Unexpected Effect of Matrix Metalloproteinase Down-Regulation on Vascular Intravasation and Metastasis of Human Fibrosarcoma Cells Selected In vivo for High Rates of Dissemination

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Abstract

The human tumor/chick embryo model involving grafting of human HT-1080 fibrosarcoma cells on the chorioallantoic membrane was used in conjunction with quantitative real-time Alu PCR to select in vivo a pair of isogenic cell lines (HT-hi/diss and HT-lo/diss), dramatically differing in their ability to disseminate from the primary tumor (i.e., intravasate into the chorioallantoic membrane vasculature and metastasize to the lungs). During an immunohistochemical time course study, HT-hi/diss cells were sequentially visualized having escaped from the primary tumors, engaged with the blood vessels, and eventually observed inside the chorioallantoic membrane capillaries, thus reflecting early intravasating events. In contrast, HT-lo/diss cells seemed restricted to their primary tumor. Importantly, after i.v. inoculation, both variants arrested, extravasated, and proliferated in host tissues with similar efficiencies, highlighting that the observed earlier events at the periphery of the primary tumor could account for their differential dissemination. In a mechanistic probing of these events, we determined that HT-hi/diss intravasation was sensitive to a broad-range matrix metalloproteinase (MMP) inhibitor. To analyze the possible role of individual MMPs, membrane-bound MMP-14 and secreted MMP-9 were individually down-regulated in HT-hi/diss cells with their corresponding small interfering RNAs. Despite efficient down-regulation of MMP-14, neither intravasation nor metastasis of HT-hi/diss cells was affected significantly. However, a substantial down-regulation of MMP-9 was accompanied by a surprising 3-fold increase in intravasation and metastasis. The results emphasize a rising awareness that targeting certain MMPs might result in an enhanced malignancy, exemplified herein at the intravasation level as this step of the metastatic cascade is dissected and quantified. (Cancer Res 2005; 65(23): 10959-69)

Introduction

Tumor cell intravasation (i.e., entering of cancerous cells into the vasculature) is likely one of the rate-limiting yet least-studied steps in the metastatic cascade. Like metastasis itself, intravasation is an inefficient process. It is estimated that <0.01% of primary tumor cells are successful in colonizing secondary tissue (1, 2); thus, tumor cell intravasation likely would be a rare event, making it difficult to analyze.

Experimental models to study tumor cell intravasation in vivo mainly include visualization of cells entering the blood vasculature by real-time or time lapse confocal microscopy (3–5). Although technically sophisticated, these methods are often time consuming, require unique and costly equipment, involve tranquilizing of live animals, and do not allow for statistically significant number of animals or intravasation events. In addition, even in those few studied systems where tumor cells have been microscopically observed entering the vasculature, the route and fate of the intravasating cells as well as the rate and extent of intravasation generally were not quantified. In this regard, grafting tissues or organ rudiments onto the chorioallantoic membrane (CAM) of a developing chick embryo, invaluable in developmental studies (6, 7), has proven useful in cancer biology, especially in analysis of human tumor cell intravasation and spontaneous metastasis (8–16).

The CAM of the chick embryo is a readily accessible tissue, which is particularly rich in blood vessels and capillaries, allowing rapid vascularization, survival, and development of tumor cells or tissues placed on the dropped CAM. Within a few days after inoculation of highly aggressive human tumor cells on the CAM, visible tumors are formed and tumor cells can be identified in distant portions of the CAM (lower CAM), as well as in the internal organs of the embryo such as lungs, liver, sternum, and brain (10, 11, 13, 14, 17). Because the human genome is uniquely enriched in Alu sequences, a semiquantitative Alu PCR method was first introduced to detect and estimate numbers of human cells within chicken tissues (11) followed by sensitive real-time Alu PCR assays (13, 18, 19).

In the CAM tumor model, comparative analysis of a series of tumor cell lines, unrelated in origin, showed that they intravasated to the vasculature with different efficiencies (11). The intravasation process at the cell and molecular levels is difficult to analyze because isogenic tumor cell lines that manifest widely differing intravasating capacities are rare (3). By using the chick embryo CAM assay and real-time Alu PCR detection system, we selected in vivo two variants of the human fibrosarcoma HT-1080 cell line, which differ 50- to 100-fold in their ability to intravasate and thus to disseminate into secondary organs. One of these cell lines is a highly disseminating variant, designated here as HT-hi/diss, whereas another, HT-lo/diss, is a low-disseminating counterpart.

In this study, we have attempted to define which intrinsic characteristics of HT-hi/diss and HT-lo/diss variants account for the pronounced difference in their intravasation capacity. Detailed immunohistochemical analysis indicated that HT-hi/diss, but not HT-lo/diss, cells were more invasive, engaged closely with
the CAM blood vessels, and eventually observed as intracapillary, indicating a differential capacity in cell-cell and cell-matrix interactions. Previously, specific serine proteases and matrix metalloproteinases (MMP), including tumor-derived MMP-9, were associated with the ability of the human tumor cells to intravasate and metastasize following grafting onto the CAM (11). In addition, membrane type 1-MMP (MT1-MMP or MMP-14) has been reported to be a critical MMP for cell invasion of the CAM tissue (20). By treatment with the hydroxamate MMP inhibitor ilomastat (GM6001), we determined that HT-hi/diss intravasation and metastasis in vivo were MMP dependent. However, down-regulation of MMP-14 in HT-hi/diss cells did not significantly affect either tumor growth or lower CAM intravasation and lung metastasis. Moreover, specific inhibition in HT-hi/diss tumors of MMP-9 expression and activity with MMP-9 small interfering RNA (siRNA) and anti-MMP-9 monoclonal antibody (mAb) resulted in an unexpected substantial increase of intravasation and metastasis, indicating that targeting of certain MMPs may lead to enhanced malignancy. This first time comparative analysis of two HT-1080 intravasation cell variants allows for mechanistic insight into the preferential route and vascular interactions that occur during the intravasation process as well as the nature of the molecules that contribute to intravasation.

