SERUM AND PLASMA M₉, 92,000 PROGELATINASE LEVELS CORRELATE WITH SPONTANEOUS METASTASIS OF RAT 13762NF MAMMARY ADENOCARCINOMA

Motowo Nakajima,³ Danny R. Welch, Dianna M. Wynn, Takashi Tsuruo, and Garth L. Nicolson

Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, 113 Japan [M. N., Z. T.]; Division of Experimental Pathology, Department of Pathology, The Pennsylvania State University, College of Medicine, The M. S. Hershey Medical Center, Hershey, Pennsylvania 17033 [D. R. W.]; and Department of Tumor Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 [D. M. W., G. L. N.]

ABSTRACT

The expression of metalloproteinases, such as type IV collagenase/gelatinase, enables tumor cells to degrade type IV collagen present in the basement membrane and correlates with metastatic potential of several tumor types. We found that increased levels of rat serum type IV collagenolytic activity are associated with increased 13762NF mammary adenocarcinoma metastases in lungs and lymph nodes of syngeneic rats. To investigate serum metalloproteinases responsible for type IV collagenolysis, we performed zymography and Western blot analysis of rat sera. A Mr 92,000 progelatinase (progelatinase B, M₉, 92,000 type IV procollagenase, MMP-9) was detected on zymograms of rat sera within 16 days after intramammary fat pad inoculation of highly metastatic MTLn3 cells. The activated serum M₉, 92,000 progelatinase degraded sodium dodecyl sulfate-denatured type I and IV collagens but was not active on casein, albumin, hemoglobin, and immunoglobulin. Sera from rats with fat pad tumors and lung metastases formed from highly metastatic clones possessed greater than 7 times higher levels of serum M₉, 92,000 progelatinase than control sera or sera from rats bearing similar size fat-pad tumors of low or nonmetastatic clones. The results were confirmed by Western blot analysis of rat sera using rabbit anti-human M₉, 92,000 progelatinase antibodies. Similar results were obtained from the analysis of rat plasma samples. The serum and plasma M₉, 92,000 progelatinase levels correlated with the extent of metastases in the lung and lymph nodes. The results indicate that high levels of serum and plasma M₉, 92,000 progelatinase are associated with the presence of disseminated metastatic adenocarcinoma cells and suggest that this enzyme may facilitate the invasion of blood-borne tumor cells through vascular basement membranes.

INTRODUCTION

The penetration of basement membranes by malignant cells is an important step in the formation of tumor metastases. Basement membranes, formed from type IV collagen, laminin, heparan sulfate proteoglycan, nidogen, and fibronectin, are relatively rigid barriers between different tissues. Several proteinases implicated in the process of tumor invasion and metastasis are capable of degrading basement membrane components (1–9). An important class of these enzymes are the metalloproteinases, such as the type IV collagenases/gelatinases (2–4). Type IV collagenolytic activity itself has been correlated with the metastatic potential of tumor cells in a variety of malignant tumors (4). For example, we found an excellent correlation between the type IV collagenolytic enzyme activities in rat 13762NF mammary adenocarcinoma cells and their spontaneous lung metastatic potentials (10). These metalloproteinases secreted from the rat mammary adenocarcinoma cells degraded both α-subunits of type IV procollagen and produced characteristic high molecular weight fragments (10). Using zymography we have identified two major metalloproteinases in the serum-free conditioned medium of highly metastatic mammary adenocarcinoma cell cultures (11). The metalloproteinases were M₉, 64,000, and M₉, 88,000 progelatinases as determined by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis under nonreducing conditions. These gelatinases specifically degraded type IV collagen and heat-denatured type I collagen (gelatin) but not serum albumin, casein, hemoglobin, or immunoglobulin (11). The production of the progelatinases by rat mammary adenocarcinoma cells was suppressed by retinoic acid treatment, paralleling a decrease in cell invasion through reconstituted basement membranes (11). These enzymes are rat homologues of the human metalloproteinases known as M₉, 72,000 progelatinase/M₉, 72,000 type IV procollagenase/gelatinase A/MMP-2 (12) and M₉, 92,000 progelatinase/M₉, 92,000 type IV procollagenase/gelatinase B/MMP-9 (13).

