Differential Regulation of MMP-9 and TIMP-2 Expression in Malignant Melanoma Developed in Metallothionein/RET Transgenic Mice

Masami Asai,1 Masashi Kato,2 Naoya Asai,1 Toshihide Iwashita,1 Hideki Murakami,1 Kumi Kawai,1 Izumi Nakashima2 and Masahide Takahashi1, 3

1Department of Pathology and 2Department of Immunology, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550

We recently established a metallothionein-I(MT)/RET transgenic mouse line in which skin melanosis, benign melanocytic tumor and malignant melanoma develop stepwise. Malignant melanoma cells but not benign melanocytic tumor cells had metastatic ability in transgenic mice. In the present study, we investigated the expression of several matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs), including MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MT1-MMP, TIMP-1 and TIMP-2, in these tumors. Western and northern blot analyses revealed that malignant transformation of melanocytic tumors developed in MT/RET transgenic mice accompanied with upregulation of MMP-9 and downregulation of TIMP-2. Expression of other MMP and TIMP genes examined was very low or undetectable in both benign and malignant tumors. Since activation of MMP-9 in malignant tumors was detected by gelatin zymography, these results suggest that imbalance of expression of the MMP-9 and TIMP-2 genes might be associated with metastatic ability of melanoma cells developed in MT/RET transgenic mice.

Key words: RET — Transgenic mice — Malignant melanoma — MMP-9 — TIMP-2

By introducing the RET recombinant oncogene (RFP/RET) fused to the mouse metallothionein-I (MT) promoter-enhancer, we previously established three transgenic mouse lines (designated lines 192, 304 and 319) in which skin melanosis and benign melanocytic tumors develop stepwise.1-3) In these lines, benign melanocytic tumors grew slowly and did not metastasize. In addition, development of malignant melanoma was very rare in MT/RET transgenic mice.

We have recently generated a new transgenic mouse line by crossing line 304 with C57BL/6 mice (designated line 304/B6) in which benign melanocytic tumors frequently progress to malignant melanoma.4) We observed that benign melanocytic tumors, which grew very slowly for several months, suddenly began to grow rapidly in more than 60% of 304/B6 mice, showing the histological appearance of malignant melanoma. The malignant tumors metastasized to lymph nodes, brain, lung, kidney, liver and spleen.4) The malignant transformation of tumor cells was accompanied with increased expression of the RFP/RET transgene, mitogen-activated protein kinases (MAPKs) and c-Jun.

In the present study, we further investigated the expression of several matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) in benign melanocytic tumors and malignant melanomas developed in 304/B6 mice. We found that, in addition to upregulation of MMP-9 expression, the TIMP-2 expression is drastically decreased in malignant melanoma cells.

MATERIALS AND METHODS

Metallothionein/RET transgenic mouse Two MT/RET transgenic mouse lines (designated lines 192 and 304) that develop skin melanosis and benign melanocytic tumors were previously described.1) By crossing line 304 with C57BL/6 mice, a new subline (designated line 304/B6) in which benign melanocytic tumors frequently progress to malignant melanoma was established.5) cDNA cDNAs for MMP-1, MMP-2, MMP-3, MMP-7, membrane-type (MT)1-MMP, TIMP-1 and TIMP-2 genes were kindly provided by Dr. Shimizu (Aichi Cancer Center Research Institute, Nagoya). E1AF cDNA was provided by Dr. Fujinaga (Sapporo Medical School, Sapporo).

RNA probes for northern blot analysis and in situ hybridization The sense- and antisense-cDNAs were inserted into the pGEM plasmids and linearized by enzyme digestion. Digoxigenin (DIG)-labelled riboprobes were generated using the SP6 and T7 promoters according to the manufacturer’s protocol (Boehringer Mannheim, Mannheim, Germany). Western blot analysis Total cell lysates were prepared from tumor tissues as described previously.5) The lysates were subjected to sodium dodecyl sulfate (SDS)/8% polyacrylamide gel electrophoresis and transferred to polyvi-
as clear zones against a blue background.

**Zymographic analysis** Zymograms demonstrating gelatinolytic activity in unreduced samples were prepared by incorporating 2 mg/ml gelatin (Difco Laboratories, Detroit, MI) into 8% polyacrylamide gels. Prior to electrophoresis, samples (50 µg) were prepared by incubating with 2% blocking reagent in 50 mM NaCl, 0.1% SDS at room temperature and with water for 5 min three times to remove SDS. Then the gels were incubated overnight in 50 mM Tris-HCl (pH 7.5) containing 2.5% Triton X-100 for 30 min at room temperature twice and with water for 5 min three times to remove SDS. Following staining with Coomassie blue, regions of proteolytic activity were visualized as clear zones against a blue background.