Materials and Methods

Chick embryo chorioallantoic membrane assay for chorioallantoic membrane intravasation and spontaneous lung metastasis. Fertilized SPAFAS White Leghorn eggs (Charles River, North Franklin, CT) were incubated in a humidified rotary incubator at 38°C for 10 days. CAM was gently dropped as described (13). Single-cell suspensions of tumor cells were prepared at 0.1 to 8 × 10⁶ cells in 25 μL of serum-free DMEM and, within 1 to 2 hours, were inoculated on the dropped CAM. The embryos were incubated for 1 to 7 days in a humidified stationary incubator at 38°C. Where indicated, the MMP inhibitor GM6001 (100 μL of a 1 mmol/L stock solution; Calbiochem, San Diego, CA), DMSO (diluent control), or mAb 7-11C specific for human MMP-9 (ref. 21; 100 μg per embryo) were applied topically onto the upper CAM on days 2 to 3. At the indicated time points, the upper CAM with the developing tumor, the portions of the lower CAM, and the lungs were excised from the embryonic chicks and snap-frozen in liquid nitrogen for biochemical analyses, frozen on dry ice for real-time Alu PCR analyses, or fixed in Zn/10% formalin for immunohistochemistry.

In vivo selection of high- and low-dissociating HT-1080 variants.

Human fibrosarcoma HT-1080 cells were obtained from the American Type Culture Collection (Rockville, MD) and routinely maintained in DMEM supplemented with 10% FCS and 10 μg/mL gentamicin (D10). To generate the HT-1080 variants, parent HT-1080 cells (HT-parental) were inoculated onto the dropped CAM, and 7 days later, the lungs from individual embryos were excised. One lung from each embryo was analyzed for the presence of human cells by quantitative Alu PCR, whereas the second lung was cultured ex ovo to generate cell lines from the metastasized tumor cells. For these purposes, individual lungs were cut into small pieces and incubated with collagenase/dispace to release cells from the tissue. Crude cell preparations from individual lungs were filtered through nylon meshes, and then cell suspensions were allowed to adhere in D10 to tissue culture plastic. After overnight incubation, nonadherent cells were washed out, and adherent cell layers were treated as separate cultures. Because the majority of cells belonged to the rapidly proliferating chick embryonic fibroblasts, the lung cultures were assessed daily for the appearance of cobblestone-like islands of human tumor cells, likely representing cell colonies developed from single cells or microscopic metastatic foci. The islands of human cells were isolated, replated, and expanded as individual cell lines. The established cell lines were analyzed for ability to intravasate and metastasize in CAM tumor assays. Two individual cell variants differing substantially from the parental HT-1080 cells were identified in further CAM assays. One cell line was distinguished by a dramatically increased ability to disseminate compared with parental and thus was named as highly disseminating or HT-hi/diss. Another cell line exhibited very low levels of intravasation and no detectable lung metastasis and hence was designated as low disseminating or HT-lo/diss.

Chick embryo experimental metastasis assay. A total of 1 × 10⁶ cells in 0.1 mL of serum-free DMEM were injected into allantoic vein of a day-10 chick embryo. At different time points (2-4 and 24 hours and 6-8 days), distant portions of the CAM were excised and analyzed with quantitative Alu PCR for numbers of human cells.

Real-time Alu PCR for quantitative detection of human tumor cells.

Human cells within chick embryo tissues were detected by real-time Alu PCR, essentially as described (13). Briefly, genomic DNA was extracted from harvested tissues using the Puregene DNA purification kit (Genta Systems, Minneapolis, MN). Human Alu sequences were amplified by real-time PCR using 30 ng of genomic DNA as template in a 10-μL reaction containing 2 mmol/L MgCl₂, 200 μmol/L deoxynucleotide triphosphate, 0.4 unit of Platinum Taq polymerase (Invitrogen Corp., Carlsbad, CA), 10⁻⁶ dilution of SYBR Green dye (Molecular Probes, Eugene, OR), and 0.4 μmol/L of each Alu sense (5'-ACGCCGTGAAATCCACGACTT-3') and Alu antisense (5'-TGCAGGCTGGTTGCA-3') primers. PCR conditions included polymerase activation at 95°C for 4 seconds followed by 30 cycles at 95°C for 30 seconds, 63°C for 30 seconds, and 72°C for 30 seconds. Each assay included a negative control (water), a positive control (human genomic DNA), and the experimental invasion cultures in duplicate. The actual number of tumor cells present in each tissue sample was determined using a standard curve generated by serial dilution of human tumor cells as previously described (13).

Immunohistochemistry. At different time points, samples of upper and lower CAM were excised, fixed in Zn-containing formalin, and paraffin embedded. Human cells within chicken CAM tissue were detected immunohistochemically with murine mAb 29-7 generated in our laboratory after immunization with human tumor Hep3 cells and reacting with a yet unidentified 75-kDa surface antigen of human tumor cells. Deparaffinized tissue sections were treated with 0.3% hydrogen peroxide and blocked with PBS/2% bovine serum albumin/5% normal goat serum. Sections were then incubated overnight at 4°C with 2 μg/mL mAb 29-7. After washing, the slides were incubated for 1 hour with secondary biotinylated goat anti-mouse IgG (1:1,000) followed by incubation with NeutrAvidin- horseradish peroxidase (HRP) conjugate (Pierce, Rockford, IL) for 30 minutes and then with a 3,3'-diaminobenzidine chromogenic substrate. Sections were counterstained with Mayer's hematoxylin. Digital images were captured using the Olympus BX60 microscope equipped with a digital DVC video camera and processed with Adobe Photoshop 6.0 software.

