Advances in Brief

Suppression of Fibrosarcoma Metastasis by Elevated Expression of Manganese Superoxide Dismutase


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Abstract

A mouse fibrosarcoma cell line (FSa-II), which exhibits low endogenous levels of manganese superoxide dismutase, was transfected with a human manganese superoxide dismutase complementary DNA. Fifty clones were screened for manganese superoxide dismutase activity by the superoxide dismutase activity gel assay. Activity of the positive clones was measured by the nitro blue tetrazolium-reduction assay in the presence of cyanide. Three cell lines exhibiting a range of activity were chosen to be transplanted into syngeneic mice. The results indicated that the metastasis rate for all transfected cells was significantly less than that of control cells.

Introduction

MnSOD is a nuclear encoded mitochondrial matrix enzyme that scavenges toxic superoxide radicals. Several lines of evidence suggest that MnSOD is a candidate tumor suppressor gene. Abnormal MnSOD activity has been found in many transformed cells (for review see Ref. 2). A correlation between loss of transformed phenotype and increased MnSOD activity has been reported for a revertant of SV40-transformed rat kidney cells (3). The loss of MnSOD activity is correlated with the loss of human chromosome 6 in human SV40-transformed fibroblast cells (4) and human melanoma cells (5). To advance our understanding of how reactive oxygen species and antioxidants are involved in cancer development, we developed mouse cell line models. We transfected a mouse fibrosarcoma cell line (FSa-II), which exhibits low endogenous levels of MnSOD, with a human MnSOD cDNA. The fibrosarcoma-II cells originally developed as a spontaneous tumor in C3Hf/Sed mice (6). The tumorigenicity and growth characteristics in vitro and in vivo of the parental cells have been studied extensively (6). The transfected cells which overexpress MnSOD can be used for in vitro and in vivo studies to further elucidate the role of MnSOD in cancer development.

In this study, we transplanted into syngeneic mice three cell lines which expressed a range of MnSOD activity. We investigated the effect of MnSOD transfection on the malignant phenotype and metastatic capabilities.

Materials and Methods

Maintenance, Transfection, and Clonal Selection. The FSa-II cells were maintained in McCoy’s Medium 5a supplemented with 10% fetal bovine serum and 5% penicillin (5 mg/ml)-streptomycin (5 mg/ml)-neomycin (10 mg/ml). For routine subculture the cells were seeded at 3 x 10^6/ml and grown at 37°C in a humidified atmosphere of 5% CO2 in air. The plasmid, pH8APR-1, used to prepare the vector carrying the human MnSOD gene was prepared as previously described (7). A monolayer of FSa-II cells was washed with serum-free media and transfected with the pSV2-Neo plasmid or co-transfected with the sense MnSOD expression plasmid plus the pSV2-Neo plasmid using lipofectin (BRL). After 48 h the cells were exposed to 400 µg/ml of genetin (G418 sulfate). Clones were obtained from single, transfected cells plated onto 24-well plates at a density of 1 cell/ml or onto 100-mm dishes at densities of 100-300 cells/plate. Clones were propagated from colonies originating from a single cell. Cell lines obtained from individual clones were maintained in McCoy’s Medium 5a with 10% fetal bovine serum and 5% penicillin-streptomycin-neomycin plus 400 µg/ml G418.

Southern Blot Analysis. To determine if the transfected MnSOD gene was stably integrated into the cellular DNA, genomic DNA from each cell line was isolated from approximately 4 x 10^7 cells by digestion with proteinase K (0.25 mg/ml proteinase K, 0.5% sodium dodecyl sulfate) at 37°C for 30 min. The DNA was extracted as described previously (8). Twenty µg of DNA from each cell line was digested overnight at 37°C with SalI and applied to a 0.8% agarose gel. The DNA was transferred to nitrocellulose paper as described by Southern (9). The blot was air dried, baked, prehybridized, subsequently hybridized with the 32P-labeled MnSOD cDNA probe, and washed as described previously (8). The blot was then exposed to X-ray film at -80°C.

Activity. Initially, MnSOD activity was assayed in cell homogenates of approximately 50 clones by the activity gel assay in the absence of cyanide. The human MnSOD migrates differently than mouse MnSOD in these gels so activity due to the human gene could be detected. The level of MnSOD activity was assayed in cell homogenates of positive clones by monitoring the reduction of nitro blue tetrazolium in the presence of 5 mM sodium cyanide which inhibits CuZnSOD as originally described by Beauchamp and Fridovich (10). One Neo-transfected clone was chosen as a control and three SOD-transfected clones were chosen based on their activity. The clones will be referred to as NEO, SOD-L, SOD-M, and SOD-H for vector alone, low-, medium-, and high-SOD activity lines, respectively.

