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Physiological and Pathological Relevance of Extracellular NM23/NDP Kinases

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The *NM23* gene is overexpressed in many hematological malignancies and other neoplasms. Some tumor cell lines that overexpress NM23 secrete this protein into extracellular environment. In this study, we found that the serum concentration of NM23-H1 protein was significantly higher in patients with various hematological malignancies. The serum level of NM23-H1 protein was clinically useful as a prognostic factor in malignant lymphoma and acute myelogeneous leukemia (AML). The level of NM23-H1 protein in all of the normal serum samples examined was lower than 10 ng/mL, while those in the tumors varied from about 0 to 1000 ng/mL. Exogenously added NM23-H1 protein did not affect the growth or survival of various leukemia and lymphoma cell lines. However, NM23-H1 protein inhibited the survival of adherent normal peripheral blood mononuclear cells (PBMNC) at 100–1000 ng/mL, and slightly stimulated the survival of nonadherent PBMNC. These results suggest that the effect of NM23-H1 protein on normal PBMNC may be associated with a poor prognosis in hematological malignancies.

KEY WORDS: Extracellular NM23; serum; prognostic factor; leukemia; lymphoma.

INTRODUCTION

We previously identified a differentiation-inhibiting factor (I-factor) in the culture medium and the membrane fraction of differentiation-resistant mouse myeloid leukemia M1 cells, but not in differentiation-sensitive M1 cells (Okabe-Kado *et al.*, 1985, 1988). The I-factor purified from the conditioned medium of M1 cells was identical to NM23 protein (Okabe-Kado *et al.*, 1992). It has been reported that the subcellular distribution of NM23 protein was 20, 65, and 15% in the membrane, cytoplasmic, and nuclear fractions of leukemia cell lines, respectively (Urano *et al.*, 1994). Furthermore, extracellular NM23 proteins have been reported in conditioned medium of some tumor cell lines, in body fluids, and on the cell surface in other tumor cell lines (Anzinger *et al.*, 2001; Niitsu *et al.*, 1999; Okabe-Kado *et al.*, 1992; Urano *et al.*, 1994). The elevated extracellular expression of NM23 has been observed only in tumor cell lines. Very little information is available concerning extracellular expression, although many studies have examined the expression of intracellular NM23 proteins.

Eight isotypes of the human *NM23* gene (*NM23-H1*, *NM23-H2*, *NM23-H3/DR-NM23*, *NM23-H4*, *NM23-H5*, *NM23-H6*, *NM23-H7*, *and NM23-H8*) have been identified (Lacombe *et al.*, 2000). Among the *NM23* family, only *NM23-H1* and *NM23-H2* have been extensively studied in human cancers. In this paper, we describe the extracellular expression of NM23-H1 protein and discuss the physiological role of extracellular NM23 proteins.

SERUM NM23-H1 LEVELS IN PATIENTS WITH HEMATOPOIETIC MALIGNANCIES AND THEIR PROGNOSTIC VALUE

The *NM23* genes are overexpressed in various hematological malignancies including acute myelogeneous leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myelogeneous leukemia in blastic crisis (CML-BC) and myelodysplastic syndrome (MDS); and a higher

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level of *NM23* expression is correlated with a poor prognosis (Wakimoto *et al.*, 1998; Yokoyama *et al.*, 1998). Since we previously detected NM23 protein in conditioned medium of leukemia cells, we made a sandwich enzyme-linked immunosorbent assay (ELISA) system for serum NM23-H1 protein and measured this protein in the plasma or serum of patients with various hematological malignancies. Nucleoside diphosphate kinase (NDPK) activity, one of the activities of NM23 protein, can be detected in human plasma, probably due to the slight in vivo lysis of red blood cells (Willems *et al.*, 1998). Therefore, to exclude the possible effects of hemolysis, we measured the content of free plasma/serum hemoglobin as a marker of hemolysis.