**RESULTS**

We previously established transgenic mouse lines that develop melanocytic tumors by introducing the RFP/RET fusion gene linked to the mouse metallothionein-I promoter- enhancer (MT/RET).1-4 Tumor tissues were obtained from two MT/RET transgenic mouse lines (lines 192 and 304/B6). Transgenic mice of line 192 develop only benign melanocytic tumors (Fig. 1a), whereas benign tumors in mice of line 304/B6 (Fig. 1b) frequently progress to malignant melanoma with metastatic ability (Fig. 1, c and d). Total cell lysates for western blotting and RNAs for northern blotting were prepared from
Fig. 1. Histopathology of tumors developed in \textit{MT/RET} transgenic mice. (a) A benign melanocytic tumor in the skin of a line 192 mouse. (b) A benign melanocytic tumor in the skin of a line 304 mouse. (c) Malignant melanoma in the skin of a line 304/B6 mouse. (d) Lung metastasis of melanoma cells in a line 304/B6 mouse. Bars represent 70 µm.

Fig. 2. Expression of Ret, MMP-9 and TIMP-2 in melanocytic tumors of \textit{MT/RET} transgenic mice. Total cell lysates (50 µg) from two benign melanocytic tumors of line 192 mice (lanes 1 and 2), two benign melanocytic tumors of line 304/B6 mice (lanes 3 and 4) and two malignant tumors of line 304/B6 mice (lanes 5 and 6) were analyzed by western blotting with anti-Ret antibody, anti-phosphotyrosine (pTyr) antibody, anti-MMP-9 antibody or anti-TIMP-2 antibody. 100 kDa Rfp/Ret, 92 kDa MMP-9 and 21 kDa TIMP-2 proteins are indicated.
benign tumors of line 192 mice and from both benign and malignant tumors of line 304/B6 mice. The sizes of benign and malignant tumors were 50–100 mm$^3$ and $>$50,000 mm$^3$, respectively.

As we have recently reported, expression of the Rfp/Ret protein and its tyrosine phosphorylation were significantly increased in malignant melanomas from line 304/B6 mice compared with those in benign tumors from lines 192 and 304/B6 mice (Fig. 2). Interestingly, when expression of MMP-9 and TIMP-2 was investigated by western blotting, marked increase of MMP-9 expression and decrease of TIMP-2 expression were observed in malignant melanomas. Decrease of the TIMP-2 expression in a malignant tumor was confirmed by in situ hybridization, whereas a benign tumor in a 304/B6 mouse showed a high level of TIMP-2 expression (Fig. 3).

We further investigated the expression of several MMPs and TIMPs including MMP-1, MMP-2, MMP-3, MMP-7, MT1-MMP, TIMP-1 and TIMP-2 by northern blotting. The expression of these genes, except TIMP-2, was undetectable in both benign and malignant tumors by northern blotting (Fig. 4a and data not shown), although the transcripts of the MMP-2 and MT1-MMP genes were detected in both by RT-PCR (Fig. 4b). In addition, since it was reported that the Ets-related E1AF gene regulates the expression of the MMP genes,7) its expression was examined in each tumor by northern blotting. As shown in Fig. 4a, E1AF was expressed at similar levels in both benign and malignant tumors.

Finally, activation of MMP-2 and MMP-9 was analyzed by gelatin zymography. Upregulation of enzymatic activity of MMP-9, but not that of MMP-2, was clearly detected in malignant tumors and their culture medium (Fig. 5, lanes 3 and 6), although the activity of MMP-2 was observed in serum from transgenic mice with either benign or malignant tumors (Fig. 5, lanes 7 to 9).
DISCUSSION

In the present study, we investigated the expression of MMPs and TIMPs in melanocytic tumors developed in two MT/RET transgenic mouse lines (lines 192 and 304/B6). Line 192 mice develop only benign melanocytic tumors whereas line 304/B6 mice frequently develop malignant melanoma by progression from benign tumors. We found that malignant transformation of melanocytic tumors in 304/B6 mice was accompanied with upregulation of MMP-9 and downregulation of TIMP-2, among various MMPs and TIMPs examined. Activation of MMP-9 was confirmed by gelatin zymography as previously described. Since many studies suggesting a correlation between malignant progression or metastatic potential of tumor cells and overexpression of MMP-9 have been

Fig. 4. Analyses of expression of MMPs and TIMPs by northern blotting and RT-PCR. (a) Total RNAs (10 µg) isolated from two benign melanocytic tumors of line 192 mice (lanes 1 and 2), two benign melanocytic tumors of line 304/B6 mice (lanes 3 and 4) and two malignant tumors of line 304/B6 mice (lanes 5 and 6) were analyzed by northern blotting with TIMP-2, E1AF and β-actin probes. The 3.5 kb and 1.0 kb transcripts of the TIMP-2 gene and 2.5 kb transcripts of the E1AF gene are indicated. (b) Expression of the MT1-MMP, MMP-2 and β-actin genes was analyzed by RT-PCR.

