Overexpression of nm23-H2/NDP Kinase B in a Human Oral Squamous Cell Carcinoma Cell Line Results in Reduced Metastasis, Differentiated Phenotype in the Metastatic Site, and Growth Factor-independent Proliferative Activity in Culture

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ABSTRACT

The metastasis suppressor activity of nm23/nucleoside diphosphate (NDP) kinase was assessed using human oral squamous cell carcinoma (SCC) cell lines. When the expression of nm23/NDP kinase was compared among several SCC cell lines, nm23-H2/NDP kinase B gene product, but not nm23-H1/NDP kinase A gene product, was reduced in the metastatic cells. Transfection of nm23-H2 into the metastatic SCC cell line LMF4 caused reduction in the lung metastasis in an experimental metastasis assay. A histological analysis of the pulmonary metastatic foci revealed that although foci of the control clones were composed of anaplastic squamous cells, those of the nm23-H2-transfected clones consisted of mostly well-differentiated cells mimicking normal stratified epithelial constitution. The transfected cells were morphologically indistinguishable from the control ones in culture, but they differed from each other in that the former cells proliferated faster than the latter, became less serum dependent, and lost responsiveness to growth factors such as platelet-derived growth factor, insulin-like growth factor I, and insulin, although both clones retained sensitivity to transferrin. These results demonstrate that nm23-H2 protein does have metastasis suppressor activity for human SCC cells and suggest that this activity may be elicited by modulating growth and/or differentiation potential in response to environmental factors.

INTRODUCTION

The process of tumor metastasis is thought to comprise sequential multiple steps (1). nm23, which was discovered as a candidate metastasis suppressor gene (2), encodes a protein identical to NDP kinase involved in the transfer of the terminal phosphate from a nucleoside triphosphate to a NDP (3, 4). Negative correlation between the expression of nm23/NDP kinase and metastatic potential has been documented for some rodent and human tumors (2, 5–8) but not in others (9–12). The antionmetastatic activity of the nm23/NDP kinase gene products has been validated for a number of human and rodent tumor cell lines (13–18) using animal model systems. In addition, these nm23/NDP kinase-transfected clones lost sensitivity to transforming growth factor β in soft agar colonization (13, 14), displayed reduced cell motility in response to insulin-like growth factor I, platelet-derived growth factor, and serum (19), and exhibited enhanced formation of basement membrane and growth arrest when cultured in reconstituted basement membrane components (20).

Although questions as to which step of the metastatic process from primary to distant metastatic sites was affected by nm23/NDP kinase still remain unanswered, there have been arguments on the possible sites of nm23/NDP kinase action. The metastasis suppressor activity of nm23/NDP kinase has been demonstrated using not only spontaneous metastasis assays but also experimental metastasis assays (13–18). All of these studies documented that nm23/NDP kinase neither affected cell growth rate in tissue culture dish nor provided consistent effect on primary tumor size when implanted into animals (13–18). In our previous study, transfection of NDP kinase α gene (rat homologue of human nm23-H2/NDP kinase B), not NDP kinase β gene (rat homologue of human nm23-H1/NDP kinase A), suppressed pulmonary metastasis of a rat mammary adenocarcinoma cell MTLn3 with no essential changes in lymph node metastasis (17). Taken together, nm23/NDP kinase seemed to suppress distant metastasis during the latter part of metastatic
process, presumably, from extravasation through secondary tumor formation.

Head and neck SCC is considered to be the sixth most common malignancy worldwide (21) and constitutes the majority of malignancies in the oral cavity. In this study, we investigated the antimetastatic action of nm23/NDP kinase using cell lines derived from human oral SCCs (22). The results demonstrate that the metastatic phenotype was well associated with the decreased expression of nm23-H2/NDP kinase B compared with nm23-H1/NDP kinase A. Forced expression of the nm23-H2/NDP kinase B gene conferred reduced metastatic phenotype on a metastatic human SCC cell when assessed in an experimental metastasis assay. Compared with control cells, the transfected cells exhibited more differentiated morphology in the metastatic sites, whereas they grew more rapidly and became less growth factor and serum-dependent in culture. These results make it likely that nm23/NDP kinase may modulate growth and/or differentiation potential of tumor cells in response to environmental factors in the metastatic sites, as a result, leading to reduction of the metastatic ability.