In vitro cell function assays.

Cells were plated in D10, 1 day before all in vitro experiments. Cells were detached with trypsin-EDTA, washed in D10, and resuspended at appropriate concentrations in D10 or serum-free DMEM. Cell proliferation assays, adhesion assays, and Transwell migration assays in two-dimensional cultures were carried out as described previously (22, 23). Proliferation in three-dimensional collagen gels was done using cell suspensions prepared at 1 × 10⁵ cells per mL of 1.5 mg/mL reconstituted type I collagen (Vitrogen 100, Cohesion, Palo Alto, CA). Cell-collagen mixtures were distributed at 0.5-μL aliquots into wells of 24-well plates and allowed to polymerize at 37°C. The gels were overlaid with D10, which was exchanged for the fresh medium daily. Cell proliferation was assessed by counting the cells released from individual gel plugs by incubation at 37°C with 0.5 mL collagenase (Sigma, St. Louis, MO). Collagen gel contraction and Matrigel invasion experiments were done essentially as described (24, 25). MMP inhibitor GM6001 was used at a final concentration of 50 μmol/L.

Matrix metalloproteinase down-regulation by RNA interference.

Gene silencing by RNA interference (siRNA) was used to down-regulate individual MMPs in HT-hi/diss cells. siRNAs specific for human MMPs were synthesized (Qiagen, Valencia, CA) against the following published target
sequences for MMP-9 5′-AACATCACCATTGGATCAAACATC-3′, nucleotides 377 to 403 (26) and MMP-14: 5′-AACAGCCTAGCTICAGGA-3′, nucleotides 228 to 248 (20). Nonsilencing siRNA (5 genes was used as a negative control. Transfection of 7 GAACGTGTCACGT-3′
zymography in the remaining siRNA-treated cells replated in D10 at 1 above. Down-regulation of MMPs was verified by Western blotting and excised from the embryos and processed for real-time Alu PCR as described above. Down-regulation of MMPs was replicated by Western blotting andzymography in the remaining siRNA-treated cells replated in D10 at 1 × 10⁶ per well of a 12-well cluster.

**Western blot analysis and zymography.** Culture medium was exchanged for serum-free DMEM 18 hours after cell plating. Following overnight incubation, conditioned media and cells were harvested. Washed cells were lysed in modified radioimmunoprecipitation assay (RIPA) lysis buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, and protease inhibitors]. To extract proteins from the primary CAM tumors, samples were snap-frozen in liquid nitrogen, ground using a mortar and pestle, and lysed in the modified RIPA buffer. The protein content in the cell and tumor lysates was determined using a bicinchoninic acid kit (Pierce). Equivalent amount of proteins were separated on 8%, 10%, or 4% to 20% SDS-PAGE precast gels (Invitrogen). Resolved proteins were transferred onto Immobilon polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk and incubated overnight at 4°C with 1 μg/mL of murine mAbs specific for human MMP-9 (7-11C) or human MMP-14 (Calbiochem). After washing, the blots were incubated with a secondary goat anti-mouse HRP-conjugated antibody (Pierce), and the proteins were visualized using SuperSignal West Pico Chemiluminescent substrate (Pierce).

For zymography, equal amounts of protein from samples of conditioned media or lysed CAM tumors were separated on 8% or 10% SDS-PAGE gels containing 0.1% gelatin. The gels were processed as described (13).

**Data analysis and statistics.** Data processing and statistical analysis were done using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). All experiments were done at least twice, and the total number of experiments and samples is indicated in the text or figure legends. Data are presented as a mean ± SE from a representative experiment or from normalized data of pooled experiments. Student’s t test (P < 0.05) was used to compare differences between the data for the two cell variants or conditions used in ex vivo and in vivo models.

**Results**

Tumor development on the chorioallantoic membrane and intravasation of HT-1080 variants. To investigate the molecular mechanisms involved in tumor cell intravasation, we generated a pair of cell lines originating from the same parental cells but widely differing in their ability to enter host vasculature. In the chick embryo model, HT-1080 fibrosarcoma exhibited relatively low rates of CAM intravasation (13), which in conjunction with an intrinsic heterogeneity of the cell line, allowed for selection of cell variants with higher (HT-hi/diss) and lower (HT-lo/diss) rates of dissemination compared with that of the parental cell line (see Materials and Methods). The quantitative nature of the Alu repeat real-time PCR assay (Alu PCR) along with its high sensitivity (as low as 10-50 human cells detectable in any given host tissue) allowed for an extensive analysis of the cell variants in the chick embryo assays.

The two newly established HT-1080 variants and the parental cell line were characterized and quantified for their ability to generate tumors, intravasate into the CAM vasculature, and metastasize to the host lungs (Fig. 1). When inoculated at 5 × 10⁵ cells per embryo, the parental HT-1080, HT-lo/diss, and HT-hi/diss cell lines gave rise to CAM tumors with the average weight of 320 ± 23, 151 ± 10, and 286 ± 12 mg, respectively. However, intravasation of the lower CAM by HT-hi/diss (5,984 ± 575 cells) was 10-fold higher than intravasation of the parental HT-1080 (592 ± 181 cells). The numbers of HT-lo/diss cells in the lower CAM (72 ± 33 cells) were ~80 times less than those of HT-hi/diss. The

![Figure 1](https://www.aacrjournals.org/doi/fig/a/666/23/65/cancerres.2005.0083-001961.large.jpg)

