The Role of Manganese Superoxide Dismutase in the Growth of Pancreatic Adenocarcinoma

Joseph J. Cullen,² Christine Weydert, Marilyn M. Hinkhouse, Justine Ritchie, Frederick E. Domann, Douglas Spitz, and Larry W. Oberley

Departments of Surgery and Radiation Oncology, University of Iowa College of Medicine, Iowa City, Iowa 52242

ABSTRACT

Chronic pancreatitis, K-ras oncogene mutations, and the subsequent generation of reactive oxygen species (ROS) appear to be linked to pancreatic cancer. ROS have also been suggested to be mitogenic and capable of stimulating cell proliferation. Cells contain antioxidant enzymes to regulate steady state levels of ROS produced by products of metabolism. The aims of our study were to determine antioxidant enzyme activity in pancreatic cancer cells and correlate enzyme activity with tumor growth, as well as determine whether tumor cell growth could be altered with antioxidant gene transfection. Western blots, enzyme activity, and enzyme activity gels were performed for manganese superoxide dismutase (MnSOD), copper/zinc, catalase, and glutathione peroxidase in normal human pancreas and in the human pancreatic cancer cell lines BxPC-3, Capan-1, MIA PaCa-2, and AsPC-1. Cell population doubling times were determined and correlated with antioxidant enzyme activity. MnSOD was overexpressed in MIA PaCa-2 using an adenoviral vector, and the effect on cell growth was determined. The cell pancreatic cancer lines BxPC-3, MIA PaCa-2, and AsPC-1 had decreased levels of MnSOD immunoreactive protein as well as activity and decreases in MnSOD levels correlated well with increased rates of tumor cell proliferation as determined by cell doubling time. No correlation could be found between cell growth and levels of copper/zinc superoxide dismutase, catalase, or glutathione peroxidase. Enforced expression of MnSOD by adenovirus transfection in the rapid growing cell line MIA PaCa-2 increased MnSOD immunoreactivity and MnSOD activity and decreased growth rate. Overexpression of MnSOD may be effective in growth suppression of pancreatic cancer.

INTRODUCTION

Pancreatic cancer is the fourth common cause of cancer death in the United States with 29,700 fatal cases annually in the United States alone (1). Although the majority of pancreatic cancers are not found in the setting of chronic pancreatitis, chronic pancreatitis is one of the most significant risk factors for pancreatic cancer yet identified (2–4). 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. Firstly, K-ras mutations have been identified in up to 95% of pancreatic cancers, implying their critical role in their molecular pathogenesis (5, 6). Similar mutations have been identified in chronic pancreatitis and ductal hyperplasia, thereby providing a genetic basis for the potential progression of chronic pancreatitis to pancreatic cancer (7). Generation of ROS (i.e., hydrogen peroxide and superoxide) occurs during both acute and chronic pancreatitis (8) exerting harmful effects on living organisms, including damage to DNA and cell membranes. 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 (9). Our group recently demonstrated similar results in human keratinocytes (10). 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. This may be because of the large increase in superoxide production caused by ras transformation. Therefore, because ras mutations are found in 95% of pancreatic cancers, this is an important system to study.

ROS has higher reactivity with biological material relative to ground state molecular oxygen. These species include not only the oxygen radicals such as superoxide (O$_2^-$) and hydroxyl radicals but also nonradical molecules like singlet oxygen and hydrogen peroxide (H$_2$O$_2$). ROS is generated during normal aerobic metabolism, and increased levels of these species are produced during various forms of oxidative stress. ROS is known to react with various intracellular targets, including lipids, proteins, and DNA. ROS-induced damage can result in cell death, mutations, chromosomal aberrations, or carcinogenesis (11). The net intracellular concentration of ROS is the result of their production and the ability of antioxidants to remove them. In recent years, much evidence has been published suggesting that ROS at high concentrations are cytotoxic and at low concentrations are involved in the regulation of several key physiological processes. These processes include cell differentiation (12), apoptosis (13), and cell proliferation (14), which may be regulated by redox-sensitive signal transduction pathways.

Cells contain a large number of antioxidants to prevent or repair the damage caused by ROS. There are three major types of primary intracellular antioxidant enzymes in mammalian cells: SOD; catalase; and peroxidase, of which GPx is the most prominent. The SODs convert O$_2^-$ into H$_2$O$_2$, whereas the catalases and peroxidases convert H$_2$O$_2$ into water. In this way, two toxic species, O$_2^-$ and H$_2$O$_2$, are converted to the harmless product water. These antioxidant enzymatic functions are thought to be necessary for life in all oxygen-metabolizing cells (15). An important feature of these enzymes is that they are highly compartmentalized. In general, MnSOD is localized in the mitochondria, Cu/ZnSOD in the cytoplasm, catalase in peroxisomes and cytoplasm, and GPx in many subcellular compartments. Each of these enzymes is also found in several isoforms. One reason for the existence of many forms of each of these enzymes is to reduce oxidative stress in the various parts of the cell; different proteins are needed for different cellular and subcellular locations.