Animals and Tumor Transplantation. C3Hf/Sed mice were obtained from Massachusetts General Hospital and were used at 7-8 weeks of age. They were housed under sterile conditions, their boxes were changed once a week, and they were provided with sterile food and water ad libitum. FSa-II- and Neo-transfected control cell lines and the three MnSOD-transfected cell lines were transplanted into the right foot at a concentration of 1 or 2 x 10^6 cells. Two experiments were performed. In the first experiment 36 animals, 9 per group, were transplanted with FSa-II- or SOD-transfected cells. Four females and five males per group were used except for the group transplanted with cells from the SOD-L line, which had 3 females and 6 males. In the second experiment all males were used as no differences were found between males and females. There were 10 animals per group except for the NEO group which had 15 animals. The tumour-bearing leg was amputated at the hip joint when the tumor reached an average diameter of 8 mm. The animals were anesthetized for amputation with sodium phenaobarbital at a dose of 60 mg/kg body weight. The experimental protocol was approved by the University of Kentucky Animal Care and Use Committee.

Immunoperoxidase Staining. All primary tumors examined by light microscopy were immunostained with polyclonal antibodies to MnSOD, CuZnSOD, CAT, and GPX. These antibodies have been characterized extensively (12, 13). All tissues were fixed in 4% formaldehyde in phosphate buffered...
anti-CAT, and 1:4(H) of anti-GPX. The time allowed for color development varied depending on the control; however, all slides for each antibody were incubated for the same amount of time. Under the conditions described above, mouse tissues have been shown to stain with these antibodies (12).2

**Light Microscopy.** Tissues to be examined by light microscopy were fixed in 10% buffered formalin for 2 h, transferred to phosphate-buffered saline, pH 7.4, and embedded in paraffin. Sections were stained with hematoxylin and eosin. Primary injection sites from randomly selected mice were examined for tumor morphology as follows: FSa-II, n = 4; NEO, n = 5; SOD-L, n = 4; SOD-M, n = 3; SOD-H, n = 4.

**Metastases.** Previous experiments with these mice transplanted with FSa-II tumor cells had shown that at least 30% of the mice would develop lung metastases 2 weeks after amputation of an 8-mm diameter tumor (metastasis formed on the surface of each lobe was examined) (14). This protocol was adapted for the present study. The median tumor growth times to reach an 8-mm diameter were 14.0 (95% confidence limit, 13.4-14.6), 13.5 (13.2-13.8), 15.2 (14.7-15.8), 20.3 (19.2-21.5), and 18.8 (18.0-19.6) days for FSa-II, NEO, SOD-L, SOD-M, and SOD-H tumors, respectively. Since the number of tumor cells injected into each foot can be varied due to small number of tumor cells injected into each foot can be varied due to small variations in injection volume and the time required for each tumor to grow from 500 to 1000 mm3 at the injected site was determined in a separate experiment. No significant difference in the time required for each tumor to grow from 500 to 1000 mm3 among all the tumors injected was found.2 Two to 4 weeks after amputation the animals were sacrificed and organs were removed. In the first experiment, lungs, liver, and spleen were removed for pathological examination. No metastases were found in any organs except lungs. Therefore, in the second experiment only lungs were examined for metastases. For pathological examination, 20 primary tumors from all treatment groups were examined by light microscopy. All tumors had similar morphology. For comparison, Fig. 3, a and b show light microscopy of tumors formed from NEO-transfected and SOD-H-transfected cells, respectively. The staining was granular and found in the cell cytoplasm as indicated by the arrows in Fig. 2b. Controls treated with normal rabbit serum did not show staining in a tumor from SOD-H transfected cells (Fig. 2c). There was no difference in the staining for CuZnSOD, CAT, or GPX between the MnSOD-transfected tumors and tumors from parental cells or NEO-transfected cells (data not shown).

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**Results**

**Human MnSOD in Transfected Cells.** The Southern blot shows that each SOD-transfected cell line had a band of 1 kilobase, the size of the cDNA insert, which hybridized to the cDNA probe (Fig. 1, Lanes C, D, and E). A similar band was not found in either the FSa-II cells (Lane A) or the NEO-transfected cells (Lane B). The intensity of the bands was in agreement with the measured activity of each clone as the cell line with the lowest activity (Lane C) had the faintest band and the middle (Lane D) and high (Lane E) activity cell lines exhibited bands of increasing intensity. The 3 clones are designated SOD-L for the low-activity cell line, SOD-M for the mid-activity cell line, and SOD-H for the high-activity cell line. Activity studies were performed by using the nitro blue tetrazolium reduction assay and were in agreement with activity gels (data not shown).

**Tumor Characteristics.** To verify that the transfected MnSOD gene is expressed in tumor tissue in vivo, tumor tissue was incubated with antioxidant enzyme antibodies. Tumors from parental and NEO-transfected cells did not show immunostaining for MnSOD. In contrast, tumors from all MnSOD-transfected cells showed staining for MnSOD. For comparison, Fig. 2, a and b show immunoperoxidase staining of tumors formed from NEO-transfected and SOD-H-transfected cells, respectively. The staining was granular and found in the cell cytoplasm as indicated by the arrows in Fig. 2b. Controls treated with normal rabbit serum did not show staining in a tumor from SOD-H transfected cells (Fig. 2c). There was no difference in the staining for CuZnSOD, CAT, or GPX between the MnSOD-transfected tumors and tumors from parental cells or NEO-transfected cells (data not shown).

