \(N_o\) = initial cell number (8).

Plating Efficiency. Adenovirus MnSOD- or AdLacZ-transduced cells (100 MOI) were plated in triplicate into 60-mm dishes in complete media. The dishes were maintained in the incubator for 6 days to allow colony formation. The colonies were then fixed and stained with 0.1% crystal violet and 2.1% citric acid, and those colonies containing greater than 50 cells were scored.

Anchorage-independent Growth in Soft Agar. Adenovirus MnSOD- or AdLacZ-transduced cells (5 × 10⁴) were suspended in 3 ml of complete medium containing a solution of 6% agar in double-distilled H₂O so that the final concen-
tration of the agar was 0.3%. This suspension was then plated over 3 ml of complete media made using a 6% agar solution in double-distilled H2O so that the final concentration of the bottom agar was 0.5%. After 16 days, colonies of greater than 0.1 mm in diameter were scored. The clonogenic fraction was determined using the following equation:

\[
\text{Soft agar plating efficiency (P.E.)} = \frac{\text{Colonies formed}}{\text{Cells seeded}} \times 100
\]

**Nude Mice.** Thirty-day-old athymic nude mice were obtained from Harlan Sprague Dawley (Indianapolis, IN). The nude mice protocol was reviewed and approved by the Animal Care and Use Committee of the University of Iowa on July 5, 2001. The animals were housed four to a cage and were fed a sterile commercial stock diet and tap water ad libitum. Animals were allowed to acclimate in the unit for 1 week before any manipulations were performed. In the first set of studies, each experimental group consisted of four to six mice. In the second set of studies, each experimental group consisted of six to eight mice.

**Adenovirus Vector-mediated MnSOD Gene Transfer.** MIA PaCa-2 tumor cells (2 \( \times 10^9 \)) were delivered s.c. into the flank region of nude mice from a 1-cc tuberculin syringe equipped with a 25-gauge needle. The tumors were allowed to grow until they reached between 3 and 4 mm in greatest dimension (from 10 days to 2 weeks), at which time they were treated with adenovirus.

The adenovirus constructs were delivered through four or more injection sites in the tumor, depending on tumor size at the time of injection. Approximately 1 \( \times 10^9 \) PFU (100 \( \mu \)l) of the adenoviral MnSOD construct were delivered to the tumor by a 25-gauge needle attached to a 1-cc tuberculin syringe. This was defined as day 1 of the experiment. In the second group of experiments, 1 \( \times 10^8 \) PFU (50 \( \mu \)l) of the adenoviral MnSOD were delivered on days 1, 7, and 14 for a total of three injections. Control tumors received serum-free medium or Ad\( \beta \)gII in similar volumes and PFU at the same time points. Tumor size was measured every 3 days by a vernier caliper, and tumor volume was estimated according to the following formula: tumor volume = \( p/6 \times L \times W^2 \), where \( L \) is the greatest dimension of the tumor, and \( W \) is the dimension of the tumor in the perpendicular direction (15). Animals were killed by CO\(_2\) asphyxiation when the tumors reached a predetermined size of 10 \( \times \) 10 mm and this was considered the time to sacrifice.

**Statistical Analysis.** Statistical analysis for the in vitro studies was performed using SYSTAT. A single factor ANOVA, followed by a post hoc Tukey test, was used to determine statistical differences between means. All means were calculated from three experiments, and error bars represent SE of mean. All of the Western blots, activity assays were repeated at least twice. For the in vivo studies, tumor volume was compared among the three groups using the data from days 1 through 26 for the first experiment and days 1 through 44 in the second experiment. To compare the treatment groups over time for tumor volume, the linear mixed model analysis (16) assuming either an autoregressive order 1 or a compound-symmetry covariance structure for within subjects was used. Selection of the covariance structure was based on the Akaike’s Information Criterion (AIC; Ref. 17). In the linear mixed-model analysis, the group was considered a fixed effect and the day was considered a continuous covariate, which means that the mean tumor volume was modeled as a linear function of days. An interaction term between day and group was also included in the model. The linear assumption seemed appropriate given that the adjusted R-squares by groups were all greater than 0.60. Survival curves were estimated by the Kaplan-Meier product limit curves. The log-rank test was used to compare survival between the groups. All of the data analyses for the in vivo data were done using SAS version 8.0.

