[CANCER RESEARCH 55, 1629–1632, April 15, 1995]

Advances in Brief

Tissue Factor-initiated Thrombin Generation Activates the Signaling Thrombin Receptor on Malignant Melanoma Cells[1]

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Abstract

The human melanoma cell line M24met expresses tissue factor, the cellular initiator of the blood coagulation cascade. Blocking of the coagulation pathways at the level of tissue factor, factor Xa, or thrombin inhibits hematogenous M24met metastasis in SCID mice, implicating a role for thrombin generation in this process. Dependent on cell surface tissue factor activity, M24met cells generate thrombin *in vitro*. Thrombin and the thrombin receptor agonist peptide TRP-14 activate a signaling pathway in M24met cells that involves an increase in intracellular calcium and induces cell proliferation. Immunofluorescence evidences expression of the signaling thrombin receptor on these cells. Thus, M24met melanoma cells express both the initiating cell surface receptor for the coagulation pathways and the central signaling receptor of the coagulation system, suggesting the *in situ* generation of proliferative signals which can contribute to the malignant phenotype.

Introduction

Expression of procoagulant activity by malignant cells is a well established phenomenon (1–5) and molecular components of the blood coagulation cascade were shown to be involved in the development of tumor metastasis (6–8). The molecular assembly of coagulation factors on the surface of tumor cells was reported to lead to the generation of thrombin *in vitro* (9). Thrombin, in turn, regulates the behavior of a variety of cell types, including endothelial cells, platelets, and leukocytes (10). Adhesion of tumor cells to platelets (7), endothelium (8, 11), and the subendothelial matrix (11) is also stimulated by thrombin.

It has been distinguished between TF[3]-dependent and -independent activation of coagulation in malignancy (3), with a cysteine proteinase referred to as cancer procoagulant among the latter (4). Human malignant melanoma cells express TF (12), also referred to as thromboplastin, which initiates the coagulation pathways by serving as the cellular receptor and cofactor of the serine protease coagulation factor VIIa. Earlier, we showed that TF activity promotes hematogenous metastasis of human melanoma cells in SCID mice (12). Here, we address the question of whether TF-driven initiation of the coagulation pathways will influence cellular functions of human melanoma. We demonstrate that: (*a*) *in vivo*, inhibition of thrombin generation or blocking of thrombin function alters hematogenous metastasis of M24met cells in SCID mice; (*b*) *in vitro*, M24met cells convert prothrombin to biologically active thrombin in the presence of the coagulation factors V, VII and X; (*c*) thrombin, as well as the thrombin receptor agonist peptide TRP-14, functions as a growth factor for these cells; and (*d*) the biological response of M24met cells to thrombin is most likely due to the presence of the signaling thrombin receptor.

Materials and Methods

Materials. Thrombin (specific activity, 4500–5500 units/mg; 1 unit/ml corresponds to 5 nM) was purchased from Calbiochem (La Jolla, CA). Human coagulation factors II (prothrombin), VII, V, and X, as well as ionomycin and pertussis toxin were obtained from Calbiochem. Recombinant hirudin, human antithrombin III, and α_2-macroglobulin were a gift from Dr. G. Schulz (Behringwerke, Marburg, Germany). Recombinant TAP was generously provided by Dr. G. P. Vlasuk (Corvas, Inc., San Diego, CA). TRP-14 and the control peptide FSLLRNPNDKYEPF were kindly provided by Dr. J. Geltosky (R. W. Johnson Research Institute, La Jolla, CA).

Antibodies. Murine anti-human TF mAbs 5G9, 10H10, and 6B4 have been described previously (12, 13). Polyclonal rabbit IgG 1809 against the hirudin-like domain of the human thrombin receptor (14) was a generous gift from Dr. S. R. Coughlin (University of California at San Francisco, San Francisco, CA).

Cell Culture. M24met human melanoma cells (15) were cultured in RPMI 1640 with 10% fetal bovine serum and 2 mM glutamine and passaged using 0.5 mM EDTA-150 mM NaCl-20 mM HEPES (Versene).

Experimental Metastasis. Female, 6-week-old C.B 17 *scid/scid* mice were given i.v. injections of 0.5×10^6 M24met cells preincubated with 1 mg mAb 5G9 or 6B4; mice were sacrificed 3 weeks later and tumor foci on the surface of the lungs were counted. Alternatively, animals were systemically anticoagulated by i.p. injection of either 10 mg TAP or 0.5 mg hirudin. After 45 min, 0.5×10^6 M24met cells were injected i.v. and lung metastasis was determined 3 weeks later.