The aims of our study were 3-fold. First, we wanted to determine the activities and levels of the cellular antioxidants, MnSOD, Cu/ZnSOD, catalase, and GPx, in normal human pancreas compared with the pancreatic cancer cell lines BxPC-3 (poorly differentiated), Capan-1 (moderately to well differentiated), MIA PaCa-2 (undifferentiated), and AsPC-1 (poorly to moderately differentiated). Although numerous studies have demonstrated altered antioxidant enzyme activity in a variety of solid tumors (16, 17), pancreatic cancer is unique because chronic pancreatitis and the subsequent generation of ROS
appears to be a risk factor for pancreatic cancer (2–4), whereas K-ras onco
genome mutations have been identified in up to 95% of pancreatic cancers (5, 6). This is a scenario that may not be found with most other malignancies. Second, we wanted to determine whether alter-
ations in antioxidant activity correlated with proliferation rates of pancreatic tumor cells. Finally, when it was discovered that decreased levels of MnSOD correlated with tumor cell growth, we wanted to determine whether augmenting levels of MnSOD could alter the proliferation of pancreatic cancer cells. Although MnSOD has been overexpressed in several tumor cell types (16–21), 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. Most importantly, de-
spite improvements in perioperative care and adjuvant therapy, the outcome of the disease has scarcely improved. The overall survival rate after 1 year is ∼10% and after 5 years <3%. At initial presenta-
tion, ∼90% of patients have metastases (22). Thus novel treatment strategies directed against this malignancy are greatly needed. This study is the first to show that overexpression of MnSOD inhibits pancreatic cancer cell growth and suggests that modulation of this protein might represent a useful therapeutic target in the treatment of pancreatic cancer.

MATERIALS AND METHODS

Human Pancreas

Human pancreatic specimens were retrieved from neurologically devas-
tated, heart-beating patients that were transplant donors where the pancreas was considered unsuitable for transplantation or no recipient was available. Pancreatic specimens were discarded and not used in this study if the donor had any history of pancreatic disease. All specimens were retrieved at the University of Iowa Hospitals and Clinics. The protocol to use the human pancreatic specimens was approved by the University of Iowa Institutional Review Board for Human Subjects on February 12, 2001.

Cell Culture

The following human pancreatic adenocarcinoma cell lines were obtained from American Type Culture Collection (Manassas, VA): BxPC-3 (poorly differentiated), Capan-1 (moderately to well differentiated), MIAPaCa-2 (un-

Cell Homogenization and Protein Determination

Cells were washed three times in PBS (pH 7.0), scraped from the dishes using a rubber policeman, 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 instructions. Western Analysis

Immunoreactive protein corresponding to antioxidant enzymes was identi-

Enzyme Activity

Superoxide Dismutase 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 (25). Sodium cyanide (5 mm) inhibits Cu/ZnSOD; therefore, activity measured in the crude homogenate in the presence of sodium cyanide indicates only MnSOD activity. The difference between total SOD activity and cyanide-insensitive activity indicates Cu/ ZnSOD activity. Errors in Cu/ZnSOD activity were determined using propaga-
tion of error theory (26). Specific activity was reported as units/mg protein.

Antioxidant Enzyme Activity Gels

In this technique, nondissociating slab gels were run essentially by the method of Davis (31) with ammonium persulfate used as the initiator in the running gel (12.0%) and riboflavin-light in the stacking gel (5%). Once run, the gels were stained for SOD activity by the method of Beauchamp and Fridovich (32). Cu/ZnSOD and MnSOD were differentiated by the presence of sodium cyanide in the staining solution, which inhibits Cu/ZnSOD. Activity gels for catalase and GPx were also used. In this case, an 8 or 10% running gel was used, and GPx and catalase were stained by a method developed in our laboratory (33). All antioxidant enzyme activity gels were done in triplicate and were performed to confirm the findings from the activity assay.

Cell Growth

Cells (1 × 10⁶) were plated in triplicate in 1.5-ml 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 was performed in triplicate and (DT) in hours was determined using the following equation: DT (hours) = 0.693(t – t₀)/ln(N divided by N₀), where t₀ = time at which exponential growth began, t = time in hours, N = cell number at time t, and N₀ = initial cell number.