**Results**

**Adenovirus Gene Transfer**

**Western Analysis.** A dose-dependent increase in MnSOD immunoreactivity was observed in cells transduced with the adenoviral MnSOD construct (Fig. 1A). MnSOD immunoreactivity was barely detectable in the parental and 100 MOI Ad\( \beta \)LacZ-transduced cells. Computerized analysis of densitometric scanning demonstrated that the amount of immunoreactive protein was increased over the parental and Ad\( \beta \)LacZ cells by 4-fold in the 50 MOI adenovirus MnSOD-transduced cells. MnSOD immunoreactivity appeared to plateau in the 100-MOI adenovirus MnSOD-transduced cells.
with a 5-fold increase in immunoreactivity in both the 100 and 200 MOI adenovirus MnSOD-transduced cells.

**SOD Activity Gel.** Enzyme activity is the most important parameter for determining the function of MnSOD because the expression of MnSOD mRNA may not necessarily mean an increase in MnSOD protein (18). Moreover, even increased MnSOD protein is not necessarily active. Therefore, we performed both enzymatic gels and activity assays to measure the activity of MnSOD. To determine the activity of the transgene-expressed MnSOD, we first performed activity gels for MnSOD on the transduced cells. The MnSOD enzyme activity gel confirmed our results from the Western blot. As demonstrated in Fig. 1B, MnSOD activity is barely seen in the parental MIA PaCa-2 cells; however, there seems to be a dose-dependent increase in activity in the adenoviral MnSOD-transduced cells. MnSOD activity appears to be maximal in the 100-MOI adenoviral MnSOD-transduced cells, and no additional increases were seen in the 200-MOI adenoviral MnSOD-transduced cells. Finally, the MnSOD activity gel correlated well with the Western blot ($r^2 = 0.98; P < 0.001$; Fig. 2).

**SOD Activity Assay.** To confirm the activity of the transgene-expressed MnSOD, we also performed activity assays for MnSOD on the transduced cells (Fig. 3; means ± SE; $n = 3$). The MnSOD enzyme activity assay confirmed our results from the native gel. The parental MIA PaCa-2 cell line expressed low constitutive MnSOD activity of 10.3 ± 2.1 units/mg protein (means ± SE; $n = 3$). MIA PaCa-2 cells transduced with AdLacZ demonstrated a small increase in MnSOD activity to 14.9 ± 2.0 units/mg protein. At 1 MOI of the adenovirus MnSOD, activity was 14.9 ± 3.9 units/mg protein, which was similar to that of the parental cell line and the cells transduced with AdLacZ. There was a dramatic increase in MnSOD activity in the cells transduced with 10–200-MOI adenovirus MnSOD with activities of 51.4 ± 15.8 units/mg protein (10 MOI), 117.7 ± 17.4 units/mg protein (50 MOI), 219.2 ± 21.8 units/mg protein (100 MOI), and, finally, 308.0 ± 55.8 units/mg protein (200 MOI). Moreover, the MnSOD activity assay correlated well with the activity as determined by the native gel ($r^2 = 0.83; P < 0.01$), and also correlated well with immunoreactive protein as determined by Western blotting ($r^2 = 0.86; P < 0.01$; Fig. 2).

**Tumor Biological Characteristics of Adenovirus-transduced Cells**

**Cell Growth.** Adenoviral MnSOD-transduced cells demonstrated slower in vitro growth compared with parental cells (Table 1). MIA PaCa-2 cell doubling time significantly increased with adenoviral MnSOD (50–200 MOI) when compared with the parental cells or with 100 MOI AdLacZ cells. Tumor cell doubling time increased from 17.5 h for the parental cell line to 18.6, 18.5, and 19.3 h with the 50-, 100-, and 200-MOI adenoviral MnSOD, respectively. For example on day 5, cell number decreased by −32% with both the 50- and 100-MOI adenoviral MnSOD, and decreased by −47% with 200 MOI or greater of adenoviral MnSOD compared with the MIA PaCa-2 cells transduced with AdLacZ (Fig. 4A).