Proliferation Assay. Assays were carried out in tissue culture-treated flat-bottomed 96-well plates (Corning, New York, NY). Cells were harvested and washed with serum-free RPMI 1640, and 7000 cells/well were incubated in the presence of coagulation factors, thrombin, peptide, and/or inhibitors for 48 h at 37°C and 7% CO_2. After incubation with 1 μCi [^{3}H]thymidine (Amersham, Arlington Heights, IL) for another 16 h, cells were harvested, and radioactivity was determined in a beta counter. Under these conditions, unstimulated cells incorporated 15,000–20,000 cpm.

Measurement of Cytosolic Free Calcium $[Ca^{2+}]_i$. M24met cells were washed in serum-free RPMI 1640–1% BSA-20 mM HEPES, pH 7.4, and loaded with 5 μM Indo-1-acetoxymethyl (AM) ester (Molecular Probes, Eugene, OR) for 30 min at 37°C. After a washing, cells were resuspended at 1×10^6 cells/ml in HBSS (containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.05). The ratio of fluorescence emission at 400 and 480 nm was detected in an SLM fluorometer (SLM Instruments, Inc., Urbana, IL) at 37°C under stirring conditions and an excitation wavelength of 380 nm. $[Ca^{2+}]_i$ was calculated as described (16).

Flow Cytometry. Cells in suspension were incubated with thrombin or TRP-14 in serum-free RPMI 1640–1% BSA-20 mM HEPES, pH 7.4, for 10 min at 37°C. Cells were then washed and incubated on ice with polyclonal anti-thrombin receptor IgG 1809 (1 μg/ml) in PBS-0.1% BSA-0.02% azide, pH 7.4, for 1 h; washed; stained with fluorescein-conjugated goat anti-rabbit IgG F(ab')$_2$ (Tago, Burlingame, CA); and analyzed by flow cytometry on a Becton Dickinson FACScan.

Received 12/21/94; accepted 3/6/95.

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[1] Supported by NIH Grants CA59692 (B. M. M.) and HL16411 (W. R.). E. G. F. was supported by a fellowship from the Deutsche Forschungsgemeinschaft and B. M. M. is the recipient of a Junior Faculty Research Award from the American Cancer Society. This is Scripps Research Institute Manuscript 9080-IMM.

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[3] The abbreviations used are: TF, tissue factor; $[Ca^{2+}]_i$ intracellular free calcium; SCID, severe combined immunodeficiency; TAP, tick anticoagulant protein; TRP-14, thrombin receptor agonist peptide.

Results

The activity of TF, factor Xa, and thrombin was found to be involved in metastasis formation in the M24met melanoma model. Preincubation of M24met cells with two different inhibitory anti-TF mAbs, 5G9, which blocks the TF-VIIa complex, and 6B4, which competes with factor VIIa binding, resulted in reduction of experimental metastasis (Table 1). Systemic anticoagulation of mice was achieved with either the factor Xa inhibitor TAP or the thrombin inhibitor hirudin. Injection i.p. of 10 mg TAP or 0.5 mg hirudin resulted after 4 h in a 94.9 or 80% inhibition, respectively, of TF-induced coagulation, as measured in a one-stage clotting assay. Experimental pulmonary M24met metastasis was significantly reduced when the melanoma cells were injected i.v. into SCID mice 45 min after i.p. administration of either 10 mg TAP or 0.5 mg hirudin (Table 1).

In order to assess *in vitro* the effect of TF-induced activation of the coagulation pathway, M24met cells were added to a mixture of the zymogen factors V, VII, and X at concentrations of 0.03, 0.1, and 0.17 nM, respectively. In the presence of prothrombin (factor II), a dose-dependent increase in proliferation (Fig. 1*a*) was observed at an optimal prothrombin concentration of 0.4 μM. Prothrombin alone (Fig. 1*a*) or solely the combination of factors V, VII, and X had no mitogenic effect (data not shown). The specific thrombin inhibitor hirudin prevented the proliferative response. Thrombin generation appeared to be dependent on TF function, because a mitogenic stimulus was not observed in the absence of factor VII or in the presence of the inhibitory anti-TF antibody 5G9. The proliferative response was not affected by the nonneutralizing mAb 10H10 (Fig. 1*a*). Thrombin itself and the thrombin receptor agonist peptide TRP-14 induced mitogenesis in M24met cells in a dose-dependent fashion (Fig. 1*b*). The maximum effect was seen at a thrombin concentration of 900 milliunits/ml or 4.5 nM. This concentration is within the range of physiological thrombin levels; *e.g.*, the thrombin concentration in human plasma is 2–5 nM (10). Hirudin prevented the thrombin-mediated melanoma cell proliferation (data not shown).

