Carbonyl Reductase: A Novel Metastasis-modulating Function

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Abstract

To explore reasons for differences in the malignancy of tumors, we have compared two cell lines derived from a mouse lung adenocarcinoma cell line that differ 10-fold in their capacity to form lung metastases from s.c. primary tumors or after i.v. injection. One mRNA encoding carbonyl reductase was identified at a relatively high abundance in the subline with low metastatic capacity but was not detectable in the highly metastatic subline. Transfection of the former subline with a plasmid construct expressing antisense carbonyl reductase rendered the cells highly metastatic. Conversely, the capacity of the highly metastatic cells to metastasize was markedly reduced after transfection with a construct expressing carbonyl reductase. We also found that human prostate cancers show loss of carbonyl reductase expression compared with normal prostate epithelia. These data suggest that carbonyl reductase has an important function in modifying the metastatic behavior of malignant tumors.

Introduction

It is clear from studies in animals and humans that metastasis is a complex multistage process orchestrated by a fine interplay between genetic, epigenetic, and environmental influences (1, 2). However, the reasons why some tumors display a more malignant phenotype than other similar ones remain obscure. This is particularly so for the question as to why one tumor is strongly metastatic, whereas another apparently identical tumor is much less capable of metastasizing. To identify genetic aberrations that modify the metastatic phenotype rather than the genes involved in metastasis per se, we have made use of two cell lines originally derived from a mouse lung adenocarcinoma (3). One of these cell lines, CMT167, has a markedly greater metastatic capacity than the other, CMT170 (3). Because both DNA fingerprinting and in-gel renaturation (4) showed the two cell lines to have a similar genetic composition, we concluded that the difference in the metastatic propensities of the two is likely to be due to differences in the expression of a gene or genes. To explore this, we used differential display (5), an unbiased PCR-based (3) method that not only detects mRNAs that differ in abundance between two populations but also allows the corresponding cDNAs to be cloned and subsequently sequenced. As a result of these studies, we identified a gene encoding an enzyme (carbonyl reductase) that had not previously been suspected of any involvement in malignant progression or the metastatic process. Moreover, we present evidence of its involvement in at least one type of human cancer, i.e., prostatic cancer.

Materials and Methods

Cell Culture and Transfection. CMT167 and CMT170 cells were cultured in DMEM containing 10% FCS. Full-length cDNA coding for mouse carbonyl reductase 1 (a gift from J. Wei) was ligated into the pBabe Puro expression vector in both orientations to generate sense and antisense carbonyl reductase. Ten μg of the sense coding plasmid were transfected into CMT167 cells, and 10 μg of antisense coding plasmid were transfected into CMT170 cells using the N-[1-(2,3-dioleoyloxyl)propyl]-N,N,N,N-tetramethyluronium-methyl sulfate lipofection protocol (Boehringer Manheim). Transfected cells were cultured in DMEM with 10% FCS containing 1.5 μg/ml puromycin. Northern blotting and Western blotting were used to confirm the expression of the appropriate carbonyl reductase constructs. As controls, 10 μg of vector only were transfected into CMT167 and CMT170 cells. For measurement of cell growth rates in vitro, replica plates of the cells were prepared, and 0.5 mg/ml 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide was added to each plate at the appropriate time point. After 4 h, the excess medium was removed, 100 μl of DMSO were added to each plate, and the absorbance was measured at 595 nm.

Differential Display. Total mRNA was isolated from CMT167 and CMT170 cells as described previously (6). First-strand cDNA synthesis was performed using the Delta RNA Fingerprinting kit (CLONTECH) according to the manufacturer’s instructions. Differential display was performed according to the manufacturer’s instructions (CLONTECH). A total of 62 different combinations of arbitrary and oligodeoxythymidylic acid primers were tested. A combination composed of the P3 arbitrary primer 5′-ATTAAACCTCACTAATTGCTGAGTGATATCTTTTTTTGC-3′ and the T8 oligodeoxythymidylic acid primer 5′-CATATTACGTAGTGATACATTTTTTTTTTTTGGC-3′ resulted in a single differentially expressed band. The band was isolated from the dried denaturing polyacrylamide gel, reamplified using the same primers, cloned into pCR 2.1 (Invitrogen), and sequenced using M13 forward and reverse primers according to the manufacturer’s instructions.

Northern and Western Blotting. For Northern analysis, total mRNA was isolated as described above, and the products were electrophoresed in a formaldehyde agarose gel and transferred to Hybond N+ (6). To generate the carbonyl reductase probe for the Northern blot, the band isolated from differential display and cloned into pCR 2.1 was recut from a bulk preparation of the plasmid, purified from an agarose gel, and labeled with [α-32P]dCTP using Ready-To-Go random-priming kit (Pharmacia). For Western analysis, protein lysates were prepared from 80% confluent CMT167 and CMT170 cells, electrophoresed through a 7.5% SDS-polyacrylamide gel, and blotted as described previously. The filter was probed with rabbit antihuman carbonyl reductase polyclonal antibody (7) overnight at 4°C. Membranes were then washed three times with PBS containing 0.1% Tween 20 and incubated for 1 h with the appropriate peroxidase-conjugated secondary antibodies. After three washes with PBS-0.1% Tween 20, bands were detected with the enhanced chemiluminescence Western blotting system (Amersham) according to the manufacturer’s instructions and visualized by exposure to Kodak X-OMAT film for various times.