Correlation plots were constructed to determine the potential for a causal relationship between MnSOD enzyme expression, activity, and cell growth. Using linear regression analysis, cell-doubling time did correlate significantly ($r^2 = 0.92; P < 0.001$) with the levels of MnSOD immunoreactivity (Fig. 4B) and activity measured by either the native gel technique (Fig. 4C; $r^2 = 0.91; P < 0.001$) or the spectrophotometric activity assay (Fig. 4D; $r^2 = 0.92; P < 0.001$).
Plating Efficiency. To determine the clonogenic capacity of adenoviral MnSOD-transduced cells, we performed a plating efficiency assay. In general, malignant cells have a higher plating efficiency than do normal cells. Plating efficiency was reduced in the transduced cells compared with the parental cells (Fig. 5). Plating efficiency was 5.45 ± 0.30% and 5.35 ± 0.10% in the AdLacZ and 0-MOI groups, respectively. Enforced expression of MnSOD with 50, 100, and 200 MOI of adenovirus MnSOD decreased the plating efficiency to 3.65 ± 0.20%, 3.80 ± 0.20%, and 3.55 ± 0.05%, respectively (P < 0.01 versus AdLacZ and 0-MOI adenovirus MnSOD).

Growth in Soft Agar. To examine anchorage-dependent growth, we performed a soft agar assay. Whereas malignant cells form colonies in soft agar, normal cells do so in far smaller numbers. Adenoviral MnSOD significantly reduced colony formation (Fig. 5). Soft agar plating efficiency was 0.30% ± 0.006 and 0.34% ± 0.001 in the 100-MOI AdLacZ and parental cells, respectively. Maximal reduction in colony formation was observed at a viral titer of 100-MOI adenovirus MnSOD, in which the plating efficiency decreased to 0.10% ± 0.02 (means ± SE; P < 0.01, 100-MOI adenoviral MnSOD versus 100-MOI AdLacZ and parental cells).

Growth of Tumor Xenografts

In the first set of nude mice tumor xenografts, the experiments were designed to test the role of MnSOD in the suppression of tumor cell growth in vivo. Mean tumor volume growth was reduced in the MIA PaCa-2 cells when the adenoviral construct were injected into the s.c. tumors (Fig. 6). The estimates for the slopes by group are provided in Table 2. A single injection of the MnSOD construct resulted in slower growth in tumors when compared with those injected with serum-free medium (P < 0.05 control versus single injection; n = 4–6/group). When the MnSOD construct was given in multiple injections on days 1, 7, and 14, a further decrease in tumor growth was seen when compared with both the control group and the single injection group (P < 0.001, control versus three injections; P < 0.05, one injection versus three injections).

In the second set of in vivo studies, we wanted to confirm our previous findings, and to determine both survival and tumor volume, for a longer period of time and in a separate group of animals. A single injection of the MnSOD construct resulted in increased time to sacrifice when compared with the control tumors (Fig. 7A; P < 0.05, control versus single injection; n = 6–8/group). The MnSOD construct given in multiple injections also had increased time to sacrifice when compared with controls (P < 0.005) and also mice given the empty vector control Ad bglII (P < 0.05, AdMnSOD-3 doses versus Ad bglII; n = 6–8/group). There were no differences in time to sacrifice between the control group and Ad bglII group (P = 0.56, control versus Ad bglII; n = 8/group), whereas there was a trend toward increased time to sacrifice for the multiple versus single injection of the MnSOD construct (P = 0.08, AdMnSOD-1 dose versus AdMnSOD-3 doses; n = 6–8/group). When determining tumor volumes, we confirmed our previous findings (Fig. 6) and found similar trends in the tumor volumes when the mice were studied to 44 days postinjection. The estimates for the slopes by group are provided in Table 3. As indicated in Fig. 7B, all of the pairwise comparisons were significantly different except the comparison between the group of animals that received a single dose of the MnSOD construct and the Ad bglII group. Rate of change in tumor volume was lowest in the group of mice that received the three injections of the MnSOD construct.

Table 1  Tumor cell doubling time*

<table>
<thead>
<tr>
<th>Adenoviral MnSOD titer</th>
<th>Doubling time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 MOI AdLacZ</td>
<td>17.5 ± 0.1</td>
</tr>
<tr>
<td>0 MOI</td>
<td>17.4 ± 0.1</td>
</tr>
<tr>
<td>1 MOI</td>
<td>17.7 ± 0.2</td>
</tr>
<tr>
<td>10 MOI</td>
<td>17.8 ± 0.3</td>
</tr>
<tr>
<td>50 MOI</td>
<td>18.6 ± 0.4^p</td>
</tr>
<tr>
<td>100 MOI</td>
<td>18.5 ± 0.1^p</td>
</tr>
<tr>
<td>200 MOI</td>
<td>19.3 ± 0.2^p</td>
</tr>
</tbody>
</table>

* Means ± SE; n = 3.
^p P < 0.01 versus 0 MOI.