**MATERIALS AND METHODS**

**Cells and Culture Conditions.** Human oral SCC cell lines, HSC-2, HSC-4, LMF4, and LMF5 were established in our laboratory (22); LMFb was newly established from HSC-3 according to the method described (22) and has metastatic potential (data not shown). These cells were maintained in DMEM supplemented with 10% FBS under a humidified atmosphere of 5% CO2/95% air at 37°C. When growth factors were tested, the cells were cultured in gelatin-coated dishes in the same medium with 0.6% FBS.

**Northern Blot Analysis.** Total RNA from subconfluent cells was isolated by the acid guanidinium thiocyanate-phenol-chloroform method using Isogen (Wako, Osaka, Japan). RNA samples (20 μg/lane), the amounts of which were adjusted by staining rRNAs (28S and 18S as indicated) with ethidium bromide. Western blot analysis was done for cell extracts (10 μg protein/lane) separated by SDS-PAGE, followed by immunological detection using isoform-specific monoclonal antibodies as described in the text.

**Preparation of Cell Extracts.** Growing cells were trypsinized and washed three times with Dulbecco’s PBS (calcium- and magnesium-free). Cell pellets were lysed in a solution containing 10 mM 3-[(3-cholamidopropyl)dimethylamino]1-propanesulfonate (Sigma Chemical Co., St. Louis, MO), 225 mM sucrose, 9 mM Tris-HCl, 1.8 mM MgCl2, and 0.9 mM EDTA.

**Fig. 1** Relationship between metastatic potential and nm23 expression in human oral SCC cell lines. Nonmetastatic (HSC-2 and HSC-4) and metastatic (LMFb, LMF4, and LMF5) cells were used for determination of mRNA and protein levels of human nm23-H1 and nm23-H2. N, nonmetastatic; M, metastatic. Northern blot analysis was performed using total RNA samples (20 μg/lane), the amounts of which were adjusted by staining rRNAs (28S and 18S as indicated) with ethidium bromide. Western blot analysis was done for cell extracts (10 μg protein/lane) separated by SDS-PAGE, followed by immunological detection using isoform-specific monoclonal antibodies as described in the text.

**Fig. 2** nm23-H2/NDP kinase B expression in control and nm23-H2-transfected cell clones. Cell clones transfected with pcDNA3 harboring nm23-H2 cDNA (H2–5 and H2–7) or vector alone (C-1 and C-10) were isolated from a parental metastatic LMF4. Top panel, nm23-H2/NDP kinase B mRNA levels determined by Northern blot analysis. Middle panel, rRNA (28S and 18S) for the reference. Bottom panel, protein levels determined by Western blot analysis using KM1121 monoclonal antibody.
(pH 7.4) at 4°C for 30 min with gentle stirring. After centrifugation at 20,000 × g for 15 min, the resulting supernatants were subjected to protein measurement using a BCA Protein Assay kit (Pierce, Rockford, IL) and Western blot analyses.

**Western Blot Analysis.** The cell extracts (10 μg of protein) were separated by SDS-PAGE on a 14% gel, transferred to a polyvinylidene difluoride membrane (24), and then incubated with either one of isoform-specific monoclonal antibodies (17), followed by detection with an ECL assay kit (Amer sham, Aylesbury, United Kingdom). The mouse monoclonal antibody specific for rat NDP kinase α (KM1121) and that for rat NDP kinase β (KM1103) specifically cross-reacted with human nm23-H2 protein and nm23-H1 protein, respectively.

**Transfection Experiments.** Full-length nm23-H2 cDNA was inserted into a mammalian expression vector pcDNA3 (Invitrogen, Cals bad, CA). The pcDNA3 plasmid harboring human nm23-H2 cDNA was linearized by PvuI and transfected into metastatic LMF4 cells by electroporation as described previously (17). G418-resistant colonies were selected after 2–3 weeks.

Integration of the transfected plasmid DNA into the chromosomes was confirmed by the PCR method. By this method, 6 of 20 and 16 of 27 randomly selected clones were found to be positive for vector plasmid and nm23-H2 gene, respectively. Among them, two randomly selected clones for the control group (C-1 and C-10) and two clones that expressed nm23-H2 mRNA and protein at their highest levels (H2–5 and H2–7) were used.