Fig. 5. MMP-9 activity in tumors of MT/RET transgenic mice. The MMP-9 and MMP-2 activities in tumor tissues (lanes 1 to 3), their culture media (lanes 4 to 6) and serum (lanes 7 to 9) were analyzed by gelatin zymography. A benign melanocytic tumor of a line 192 mouse (lanes 1, 4 and 7), a benign melanocytic tumor of a line 304/B6 mouse (lanes 2, 5 and 8) and a malignant tumor of a line 304/B6 mouse (lanes 3, 6 and 9) were used in this assay. Proenzyme (a) and activated form (b) of MMP-9 and proenzyme (c) of MMP-2 are indicated.
reported,6–14) it is possible that upregulation of MMP-9 is associated with the metastatic ability of malignant tumors of 304/B6 mice.

It is noteworthy that expression of the TIMP-2 gene was drastically downregulated in malignant melanoma of 304/B6 transgenic mice. TIMP-2 is known to regulate the function of MT1-MMP, that can activate MMP-2.15,16) Thus, we examined the expression of MT1-MMP and MMP-2 in tumors of MT1 RET transgenic mice. Analyses by northern blotting and RT-PCR revealed that expression of these two genes was low in both benign and malignant tumors. Although we have recently reported the activation of MMP-2 in malignant tumors of MT1 RET transgenic mice by gelatin zymography,17) its activation was unclear in the tumors as well as in their culture medium in the present study, suggesting that it may not be crucial for the metastatic ability of malignant melanoma cells in our transgenic mice. Rather, the expression and activation level of MMP-2 could depend on the individual tumors examined and downregulation of TIMP-2 may play a role in the activation of MMP-2.

At present, we do not know how MMP-9 and TIMP-2 gene expression is regulated in tumors of transgenic mice. Although we investigated the expression of the EIAF gene, that was reported to regulate MMP expression,7) it was expressed at similar levels in both benign and malignant tumors. Thus, it seems unlikely that the EIAF gene is involved in the regulation of MMP-9 and TIMP-2 expression in malignant transformation of tumor cells in our transgenic mice. Since the Rfp/RET, MAPK and c-Jun expression was markedly upregulated in malignant tumors of 304/B6 mice,9) this suggests that the intracellular signaling through Rfp/RET may be important for upregulation of MMP-9 and downregulation of TIMP-2. Further investigation will be required for elucidation of the mechanisms of the aberrant expression of MMP-9 and TIMP-2 in melanoma cells.

Our transgenic mice are interesting model animals in which benign melanocytic tumors spontaneously progress to malignant melanoma. This transgenic line should provide a useful system to study the mechanisms of malignant transformation and metastasis of tumor cells in vivo.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for COE Research, Scientific Research and Cancer Research from the Ministry of Education, Science, Sports and Culture of Japan. We are grateful to K. Kozaki for helpful discussions and to K. Imaizumi, K. Uchiyama and M. Kozuka for technical assistance.

(Received August 24, 1998/Revised October 7, 1998/Accepted

REFERENCES

1) Iwamoto, T., Takahashi, M., Ito, M., Hamatani, M., Ohbayashi, M., Wajiwalku, W., Isobe, K. and Nakashima, I. Ablerrant melanogenesis and melanocytic tumor development in transgenic mice that carry a metallocysteine/ret fusion gene. EMBO J., 10, 3167–3175 (1991).

2) Iwamoto, T., Takahashi, M., Ohbayashi, M. and Nakashima, I. The ret oncogene can induce melanogenesis and melanocyte development in W/Wv mice. Exp. Cell Res., 200, 410–415 (1992).

3) Takahashi, M., Iwamoto, T. and Nakashima, I. Proliferation and neoplastic transformation of pigment cells in metallocysteine/ret transgenic mice. Pigment Cell Res., 5, 344–347 (1992).