Serum NM23-H1 levels were significantly higher in patients with any of the hematological neoplasms tested (AML, CML, ALL, MDS, Hodgkin's disease, non-Hodgkin's lymphoma) than in normal controls (Ito *et al.*, 2001; Niitsu *et al.*, 1999, 2000, 2001a,b). For example, the serum NM23-H1 level in patients with indolent and aggressive non-Hodgkin's lymphoma or AML was significantly higher than that in normal controls (Mann Whitney U-Test, $p = 0.00001$, Fig. 1(A)). The serum NM23-H1 level in patients with aggressive non-Hodgkin's lymphoma was also significantly higher than that in indolent non-Hodgkin's lymphoma ($p = 0.00001$, Fig. 1(A)).

The patients were divided into two groups with different serum NM23-H1 levels using various cutoff values above the upper limit in the controls. The cutoff values tested were as follows: 80 ng/mL for aggressive non-Hodgkin's lymphoma (Fig. 1(C)) and AML (Fig. 1(D)), and 25 ng/mL for indolent non-Hodgkin's lymphoma (Fig. 1(B)). Patients with a higher NM23-H1 level had a worse prognosis than those with lower NM23-H1 levels in each disease (Fig. 1(A)–(D)). Thus, an elevated serum NM23-H1 protein concentration predicted a poor outcome for these diseases independent of the type of hematological tumor (Fig. 1 and Niitsu *et al.*, 2001a,b). Especially in aggressive non-Hodgkin's lymphoma (Fig. 1(C)), the serum NM23-H1 protein levels were an important prognostic factor in planning an appropriate treatment strategy. Furthermore, we could identify a risk group even in indolent non-Hodgkin's lymphoma (Fig. 1(B)), which is associated with a relatively low serum level compared with aggressive non-Hodgkin's lymphoma (Fig. 1(A)).

It is unclear whether serum NM23-H1 protein is directly produced by lymphoma or leukemia cells. As mentioned above, *NM23* genes were overexpressed in AML cells and a higher level of *NM23-H1 mRNA* expression was correlated with a poor prognosis in AML (Wakimoto *et al.*, 1998; Yokoyama *et al.*, 1998). There was a significant correlation between the serum NM23-H1 level and the product of the *mRNA* level and the blast cell count (Niitsu *et al.*, 2000; Okabe-Kado, 2002). The serum NM23-H1 protein level probably depends on the total mass of leukemia cells overexpressing *NM23-H1*. These results suggest that serum NM23-H1 protein might be derived from leukemia cells (Niitsu *et al.*, 2000).

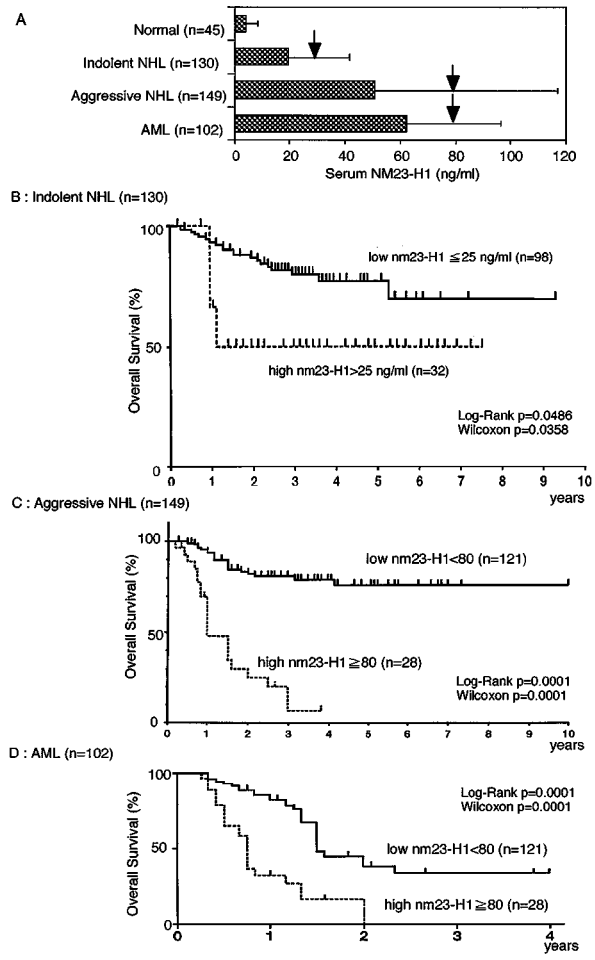

Fig. 1. Serum NM23-H1 protein level in various hematological malignancies and its association with a poor prognosis. (A) Serum NM23-H1 protein levels in normal healthy control serum, indolent and aggressive non-Hodgkin's lymphoma (NHL), and acute myelogeneous leukemia (AML). Arrows show the cutoff values used for the survival analysis. Bars: SD. (B)–(D) Overall survival curves of patients with indolent NHL (B), aggressive NHL (C), and AML (D). Cutoff value of the serum NM23-H1 concentration, 80 ng/mL for AML and aggressive NHL; 25 ng/mL for indolent NHL.