Since rat mammary adenocarcinoma cells secrete type IV collagenolytic metalloproteinases in direct relation to their metastatic potentials (10), we assayed sera from rats bearing mammary adenocarcinoma cells of differing metastatic potentials for the collagenolytic metalloproteinases using metabolically [³H]proline-labeled type IV collagen. We found that serum type IV collagenolytic activity correlated with the extent of metastases in the lung and lymph nodes (14). Here, we demonstrate that the expression of serum and plasma M₉, 92,000 progelatinase correlates with the metastasis formation of rat 13762NF mammary adenocarcinoma cells.

MATERIALS AND METHODS

Cells and Cell Culture. Doubly cloned cell lines were derived from the 13762NF mammary adenocarcinoma growing at local mammary fat pad implant sites (MT and MTF) and from spontaneous lung metastases (MTLn2 and MTLn3) (15). Subclones MTLn3(T44).5 and MTF7(T35).3 were selected by in vitro cloning from MTLn3 and MTF7, respectively (16). Cells were grown on 100-mm plastic tissue culture plates (Coming Glass Works, Corning, NY) containing α-minimal essential medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (Biocell, Carson, CA) at 37°C in a humidified incubator (5% CO₂; 95% air). Cells from subconfluent cultures were used for all assays.

Spontaneous Metastasis Assay. 13762NF Mammary adenocarcinoma cells used for all assays were harvested from subconfluent cultures by 0.25% trypsin treatment, and their viability was determined by trypsin blue dye exclusion. Only cells suspensions with viability greater than 95% were used for animal inoculations. Age-matched F344 rats received 1× 10⁶ tumor cells suspended in 0.5 ml phosphate-buffered saline s.c. in the left posterior inguinal mammary fat pad. Blood was collected by cardiac puncture at various periods after tumor cell injection. Rats were killed using Metofoane anesthesia and were subjected to complete gross necropsies. The numbers of metastases were related to enzyme activities using a one-way analysis of variance and the Student-Newman-Keuls procedure for group significance analysis.

Type IV Collagenolysis Assay. The type IV collagenolysis assay was performed as previously reported (10, 11). Briefly, [³H]Proline-labeled type IV collagen was purified from EHS tumors that were cultured for 48 h in proline-free Dulbecco's modified Eagle's medium containing 100 μg/ml ascorbic acid, 100 μg/ml β-aminopropionitrile fumate, and 25 μg/ml t-[²,³,⁴,⁵-³H]prolinetreatment in a humidified incubator (5% CO₂; 95% air). Cells from subconfluent cultures were used for all assays.

The abbreviations used are: MMP, matrix metalloproteinase; EHS, Engelbreth-Holm-Swarm; ELISA, enzyme-linked immunosorbent assay.

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2 To whom requests for reprints should be addressed, at Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, 113 Japan.
were measured by use of a liquid scintillation counter. In some experiments diluted 10-fold with 50 mM Tris-HCl, 0.1 M sodium chloride, 10 mM calcium chloride, and 0.05% sodium azide (pH 7.5) and incubated with 40 μg of [3H]proline-labeled type IV collagen at 37°C. Incubation was terminated by chilling on ice, and undigested proteins were precipitated by incubation with 10% trichloroacetic acid and 0.5% tannic acid at 4°C for 30 min. After a centrifugation at 10,000 × g for 5 min, degradation products in the supernatant were measured by use of a liquid scintillation counter. In some experiments serum (10 μl) was pretreated with 10 μg/ml soybean trypsin inhibitor. In the analysis of the degradation products, 2 mM phenylmethylsulfonl fluoride, 5 mM N-ethylmaleimide were added to the assay buffer, and the incubation was performed at 27°C for 24 h to avoid degradation by serine and cysteine proteinases. The incubation products were analyzed by 6% polyacrylamide slab gel electrophoresis in the presence of 0.5 M urea and 5 mM dithiothreitol. Autoradiography was carried out using an autoradiography enhancer, ENHANCE (New England Nuclear, Boston, MA).