4) Kato, M., Takahashi, M., Akhand, A. A., Liu, W., Shimizu, S., Iwamoto, T., Suzuki, H. and Nakashima, I. Transgenic mouse model for skin malignant melanoma. Oncogene, 17, 1885–1888 (1998).

5) Asai, N., Iwashita, T., Matsuyama, M. and Takahashi, M. Mechanism of activation of the ret proto-oncogene by multiple endocrine neoplasia 2A mutations. Mol. Cell. Biol., 15, 1613–1619 (1995).

6) Sakata, K., Kozaki, K., Iida, K., Tanaka, R., Yamagata, S., Utsumi, K. R., Saga, S., Shimizu, S. and Matsuyama, M. Establishment and characterization of high- and low-lung-metastatic cell lines derived from murine colon adenocarcinoma 26 tumor line. Jpn. J. Cancer Res., 87, 78–85 (1996).

7) Higashino, F., Yoshida, K., Norumi, T., Seiki, M. and Fujinaga, K. Ets-related protein EIA-F can activate three different matrix metalloproteinase gene promoters. Oncogene, 10, 1461–1463 (1995).

8) Bernhard, E. J., Muschel, R. J. and Hughes, E. N. Mr 92,000 gelatinase release correlates with the metastatic phenotype in transformed rat embryo cells. Cancer Res., 50, 3872–3877 (1990).

9) Houde, M., De Bruyne, G., Bracke, M., Ingelman-Sundberg, M., Skoglund, G., Masure, S., Van Damme, J. and Opdenakker, G. Differential regulation of gelatinase B and tissue-type plasminogen activator expression in human bowes melanoma cells. Int. J. Cancer, 53, 395–400 (1993).

10) Hujanen, E. S., Vaisanen, A., Zheng, A., Tryggvason, K. and Turpeenniemi-Hujanen, T. Modulation of Mr 72,000 and Mr 92,000 type-IV collagenase (gelatinase A and B) gene expression by interferons alpha and gamma in human melanoma cells. Int. J. Cancer, 58, 582–586 (1994).

11) Kawashima, A., Nakanishi, I., Tsuchiya, H., Roessner, A., Obata, K. and Okada, Y. Expression of matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) induced by tumour necrosis factor α correlates with metastatic ability in a human osteosarcoma cell line. Virchows Arch., 424, 547–552 (1994).
12) MacDougall, J. R., Bani, M. R., Lin, Y., Rak, J. and Kerbel, R. S. The 92-kDa gelatinase B is expressed by advanced stage melanoma cells: suppression by somatic cell hybridization with early stage melanoma cells. *Cancer Res.*, 55, 4174–4181 (1995).

13) Gouon, V., Tucker, G. C., Kraus-Berthier, L., Atassi, G. and Kieffer, N. Up-regulated expression of the β3 integrin and the 92-kDa gelatinase in human HT-144 melanoma cell tumors grown in nude mice. *Int. J. Cancer*, 68, 650–662 (1996).

14) van den Oord, J. J., Paemen, L., Opdenakker, G. and De Wolf-Peeters, C. Expression of gelatinase B and the extracellular matrix metalloproteinase inducer EMMPRIN in benign and malignant pigment cell lesions of the skin. *Am. J. Pathol.*, 151, 665–670 (1997).

15) Stetler-Stevenson, W. G., Krutzsch, H. C. and Liotta, L. A. Tissue inhibitor of metalloproteinase (TIMP-2). *J. Biol. Chem.*, 264, 17374–17378 (1989).

16) Tokuraku, M., Sato, H., Murakami, S., Okada, Y., Watanabe, Y. and Seiki, M. Activation of the precursor of gelatinase A/72 kDa type IV collagenase/MMP-2 in lung carcinomas correlates with the expression of membrane-type matrix metalloproteinase (MT-MMP) and with lymph node metastasis. *Int. J. Cancer*, 64, 355–359 (1995).

17) Sato, H., Takino, T., Kinoshita, T., Imai, K., Okada, Y., Stetler-Stevenson, W. G. and Seiki, M. Cell surface binding and activation of gelatinase A induced by expression of membrane-type-1 matrix metalloproteinase. *FEBS Lett.*, 385, 238–240 (1996).

18) Imai, K., Ohuchi, E., Aoki, T., Nomura, H., Fujii, Y. Sato, H., Seiki, M. and Okada, Y. Membrane-type matrix metalloproteinase 1 is a gelatinolytic enzyme and is secreted in a complex with tissue inhibitor of metalloproteinase 2. *Cancer Res.*, 56, 2707–2710 (1996).