We transplanted a human B-lymphocytic lymphoma cell line that overexpressed *NM23-H1* into immunosuppressed nude mice and examined the tumor size and serum human NM23-H1 protein levels. NM23-H1 protein was detected in the serum of nude mice depending on the tumor size (Okabe-Kado, 2002). These results

strongly suggest that serum NM23-H1 protein is directly produced by lymphoma cells and its level depends on the total mass of malignant cells overexpressing *NM23-H1* (Okabe-Kado, 2002).

NM23 PROTEINS IN BODY FLUIDS OF PATIENTS WITH SOLID TUMORS AND IN CONDITIONED MEDIUM OF TUMOR CELL LINES

The *NM23-H1* gene is overexpressed in many hematological malignancies. Overexpression of the *NM23-H1* gene has also been reported in many other neoplasms including pancreatic, lung, ovarian, and gastric cancer (Lacombe *et al.*, 1991). Overexpression of *NM23-H1* is indicative of a poor patient prognosis in neuroblastomas, and mutations of NM23-H1 protein are detected in advanced neuroblastoma (Leone *et al.*, 1993). Therefore, *NM23-H1* might be a useful prognostic factor for many other neoplasms that overexpress *NM23-H1* as well as for AML and malignant lymphoma. We previously measured the serum NM23-H1 levels in about 2000 patients with various tumors (Fig. 2(A)). The NM23-H1 protein levels in all of the normal serum samples examined were lower than 10 ng/mL, while those in the tumors varied from about 0 to 1000 ng/mL (Fig. 2(B)). It would be interesting to examine whether the serum NM23-H1 level generally predicts a poor outcome for patients with tumor.

Huwer *et al.* (1997) showed that NM23 expression is considerably elevated in squamous cell lung carcinoma. Using western blot analysis, they found a 2–7-fold increase in the amount of NM23 protein in bronchial lavage fluid of tumor-bearing lung compared to the healthy side. This finding was not related to either the tumor stage or location. They suggested that NM23 protein is secreted by bronchogenic squamous cell carcinoma and NM23 protein in bronchial lavage fluid might be useful for establishing a diagnosis when pulmonary nodules of unknown etiology are found.

In a variety of human tumor cell lines (breast, lung, colon, and prostate), the secretion of NM23 protein can be detected. Anzinger *et al.* (2001) found NM23-H2/NDPK-B protein as a phosphoprotein in the extracellular environment. They suggested that the localized production of extracellular ATP by tumor-derived NM23-H2/NDPK-B may facilitate the process of metastasis since it may support tumor cell transit, intravasation, and angiogenesis. We have not tried to measure the concentration of serum NM23-H2, since we have not yet made an ELISA system specific for NM23-H2. It has also been reported that several compounds that reportedly

A

	No. of cases
Healthy	151
AML	126
Malignant lymphoma	937
Others	137
Lung Cancer	305
Neuroblastoma	374
Total	2,030

B

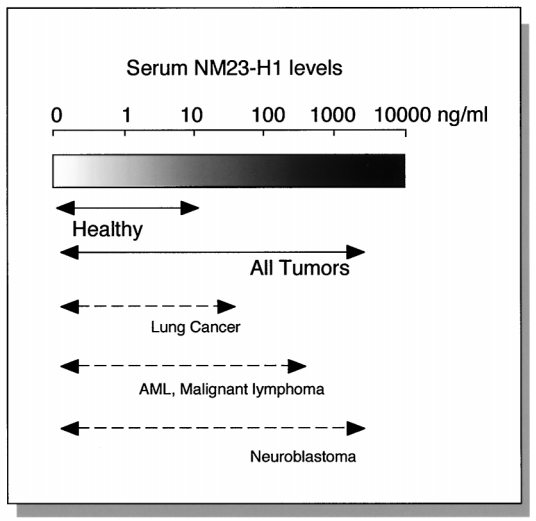

Fig. 2. Serum NM23-H1 protein levels in various tumors. (A) We measured the NM23-H1 protein levels in various tumors. Others, hematological malignancies except AML and malignant lymphoma. (B) The range of serum NM23-H1 concentrations observed. The NM23-H1 levels in all of the normal serum samples used were lower than 10 ng/mL, while those in the tumors varied (0–1000 ng/mL).