**Experimental Pulmonary Metastasis Assay.** To examine pulmonary metastasis of these clones, freshly trypanized cells (4 × 10^5) suspended in 0.1 ml of serum-free DMEM were injected into the lateral tail vein of 4–6-week-old female nude mice [Crl:CD-1 (ICR) nu/nu; Charles River Japan]. Seven weeks later, mice were killed, and the lungs were resected and fixed with Bouin’s fixative. The number of metastatic colonies on the surface of the lung was counted with the aid of a stereo microscopic.

**Histological Analysis.** Dissected lung specimens fixed with Bouin’s fixative were soaked in 50% ethanol and then embedded in paraffin. Paraffin sections (4–5 μm) were stained with H&E for microscopic examination. For measurement of mitotic cells, exponentially growing cells cultured without Colcemid were treated with 0.56% (w/v) KCl for 20 min at 37°C, fixed with methanol:acetic acid mixture (3:1), and then stained with Giemsa solution (25).

### RESULTS

**Expression of nm23-H1 and nm23-H2 in Oral SCC Cell Lines.** Nonmetastatic (HSC-2 and HSC-4) and metastatic (LMFb, LMF4, and LMF5) human oral SCC cell lines were compared in terms of their expression of nm23-H1 and nm23-H2 genes (Fig. 1). A Northern blot analysis using full-length cDNAs showed that both transcripts were drastically reduced in metastatic three cell lines relative to nonmetastatic ones. The reduced expression of nm23-H2 was consistently observed at the protein level by a Western blot analysis using an isoform-specific monoclonal antibody. In contrast, nm23-H1 protein levels did not parallel their transcript levels and were essentially the same among these cell lines tested. These results led us to speculate that the metastatic phenotype of the SCC cell lines might be related to the altered expression of nm23-H2 gene rather than that of nm23-H1 gene.

**Isolation of nm23-H2 cDNA-transfected Cell Clones and Their Decreased Metastatic Potential.** To investigate whether a causal relationship existed between the metastatic phenotype and the nm23-H2 gene expression, a plasmid construct harboring nm23-H2 cDNA in pcDNA3 vector was made and transfected into a metastatic LMF4 cell, and then clones that stably expressed nm23-H2 protein were isolated. Fig. 2 illustrates the representative data in which two nm23-H2 cDNA-transfected clones (H2–5 and H2–7) displayed elevated levels of the transcripts as well as its translated products, compared with the control clones transfected with pcDNA3 alone (C-1 and C-10).

To examine the metastatic potential of these clones, 4 × 10^5 cells of each clone were injected into the tail vein of each athymic nude mouse, and metastatic foci on the surface of the lung were counted macroscopically 49 days after injection. As shown in Table 1, the control clones (C-1 and C-10) formed numerous foci (110–230 foci/lung), whereas both of the nm23-H2-transfected clones (H2–5 and H2–7) gave rise to a remarkably reduced number of foci (10–30/lung). In this experiment, we examined in parallel the metastatic potential of the nm23-H1-transfected clones (H1–8 and H1–25) in which its mRNA level was elevated but protein level was unchanged (data not shown) and found that there was no clear reduction in their metastatic ability. The results unambiguously demonstrate that the nm23-H2 gene product does have the ability to suppress metastatic potential of certain human SCC cells under these conditions.