**Figure 1.** In vivo growth characteristics of HT-1080 cell variants. A total of 5 × 10⁵ cells from the parental HT-1080 line (HT-parental) and two HT-1080 variants (HT-hi/diss and HT-lo/diss) were grafted on the dropped CAM of day-10 chick embryos. Following 6 to 7 days of incubation, tumors were excised and weighed (A), and lower CAM (LCAM) and lung tissues were harvested for Alu PCR analysis done as described in Materials and Methods. The numbers of human cells in the lower CAM (B) and lungs (C) were determined after subtraction of background levels of Alu PCR signal, which were calculated from the C₅ values obtained for the tissues from control embryos that did not receive any human tumor cells (21 ± 4 cell equivalents in the lower CAM, n = 27 and 27 ± 7 cell equivalents in the lungs, n = 5). Columns, means from nine experiments for HT-parental (n = 58), seven experiments for HT-lo/diss (n = 53), and 32 experiments for HT-hi/diss (n = 176) cell variants; bars, SE. n = number of embryos analyzed.
high intravasation rate of HT-hi/diss yielded significant levels of lung metastasis (188 ± 28 cells), whereas no lung metastasis was observed in the embryos with HT-lo/diss tumors (0 ± 1 cell).

**Cell dose dependency of chorioallantoic membrane tumor growth and intravasation.** Because HT-lo/diss cells generated tumors ~50% smaller than the tumors produced by HT-hi/diss cells (Fig. 1A), we questioned whether HT-lo/diss cells would yield intravasation and metastasis if their tumors would be equal in size or exceed that of HT-hi/diss tumors. Inoculation of increasing numbers of HT-hi/diss cells resulted in a corresponding increase in tumor size as well as frequency of human tumor cells detected in the lower CAM tissue (Fig. 2A). The size of HT-lo/diss tumors also increased with an increase of cell dose, indicating that HT-lo/diss cell line was not just incapable of growing progressively on the CAM (Fig. 2B, top). However, even when the HT-lo/diss inoculum exceeded 16-fold that of HT-hi/diss, and HT-lo/diss tumors grew to more than twice that of HT-hi/diss tumors, little or no intravasation into the lower CAM or metastasis to the lungs was detected (Fig. 2B, bottom). Therefore, HT-lo/diss cells within a progressively expanding primary tumor seem severely impaired in their ability to enter the CAM vasculature, which ultimately results in the lack of tumor dissemination to secondary tissues or organs.

**Kinetics of HT-hi/diss chorioallantoic membrane intravasation and lung metastasis.** We next analyzed whether intravasation of HT-hi/diss occurred within a certain time frame. A detailed time course analysis of tumor growth, lower CAM intravasation, and lung metastasis was done after grafting 0.5 × 10⁶ HT-hi/diss cells on the CAM (Fig. 3). Although CAM tumors grew steadily over the 6-day incubation (Fig. 3A), intravasation by HT-hi/diss showed a substantial increase in the frequency of tumor cells appearing in the lower CAM 3 to 5 days after cell grafting (Fig. 3B). Quantifying the number of human cells in the lungs indicated that HT-hi/diss cells were detectable on days 5 and 6 (Fig. 3C; i.e., 1-2 days after their initial appearance in the lower CAM when apparently a sizable portion of tumor cells enter the vasculature, reach distant parts of the CAM, and expand to detectable levels).

In contrast to HT-hi/diss, no significant numbers of HT-lo/diss cells were identified in the lower CAM or lungs at any time up to 7 days after grafting as much as 2.5 × 10⁶ cells on the CAM (data not shown), thus confirming an intrinsic inability of HT-lo/diss variant to penetrate the vasculature barrier as originally indicated (Fig. 1B and Fig. 2B). Importantly, the substantial difference in the disseminating ability between HT-lo/diss and HT-hi/diss was preserved at least up to 36 passages in vitro and also following generation of new cell lines from the corresponding primary CAM tumors (data not shown). Altogether, these findings indicate that the two established HT-1080 variants are stable and yield reproducible dose-dependent and time-dependent results, making this unique pair of cell lines suitable for further probing of the mechanisms critical for tumor cell intravasation and metastasis.

**In vivo characterization of HT-1080 variants in the experimental metastasis model.** The lack of intravasation by HT-lo/diss could be associated with its inability to survive in the circulation, or attach to the endothelium of the lower CAM, or extravasate and proliferate within the surrounding tissues. To analyze these possibilities, HT-hi/diss and HT-lo/diss were compared in the experimental metastasis model involving a direct inoculation of the cells into the chick embryo circulation. Equal numbers of HT-hi/diss and HT-lo/diss cells (1 × 10⁶ per embryo) were inoculated into the allantoic vein. At 2 to 4 hours, 24 hours, and 6 to 7 days following inoculation, the portion of the CAM, most distant to the site of inoculation, was analyzed by Alu PCR for the actual numbers of human cells. A representative experiment is shown in Table 1. Although the mean numbers of HT-lo/diss cells detected in the CAM within the first day (at 4 and 24 hours) were lower than for HT-hi/diss, this difference was not significant.
indicated high levels of tumor cell proliferation. The inner cell layer
of tumor mass was directly associated with the CAM (arrows), enriched in a capillary network. The middle layer of the CAM, the mesoderm, transversed by a network of small capillaries as well as large blood vessels, was free of tumor cells and was underlined by the endoderm (Fig. 4A and G, arrowheads).