Stimulation of human melanoma cell growth by thrombin and TRP-14 suggested the presence of the signal-transducing thrombin receptor on these cells. To support this contention, mobilization of intracellular Ca^{2+} was measured as an early event in the activation of cells by the thrombin receptor. Stimulation with thrombin or TRP-14 revealed a dose-dependent transient $[Ca^{2+}]_i$ increase (Fig. 2, *a* and *b*). The thrombin effect was abolished by hirudin (Fig. 2*c*) and, as well, by two other thrombin inhibitors α_2-macroglobulin and antithrombin III (in the presence of heparin). The $[Ca^{2+}]_i$ response was characterized by an initial sharp peak and a second sustained phase (Fig. 2*a*). The initial peak is most likely mobilization of intracellular Ca^{2+} stores. The second sustained phase, which could be prevented by addition of 5 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid to the buffer (Fig. 2*d*) is due to an influx of Ca^{2+} across the cell membrane (17). Pretreatment of the cells with pertussis toxin (100 ng/ml, 24 h) yielded an unimpaired response. Mobilization of $[Ca^{2+}]_i$ was not observed after addition of the control peptide; prothrombin; factors VII, VIIa, X, and Xa; or mAbs 10H10 and 5G9 (data not shown).

Table 1 *Experimental pulmonary metastasis of M24met melanoma cells in SCID mice in the presence of anti-TF antibodies, the factor Xa inhibitor TAP, or hirudin*

Treatment	No. of pulmonary foci	P^a
Experiment 1		
Saline	17, 19, 29, 51, 63, 75	
mAb 5G9	0, 0, 0, 0, 0, 1	0.00216
mAb 6B4	2, 4, 6, 7, 8, 9	0.00216
Experiment 2		
Saline	18, 19, 20, 39, 48, 59	
TAP	0, 1, 7, 7, 7, 15	0.00216
Hirudin	0, 1, 1, 3, 5, 11	0.00216

[a] Probability of no difference to the saline-treated control group in the nonparametric Wilcoxon rank sum test.

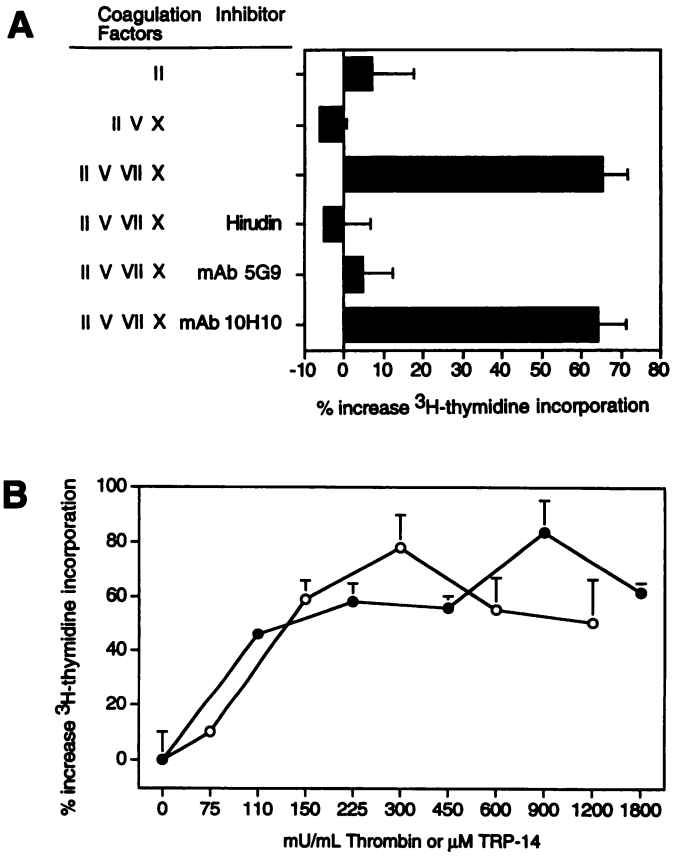

Fig. 1. Proliferation of M24met melanoma cells. Cells were incubated with 0.4 μM prothrombin (factor II) and additional coagulation factors, as indicated. Factor V was added at 0.03 nM, VII at 0.1 nM, X at 0.17 nM, hirudin at 1 μM, and anti-TF-mAbs 5G9 and 10H10 at 20 μg/ml (*A*). Alternatively, cells were incubated with thrombin (●) or TRP-14 peptide (○) (*B*). [^{3}H]Thymidine incorporation was determined, as described. Values are given as percentage of untreated controls and represent means of triplicate samples. *Bars*, SD.