### Table 1  Metastatic potential of control, nm23-H2- and nm23-H1-transfected LMF4 cell clones

<table>
<thead>
<tr>
<th>Cell clone</th>
<th>No. of foci/lung</th>
<th>Mean ± SE</th>
<th>vs. C-1</th>
<th>vs. C-10</th>
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<tr>
<td>C-1</td>
<td>53, 83, 79, 54, 102, 143, 314, 71, 113</td>
<td>112 ± 27</td>
<td>0.023</td>
<td>0.023</td>
</tr>
<tr>
<td>C-10</td>
<td>145, 115, 276, 104, 185, 169, 292, 453, 365</td>
<td>234 ± 40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2-5</td>
<td>6, 2, 5, 20, 3, 1, 35, 5, 4</td>
<td>9 ± 4</td>
<td>0.0049</td>
<td>0.0005</td>
</tr>
<tr>
<td>H2-7</td>
<td>2, 6, 0, 9, 10, 2, 77, 50, 118</td>
<td>30 ± 14</td>
<td>0.019</td>
<td>0.0008</td>
</tr>
<tr>
<td>H1-8</td>
<td>121, 40, 60, 117, 104, 464, 805, 611</td>
<td>290 ± 104</td>
<td>0.14</td>
<td>0.62</td>
</tr>
<tr>
<td>H1-25</td>
<td>172, 207, 119, 50</td>
<td>137 ± 34</td>
<td>0.61</td>
<td>0.17</td>
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</table>
Histological Analysis of the Metastatic Foci. The parental LMF4 cell line, according to the original report (22), manifested high frequency of pulmonary colonization in an experimental metastasis assay and produced small nodules with no sign of keratinization. We, therefore, examined histological features of the pulmonary metastatic foci formed in the experimental metastasis assay as described above. Control clones (C-1 and C-10) formed numerous metastatic foci composed of anaplastic squamous cells (Fig. 3, A, B, E, and F). They proliferated with small islet-like clusters and expanded invasively, forming discrete small nests or single cells with no massive keratinized foci. These histological features were essentially identical to those of the parental LMF4 cells. In contrast, the nm23-H2-transfected clones (H2–5 and H2–7) were composed of well-differentiated SCC (Fig. 3, C, D, G, and H); they were demarcated by lined up cells resembling basal layer arrangement and grew in a laminal concentric form with horn pearls mimicking normal stratified epithelial constitution (Fig. 3H). Vascularization into the metastatic foci was remarkable for the control but was scarcely observed for the transfected clones. Furthermore, the foci of nm23-H2-transfected clones were often accompanied by a large number of infiltrating small nuclear cells, compared with those of the control cells. Some of the small differentiated tumor nests of the nm23-H2 transfectants showed degenerative feature (Fig. 3I).

Growth Properties of nm23-H2-transfected Clones in Culture. Because cell growth potential in response to a milieu of a metastatic site is considered to be one of the crucial factors that determine metastasis formation (26, 27), we investigated whether the metastatic potential of the transfected cells correlated with their proliferative activity. Although the transfection of nm23-H2 gene caused no major change in the cell morphol-
Cells (13B, morphology taken under a phase-contrast microscope. was enhanced to grow clone was able to proliferate 2–3-fold (Table 2). The C-10 clone

Properties of control and \( \text{nm23-H2} \)-transfected clones. A, cell morphology taken under a phase-contrast microscope. B, growth rate. Cells (1 \( \times 10^5 \)) of each clone (C-1, C-10, H2–5, and H2–7) were inoculated and cultured in DMEM-10% FBS, and then cell numbers were counted at various times. Each point denotes the mean values of triplicate samples. Inset, doubling time of these cell clones.

DISCUSSION

This study presented evidence for the metastasis suppressor activity of \( \text{nm23/NDP kinase} \) in human oral SCC cell LMF4. This suppression was elicited by \( \text{nm23-H2/NDP kinase B} \), the action of which, in contrast to \( \text{nm23-H1/NDP kinase A} \), has been unsettled for quite a while. Although previous studies documented that the antimetastatic action of \( \text{nm23} \) occurs independently of the proliferative activity in culture and primary tumor size of the transfected cells, the reduced metastatic potential of the \( \text{nm23-H2}-\text{transfected clones} \) was accompanied by higher growth rate, less serum dependency and diminished responsiveness to growth factors in culture. Histological analysis of the pulmonary metastatic foci revealed, however, that the transfected cells showed more differentiated morphology compared with the control cells. These data lead to the speculation that the reduced metastasis might have occurred as a result of decreased colonization ability attributable to altered responsiveness to environmental factors at the metastatic sites.

The enhanced growth rate of LMF4 cells induced by \( \text{nm23-H2 gene transfection} \) was unexpected because there have been no such precedent reports (13–18). Presumably, this arose in part because of the relatively slow growth characteristics of the cell line (doubling time, 47 h; see also Ref. 22). Under low serum conditions, the growth rate of the transfected clones was comparable with that of the control clones stimulated by either one of the growth factors and no longer stimulated by growth factors. The simplest explanation for the \( \text{nm23/NDP kinase} \) action is that a common locus existing downstream of these growth-signaling pathways may be up-regulated by \( \text{nm23-H2 protein} \) to near a maximum level. Identification of the target site affected by \( \text{nm23/NDP kinase} \) would be essential to substantiate this view. Although transferrin was identified as a preferential growth-promoting factor for lung-metastasizing tumor cells (27), both control and \( \text{nm23-H2}-\text{transfected clones} \) showed similar responsiveness to this compound, ruling out the possibility that the decreased lung metastasis of the transfected clones resulted from altered response to transferrin. The present observations, as an extension of previous investigations, at least demonstrate that \( \text{nm23 protein} \) has the ability to modulate responsiveness to a number of growth factors in terms of cell proliferation.