Zymography. Zymography was performed as described previously (11). An aliquot of the serum or plasma (1–6 μl) was dissolved in electrophoresis sample buffer containing sodium dodecyl sulfate and subjected to electrophoresis in a 7.5% polyacrylamide gel embedded with 1 mg/ml protein substrate in the absence of β-mercaptoethanol using a Bio-Rad Protean II System equipped with a cooling core (Bio-Rad, Richmond, CA). After electrophoresis at 16°C, enzymes were renatured by incubation with Triton X-100, and the enzyme reaction was allowed to proceed at 37°C for 12 h. Proteinases were detected as transparent bands on the blue background of Coomassie blue-stained slab gels. To quantify relative enzyme activities, stained gels were scanned for absorbance at 560 nm using a Beckman DU-8 spectrophotometer (Beckman, Irvine, CA).

Western Blot Analysis. Rabbit polyclonal antibodies were raised against synthetic peptides of the NH2-terminal sequences of M, 92,000 progelatinase (LRTNLDQRAEELYYRAG) (13) and M, 72,000 progelatinase (APSPPIK-FPGDVPKTD) (12). The antibodies were purified by affinity chromatography using columns of the NH2-terminal peptides covalently linked to Affi-Gel 10 (Bio-Rad). Samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis in 7.5% polyacrylamide gels and electroblotted on nitrous cellulose membranes followed by hybridization with polyclonal antibodies raised against gelatinase amino terminal sequences. Immunostaining was further carried out using anti-rabbit IgG-horse radish peroxidase conjugates and the enhanced chemiluminescence Western blotting detection system (Amersham Corporation, Arlington Heights, IL).

RESULTS

Type IV Collagenolytic Activity in Sera of Normal and Tumor-bearing Rats. When it was diluted more than 10-fold with 50 mM Tris-HCl, 0.1 M sodium chloride-10 mM calcium chloride (pH 7.5), untreated sera from normal female F344 rats contained low levels of type IV collagenolytic activity (Fig. 1). The degradation of type IV collagen by the diluted normal sera was constant over a 9-h incubation period under the conditions used. Sera from rats with mammary fat pad tumors and lung metastases formed from MTLn3 mammary adenocarcinoma cells possessed 4-5 times higher levels of type IV collagenolytic activity than did normal rat sera (Fig. 1). The degradation of type IV collagen by diluted sera from tumor-bearing rats was completely suppressed by 10 mM 1,10-phenanthroline or 20 mM EDTA but not by 2 mM phenylmethylsulfonyl fluoride or 5 mM N-ethylmaleimide, suggesting that the serum enzyme responsible for type IV collagenolysis is a metalloproteinase. Treatment of rat sera with N-tosyl-l-phenylalanine chloromethyl ketone-treated trypsin markedly enhanced type IV collagenolytic activity, indicating that the sera contained type IV collagenolytic metalloproteinases in both latent and active forms. The maximal activation of the latent enzyme was achieved by 5-min treatment with 1 μg/ml N-tosyl-l-phenylalanine chloromethyl ketone-treated trypsin. Under these conditions we detected a 3.7× increase in normal rat serum enzyme activity and a 2.5× increase in serum enzyme activity in metastatic tumor-bearing rats (Fig. 1). Since the results from the experiments with untreated sera were more reproducible than those with trypsinized sera, untreated sera were used for further studies.

To characterize serum type IV collagenolytic enzymes, the [3H]proline-labeled type IV collagen was incubated with the untreated serum from a MTLn3 tumor-bearing rat in the presence of 2 mM phenylmethylsulfonyl fluoride and 5 mM N-ethylmaleimide at 27°C for 24 h, and the degradation products were analyzed by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (Fig. 2). Both pro-a1(IV) and pro-a2(IV) subunits of type IV collagen, of Mr 185,000 and Mr 170,000, respectively, were degraded by serum metalloproteinases, and characteristic fragments with molecular weights of 135,000, 120,000, 110,000, 92,000, and 85,000 were produced. These fragments were similar to those produced by the metalloproteinases secreted from MTLn3 cells in serum-free culture (10).