To determine whether thrombin and TRP-14 interact with the same receptor, we assessed for heterologous receptor desensitization. Stimulation of M24met cells with TRP-14 prior to the addition of thrombin prevented an appropriate $[Ca^{2+}]_i$ response, whereas the influx by the ionophore ionomycin was not affected (Fig. 2*e*). We also observed heterologous desensitization for TRP-14 after thrombin stimulation. Homologous desensitization for thrombin was seen after thrombin stimulation, as well as for the TRP-14 peptide after peptide stimulation (data not shown).

Indirect immunofluorescence demonstrated the presence of the thrombin receptor molecule on the surface of M24met cells (Fig. 3). Flow cytometry analysis showed staining of M24met cells with anti-thrombin receptor antibodies that decreased after stimulation of the cells with TRP-14 or thrombin consistent with receptor internalization (18, 19).

Discussion

Functional intervention in the blood coagulation pathway at the level of TF, factor Xa, or thrombin affects experimental melanoma metastasis. As recently demonstrated, blocking of the TF-VIIa complex by mAb 5G9 inhibits pulmonary metastasis of human melanoma cells in SCID mice (12). We demonstrate here that competition of factor VIIa binding to TF by mAb 6B4 is also sufficient to inhibit

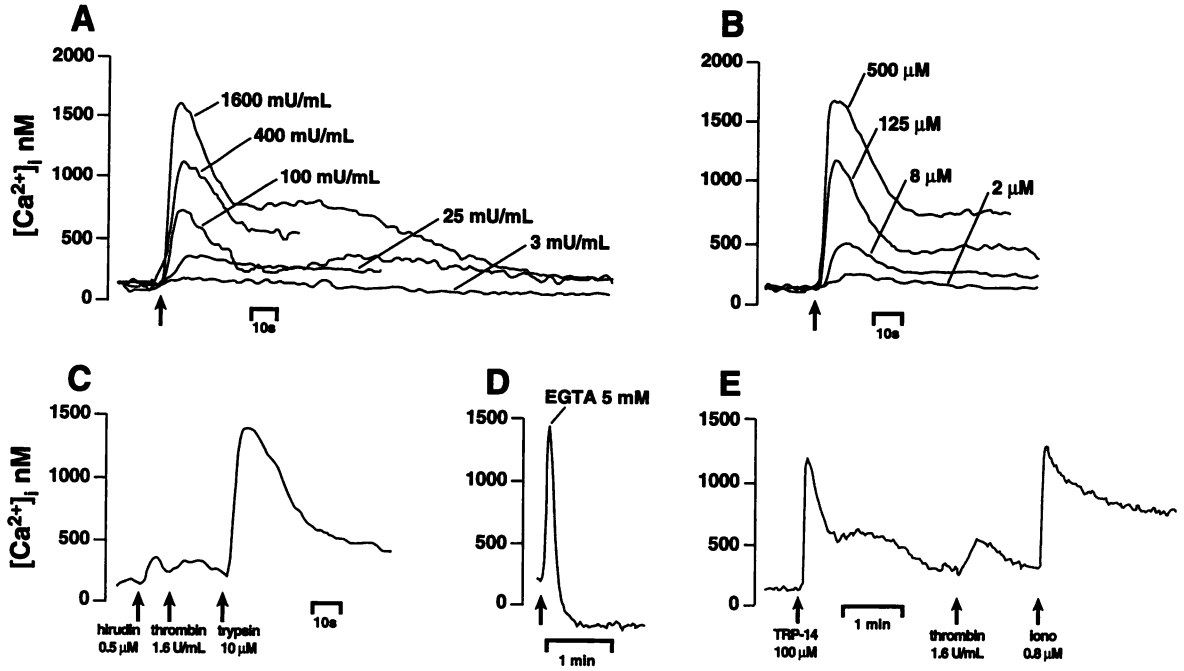

Fig. 2. Increase of $[Ca^{2+}]_i$ in Indo-1-AM-loaded M24met melanoma cells. Dose-dependent response to thrombin (*A*) or TRP-14 (*B*). Hirudin (0.5 μM) prevents thrombin- but not trypsin-induced $[Ca^{2+}]_i$ increase (*C*). Thrombin-induced $[Ca^{2+}]_i$ increases with addition of 5 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (*D*). *E*, $[Ca^{2+}]_i$ response to thrombin after prior stimulation with TRP-14.