At day 2, despite close proximity to the mesodermal capillary plexus, tumor cells seemed still separated from it by the ectoderm (Fig. 4B and H). This was changed at day 3, when the inner edges of tumor mass started to protrude into the CAM mesoderm, rupturing the ectoderm layer (Fig. 4C and I). This ectoderm breaching was more pronounced in the case of HT-hi/diss tumors. Although HT-lo/diss tumors displayed some cell invasion activity at the ectoderm/mesoderm interface, most of the migrated HT-lo/diss cells did not form protrusions in close proximity to blood vessels (Fig. 4D). In contrast, HT-hi/diss tumor cells formed invasion trails at the outermost periphery of the expanding tumor (Fig. 4F), where they displayed a distinctive motile phenotype with long invadopodia-like protrusions. Here, HT-hi/diss cells migrating deeper into the CAM mesoderm were frequently found in apparent close contact with capillaries or engaging with the blood vessel wall (Fig. 4F). These invasion trails become a major feature of the distinct morphologic differences between HT-hi/diss and HT-lo/diss tumors.

By day 4, both types of tumors grew considerably. The overall morphology of HT-lo/diss tumors did not change significantly, as they remained compact and smooth edged. Individual HT-lo/diss cells could be found slightly separated from the primary tumor, but they still did not show close associations with the blood vessels (Fig. 4E). In contrast, HT-hi/diss cells were identified further from the edge of the primary tumor compared with day 3 (Fig. 4K). Moreover, these cells often were visualized being tightly congregated around blood vessels and also localized individually or as small groups within the capillaries, likely reflecting the result of intravasation events. A number of such intracapillary tumor cells are

More importantly, that by day-6 HT-lo/diss and HT-hi/diss exhibited similar levels of CAM colonization. Thus, the two cell variants apparently do not significantly differ in their ability to survive, arrest, and grow in vivo during the later steps of tumor dissemination, thus indicating that the inability of HT-lo/diss to efficiently intravasate earlier at the site of primary tumor development accounts for their lack of detectable spontaneous metastasis into secondary organs.

**Immunohistochemical analysis of tumor development and intravasation.** A detailed immunohistochemical analysis of developing HT-hi/diss and HT-lo/diss CAM tumors was done using mAb 29-7 directed against a human cell surface antigen. Following inoculation onto the CAM and overnight incubation of the embryos, both cell types were observed as multicellular layers (up to 10 cells in cross-sections) spread over large areas of the upper CAM (Fig. 4A and G). Numerous mitoses in both variants indicated high levels of tumor cell proliferation. The inner cell layer

### Table 1. Colonization of chorioallantoic membrane after i.v. inoculation of HT-1080 variants

<table>
<thead>
<tr>
<th>Time after i.v. inoculation</th>
<th>Chorioallantoic membrane colonization</th>
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<tr>
<td></td>
<td>HT-lo/diss</td>
</tr>
<tr>
<td>4 h</td>
<td>3,602 ± 623</td>
</tr>
<tr>
<td>24 h</td>
<td>4,662 ± 644</td>
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<tr>
<td>6 d</td>
<td>44,063 ± 464</td>
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**NOTE:** A total of 1 × 10⁵ cells of the two HT-1080 variants (HT-hi/diss and HT-lo/diss) were injected into allantoic vein of 10-day-old embryos. At 4 and 24 hours and 6 days, the portions of the CAM (most distant from the site of inoculation) were harvested and analyzed by Alu PCR analysis done as described in Materials and Methods. The numbers of human cells in the tissues were determined after subtraction of background levels of Alu PCR signal obtained for the tissues from control embryos that did not receive any human tumor cells (23 ± 11 cell equivalents in the CAM and 6 ± 1 cell equivalents in the lungs). For statistical significance (P < 0.05), Ci values were compared using a nonpaired Student’s t test to determine Ps. Mean number of cells ± SE from one of two independent experiments involving three to four embryos per time point.
shown across different fields in Fig. 4M. No such widespread and close association with and within the vasculature was seen in HT-lo/diss tumors.

On day 5, HT-hi/diss cells could be still observed within the capillaries or tightly engaged with the blood vessel wall, whereas the CAM vasculature around the HT-lo/diss tumors was relatively clear of tumor cells (Fig. 4F and L, respectively). Importantly, only HT-hi/diss cells were identified within the CAM blood vessels or capillaries (Fig. 4L, inset), as well as outside some capillaries located relatively far from the tumor edge, possibly indicating an extravasation event (Fig. 4N). At day 6, HT-hi/diss cells, could be identified in the portions of mesoderm quite far from the edge of the primary tumor (Fig. 4O), more consistent with intravasation/extravasation processes than extraordinary long-distance cell migration along the extracellular matrix or vasculature of the mesoderm.

In the portions of lower CAM, maximally remote from the primary tumor, some rare intravascular HT-hi/diss cells were shown by immunohistochemistry on day 5 after inoculation (Fig. 5A), whereas abundant HT-hi/diss cells were readily observed on day 6 (Fig. 5B). Most cells were localized inside or juxtaposed to the capillaries of the ectoderm (Fig. 5C-E), possibly reflecting the preferential route of their intravascular migration via the ectoderm capillary network. Single cells were also observed at some distance from capillary walls (Fig. 5F-H), probably as a result of extravasation into the lower CAM mesoderm. Rarely, larger clusters of HT-hi/diss cells were identified (Fig. 5I-F), suggesting extensive proliferation and/or migration in cell groups. In full agreement with the Alu PCR data, no HT-lo/diss cells were detected in the lower CAM by immunohistochemistry.

Overall, the immunohistochemical analysis indicates that at least partially, HT-hi/diss intravasation occurs at the periphery of the primary tumor where individual tumor cells migrate toward the capillaries, converge on, and/or attract the developing blood vessel. For HT-lo/diss tumors, the lack of distinct invasion trails at the periphery of CAM tumors and the absence of extensive vessel engagement by tumor cells were the major morphologic differences distinguishing them from the HT-hi/diss variant.