have antiangiogenic or antitumorigenic effects inhibit secreted NM23-H2/NDPK-B (Malmquist *et al.*, 2001). They hypothesized that the inhibition of extracellular NM23-H2/NDPK-B may be mechanistically associated with the inhibition of metastasis by multiple human tumors.

POSSIBLE EFFECTS OF EXTRACELLULAR NM23 PROTEINS ON THE GROWTH AND SURVIVAL OF LEUKEMIA/LYMPHOMA CELL LINES AND NORMAL BLOOD CELLS

As described above, patients with a higher NM23-H1 level had a worse prognosis than those with lower NM23-H1 levels for each disease (Fig. 1(A)–(D)), although the

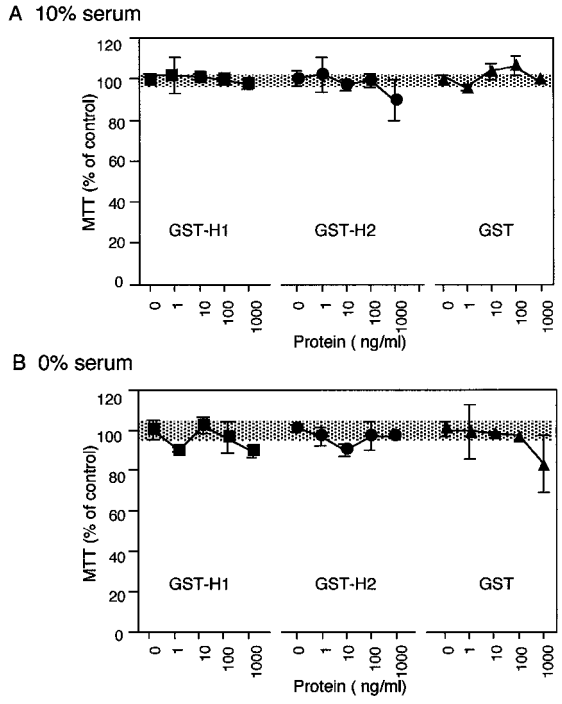

Fig. 3. Effects of recombinant NM23 proteins on the growth and survival of a lymphoma cell line BALM-3. The effects of GST-fused NM23 proteins on the growth of the lymphoma cell line with (A) or without (B) fetal bovine serum.

level of serum NM23-H1 depended on the type of tumor. The mechanisms by which NM23-H1 protein is secreted into the serum and affects the patient outcome are unclear. We examined the possibility that a high concentration of serum NM23-H1 may positively affect tumor cell growth or negatively affect normal cells. We investigated the extracellular effects of NM-23 proteins (GST-H1, GST-H2, and GST as a control) on the growth and survival of tumor cells and normal cells at serum NM23-H1 concentrations of 0 to 1000 ng/mL, which were detected in the serum of patients with malignant hematological neoplasms. We used cell lines as tumor cells and peripheral blood mononuclear cells (PBMNC) as normal cells. Cell growth and survival were measured by the MTT assay. Figure 3(A) shows the effects of GST-H1, GST-H2, and GST proteins on the growth of the human malignant lymphoma cell line BALM-3 in medium with (Fig. 3(A)) or without (Fig. 3(B)) fetal calf serum. NM23 proteins did not have any effects on the growth of BALM-3 cells at these concentrations of NM23 protein. We examined the effects of NM23 proteins on various hematopoietic cell lines (BALM-3, SKW-4, Raji, Jurkat, HL-60, THP-1, K562, and YNH-1) (data not shown). We did not detect any stimulatory or inhibitory effects on the tumor cell lines