metastasis. Furthermore, the Xa-specific inhibitor TAP and the thrombin inhibitor hirudin can effectively prevent experimental melanoma metastasis. Similarly, antimetastatic properties of hirudin (6) and antistasin, a protein from the Mexican leech with Xa-inhibiting potential (20), have been reported earlier. Together, these results suggest that hematogenous melanoma metastasis requires cell surface TF activity for localized thrombin generation.

TF activity on the surface of M24met melanoma cells initiates the assembly of the molecular machinery required for thrombin generation *in vitro*. Thrombin itself can elicit a broad spectrum of biological effects. Mechanisms like the activation of platelets and endothelial cells, as well as the generation of fibrin, have been implicated in cancer metastasis (1–5, 7, 8, 11). Here, we focus on direct effects of thrombin on metastatic tumor cells. We observed that thrombin, as well as TRP-14, has growth factor activity for M24met cells, suggesting that the signaling thrombin receptor is involved in the biological responses of these cells.

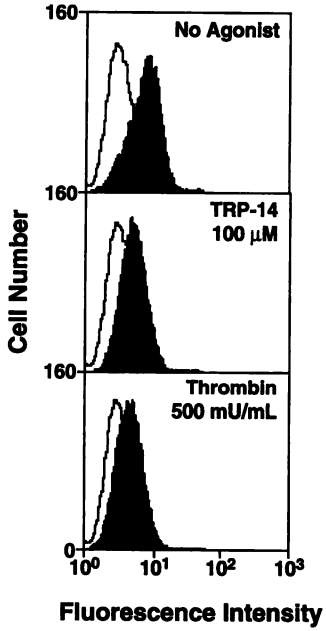

Fig. 3. Flow cytometry analysis. M24met melanoma cells were stained with either secondary goat anti-rabbit antibodies alone (*white histograms*) or polyclonal rabbit anti-thrombin-receptor IgG 1809 followed by secondary antibodies (*dark histograms*). Cells were pretreated, as indicated, with buffer, TRP-14, or thrombin for 10 min.

Molecular cloning of the signaling thrombin receptor (21, 22), a member of the seven transmembrane domain receptor family, revealed that thrombin activates its receptor by limited proteolysis. This exposes a new NH_2 terminus, which then activates the receptor as a tethered ligand. The peptide TRP-14 represents the 14 NH_2-terminal amino acids of the cleaved receptor and is sufficient for receptor activation (21). The pathway of cellular activation and induction of mitogenesis by thrombin is well characterized for fibroblasts and other cell types. It involves increase in $[Ca^{2+}]_i$ and activation of protein kinase C via the messengers inositol 1,4,5-triphosphate and diacylglycerol (23). In many cell types, thrombin and the agonist peptide evoke different biological responses in the same cell (17, 23, 24), suggesting involvement of distinct signaling pathways triggered by thrombin or TRP-14. In our experiments, the receptor agonist peptide and thrombin were similarly effective in triggering mitogenic responses and $[Ca^{2+}]_i$ mobilization. TRP-14 is mitogenic for fibroblasts only in association with fibroblast growth factor (23), due to its inability to trigger persistent stimulation of a mitogen-activated protein kinase (25). In contrast, M24met cells do not require an additional external stimulus to utilize the peptide as a growth factor. This may be due to the fact that melanoma cells produce a whole array of growth factors, including basic fibroblast growth factor, in an autocrine fashion (26, 27).

In summary, we demonstrate that M24met melanoma cells express the signaling thrombin receptor and that through this receptor, thrombin acts as a growth factor for these cells. This finding adds a new

aspect to our understanding how procoagulant activity of tumor cells can contribute to the malignant phenotype.

Acknowledgments

We are indebted to Drs. R. A. Reisfeld and T. S. Edgington for their support and encouragement. We thank all our colleagues who provided reagents for this study and Dr. I. U. Schraufstätter for discussion and generous access to the fluorometer. We are grateful to M. D. Pfeifer for technical assistance and to L. A. Kottel for preparation of the manuscript.

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Cancer Res 1995;55:1629-1632.