Discussion

Criteria for measuring cell growth to determine the characteristics of the tumor cell malignant phenotype include in vitro cell growth measured by growth rate and clonogenic ability, in vitro growth in soft agar, and, finally, in vivo tumor formation in nude mice. Our study demonstrates that enforced expression of MnSOD changes the in vitro biological characteristics of pancreatic cancer, specifically increasing doubling time and decreasing both plating efficiency and growth in soft agar. The injection of the MnSOD construct into established tumors also demonstrated promising results. Tumors grew slower in nude mice injected with the adenoviral MnSOD construct compared with the parental cell line. Multiple injections of the adenoviral MnSOD construct...
had even more dramatic results in inhibiting tumor cell growth.

Because of the presence of K-ras oncogene mutations, the tumor biology of pancreatic cancer has a number of unique features that are not seen in other malignancies. First, K-ras mutations have been identified in up to 95% of pancreatic cancers, implying their critical role in the molecular pathogenesis (19, 20). Recent studies demonstrate that fibroblasts transfected with the viral ras oncogene have increased superoxide (O$_2^-$) production and the generated O$_2^-$ may act as a second messenger molecule to promote cell proliferation (21). Our group recently demonstrated similar results in human keratinocytes (22, 23). In ras-transformed keratinocytes, increased O$_2^-$ production was demonstrated, and this increased production could be blocked efficiently by adenoviral MnSOD transduction. Most interestingly, these initial results showed that the transduction of adenoviral MnSOD alone was enough to kill ras-transformed cells, whereas it did not kill any of the other cancer types that we have examined. Aoki et al. (24) have previously demonstrated K-ras point mutations in the MIA PaCa-2 cell line, and recent studies from our laboratory have indicated that ras-immunoreactive protein is abundant in the MIA PaCa-2 pancreatic cancer cell line used in this present study and in other pancreatic cancer cell lines that we have studied (25). Therefore, because ras mutations are found in 95% of pancreatic cancers, the role of reactive oxygen species is an important system to study. Altered MnSOD levels have been found in many cancer cells including pancreatic cancer. In pancreatic cancer, MnSOD is decreased in primary pancreatic tumors (6) and also in a variety of pancreatic cancer cell lines (7) when compared with normal human pancreas. Overexpression of MnSOD has been shown to decrease the rate of tumor cell growth in many tumors including pancreatic cancer cell lines (7). Transfection of human MnSOD cDNA into human melanoma cells (26), human glioma cells (27), and human prostate carcinoma cells (28) suppressed the malignant phenotype. The rationale for overexpressing MnSOD in pancreatic cancer includes the fact that many of the known oncogenes and tumor suppressor genes are cell-type-specific, and we
wanted to determine whether pancreatic cancer is also responsive to MnSOD overexpression. Our study adds human pancreatic cancer to the list of tumors responsive to the in vitro manipulations of increasing expression of MnSOD to suppress the malignant phenotype.

The most definitive experimental test of malignancy, the growth of tumor cells in nude mice, demonstrates direct evidence that MnSOD behaves as a tumor suppressor gene. Previous studies have transfected human MnSOD cDNA into human melanomas followed by injection of these cells into the flanks of nude mice (25). These studies have demonstrated little or no growth in the tumors that had enforced expression of MnSOD before s.c. injection. Although these studies are important in defining the in vivo role of MnSOD in the suppression of tumor growth, they may have little clinical relevance. Recently, direct injection of the human MnSOD gene has been delivered to solid hamster-cheek-pouch carcinoma xenografts, demonstrating 50% reduction in tumor growth compared with untreated controls (15). Our present study is important for two reasons. First, the present study is clinically applicable with the demonstration that pancreatic tumors can be treated by either single or multiple direct injections of the MnSOD cDNA. Secondly, and perhaps more importantly, multiple injections of the adenoviral construct appear to cause tumor regression. Tumor size decreased from 69 mm³ (minimum to maximum: 0 – 154 mm³) to 16 mm³ (minimum to maximum: 0 – 60 mm³) when injections were administered every 7 days for a total of three injections, which suggests a regression of tumor volume. Multiple injections resulting in tumor regression is also supported by the slope estimation being negative (−2.2) and both end-points of confidence interval being negative (−4.0 to −0.4). This also suggests tumor regression.