In vitro characteristics of HT-1080 variants. Immunohistologic studies suggested that HT-lo/diss cells could be impaired in their invasive capacity or their ability to interact and modify the tissue matrix of the CAM. Therefore, a series of in vitro functional assays was done to analyze whether differences in the cell line characteristics could be ascertained. In two-dimensional culture conditions, proliferation potential of HT-lo/diss cells was nearly identical to that of HT-hi/diss as determined in five independent experiments (doubling time of 18.8 ± 0.34 and 19.69 ± 1.64 hours, respectively). Similarly, the two cell lines did not differ in their adhesion to, or migration on type I collagen- and vitronectin-coated surfaces (data not shown). In contrast to two-dimensional
cultures, proliferation of HT-hi/diss cells in three-dimensional collagen was more efficient than that of HT-lo/diss, manifesting a doubling time of 23.0 ± 1.9 versus 29.5 ± 1.4 hours, respectively. This finding could reflect the observed slower development of HT-lo/diss primary tumors within the in vivo collagenous environment of the CAM (Figs. 1A and 2B).

A differential in three-dimensional cell behavior was further indicated when HT-lo/diss cells were shown to be significantly diminished in their ability to contract collagen gels compared with HT-hi/diss cells, which contracted the collagen gels down to 25% of their original area (Fig. 6A). Gel contraction by both cell variants was sensitive to a broad range MMP inhibitor GM6001 (Fig. 6f). In addition, three-dimensional Matrigel invasion was substantially lower for HT-lo/diss than for HT-hi/diss (Fig. 6B). Matrigel invasion of both HT-1080 variants was also very sensitive to GM6001, which inhibited Matrigel invasion of HT-hi/diss cells 60% to 70% and yielded almost complete inhibition of the slower invading HT-lo/diss cells (Fig. 6b). Altogether, the differential behavior and protease inhibitor sensitivity of those assays involving remodeling of three-dimensional extracellular matrix (i.e., fibrillar collagen and Matrigel) suggest that undefined MMPs may contribute to the phenotypic differences between the two HT-1080 cell variants.

**HT-hi/diss lower chorioallantoic membrane intravasation and lung metastasis is matrix metalloproteinase dependent.** Because in vitro matrix remodeling and invasion showed sensitivity of HT-1080 cell variants to MMP inhibitors, we tested for the in vivo involvement of MMP activity in intravasation and metastasis of HT-hi/diss cells using the same MMP inhibitor GM6001. The inhibitor was applied topically on the upper CAM of the chick embryos inoculated with HT-hi/diss cells (Fig. 7). Although tumor growth was affected only marginally (P > 0.05), the MMP inhibitor decreased lower CAM invasation by 70% (P < 0.05) and lung metastasis by 67% (P < 0.05). Systemic (i.v.) delivery of GM6001 to the tumor-bearing embryos yielded similar inhibition of intravasation and lung metastasis (data not shown). These findings suggested that the activity of undefined metalloproteinases plays some role in the dissemination of HT-hi/diss variant.

**Effects of down-regulation of matrix metalloproteinases on HT-hi/diss intravasation and metastasis.** To analyze the specific role of individual MMPs in intravasation and metastasis of HT-hi/diss cells, we used a siRNA approach to down-regulate two MMPs (i.e., MMP-9 and MMP-14), down-regulation of which has been previously shown to diminish tumor angiogenesis and invasion (26–28). Down-regulation of MMP-14 protein and activity with a specific siRNA were efficiently achieved in the HT-hi/diss variant, as indicated by the reduction of MT1-MMP protein in Western blots of cell lysates and concomitant reduction of MT1-MMP-dependent MMP-2 processing/activation in zymograms of the conditioned medium (Fig. 8A, top). However, down-regulation of MMP-14 did not result in any significant inhibition of lower CAM intravasation or lung metastasis by HT-hi/diss cells (Fig. 8B).

Treatment of HT-hi/diss cells with human MMP-9 siRNA resulted in significant down-regulation of MMP-9 protein expression in vitro, shown by both zymography and Western blotting (Fig. 8A, bottom). The specificity of down-regulation was indicated by the lack of changes in MMP-2 levels when cells were treated with MMP-9 siRNA. Western blot and zymographic analysis confirmed that MMP-9 expression was still substantially reduced in HT-hi/diss cells treated with MMP-9 siRNA.

**Figure 5.** Immunohistochemical analysis of HT-hi/diss cell intravasation into the lower CAM. Samples of lower CAM from the embryos engrafted with 1 × 10⁶ HT-hi/diss cells were harvested daily during days 2 to 6, fixed with Zn/formalin, and embedded into paraffin. Human cells were visualized by immunohistochemical staining with mAb 29-7 (brown) and counterstained with Mayer’s hematoxylin. Original magnifications, ×400 (A), ×200 (B), and ×400 (C–J). Detailed explanation is incorporated in the text.
Intravasation \((= 0.09; \text{Fig. 8})\) with mAb 7-11C led to a 2.9-fold increase in HT-1080 cell infiltration into the CAM, which substantially differ in their in vivo ability to intravasate into the circulation of the host. The differential intravasative capacity of the two variants, HT-hi/diss and HT-lo/diss, does not depend on the growth or size of their primary tumors but on their apparent ability to escape from the primary tumor and interact with the host’s vasculature. Most importantly, following i.v. inoculation, the two variants survive in the circulation and arrest and grow at secondary sites similarly. Therefore, their phenotypic dissimilarities are likely attributed to those early metastatic events occurring in-between the establishment of the primary tumor and the appearance of tumor cells in the circulation.