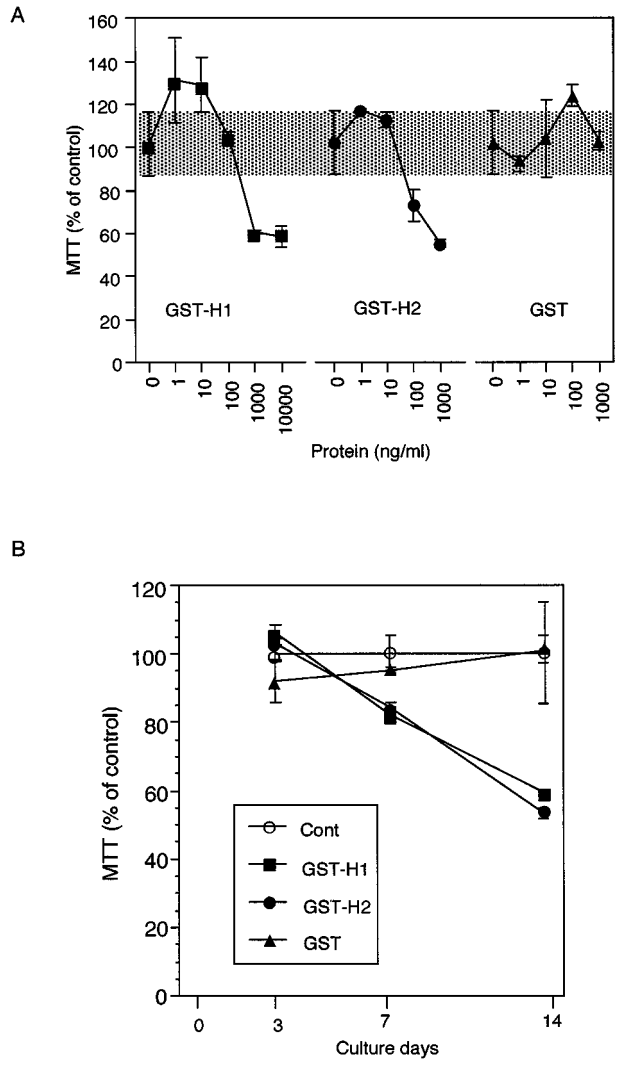

Fig. 4. Effects of NM23 proteins on the survival of normal peripheral blood mononuclear cells (PBMNC). Both NM23-H1 and NM23-H2 proteins inhibited the survival of PBMNC in a dose-dependent manner at day 14 (A) and in a time-dependent manner with 1000 ng/mL (B).

tested. Therefore, these results suggest that extracellular NM23 proteins do not affect tumor cells in vitro.

Next, we examined the effect of NM23 proteins on the survival of normal PBMNC. PBMNC were obtained from healthy volunteers and mononuclear cells were separated over Ficoll-Hypaque. PBMNC contain mainly lymphocytes and monocytes. Both the NM23-H1 and NM23-H2 proteins, but not GST, inhibited the survival of PBMNC (mainly CD68-positive monocytes) in a dose-dependent (Fig. 4(A)) and time-dependent manner (Fig. 4(B)). On the other hand, they slightly stimulated the survival of nonadherent PBMNC (mainly lymphocytes) (data not shown). These results suggest that the effect of

NM23-H1 protein on the survival of normal monocytes and lymphocytes may be associated with a poor prognosis in hematological malignancies. We are now more precisely analyzing the effect of extracellular NM23-H1 protein on normal PBMNC.

CONCLUDING REMARKS

In this study we focused on extracellular NM23-H1 protein derived from tumor cells. Some tumor cell lines that overexpress intracellular NM23 secrete this protein into the extracellular environment. High concentrations of NM23 protein were found in the serum and body fluid of patients with tumors overexpressing NM23. Tumor cells may secrete this protein through some unknown mechanism, since there is no signal peptide sequence for secretion in the NM23 molecule. The serum level of NM23-H1 protein is clinically useful as an important prognostic factor in malignant lymphoma and AML. We hypothesize that the extracellular NM23-H1 proteins secreted from tumor cells may positively affect tumor cells as an autocrine growth factor or negatively affect normal cells, like some cytokines. Our preliminary data show that extracellular NM23 proteins affect the survival of normal PBMNC, but not tumor cells.

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