The mechanisms involved in the MnSOD growth suppression of pancreatic cancer remain unclear. In previous studies and in different tumor cells, MnSOD overexpression does not involve a reduction in cell number by necrosis or apoptosis (27, 29, 30). Our present study does not address the mechanisms involved in the growth suppression of the MIA PaCa-2 cells. However, the fact that multiple injections of MnSOD into established MIA PaCa-2 tumors causes tumor regression in vivo suggests that a different mechanism may be involved than was seen in other tumor cells.

Table 2  In vivo tumor volume slopes over time for the data shown in Fig. 6

<table>
<thead>
<tr>
<th>Group</th>
<th>Slope (CI* 95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.8 (3.7–8.9)</td>
</tr>
<tr>
<td>One injection</td>
<td>1.7 (−0.3–3.7)</td>
</tr>
<tr>
<td>Three injections</td>
<td>−2.2 (−4.0–0.4)</td>
</tr>
</tbody>
</table>

Table 2  In vivo tumor volume slopes over time for the data shown in Fig. 6

* CI, confidence interval.
Surgical resection of the primary tumor remains the only potentially curative treatment for pancreatic cancer, leading a number of investigators to study preoperative chemoradiation for its ability to convert locally unresectable pancreatic cancer to resectable disease. In an earlier study of 16 patients who had locally advanced, unresectable pancreatic cancer treated with preoperative chemoradiation therapy to enhance resectability, only 2 patients were able to undergo resection (31). A similar study by White et al. (32) with 25 patients noted that only 8% of patients with locally advanced pancreatic cancer treated with preoperative chemoradiation were able to undergo complete resection with negative margins. Thus, it is unlikely that neoadjuvant chemoradiation can convert unresectable lesions to resectable ones and thereby increase the number of patients who can be cured with combined-modality therapy. Our study presents an attractive alternative in the treatment of pancreatic tumors that at first seem unresectable, by directly injecting an adenoviral vector containing the MnSOD gene to reduce tumor size. Effectiveness of in situ gene delivery using adenoviral vectors has been demonstrated by a number of investigators (33, 34). Diffusion of the adenoviral vectors has been demonstrated from 1 mm to 1 cm from the injection site (35, 36). Gene transfer efficiencies are typically between 10 and 30% (36, 37), although some investigators have reported gene transfer efficiencies as high as 50% (38).

In summary, enforced expression of MnSOD changes the malignant phenotype of pancreatic cancer by decreasing cell growth, plating efficiency, and growth in soft agar. Injection of the MnSOD construct into established pancreatic tumors resulted in slower growth in nude mice when compared with tumors injected with serum-free medium. Additionally, multiple injections of the adenoviral MnSOD construct further inhibited tumor cell growth. With the lack of treatment options for pancreatic cancer, clinical application of direct injection of the MnSOD gene into pancreatic tumors may offer benefits to these patients when survival is measured in months.

Table 3  In vivo tumor volume slopes over time for the data shown in Fig. 7B

<table>
<thead>
<tr>
<th>Group</th>
<th>Slope (CI* 95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.6 (8.3–10.9)</td>
</tr>
<tr>
<td>Adbgll</td>
<td>5.4 (4.1–6.7)</td>
</tr>
<tr>
<td>One injection</td>
<td>4.7 (3.4–6.0)</td>
</tr>
<tr>
<td>Three injections</td>
<td>1.3 (–0.2–2.7)</td>
</tr>
</tbody>
</table>

* CI, confidence interval.

References


Molecular Cancer Therapeutics

Suppression of the Malignant Phenotype in Human Pancreatic Cancer Cells by the Overexpression of Manganese Superoxide Dismutase

Christine Weydert, Benjamin Roling, Jingru Liu, et al.


Updated version
Access the most recent version of this article at:
http://mct.aacrjournals.org/content/2/4/361

Cited articles
This article cites 31 articles, 10 of which you can access for free at:
http://mct.aacrjournals.org/content/2/4/361.full#ref-list-1

Citing articles
This article has been cited by 11 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/2/4/361.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.