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**Metastasis.** Metastases, when present, showed similar morphology in each treatment group. For example, metastases in NEO-transplanted (Fig. 3c) and SOD-L-transplanted (Fig. 3d) mice were identical. However, as shown in Table 1, the incidence of tumor metas-

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5 Unpublished observations.

6 M. Urano, et al., unpublished observation.
MnSOD suppresses tumor metastasis

Detection of MnSOD activity by immunoperoxidase analysis

Fig. 2. Immunoperoxidase analysis of tumors formed from transfected cells. a, a primary tumor from an animal transplanted with NEO-transfected cells. No staining was seen after treatment with anti-MnSOD. b, a primary tumor from an animal transplanted with the high-dose MnSOD-transfected cells. The cells showed a large amount of reaction product (arrows) after treatment with anti-MnSOD. c, a primary tumor from an animal transplanted with the high-dose MnSOD-transfected cells. The cells were incubated with normal rabbit serum (NRS) and no labeling was seen.

The results from this investigation indicate that an increase in MnSOD activity is correlated with a loss of metastatic capabilities. Tumors from FSa-II- or NEO-transfected cells, which have no measurable level of MnSOD activity, exhibited up to a 90% metastatic rate, while the maximum rate for SOD-transfected cells was 50%. Furthermore, tumors from the cell line with the highest activity did not metastasize in this experiment. It is also interesting to note that in the second experiment the reduction of tumor metastasis in MnSOD-transfected cells was dose dependent, i.e., the higher the MnSOD activity, the lower the frequency of metastasis. The differences in metastatic rate of the FSa-II and SOD-L cells between the two experiments is probably due to differences in the number of cells injected in the two experiments. In the first experiment, 50% fewer cells were injected and this may account for the lower number of metastases observed in FSa-II parental cells. Previous work by Urano and Kahn (6) showed that this magnitude of difference in injected cells did not affect the growth rate of the tumors. However, the relationship between the number of tumor cells injected and the metastatic rate is not known and is currently being explored in our laboratory.

Discussion

The mechanism by which MnSOD suppresses tumor metastasis is not known. Morphological results rule out an effect of inflammation or tissue necrosis on metastasis suppression. Ultrastructural studies did not reveal cell injury. In particular, mitochondria showed no evidence of damage; this is important since MnSOD is a mitochondrial protein and one of its products, H$_2$O$_2$, has the potential to cause oxidative damage. The role of MnSOD in suppressing metastasis may be related to the permissive role that MnSOD has in promoting cell differentiation (15). We have previously shown that expression of human MnSOD in mouse embryonic fibroblast cells promotes the differentiation of fibroblasts into muscle cells (15). Although cells from tumors derived from SOD-transfected cells were not morphologically distinguishable from FSa-II tumor cells, it is possible that tumor cells with high MnSOD activity were beginning to functionally differentiate. There is little evidence to support this hypoth-
Fig. 3. Light microscopy of fibrosarcoma cells. Primary tumors from animals transplanted with a, NEO-transfected, and b, high-dose MnSOD-transfected cells showed identical morphology. The cells were spindle shaped and had large nuclei which were either spindle or oval shaped. Mitoses were identified in both types of primary tumors (arrows). Metastatic lesions (T) in lungs of animals transplanted with c, NEO-transfected, or d, low-dose MnSOD-transfected cells had identical morphology. Metastatic lesions were not present in animals transplanted with cells expressing high levels of MnSOD. No inflammation was identified in metastatic lesions.

esis. However, an extremely low frequency of spontaneous differentiated cells, defined as the presence of multinucleated cells, was found in cultured SOD-transfected cells. Identification of the mechanisms by which MnSOD suppressed metastasis is currently in progress in our laboratory.

Church et al. (5) reported that transfection of a cDNA for MnSOD into the human melanoma cell line UACC-903 suppressed the malignant phenotype when evaluated in nude mice tumorigenicity assays. Obviously, in the present report, the fibrosarcoma cells formed tumors in syngeneic mice. The reason for this discrepancy is not clear.

Perhaps, tumor “take” is different in nude mice versus syngeneic mice. A second possibility is that MnSOD could affect different cell types differently. A recent publication by Negrini et al. (16) on lack of suppression of tumorigenicity of a human breast cancer cell line MDA-MB-231 by complementation of the tumor cells with human chromosome 6 containing the MnSOD locus supports this idea. Thus, expression of MnSOD may only be effective in suppressing the growth of cancer that has a defect in human chromosome 6q25, where the MnSOD gene resides.

Our results indicate clearly that expression of MnSOD suppresses the metastasis of fibrosarcoma. This finding is important because most cancer deaths are not a result of primary tumor growth, but rather by metastasis of tumor cells to secondary sites. If expression of MnSOD can significantly suppress the metastasis of various human tumors, then successful manipulations of MnSOD activity in tumor cells could lead to an increase in the cure rate of cancer patients.

References
3. Fernandez-Pol, J. A., Hamilton, P. D., and Klos, D. J. Correlation between the loss of


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