Adenovirus Gene Transfer

The adenovirus constructs used were replication-defective, E1- and E3-deleted recombinant adenovirus (34). Inserted into the E1 region of the adenovirus genome was either the human MnSOD gene or LacZ reporter gene, both of which are driven by a cytomegalovirus promoter. Adenoviruses were made at the University of Iowa Vector Core Facility. Approximately 10⁽ MIA PaCa-2 cells were plated in 10 ml of complete media in a 100-mm plastic culture dish and allowed to attach for 24 h. Cells were then washed three times in serum- and antibiotic-free media. The adenovirus-MnSOD construct, suspended in 3% sucrose, was then applied to cells suspended in 4 ml of antibiotic-free media at 0, 10, 25, 50, and 100 MOI. Control cells were treated with 100 MOI of the adenovirus-LacZ construct. Cells were incubated with the adenovirus constructs for 24 h. The media was then replaced with 4 ml of complete media for an additional 24 h before cells were harvested.
Statistical Analysis

Statistical analysis was performed using SYSTAT. A single factor ANOVA followed by post-hoc Tukey test, or Student’s t test when appropriate, was used to determine statistical differences between means. To examine the relationship between two quantitative variables, the Pearson’s correlation coefficient and linear regression were examined. For Fig. 3 data, to adjust for the possible influential point, Spearman’s correlation coefficients were also computed with the corresponding probability values based on exact methods for small sample situations. The correlation coefficients were calculated within the SAS statistical package. All means were calculated from three experiments, and error bars represent SE of mean (SE). All Western blots and activity gel assays were performed in triplicate.

RESULTS

Antioxidant Enzyme Determination

Western Analysis. MnSOD immunoreactivity was increased in normal human pancreas when compared with the poorly to undifferentiated primary tumors BxPC-3 (0.7), MIA PaCa-2 (0.6), and AsPC-1 (0.7; Fig. 1). The metastatic pancreatic cancer cell line Capan-1 (moderately to well-differentiated) pancreatic cell line had increased MnSOD immunoreactivity (1.2) when compared with human pancreas and the other pancreatic tumor cell lines. As mentioned in the “Materials and Methods” section, the data reported on the Western blot is one of three blots performed. These three blots were essentially identical.

Cu/ZnSOD demonstrated a different pattern of immunoreactivity on Western analysis (Fig. 1). The cell line BxPC-3 had decreased Cu/ZnSOD immunoreactivity (0.8), but Cu/ZnSOD immunoreactivity was slightly increased in the Capan-1 cell line (1.1) and additionally increased in both the MIA PaCa-2 (1.6) and the AsPC-1 (1.6) cell lines compared with normal human pancreas. Catalase immunoreactivity (Fig. 1) was decreased in human pancreas compared with the pancreatic tumor cell lines, which had very similar levels of catalase protein ranging from 1.3 (BxPC-3) to 1.6 (AsPC-1). In general, GPx immunoreactivity was decreased in the pancreatic cell lines when compared with normal pancreas (Fig. 1). When compared with normal pancreas, Capan-1 had the lowest GPx immunoreactivity (0.3), followed by BxPC-3 (0.4), MIA PaCa-2 (0.4), and AsPC-1 (0.7).

Enzyme Activity. Activity is believed to be the most important parameter determining the biological impact of the antioxidant enzymes. The expression of the antioxidant enzyme mRNA does not necessarily result in an increase in antioxidant enzyme protein or activity (35). Even increased immunoreactive antioxidant enzyme protein is not necessarily indicative of activity. With this in mind, enzymatic assays and native gels were used to measure the activity of the antioxidant enzymes. Using enzymatic activity assays, total SOD activity was greatest in the Capan-1 cell line with a total SOD activity of 83.1 ± 8.5 units of activity/mg protein, whereas BxPC-3 was lowest with a total SOD activity of 22.1 ± 3.1 (Table 1). Both of these cell lines were significantly different in total SOD activity when compared with normal human pancreas (57.8 ± 3.2). MIA PaCa-2 and AsPC-1 had total SOD activity of 49.6 ± 4.3 and 50.4 ± 10.4, respectively (means ± SE, units of activity/mg protein).

The MnSOD enzyme activity assays correlated well ($r^2 = 0.87$, $P < 0.05$) with the results obtained with MnSOD immunoblot (Fig. 1 and Table 1). It should be emphasized at this point that enzymatic activity data were calculated from multiple samples, whereas the Western blots and activity gels to be discussed later were from only one sample (different samples for the Western blots and activity gels). In general, the primary pancreatic tumor cell lines (BxPC-3, MIA PaCa-2, AsPC-1) had decreased MnSOD activity (~50%) compared with normal pancreas, whereas the metastatic cell line (Capan-1) demonstrated a 2-fold increase in MnSOD activity relative to normal pancreas (Table 1). These results suggest that MnSOD activity may be reduced in primary human pancreatic cancer cell lines.

Cu/ZnSOD enzyme activity (Table 1) also correlated well with the immunoblot in Fig. 1 ($r^2 = 0.80$, $P < 0.05$). Cu/ZnSOD was greatest in the MIA PaCa-2 cell line (36.7 ± 4.3) and lowest in the BxPC-3 cell line (11.3 ± 3.7; Means ± SE, units of activity/mg protein). In contrast to the results with MnSOD activity, Cu/ZnSOD activity did not appear to be altered in the majority of human pancreatic cancer cell lines, relative to normal pancreas.