Relationship between Serum Type IV Collagenolytic Activity and Metastasis. Type IV collagenolytic activity in rat sera slowly increased with time during the first 2 weeks after injection of highly metastatic MTLn3 cells into mammary fat pads (Fig. 3A). Between day 16 and 23 the metalloproteinase activity rose faster and continuously increased until day 30. No significant correlation was observed between serum type IV collagenolytic activity and size of mammary fat pad tumors (r² = 0.683) (Fig. 3B). The sera from rats bearing fat pad tumors and metastases in the lung and lymph nodes had significantly higher type IV collagenolytic activity than those from rats with only local tumors (P < 0.05) (Fig. 3C).

Zymography of Serum Metalloproteinases. The molecular size of serum type IV collagenase/gelatinase was investigated by means of protein-embedded polyacrylamide gel electrophoresis followed by incubation. When heat-denatured type I collagen (gelatin) (1.0 mg/ml) was used as a substrate, no significant degradation activity was discernible in the sera from normal control rats or in the sera of rats with mammary fat pad tumors formed from poorly metastatic MTC or MTLn2 cells (Fig. 4). In contrast, M, 92,000 progelatinase activity was found in the sera of rats bearing highly metastatic MTLn3 tumors. This serum gelatinase comigrated with the high molecular weight gelatinase secreted by cultured MTLn3 cells (previously described as M, 88,000 metalloproteinase in Ref. 11) in a 7.5% polyacrylamide gel containing gelatin under the conditions used in the present study. The serum proteinase activity was observable 16 days after intra-fat pad injection of MTLn3 cells (Fig. 4). When 0–6-μl aliquots of the serum
from the rat bearing MTLn3 tumor were analyzed by gelatin zymography, a linear correlation was observed between the serum volume (up to 4 μl) and the Mr 92,000 progelatinase activity.

The serum Mr 92,000 progelatinase activity detected by zymography was completely suppressed by 5 mM 1,10-phenanthroline or 10 mM EDTA but not 2 mM phenylmethyl-sulfonyl fluoride or 5 mM N-ethylmaleimide, confirming that the enzyme is a metalloproteinase. Gelatins prepared from rat tail collagen type I and porcine skin were better substrates for the Mr 92,000 progelatinase than gelatin from EHS type IV collagen, whereas rat casein, rat albumin, rat hemoglobin, and rat immunoglobulin were not susceptible to this enzyme (data not shown). The degradation rate for gelatin from rat tail type I collagen was 7 to 8 times higher than that denatured EHS type IV collagen.

In casein zymograms three metalloproteinase bands with Mr 85,000, 82,000, and 78,000 were detected in the sera of rats with MTLn3 or MTC tumors (data not shown). These enzymes did not degrade gelatin and there was no correlation of caseinolytic activity with either primary tumor size or number of metastases.

**Serum Mr 92,000 Progelatinase Level and Extent of Metastasis.**

The relationship between Mr 92,000 progelatinase level and extent of metastasis was quantitatively analyzed as shown in Fig. 5. Very low levels of Mr 92,000 progelatinase were detected in the sera of rats injected with tumor cells or phosphate-buffered saline at day 0. (Fig. 3A). The levels did not change during the first 9 days after intra-fat pad injection of highly metastatic MTLn3 cells. At day 16, however, the enzyme activity rose and continuously increased until at least 30 days (Fig. 3A). Rats with mammary fat pad tumors plus lung metastases formed from highly metastatic clones, such as MTLn3, MT7-F7(T35), or MTLn3(T44), had 7-times higher levels of serum Mr 92,000 progelatinase by day 30 after tumor cell inoculation (Fig. 3A).

In contrast, the sera from rats bearing only mammary fat pad tumors of low or nonmetastatic clones, such as MTLn2 or MTC, had low gelatinase activities, ones similar to those of control rat sera (Table 1). When serum Mr 92,000 progelatinase levels of rats injected with highly metastatic MTLn3 cells were compared, the size of primary tumors correlated with the gelatinase activity ($r^2 = 0.882$) (Fig. 3B). Also there was a significant difference in the serum Mr 92,000 progelatinase level between the rats with only fat pad tumors and the rats bearing metastases in axillary and inguinal lymph nodes ($P < 0.01$).