Immunocytochemistry. This was performed as described by Suto et al. (7). Sections (5 μm) were cut and mounted on 3-(triethoxysilyl)-propylamine-coated slides (Merck). Immunostaining was performed using a Biogenex Optimax Plus automated immunostainer. All incubations were performed at room temperature and were followed by a wash in Optimax Buffer (Biogenex).

Endogenous peroxidase activity was blocked using 1% aqueous H2O2 for 10 min. Nonspecific staining was blocked using 20% normal goat serum for 20 min. Sections were then incubated for 1 h in a 1:1000 dilution of primary antihuman carbonyl reductase antibody (7), followed by a 30-min incubation
in prediluted biotinylated secondary antibody (Dako Chemmate detection kit) and finally by a 30-min incubation in peroxidase-labeled streptavidin (Dako Chemmate detection kit). The peroxidase label was visualized with 3,3'-diaminobenzidine for 10 min, followed by nuclear staining with Gill’s triple strength hemalum. All experiments included negative controls in which the primary antibody was omitted.

**Mice.** All animals used in this study were treated according to the Home Office license standards. C57 B/T syngeneic mice were used in all experiments. A single cell suspension of $1 \times 10^5$ cells in 0.1 ml of DMEM was injected s.c. into the right flank of mice (for overall metastasis assay), or $1 \times 10^4$ cells in 0.1 ml of DMEM were injected into the tail vein of mice (for the colonization assay). Primary tumor growth after s.c. injection was monitored regularly, and once growth was palpable, measurements of primary tumor volume were made. Animals were sacrificed 20 days after treatment. The weight and volume of the primary tumors were measured, and the lungs were subsequently inflated with 1.5 ml of black India ink solution [15% (v/v) black India ink, 0.5% (v/v) ammonia solution]. The lungs were dissected out, rinsed briefly in water, and stored in Fekete’s solution (100 ml of 70% ethanol, 10 ml of 38% formaldehyde solution, and 5 ml of glacial acetic acid). Lungs were stored for a minimum of 24 h for bleaching and fixation before the lobes of the lungs were examined, and metastatic deposits were counted and confirmed histologically as metastatic tumors.

**Results and Discussion**

The derivation of two mouse lung adenocarcinoma cell lines that are both metastatic but differ significantly in their metastatic capability has been described previously (3). There is an approximately 10-fold greater number of lung metastases in syngeneic mice from the highly metastatic CMT167 subline than from the low metastatic CMT170 subline (Fig. 1, a and b) when assayed by either s.c. injection, a measure of the overall metastatic capacity of cells from invasion to colonization, or i.v. injection, which largely assays the capacity of the cells to colonize the lungs. Although tumors formed by CMT167 cells grew faster than those from CMT170 cells (Fig. 1c), the difference in metastatic capacity of the CMT167 cells was not dependent on their higher growth rate (Fig. 1d) and remained significant even when adjusted for the difference in the growth rates of the primary tumors (data not shown). Interestingly, the difference in the growth rates of the tumors in vivo was not a reflection of the growth rates of the sublines in vitro, which were identical (data not shown). This suggested that the difference in vivo is not an intrinsic phenomenon of these cell lines.

Because the two cell lines have a similar genetic composition (data not shown), it appeared likely that the difference in metastatic behavior between the two cell lines is due to differences in gene expression rather than in the genetic make up of the cells.
Comparison of the mRNA populations of the two cell lines by differential display (5) with several pairs of primers detected four differentially expressed mRNAs, the differential expression of which was subsequently confirmed by Northern blotting. Of these four mRNAs, one was not detectable in the highly metastatic CMT167 cells but was present at a relatively high level in CMT170 cells (Fig. 2). Subsequent cloning and sequencing of the corresponding cDNA showed a 99.9% and 80% homology to mouse and human carbonyl reductase mRNA, respectively. To confirm that the differential expression of carbonyl reductase at the mRNA level is also reflected at the protein level, a polyclonal antibody raised against the whole Mr 33,000–34,000 human carbonyl reductase protein (7) was used in Western blotting analysis of cell lysates. The results confirmed the Northern blot analysis data (Fig. 2). Southern blotting experiments showed that the failure of CMT167 cells to express carbonyl reductase is not due to deletion.