Catalase activity was similar in the human pancreas and the pancreatic cancer cell lines except for one notable exception. The MIA PaCa-2 cell line had decreased catalase activity when compared with human pancreas (human pancreas: 30.5 ± 9.2 k/g versus MIA PaCa-2: 6.8 ± 0.3 k/g, $P = 0.06$ versus normal). As shown in Fig. 1, there were similar levels of catalase immunoreactive protein among all of the pancreatic cancer cell lines; thus catalase activity did not correlate well with catalase immunoreactive protein ($P > 0.05$). This is likely because there was little variation in either catalase activity or protein among the various samples. In general, there were only small differences in GPx activity between the pancreatic cancer cell lines, and there were no consistent alterations in normal pancreas versus pancreatic cancer cell lines. Also, GPx activity did not correlate significantly with GPx immunoreactive protein ($P > 0.05$).

Antioxidant Enzyme Activity Gels. To confirm the levels of MnSOD and Cu/ZnSOD activity in the cell lines, enzymatic activity of these antioxidant enzymes were determined using the native gel technique. As was seen in the activity assays, the enzymatic activities of MnSOD were decreased in the cell lines BxPC-3, MIA PaCa-2, and AsPC-1 when compared with human pancreas (Fig. 2). Consistent with the activity assay and the immunoblots, there was increased MnSOD enzymatic activity in the cell line Capan-1 when compared with human pancreas. The values obtained from the MnSOD activity gel correlated well with the MnSOD immunoreactivity from the Western blot ($r^2 = 0.96$, $P < 0.01$) and the activity assay ($r^2 = 0.85$, $P < 0.05$). As mentioned in the “Materials and Methods” section, the

![Fig. 1. Western blot analysis of MnSOD, Cu/ZnSOD, catalase, and GPx in human pancreas and in the pancreatic cancer cell lines BxPC-3, Capan-1, MIA PaCa-2, and AsPC-1. Total protein was electrophoresed in a 12.5% SDS-polyacrylamide running gel and a 5% stacking gel. After blocking in 20% FBS for 1 h, the sheets were washed and then treated with polyclonal rabbit-antihuman antibodies to MnSOD, Cu/ZnSOD, catalase, and GPx. The area of the bands relative to the human pancreas cells as measured by densitometric analysis is shown at the bottom of the lanes; these numbers indicate the relative amount of immunoreactive protein.](Image)
data reported on the activity gels is one of gels performed. These three gels were essentially identical.

In general, the native gel for Cu/ZnSOD as shown in Fig. 2 reflected the levels of this antioxidant enzyme seen with the immunoblots in that the cell line BxPC-3 had lower levels of this antioxidant enzyme when compared with human pancreas and the other pancreatic cancer cell lines. However, there was not a positive correlation between the Cu/ZnSOD activity gel and immunoreactive protein (r² = 0.72, P = 0.07), but there was a positive correlation between the Cu/ZnSOD activity gel and activity assay (r² = 0.81, P < 0.05).

The native gel for catalase activity demonstrated roughly equivalent amounts of catalase activity between the human pancreas and the pancreatic cancer cell lines (0.8 to 1.2 when compared with normal pancreas). This differed from the activity assays only that the cell line, MIA PaCa-2, which showed very little activity in the spectrophotometric assay, demonstrated more activity in the native gel assay. Two possible reasons for this are that only one sample is examined in the native gels or an inhibitor of catalase could be present that is separated during electrophoresis. In this regard, the catalase activity gel did not correlate with the findings from the immunoblot (P > 0.05). However, both Western blotting and activity gels showed roughly equal levels of catalase among all of the samples; the lack of correlation is probably because of this lack of variability in catalase levels. When measured using native gels, GPx activity was present in the human pancreas but decreased in all of the other pancreatic cancer cell lines. The GPx native gel did correlate with the respective immunoreactive protein (r² = 0.79, P < 0.05), however, the GPx native gel did not correlate with the activity assay (P > 0.05). Again, one possibility here is that an inhibitor is present in the homogenate used in the activity assay that is separated in the activity gel.

Cell Growth Characteristics

Cell Growth. To determine the tumor proliferation rates of the pancreatic cancer cell lines, cell growth was measured and doubling time determined. The Capan-1 cell line had the slowest growth rate with a doubling time of 97 h. The other cell lines had more rapid doubling times of 24 h for BxPC-3, 19 h for MIA PaCa-2, and 34 h for AsPC-1.

Correlation of Cell Growth with Antioxidant Enzyme Content. Correlation plots were constructed to determine the potential for a causal relationship between antioxidant enzyme expression and cell growth. There was no significant correlation between cell doubling time and the levels of Cu/ZnSOD, catalase, or GPx as determined by Western immunoblotting, activity assays, or the native gel technique (data not shown). However, using linear regression analysis, cell doubling time did correlate significantly (P < 0.01) with the levels of MnSOD immunoreactivity (Fig. 3A) and activity measured by either the spectrophotometric activity assay (Fig. 3B) or the native gel technique (Fig. 3C). The Pearson’s correlation coefficient for MnSOD immunoreactivity and doubling time was 0.99, whereas Spearman’s correlation coefficient was 0.80 (P = 0.33). The Pearson’s correlation coefficients were 0.98 and 0.99 for MnSOD activity and cell growth as measured by the activity assay and native gel technique, respectively, with the Spearman correlation coefficients being only slightly lower at 0.80 (P = 0.33) and 0.95 (P = 0.17). The lack of statistical significance in the Spearman correlation coefficients is partly because of the small sample size.