**Number of lung colonies**

- **A:** increase in serum type IV collagenolytic activity and Mr 92,000 progelatinase level.
- **B:** relationship between size of primary tumor and serum type IV collagenolytic activity or Mr 92,000 progelatinase level.
- **C:** relationship between number of lung metastases and serum type IV collagenolytic activity or Mr 92,000 progelatinase level (*, lymph node metastasis positive).
were detected as transparent bands on the blue background of Coomassie blue-stained slab polyacrylamide gel embedded with 1 mg/ml rat tail type I collagen-derived gelatin. After metastasize, even after 30 days. Serum (2.5.d) was subjected to electrophoresis in a 7.5% gelatinolytic enzymes were visualized using goat anti-rabbit IgG-horse radish peroxidase followed by incubation with anti-human Mr 92,000 progelatinase antibodies. Immunoreactive bands were revealed by enhanced chemiluminescence (ECL). Anti-human Mr 92,000 progelatinase antibodies bound to a rat serum gelatinolytic activity is associated with high numbers of 13762NF mammary adenocarcinoma metastases in lungs and lymph nodes (14). We now demonstrate that the Mr 92,000 metalloproteinase activity in the sera and plasma of female F344 rats increases with time after injection of highly metastatic but not poorly metastatic 13762NF cells into the mammary fat pad. This metalloproteinase was identified as a Mr 92,000 progelatinase with the same substrate specificity and reactivity of anti-human M 92,000 progelatinase (MMP-9). The Mr 92,000 progelatinase levels in the sera and plasma of rats bearing MTLn3 fat pad tumors correlated with the extent of metastases. The sera from rats with large numbers of metastases in the lungs and/or lymph nodes had much higher levels of Mr 92,000 progelatinase than the sera from rats with few or no metastases. In contrast, sera from rats bearing mammary adenocarcinoma of low metastatic potential, such as clones MTC and MTLn2, contained low levels of this enzyme, even 30 days after tumor cell injection and extensive growth of the tumor at the primary site in the mammary fat pad. These results suggest that the high levels of serum and plasma Mr 92,000 progelatinase are probably due to the presence of metastatic adenocarcinoma cells. Since proenzymes of metalloproteinases can be activated by tumor cell surface serine proteinases or cysteine proteinases (1–8), a high level of Mr 92,000 progelatinase in plasma could contribute to extravasation of tumor cells circulating in blood and hence enhance blood-borne metastasis. A Mr 72,000 type IV collagenolytic activity is produced by a variety of normal and malignant cells (4) and has been purified from (Fig. 5). It is not known whether the Mr 72,000 progelatinase, because polyclonal antibodies raised against the NH2-terminal peptide of human Mr 72,000 progelatinase did not show any reactivity with rat serum components (data not shown). There was a significant difference in the intensity of anti-human Mr 92,000 progelatinase-immunostained bands between the sera of rats bearing poorly metastatic tumors (MTC = 1.0; MTLn2 = 1.8) and the sera of rats bearing highly metastatic tumors (MTLn3 = 4.2; MTLn3(T44).5 = 5.2, MTF7(T35).3 = 5.0) (P < 0.05), consistent with the results obtained from zymography.

**Plasma Mr 92,000 Progelatinase Activity and Extent of Metastasis.** Plasma from rats bearing tumors of poorly metastatic MTC and highly metastatic MTLn3 clones were assayed for gelatinase (Fig. 6). The rat plasma contained gelatinolytic metalloproteinases of Mr 92,000 and Mr 64,000, and their levels did not change over the 40 days after intra-fat pad injection of poorly metastatic MTC cells. In contrast, the Mr 92,000 progelatinase level continuously increased until 30 days after cell inoculation or until rats became moribund (Fig. 6).