Increased \( \text{nm23 gene expression} \) reportedly occurs in association with enhanced cell growth (29), immortalization (30), and tumorigenesis (9, 10, 12). Microinjection of an anti-nm23 antibody inhibited cell division (31). Furthermore, partial cell growth inhibition was noted by treatment with antisense oligonucleotide or antisense mRNA of \( \text{nm23-H1} \) (32). All of these observations implicate the significance of \( \text{NDP kinase/nm23} \) in cell growth. On the other hand, as discussed above, there have been no clear data demonstrating growth potentiation by the gene transfection. The literature suggests a positive role of \( \text{nm23/NDP kinase} \) during development and differentiation of cells and tissues (23, 33–35). We observed previously that high expression of \( \text{nm23/NDP kinase} \) occurs in postmitotic tissues such as heart and brain (23). In bone tissues, \( \text{nm23/NDP kinase} \) accumulated in differentiated osteoblasts in parallel with production of a differentiation marker, alkaline phosphatase, rather than osteoprogenitor cells that possessed proliferative activity.
(34). Furthermore, in the case of PC12D cells, neurite outgrowth was induced by transfection with NDP kinase β in the presence of no differentiation inducers, which accompanied prolongation of doubling time (35). The differentiation signals triggered by nerve growth factor and a cyclic AMP analogue were downregulated by overexpressing inactive mutant forms of NDP kinase (Hs118 was replaced with Ala) in the cell. In addition, high expression of NDP kinase protein is associated with differentiated SCCs (36, 37). Taking these into account, it could be possible that nm23/NDP kinase may elicit dual action in cells by virtue of, most probably, modulating responsiveness to environmental factors: one that leads to cell growth potentiation and the other that contributes to cell development and differentiation. The present finding that although the nm23-transfected cells displayed increased growth rate in *in vitro* culture, the fact that their morphology in the pulmonary metastatic sites was more differentiated seems to be well interpreted from this point of view, *i.e.*, the nm23-transfected LMF4 cells may have a potential to undertake both directions, but their fate appears to be determined by surrounding environments. Metastasis suppression would happen as a result of such a pleiotropic effect of nm23/NDP kinase in the cell. Relevant to this consideration, Howlett et al. (20) reported that *nm23-H1* gene transfection into a human breast cancer cell MDA-MB-435, which resulted in reduced metastasis with no change in growth potential in a plastic dish, led to regaining several aspects of normal phenotype such as acini formation, deposition of basement membrane components, expression of sialomucin, and growth arrest when cultured in the milieu of the reconstituted basement membrane components.

Although *nm23-H1*/NDP kinase A protein has been observed as a positive correlate to the lack of metastasis in human oral squamous cell carcinomas (37), it remains ambiguous in this study whether *nm23-H1* has antimetastatic activity for LMF4 cell. This was mainly because of the failure of obtaining high expression clones. Most of the *nm23-H1* gene-transfected, G418-selected clones showed increased *nm23-H1* mRNA but with no obvious changes in the protein level (data not shown), even with use of a plasmid vector with strong cytomegalovirus promoter. Considering the essential housekeeping nature of this protein family, organisms may be protected from fluctuation of the protein levels. But still there exist uncertainties on the discrepant levels between mRNA and protein. Because dissociation between mRNA and protein levels often occurs as shown in Fig. 1 of this study and in some literature from other laboratories (28), nm23/NDP kinase protein levels are likely to be regulated more rigidly compared with its mRNA at the posttranscriptional level. In this respect, our recent discovery (38) that rat NDP kinase mRNAs produced from multiple transcription initiation sites have distinct translational efficiencies may help understand how the dissociation between mRNA and protein levels takes place under physiological conditions.

**ACKNOWLEDGMENTS**

We are grateful to N. Shimada, H. Seto, and A. Ishii, Tokyo Metropolitan Institute of Gerontology, for technical advice; to Dr. H. Iwaki, Faculty of Dentistry, Tokyo Medical and Dental University; and to Dr. F. Momose, Department of Oral Surgery, Toride Kyodo General Hospital, for providing the cell lines.

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