Effect of Adenovirus MnSOD Gene Transfer on Cell Growth

Adenovirus Gene Transfer. To determine whether a causal relationship existed between alterations in MnSOD activity and cell growth increasing, the amounts of MnSOD activity were expressed using an adenoviral expression vector in a pancreatic tumor cell line (MIA PaCa-2) that had previously been shown to have both low MnSOD immunoreactive protein and rapid growth. After exposure of MIA PaCa-2 cells to increasing MOI of replication incompetent adenovirus containing MnSOD cDNA, MnSOD immunoreactive protein was determined using Western analysis, and MnSOD activity was measured using the native gel technique. A dose-dependent increase in MnSOD immunoreactivity in cells exposed to 0–200 MOI adenovirus MnSOD was observed by Western analysis (Fig. 4A). The native gel technique was used to determine whether MnSOD activity was also increased in the adenovirus-MnSOD-transduced cells. MnSOD activity

Table 1 Antioxidant enzyme activity in the pancreatic cancer cell lines BxPC-3, Capan-1, MIA PaCa-2, and AsPC-1.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Total SOD (units/mg protein)</th>
<th>MnSOD (units/mg protein)</th>
<th>Cu/ZnSOD (units/mg protein)</th>
<th>Catalase (k/g protein)</th>
<th>GPx (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>57.8 ± 3.2</td>
<td>25.7 ± 1.3</td>
<td>29.2 ± 3.4</td>
<td>30.5 ± 9.2</td>
<td>21.0 ± 6.0</td>
</tr>
<tr>
<td>BxPC-3</td>
<td>22.1 ± 3.4</td>
<td>11.9 ± 2.1</td>
<td>11.3 ± 3.7</td>
<td>23.3 ± 2.1</td>
<td>18.3 ± 5.0</td>
</tr>
<tr>
<td>Capan-1</td>
<td>83.1 ± 8.5</td>
<td>58.8 ± 9.6</td>
<td>24.3 ± 12.8</td>
<td>26.8 ± 12</td>
<td>27.7 ± 0.9</td>
</tr>
<tr>
<td>MIA PaCa-2</td>
<td>49.6 ± 4.3</td>
<td>12.9 ± 0.5</td>
<td>36.7 ± 4.3</td>
<td>6.8 ± 0.3</td>
<td>14.7 ± 2.6</td>
</tr>
<tr>
<td>AsPC-1</td>
<td>50.4 ± 10.4</td>
<td>14.4 ± 2.9</td>
<td>36.0 ± 10.8</td>
<td>20.4 ± 1.3</td>
<td>24.0 ± 3.5</td>
</tr>
</tbody>
</table>

* Means ± SE of three to five individual samples.

| p | < 0.05 versus normal.

| p | 0.06 versus normal.

Fig. 2. Native gel assay for Cu/ZnSOD and MnSOD activity in human pancreas and in the pancreatic cancer cell lines BxPC-3, Capan-1, MIA PaCa-2, and AsPC-1. Proteins were separated on native polyacrylamide gels and stained for SOD. The area of the bands relative to the human pancreas cells as measured by densitometric analysis is shown at the bottom of the lanes; these numbers indicate the relative amount of activity.
was clearly increased in the 50, 100, and 200 MOI adenovirus-MnSOD infected cells (Fig. 4B). Thus, the enforced expression not only increased MnSOD protein but also increased MnSOD activity.

**Tumor Biological Characteristics of Adenovirus-transduced Cells.** Adenovirus-MnSOD-transduced cells demonstrated slower growth, compared with parental cells (Fig. 5A). For example, cell number at 96 h was 56% with 100 MOI of adenovirus-MnSOD compared with the 100 MOI AdLacZ group. Tumor cell doubling time increased from 19.3 and 18.7 h for the parental cell line and 100 MOI AdLacZ groups, respectively, to 23.4 and 24.5 h with adenovirus-MnSOD at 100 and 200 MOI, respectively ($P < 0.05$ versus MIA PaCa-2 parental cell line and 100 MOI AdLacZ; Table 2). It was found that increases in tumor cell doubling times significantly correlated with MnSOD immunoreactive protein (Fig. 5B; $r^2 = 0.84$, $P < 0.05$) and significantly correlated with MnSOD activity as measured by the native gel technique (Fig. 5C; $r^2 = 0.92$, $P < 0.01$) as determined by linear regression analysis.

Fig. 3. Linear correlation analysis of MnSOD expression versus tumor cell doubling time for BxPC-3, Capan-1, MIA PaCa-2, and AsPC-1. Cells ($2 \times 10^4$/well) were seeded in 24-well plates, and cell numbers were counted daily using a hemocytometer. Linear regression analysis indicates a positive dependence of cell growth on MnSOD immunoreactivity and MnSOD enzyme activity detected by activity assay or native gels.