**DISCUSSION**

We previously reported that increased amounts of rat serum type IV collagenolytic activity is associated with high numbers of 13762NF mammary adenocarcinoma metastases in lungs and lymph nodes (14). We now demonstrate that the Mr 92,000 metalloproteinase activity in the sera and plasma of female F344 rats increases with time after injection of highly metastatic but not poorly metastatic 13762NF cells into the mammary fat pad. This metalloproteinase was identified as a Mr 92,000 progelatinase with the same substrate specificity and reactivity of anti-human Mr 92,000 progelatinase (MMP-9). The Mr 92,000 progelatinase levels in the sera and plasma of rats bearing MTLn3 fat pad tumors correlated with the extent of metastases. The sera from rats with large numbers of metastases in the lung and/or lymph nodes had much higher levels of Mr 92,000 progelatinase than the sera from rats with few or no metastases. In contrast, sera from rats bearing mammary adenocarcinoma of low metastatic potential, such as clones MTC and MTLn2, contained low levels of this enzyme, even 30 days after tumor cell injection and extensive growth of the tumor at the primary site in the mammary fat pad. These results suggest that the high levels of serum and plasma Mr 92,000 progelatinase are probably due to the presence of metastatic adenocarcinoma cells. Since proenzymes of metalloproteinases can be activated by tumor cell surface serine proteinases or cysteine proteinases (1–8), a high level of Mr 92,000 progelatinase in plasma could contribute to extravasation of tumor cells circulating in blood and hence enhance blood-borne metastasis. A Mr 72,000 type IV collagenase/progelatinase is produced by a variety of normal and malignant cells (4) and has been purified from...
baggy cells (28). The expression of M, 95,000 progelatinase was also shown to be associated with high metastatic potential of murine colon carcinoma cells (29).

The levels of Mf, 92,000 and Mf, 72,000 progelatinases secreted by human colon carcinoma KM12 cells correlated with the spontaneous metastatic potential of KM12 cells (30). KM12SM cells spontaneously metastasize to liver from the intracaval wall but not from the subcutis in nude mice. We have found that the expression of both Mf, 92,000 and Mf, 72,000 progelatinases is modulated by organ microenvironment: down-regulated in the subcutis and up-regulated in the cecal wall (31). These results suggest that the regulation of colon carcinoma metalloproteinase expression by organ-derived factors may affect the ability of the colon carcinoma cells to degrade basement membrane and metastasize. We also found that transforming growth factor-β treatment of MTLn3 cells stimulated progelatinase production 2- to 4-times and resulted in a significant enhancement of lung colonization after i.v. MTLn3 cell injection (32).

The Mf, 92,000 and Mf, 72,000 progelatinases are normal components of human plasma (33), but whether a change in their levels is associated with any physiological or pathogenic condition has not been established. We detected both Mf, 92,000 and Mf, 72,000 progelatinases in sera and plasma of breast cancer patients as well as normal adults by zymography. Unlike human serum and plasma, rat serum and plasma contain only low levels of Mf, 92,000 progelatinase as a normal component, and no significant Mf, 72,000 progelatinase activity was detected in sera and plasma from rats bearing mammary adenocarcinoma metastases.

Using ELISA, a correlation of serum Mf, 72,000 progelatinase (MMP-2) levels with lung cancer metastases has been found by Garbisa et al. (34). They noticed that the plasma showed higher variability compared to the corresponding sera and performed their assays using the 10X diluted serum samples. Zucker et al. (35) developed the reliable assay of human plasma Mf, 72,000 progelatinase using ELISA and showed significant increase of plasma Mf, 72,000 progelatinase in the second half of pregnancy. Zucker et al. (36) have utilized their newly developed ELISA for plasma Mf, 92,000 progelatinase measurements and have recently reported that the plasma Mf, 92,000 progelatinase was significantly increased in patients with gastrointestinal tract and breast cancers. However, the concentrations of Mf, 92,000 progelatinase were not significantly higher in patients with metastatic disease than those with nonmetastatic cancer.

We found that the Mf, 92,000 progelatinase was quite stable in serum and plasma. There was no significant loss of activity after a 30-min treatment at 56°C or during storage at 23°C for 3 days, 4°C for 3 months, -23°C for 2 years, or -70°C for 6 years. We were also able to detect high activities of Mf, 92,000 progelatinase in the sera of breast cancer patients that had been stored at -60°C for 29 years. Therefore, the serum Mf, 92,000 progelatinase may be a useful marker for monitoring patients with certain types of malignant disease, such as breast cancer. Since normal human plasma also contains variable amounts of Mf, 92,000 progelatinase, further studies are necessary to evaluate the potential significance of the Mf, 92,000 progelatinase in the diagnosis of human malignant disease.

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Motowo Nakajima, Danny R. Welch, Dianna M. Wynn, et al.


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