Fig. 3. Carbonyl reductase modulates the metastatic behavior and growth rate of CMT167 and CMT170 cells in vivo. a, transfection of CMT167 with plasmid coding for sense carbonyl reductase mRNA (CMT 167 S) significantly inhibited the metastatic potential of these cells compared with untransfected cells after i.v. (□) or s.c. (■) injection (P = 0.0001, Mann-Whitney rank-sum test). Conversely, transfection of CMT170 with plasmid coding for antisense carbonyl reductase mRNA (CMT 170 AS) had the opposite effects (P = 0.0001), whereas transfection with vector only had no effect on the cell behavior. The results represent the mean ± SE for three independent experiments, each of which contained 15 mice for i.v. injections and 15 mice for s.c. injections. b, as in a, changes in primary tumor volume after s.c. injection of cells (P = 0.036). c, densitometric quantification of the carbonyl reductase Western blot shown on the right.

Fig. 4. Carbonyl reductase expression is reduced in advanced prostate cancers. Carbonyl reductase was measured in sections of (A) normal prostate or (B) advanced prostate cancer tissue by immunocytochemistry. The normal prostate section (A) shows staining of the glandular epithelium (orange), which was not seen in the prostate cancer section (B). All experiments included negative controls in which the primary antibody was omitted.
of the gene (data not shown). We cannot, however, rule out mutation of the gene’s promoter as the mechanism.

To evaluate the role of carbonyl reductase in modifying the metastatic behavior of cells, we transfected CMT170 cells with an expression vector encoding antisense carbonyl reductase mRNA and analyzed the behavior of the cells after s.c. and i.v. injection into syngeneic mice. Expression of antisense carbonyl reductase mRNA and the subsequent elimination of carbonyl reductase protein from the CMT170 cells (Fig. 3c) increased the metastatic capacity of the CMT170 cells to levels similar to those of CMT167 cells (Fig. 3a), whereas transfection with the empty expression vector had no effect. Conversely, expression of sense carbonyl reductase mRNA in CMT167 cells (Fig. 3c) significantly reduced their metastatic capacity to match that of CMT170 cells (Fig. 3a). Interestingly, transfection of the cell lines with carbonyl reductase sense or antisense constructs had a lesser effect on the growth rates of the tumors in vivo (Fig. 3b).

Our data suggest that, in this mouse model, carbonyl reductase has a novel function in modulating the metastatic behavior of cells, particularly in the latter stages of the process (because of its effect on i.v. injected cells), and that its silencing by whatever mechanism permits a greater tumor growth rate and a more aggressive behavior in vivo. Carbonyl reductase (E.C.1.1.1.184) is one of several monomeric, NADPH-dependent oxidoreductases with wide specificity for carbonyl compounds that are generally referred to as aldoketoreductases (8, 9). It has been extensively studied in relation to its ability to reduce a great variety of carbonyl compounds, such as quinones, the antitumor anthracycline antibiotics daunorubicin and doxorubicin, and 9-ketoprostaglandins (10–12). The latter property could be directly relevant to the phenomenon we have described because carbonyl reductase has been found to be biochemically, immunologically, and functionally identical to prostaglandin 9-ketoreductase, which oxidizes prostaglandin E2, F2α, and D2 to their corresponding, biologically inactive, 15-keto metabolites (13, 14). Prostaglandins, especially prostaglandin E2, play an important role in modulating tumor growth and metastasis in a variety of human tumors (15–17). Moreover, prostaglandin E2 has important functions not only in modulating apoptosis in cancer cells (18) but also in regulating angiogenesis (19, 20), an essential prerequisite for the establishment of viable metastases.

We appreciate that the novel function for carbonyl reductase we have described here may be restricted to the model system used in this study, especially because carbonyl reductase has never before been associated with tumor growth or metastasis. To determine whether loss of carbonyl reductase expression is a feature of human cancers, we have screened a limited number of normal and malignant tissues for carbonyl reductase expression by immunocytochemistry. Carbonyl reductase is not expressed in some normal tissues, or its expression may be variable, as in normal lung tissue, for reasons that have yet to be clarified. However, a series of 20 prostatic adenocarcinomas was found to have a reduced or total absence of carbonyl reductase expression, irrespective of stage or grade, compared with normal prostate epithelium (Fig. 4). A much larger series of cases will need to be investigated to more precisely define the clinical features associated with loss of carbonyl reductase expression. Recent evidence has also shown that carbonyl reductase expression is significantly reduced in hepatocellular cancer (7). These preliminary results support a more important role for carbonyl reductase in modifying the behavior of human malignant tumors than previously suspected.

Our results demonstrate a novel function for carbonyl reductase in tumor growth and metastasis. We believe that loss of this enzyme by genetic or epigenetic mechanisms in a metastatic cell renders it more malignant in vivo than a cancer cell that expresses the enzyme. Thus, an investigation of the precise mechanisms by which carbonyl reductase modulates malignant progression, in particular, the metastatic process, is now indicated.

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References

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