Fig. 4. A, MIA PaCa-2 cells transduced with 100 MOI AdLacZ or 0–200 MOI adenovirus-MnSOD demonstrate increases in MnSOD immunoreactivity with increasing viral titer. No difference was seen with 100 MOI AdLacZ transfer compared with parental cells. B, detection of MnSOD activity by the activity gel assay in the MIA PaCa-2 cells transduced with 100 MOI AdLacZ or 0–200 MOI adenovirus-MnSOD. Increases in MnSOD activity are demonstrated with increasing viral titer. Proteins (250 μg each) were separated on native polyacrylamide gels and stained for MnSOD activity by the photo-induced nitroblue tetrazolium reaction in the presence of 0.75 mM sodium cyanide.

Fig. 5. A, MIA PaCa-2 cells transduced with 0–200 MOI adenovirus-MnSOD or 100 MOI AdLacZ demonstrate reductions in cell growth. Abscissa, days postinfection. No significant changes were seen with 100 MOI AdLacZ transfer compared with parental cells. Mean in vitro cell growth of adenovirus-MnSOD- or AdLacZ-transduced MIA PaCa-2 cells are shown. Each point was determined in triplicate from the same culture. *$P < 0.05$ versus 0 MOI AdMnSOD. B, linear regression analysis indicates positive correlation of cell doubling time versus MnSOD immunoreactivity. MIA PaCa-2 cells transduced with 100 MOI AdLacZ or 0–200 MOI adenovirus-MnSOD demonstrated increases in MnSOD immunoreactivity with increasing viral titer (demonstrated in Fig. 4A). No difference was seen with 100 MOI AdLacZ transfer compared with parental cells. After transfection with adenovirus-MnSOD, linear regression analysis indicates a positive dependence of cell growth on MnSOD immunoreactivity. C, linear regression analysis also indicates positive correlation of cell doubling time versus MnSOD activity as measured by the native gel technique. MIA PaCa-2 cells transduced with 100 MOI AdLacZ or 0–200 MOI adenovirus-MnSOD demonstrated increases in MnSOD activity with increasing viral titer (demonstrated in Fig. 4B). No difference was seen with 100 MOI AdLacZ transfer compared with parental cells. After transfection with adenovirus-MnSOD, linear regression analysis indicates a positive dependence of cell growth on MnSOD activity.
Our present study demonstrates that various pancreatic cancer cell lines have altered levels of antioxidant enzymes. In general, primary (nonmetastatic) pancreatic cancer cell lines had decreased levels of MnSOD when compared with normal human pancreas. Additionally, cell doubling time was most rapid in the cell lines with the low levels of MnSOD protein and activity. There was no correlation between cell growth and the levels of Cu/ZnSOD, catalase, or GPx. In contrast, MnSOD activity and immunoreactive protein significantly correlated with pancreatic tumor cell doubling time. Transfection of MnSOD cDNA into a rapidly growing cell line MIA PaCa-2 increased MnSOD immunoreactivity and MnSOD activity and decreased growth rate. Overall, these results demonstrate that MnSOD activity was decreased in primary pancreatic cancer, and MnSOD appears to play a role in regulating the growth of pancreatic cancer cells in vitro. In addition, the results using enforced expression of MnSOD suggest that this protein may be effective in tumor growth suppression of pancreatic cancer.

For 25 years now, it has been known that the activity of MnSOD was diminished in transformed cells when compared with an appropriate normal cell control. Because of these initial observations, numerous papers have been published showing altered levels of antioxidant enzymes in cancer cells (16–21). Cancer cells are generally low in MnSOD activity and sometimes low in Cu/ZnSOD activity (16–21). GPx activity is variable. In pancreatic cancer cells, a similar situation was observed. Using immunohistochemical techniques on previously fixed specimens, we recently demonstrated that cytoplasmic values of MnSOD, Cu/ZnSOD, catalase, and GPx were decreased in human pancreatic carcinoma specimens when compared with normal pancreas specimens (37). Our present study confirms this finding with MnSOD activity and immunoreactive protein in pancreatic cancer cells in culture when compared with normal pancreas. The lone exception was a metastatic cell line, Capan-1, which suggests that metastases may affect MnSOD levels.

Although we attempted to harvest main pancreatic ducts alone for our study, our samples of normal pancreas may contain different cell types, whereas pancreatic cancer arises from ductal epithelium. Although we used normal human pancreas from heart-beating transplant donors as our controls, the human pancreas contains numerous cell types of which acinar cells comprise 80% of the cells in the pancreas (38). Ductular cells and blood vessels form ~4% of the gland volume, whereas endocrine cells comprise ~2%. Recently, we demonstrated that immunohistochemical staining intensity for the antioxidant enzymes is similar in ductal and acinar cells (37). Methods for the isolation of pancreatic ducts, including dissociation of the gland with enzymes and mechanical shearing, followed by isolation of ducts by either manual selection, centrifugation, microdissection, or microdissection without prior tissue dissociation have substantial problems, including low yields of ducts or morphologically poorly preserved epithelial cells (39). Furthermore, ducts yielded with these methods usually contain adherent acinar tissue (39–41). Therefore, we did not attempt such isolation in this study.