To corroborate this unexpected finding, we verified whether an independent method of down-regulation of MMP-9 activity using human MMP-9-specific function-blocking mAb 7-11C would also result in an increase in lower CAM intravasation and lung metastasis of HT-hi/diss cells. Zymographic analysis of HT-hi/diss tumors confirmed that MMP-9 enzyme expression was significantly down-regulated in the embryos treated with mAb 7-11C (Fig. 8E). However, whereas having no significant effect on the growth of the primary tumor \((P = 0.24)\), treatment with mAb 7-11C led to a 2.9-fold increase in HT-hi/diss cell intravasation \((P = 0.02)\) and 2.4-fold increase in lung metastasis \((P = 0.09; \text{Fig. 8F})\). Thus, specific inhibition of tumor MMP-9 expression and activity by two independent approaches unexpectedly provided an enhancing effect on tumor cell intravasation in the herein described model. These results highlight a specific and unique role of this MMP and emphasize the existence of complex, MMP-dependent mechanisms involved in tumor cell intravasation.

**Discussion**

In a view that intravasation is a key step in systemic dissemination of cancer, understanding the complex mechanisms determining the ability of tumor cells to migrate and enter the vasculature is necessary to control metastatic spread. The present study documents the first characterization of a pair of HT-1080-derived cell lines, which substantially differ in their in vivo ability to intravasate into the circulation of the host. The differential intravasative capacity of the two variants, HT-hi/diss and HT-lo/diss, does not depend on the growth or size of their primary tumors but on their apparent ability to escape from the primary tumor and interact with the host’s vasculature. Most importantly, following i.v. inoculation, the two variants survive in the circulation and arrest and grow at secondary sites similarly. Therefore, their phenotypic dissimilarities are likely attributed to those early metastatic events occurring in-between the establishment of the primary tumor and the appearance of tumor cells in the circulation.

The nature of the described chick embryo model system combined with Alu PCR sensitivity contributed substantially to both the isolation of the unique HT-1080 tumor variants and further analysis of their intravasative properties. Distal to the primary tumor, highly vascularized lower CAM is an ideal repository for intravasated tumor cells (11, 29), which can be easily and temporally dissected out and analyzed at the tissue, cell, and molecular levels. The apparent lack of such repository organ in the mouse, the dominant animal model used in...
metastasis studies, has limited the experimental analysis of intravasation in mammals.

The human tumor/chick embryo model, in addition to uniquely providing a means for examining directly the process of intravasation, also allows for easy access and early temporal studies of the developing primary tumor, adjacent stromal tissue, and emerging vasculature. At least two distinct features in the in vivo behavior of HT-lo/diss and HT-hi/diss were elucidated during a kinetic immunohistochemical analysis of primary tumor expansion and intravasation. One difference was that the HT-lo/diss cells seemed more restricted at the leading edge of the tumor, whereas distinct invasive cell trails into the mesoderm were characteristic of HT-hi/diss primary tumors. A second in vivo characteristic was manifested by the close proximity and apparent attraction of human tumor cells in HT-hi/diss tumors to the CAM.

Figure 8. Effects of down-regulation of MMP-14 and MMP-9 on tumor growth, intravasation, and metastasis of HT-hi/diss cells. A, down-regulation in vitro of human MMP-14 and MMP-9 by corresponding siRNAs. HT-hi/diss cells were transfected with 100 nM of MMP-14 siRNA (+, top), MMP-9 siRNA (+, bottom), or control siRNA (–). The following day, the cells were harvested and prepared for CAM assays. Aliquots (1 × 10⁶ cells) of the cell suspension were replated in D10 into wells of a 12-well plate. The following day, culture medium was exchanged for serum-free DMEM with 0.1 μM phorbol 12-myristate 13-acetate for MMP-14 siRNA-treated cells or without phorbol 12-myristate 13-acetate for MMP-9 siRNA-treated cells. After overnight incubation, the cells were lysed and analyzed by Western blotting for expression of the corresponding MMP, and conditioned medium was harvested and analyzed by gelatin zymography. B, MMP-14 siRNA-treated cells were grafted onto the CAM at 1 × 10⁶ per embryo. On day 5, tumors were excised and weighed, and lower CAM (LCAM) and lungs were harvested and analyzed by Alu PCR analysis for the numbers of human cells. Columns, mean fold difference between the embryos engrafted with the cells transfected with MMP-14 siRNA (n = 24) versus control siRNA (n = 36) from five independent experiments; bars, SE. n = total number of embryos per treatment. P > 0.05 for all variables compared. C, HT-hi/diss cells were transfected with MMP-9 siRNA or control siRNA as described in (A) for MMP-14 siRNA-treated cells. Down-regulation of MMP-9 in day-5 CAM tumors, individually lysed in modified RIPA buffer, was confirmed by Western blotting with mAb 7-11C specific for human MMP-9 (top) and zymographic analysis (bottom). Lane, individual tumor for the treatment indicated. D, effects of MMP-9 down-regulation on HT-hi/diss cells were assessed in vivo exactly as described in (B) for the cells treated with MMP-14 siRNA. Columns, mean difference between the embryos grafted with HT-hi/diss cells treated with MMP-9 siRNA (n = 33) versus treated with control siRNA (n = 59) from eight independent experiments. n = total number of embryos per treatment. *, P < 0.05. E, down-regulation of human MMP-9 in HT-hi/diss tumors treated with MMP-9-specific mAb. Embryos with developing tumors were treated topically with 100 μg of control mAb or mAb 7-11C on days 2 and 4. On day 6, individual tumors were excised and lysed in modified RIPA buffer for zymography analysis of MMP-9 protein levels. F, effects of anti-MMP-9 mAb. Tumors from embryos treated with control mAb or mAb 7-11C were weighed, and lower CAM and lungs analyzed for the number of human cells by Alu PCR. Columns, mean fold difference from two independent experiments, involving a total of 12 (control mAb) and 13 (mAb 7-11C) embryos per treatment; bars, SE. *, P < 0.05 (lower CAM intravasation) and P = 0.09 (lung metastasis).
invading HT-hi/diss cells to the capillaries and blood vessels in the tumor-adjacent mesoderm. These observations are reminiscent of the time lapse microscopy data on metastatic and nonmetastatic variants of rat mammary carcinoma: both variants were motile and showed provocative activity in the tumor stroma, but only metastatic cells were oriented toward and intravasated into tumor blood vessels (3).