There are several lines of evidence linking free radicals and antioxidant enzymes to pancreatic cancer. Chronic pancreatitis is one of the most significant risk factors for pancreatic cancer yet identified (2–4). K-ras mutations have been identified in up to 95% of pancreatic cancers, and similar mutations have been identified in chronic pancreatitis and ducal hyperplasia, thereby providing a basis for the potential progression of chronic pancreatitis to pancreatic cancer (5, 6). Additionally, generation of ROS occurs during acute and chronic pancreatitis (8), as well as in cell lines, which overexpress the ras oncogene (42). Studies from our laboratory and others (43) demonstrate that the four cell lines that we used in this study all express K-ras. Local oxidative stress produced by inflammation and/or ras overexpression could be one mechanism regulating cell growth contributing to tumor progression. MacMillan-Crow et al. (44) have demonstrated 21–97-fold increases in levels of nitrotyrosine, a footprint of the reactive nitrogen species peroxynitrite (formed by the reaction of superoxide with nitric oxide), in pancreatic cancer specimens compared with normal pancreas. Therefore, increased oxidative stress associated with the induction of pancreatic cancer correlates well with other studies in various model systems that demonstrate that ROS can initiate and promote carcinogenesis as well as findings that antioxidants in general, and SOD and SOD-mimetics in particular, inhibit malignant transformation (16, 21). For example, the role of ROS, antioxidants, and malignant transformation was suggested by St. Clair et al. (45) by demonstrating that overexpression of MnSOD in a mouse fibroblast cell line by cDNA transfection leads to inhibition of radiation-induced transformation.

If antioxidant enzymes are important in the genesis or progression of pancreatic cancer, then normalization of the levels of these enzymes should result in reversal of at least part of the cancer cell phenotype. Our present study demonstrates that overexpression of MnSOD in the pancreatic tumor cell line MIA PaCa-2, which is low in MnSOD, slowed cell growth. This hypothesis has also been tested in other cancer cell lines by elevation of SOD, particularly MnSOD, by cDNA transfection, as first demonstrated in 1993 (46) where transfection of MnSOD cDNA into cultured human melanoma cells resulted in the loss of the malignant phenotype. In addition, consistent with the current findings, melanoma cells overexpressing MnSOD demonstrated a significant reduction in proliferating cell nuclear antigen staining.

The mechanism of the tumor suppression by MnSOD overexpression in the pancreatic cancer cell line MIA PaCa-2 is unknown. Previous studies have suggested that effects of MnSOD overexpression on cancer cells are because of a nococtotoxic tumor suppressive effect. Changes in cell cycle parameters after MnSOD overexpression using flow cytometry have been demonstrated (47). MnSOD overexpression may lead to changes in the O$_2^-$/$\text{H}_2\text{O}_2$ balance, and this causes changes in the redox state that affects signal transduction pathways modulating cell proliferation. This is a reasonable hypothesis because in the last several years, it has been shown that kinases, phosphatases, and transcription factors are all redox-regulated by ROS (48–52).

In summary, the pancreatic cancer cell lines BxPC-3, MIA PaCa-2, and AsPC-1 have decreased levels of MnSOD immunoreactivity and enzyme activity and rapid cell doubling times. Although there was no correlation between cell growth and the levels of other antioxidant enzymes, the levels of MnSOD activity, correlated with pancreatic tumor cell doubling time. Enforced expression of MnSOD into the rapid growing cell line MIA PaCa-2 increased MnSOD immunoreactivity and MnSOD activity. Most importantly, enforced expression of MnSOD into pancreatic cancer cells decreased growth rate. Our study

### Table 2. Tumor cell doubling time

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Doubling time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 MOI AdLacZ</td>
<td>18.7 ± 0.2</td>
</tr>
<tr>
<td>0 MOI</td>
<td>19.3 ± 0.1</td>
</tr>
<tr>
<td>50 MOI</td>
<td>20.6 ± 0.5</td>
</tr>
<tr>
<td>100 MOI</td>
<td>23.4 ± 0.8$^a$</td>
</tr>
<tr>
<td>200 MOI</td>
<td>24.5 ± 0.8$^a$</td>
</tr>
</tbody>
</table>

$^a$ Means ± SE, n = 3.

$^b$ P < 0.05 versus 100 MOI AdLacZ and 0 MOI adenovirus-MnSOD.

### DISCUSSION

Although we attempted to harvest main pancreatic ducts alone for our study, our samples of normal pancreas may contain different cell types, whereas pancreatic cancer arises from ductal epithelium. Although we used normal human pancreas from heart-beating transplant donors as our controls, the human pancreas contains numerous cell types of which acinar cells comprise 80% of the cells in the pancreas (38). Ductular cells and blood vessels form ~4% of the gland volume, whereas endocrine cells comprise ~2%. Recently, we demonstrated that immunohistochemical staining intensity for the antioxidant enzymes is similar in ductal and acinar cells (37). Methods for the isolation of pancreatic ducts, including dissociation of the gland with enzymes and mechanical shearing, followed by isolation of ducts by either manual selection, centrifugation, microdissection, or microdissection without prior tissue dissociation have substantial problems, including low yields of ducts or morphologically poorly preserved epithelial cells (39). Furthermore, ducts yielded with these methods usually contain adherent acinar tissue (39–41). Therefore, we did not attempt such isolation in this study.

There are several lines of evidence linking free radicals and antioxidant enzymes to pancreatic cancer. Chronic pancreatitis is one of the most significant risk factors for pancreatic cancer yet identified (2–4). K-ras mutations have been identified in up to 95% of pancreatic cancers, and similar mutations have been identified in chronic pancreatitis and ducal hyperplasia, thereby providing a basis for the potential progression of chronic pancreatitis to pancreatic cancer (5, 6). Additionally, generation of ROS occurs during acute and chronic pancreatitis (8), as well as in cell lines, which overexpress the ras oncogene (42). Studies from our laboratory and others (43) demonstrate that the four cell lines that we used in this study all express K-ras. Local oxidative stress produced by inflammation and/or ras overexpression could be one mechanism regulating cell growth contributing to tumor progression. MacMillan-Crow et al. (44) have demonstrated 21–97-fold increases in levels of nitrotyrosine, a footprint of the reactive nitrogen species peroxynitrite (formed by the reaction of superoxide with nitric oxide), in pancreatic cancer specimens compared with normal pancreas. Therefore, increased oxidative stress associated with the induction of pancreatic cancer correlates well with other studies in various model systems that demonstrate that ROS can initiate and promote carcinogenesis as well as findings that antioxidants in general, and SOD and SOD-mimetics in particular, inhibit malignant transformation (16, 21). For example, the role of ROS, antioxidants, and malignant transformation was suggested by St. Clair et al. (45) by demonstrating that overexpression of MnSOD in a mouse fibroblast cell line by cDNA transfection leads to inhibition of radiation-induced transformation.

If antioxidant enzymes are important in the genesis or progression of pancreatic cancer, then normalization of the levels of these enzymes should result in reversal of at least part of the cancer cell phenotype. Our present study demonstrates that overexpression of MnSOD in the pancreatic tumor cell line MIA PaCa-2, which is low in MnSOD, slowed cell growth. This hypothesis has also been tested in other cancer cell lines by elevation of SOD, particularly MnSOD, by cDNA transfection, as first demonstrated in 1993 (46) where transfection of MnSOD cDNA into cultured human melanoma cells resulted in the loss of the malignant phenotype. In addition, consistent with the current findings, melanoma cells overexpressing MnSOD demonstrated a significant reduction in proliferating cell nuclear antigen staining.

The mechanism of the tumor suppression by MnSOD overexpression in the pancreatic cancer cell line MIA PaCa-2 is unknown. Previous studies have suggested that effects of MnSOD overexpression on cancer cells are because of a nococtotoxic tumor suppressive effect. Changes in cell cycle parameters after MnSOD overexpression using flow cytometry have been demonstrated (47). MnSOD overexpression may lead to changes in the O$_2^-$/$\text{H}_2\text{O}_2$ balance, and this causes changes in the redox state that affects signal transduction pathways modulating cell proliferation. This is a reasonable hypothesis because in the last several years, it has been shown that kinases, phosphatases, and transcription factors are all redox-regulated by ROS (48–52).

In summary, the pancreatic cancer cell lines BxPC-3, MIA PaCa-2, and AsPC-1 have decreased levels of MnSOD immunoreactivity and enzyme activity and rapid cell doubling times. Although there was no correlation between cell growth and the levels of other antioxidant enzymes, the levels of MnSOD activity, correlated with pancreatic tumor cell doubling time. Enforced expression of MnSOD into the rapid growing cell line MIA PaCa-2 increased MnSOD immunoreactivity and MnSOD activity. Most importantly, enforced expression of MnSOD into pancreatic cancer cells decreased growth rate. Our study
suggests that decreased MnSOD was found in primary pancreatic cancer cell lines but not in a metastatic cell line. Also, MnSOD appears to play a role in the growth of pancreatic cancer in vitro and therefore demonstrates the characteristics necessary to be effective at tumor growth suppression in pancreatic cancer in vivo.

REFERENCES

The Role of Manganese Superoxide Dismutase in the Growth of Pancreatic Adenocarcinoma

Joseph J. Cullen, Christine Weydert, Marilyn M. Hinkhouse, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/63/6/1297

Cited articles
This article cites 46 articles, 12 of which you can access for free at:
http://cancerres.aacrjournals.org/content/63/6/1297.full#ref-list-1

Citing articles
This article has been cited by 16 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/63/6/1297.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.