In the stroma of CAM mesoderm, starting on day 4, many of HT-hi/diss cells were clearly observed intravascularly, indicating active intravasation and migration through the circulatory system. This notion is supported first by findings of rare but unambiguously intracapillary HT-hi/diss cells far distant from the leading edge of the primary tumor, and then, by intravascular and extravascular localization of tumor cells in the capillary plexus of the lower CAM. This comparative analysis of cell dissemination from HT-hi/diss versus HT-lo/diss tumors supports neither the newly proposed mechanism of extravascular migratory metastasis recently described for human tumor cells in the CAM model (30) nor invasion-independent intravasation of human tumor cell nests surrounded by vascular endothelial cells in a murine mammary model (31, 32). More likely, the cellular mechanisms that are responsible for the differential behavior of HT-1080 variants involve distinct cell-cell or cell-matrix adhesion reactions and specific migratory responses, resulting in HT-hi/diss cells physically converging on blood vessels or being attracted to signals derived from the angiogenic vessels.

These distinct morphologic features in tumor expansion prompted in vitro studies to link specific in vivo characteristics with cell culture properties of the two tumor variants. Cell function assays indicated that HT-lo/diss and HT-hi/diss variants are similar in their proliferative potential, as well as in migratory and adhesion properties in two-dimensional conditions. However, in three-dimensional cultures, the two variants did differ significantly, in particular in Matrigel invasion and collagen remodeling. Both of these properties could be conceptually related to the variants' in vivo intravasation behavior, and both were partially sensitive to MMP inhibition. Confirmation that a role exists for MMPs in the intravasation process came about with the observed in vivo sensitivity of HT-hi/diss cell dissemination to the same broad-spectrum MMP inhibitor.

To analyze the role of specific MMPs in HT-hi/diss intravasation, we chose to down-regulate two candidate MMPs (i.e., the membrane-bound MMP-14 and the secreted MMP-9). MMP-14 was selected because its expression had been extensively linked to the aggressive malignant phenotype (33–35). MMP-9 was chosen because it had been strongly associated with tumor progression and tumor-induced angiogenesis in vivo (36–41). Because of the compelling historical links, we expected that down-regulation of one or both selected MMPs would result in a substantial enhancement of HT-hi/diss tumor intravasation, now repeated in 10 separate experiments using two independent means of MMP-9 down-regulation. Because of the compelling historical links, we expected that the strong causal link between MMP-14 and cancer progression may have to be reevaluated for specific early steps in the metastatic cascade. However, the striking result of MMP-9 down-regulation in HT-hi/diss cells is not easily explained because of an inhibitory effect of GM6001 on intravasation shown in this study. Nevertheless, the substantial enhancement of HT-hi/diss tumor intravasation, now repeated in 10 separate experiments using two independent means of MMP-9 down-regulation, emphasizes the multilayered complexity of the metastatic process and the possible contrasting involvement of different MMP family members.

The unexpected effects of tumor MMP down-regulation could indicate that mainly host MMPs and possibly only a select few tumor MMPs constitute a restricted set of enzymes, which are positive contributors to the metastatic process and targets of some of the broad-range inhibitors. Furthermore, if the levels of secreted MMPs, such as MMP-9, positively correlate with the expression levels of natural MMP inhibitors, including tissue inhibitor of metalloproteinase-1 (TIMP-1) and TIMP-2, down-regulation of MMP-9 might function as a key negative regulator of the intravasation step (e.g., by mediating the proteolytic generation of specific intravasation inhibitors or catalytic degradation of positive signals that drive the tumor cells entry into the vasculature). That certain MMPs, including MMP-9, are able to generate collagen- or plasma-derived biologically active proteolytic fragments possessing strong antiangiogenic characteristics has been shown in numerous mammalian models (43–46) and thus could well represent a basic, evolutionary preserved mechanism of angiogenesis regulation.

Overall, our results with MMP-9 down-regulation highlight an apparent fine balance between positive effector MMPs and negative regulator MMPs that might at least partially control the process of metastasis. This suggestion is strengthened by studies showing that the reduction of plasma levels of MMP-9 or genetic loss of MMP-8 increased tumor progression and vascularization (47–49). The results also bring up a clear cautionary note (i.e., that targeting certain MMPs might result in an enhancement of malignancy). Such notes of caution may indeed fit well following the retrospective analysis of failed clinical trials (50) but certainly belong to the plans for elucidating the identities of the putative positive and negative regulatory MMPs.

It should be emphasized that the involvement of MMPs in the intravasation process is apparently only partial. Inhibition of MMPs did not reduce the level of HT-hi/diss intravasation down to the baseline level of HT-lo/diss. It is also unlikely that up-regulation of MMPs in HT-lo/diss tumors would enhance intravasation 50- to 100-fold. Indeed, other gene products could be responsible for the high levels of HT-hi/diss intravasation, as well as putative suppressor factors might be linked to the severely impaired intravasation of HT-lo/diss cells. The model system described in this study offers an experimental means to identify these gene products and to critically and quantitatively analyze the complex and understudied process of tumor cell intravasation.

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References

Unexpected Effect of Matrix Metalloproteinase Down-Regulation on Vascular Intravasation and Metastasis of Human Fibrosarcoma Cells Selected In vivo for High Rates of Dissemination
