Reduction of Experimental Human Fibrosarcoma Lung Metastasis in Mice by Adenovirus-Mediated Cystatin C Overexpression in the Host

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Abstract
Tumor cell invasion and metastasis are associated with degradation of components of the extracellular matrix by different proteinases. Among those, papain-like cysteine proteinases, such as cathepsin B, seem to play an important role, as they are associated with poor clinical outcome in different cancers. In this study, we tested whether cystatin C, a natural extracellular inhibitor of papain-like cysteine proteinases, can inhibit metastasis when overexpressed at the tumor-host interface. Local overexpression of cystatin C in liver and lungs of CD1 nu/nu mice was achieved by gene transfer with a novel adenoviral construct, which also led to the presence of 60 ng/mL of cystatin C in the serum. Three days after gene transfer, these mice were challenged by i.v. inoculation of lacZ-tagged human fibrosarcoma cells (HT1080/lacZ-K15), leading to the formation of experimental lung and liver metastases. In this model, formation of experimental metastatic foci correlated with expression of cathepsin B in lungs, whereas there was no correlation with metastasis to the liver. In mice overexpressing cystatin C, the number of lung metastases was significantly reduced by 92%, as compared with mice receiving control adenovirus. The efficacy of extravasation of HT1080/lacZ-K15 cells into the liver was not affected, indicating the independence of this process from the activity of cysteine-cathepsins. The present report is the first evidence of successful reduction of metastasis by inhibition of cysteine-cathepsins by cystatin C overexpression in the host microenvironment. Furthermore, organ-specific protease expression during tumor-host cell interactions could affect the success of antiproteolytic intervention against metastasis. (Cancer Res 2005; 65(19): 8608-12)

Introduction
Metastatic spread of tumor cells is the main cause of death in cancer patients. One prerequisite for tumor progression and metastasis is the proteolytic degradation of components of the extracellular matrix. The well-characterized tumor-associated proteinases cathepsins B and L belong to the 11 known human papain-like cysteine proteinases (1). Cathepsins normally occur as lysosomal proteinases, but were found to be secreted during tumor cell invasion and metastasis as extracellular contributors to the degradation of components of the extracellular matrix or by activation of other proteinases such as the urokinase-type plasinogen activator (2). Papain-like cysteine proteinases were found to be secreted and detectable in blood or other body fluids of cancer patients with poor prognosis (3–5), leading to the selection of extracellular cysteine-cathepsins as potential drug targets. Activity of cysteine-cathepsins is naturally regulated by cystatins, a group of natural inhibitors, of which 12 are known in humans (6). Although cystatins A and B (stefin A and B) are located intracellularly, cystatin C and other cystatins (e.g., cystatin D, E/M, and F) are secreted and can regulate extracellular tumor-associated cysteine-cathepsins. Initial studies in vitro have shown inhibition of tumor cell invasion by stable transfection of human cystatin C cDNA (7) or human cystatin M/E cDNA (8) into tumor cells. Overexpression of cystatin C by tumor cells can suppress tumor growth in vivo (9). In the clinic, however, cancer patients with increased levels of cystatin C or stefins, have a poor prognosis (4). The association between increased cystatin levels and poor prognosis in cancer patients was related to the dual role of cathepsin B in tumor progression, namely, in addition to promotion of invasion, its proapoptotic activity, when overexpressed in tumor cells (10). Overexpression of stefin A (intracellular cystatin A) by tumor cells inhibited cathepsin B–induced apoptosis (10). In contrast with previous studies, where cystatin expression was manipulated in the tumor cells, we here aimed to test the influence of prophylactic overexpression of the natural extracellular inhibitor cystatin C by the host on cathepsin B–correlated colonization of human fibrosarcoma cells to lungs. This study shows for the first time that overexpression and secretion of a natural inhibitor of cysteine-cathepsins by host cells can convey protection against metastasis.

Materials and Methods
Adenoviral constructs and cell culture. Virus Addl70-3 encoding no transgene (11) was used as control. For generation of cystatin C encoding adenovirus (AdCysC), PCR was used to add restriction sites XhoI and HindIII to the synthetic cDNA of human cystatin C (12) to facilitate cloning. Cystatins are able to inhibit the adenoviral cysteine-protease (13), whose activity is necessary for the generation of viral particles (14). Therefore, we exchanged the endogenous leader sequence of cystatin C with the strong Igk-leader sequence, including a stop codon at the 3′-end. Amplified DNA was inserted into pSecTag2/Hygro B, Cystatin C cDNA together with start codon, Igk-leader sequence, and stop codon was cloned into adenoviral shuttle plasmid pKAI containing the human cytomegalo-virus promoter, SV40 polyadenylation signal, and a loxp site for site-specific recombination with the adenoviral backbone plasmid, pBH1loxDel13Cre (AdMax System, Microbix, Toronto, Canada). Recombinant adenovirus was generated by cotransfection of backbone plasmid and pKAI, encoding...
secretion-optimized cystatin C, into low-passage HEK293 cells. Adenovirus recombinants were plaque-purified, propagated on HEK293 cells, cesium chloride-banded, and plaque-titered using standard protocols (15). HT1080 cells, kindly provided by Agnes Noël (Université Liège, Liège, Belgium), and HT1080lacZ-K15 cells were cultured as previously described (16). Infection of 2 × 10^6 HT1080 cells per well of a six-well plate was done using standard protocol (15). Medium was replaced 12 hours after infection, cells were washed twice with PBS, and 2 mL of FCS-free culture medium were added in each well. After 48 hours, medium was collected, cells from each well were counted, medium was centrifuged (400 × g, 10 minutes, 4°C), and snap-frozen in liquid nitrogen. Cell supernatant samples were kept at −80°C until use.

**Experimental metastasis assay.** Experimental metastasis assay was done as described previously (16) with the following modifications: mice were injected i.v. with either 2 × 10^6 plaque-forming units (pfu) AdCysC or Addl70-3 (control virus) 3 days prior to i.v. tumor cell inoculation (1 × 10^6 HT1080lacZ-K15). Mice were sacrificed 21 days after inoculation of tumor cells. Lungs and livers were removed and blood samples collected. Blood was incubated for 12 hours at 4°C. Serum was separated from blood clot by centrifugation (4°C, 200 × g, 1 hour) and stored at −20°C. The right lobe from each lung and three samples from each liver with a volume of 0.5 cm^3 were snap-frozen in liquid nitrogen for biochemical analysis, left lung lobes and remaining liver and serum were frozen on X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Roche Diagnostics, Penzberg, Germany; ref. 16), which enables detection of metastatic foci at the single-cell level (17). Blue metastatic foci on the surface of the lungs were counted and all organs were screened for the presence of metastasis. To quantify metastasis in lungs, in which only very small (single cell) metastases appear, we did quantitative real-time PCR (qRT-PCR) of lacZ mRNA. To determine the possible influence of cystatin C on the growth of lung metastases, metastatic foci were categorized according to their respective diameter (>0.4, >0.1, or <0.1 mm, respectively) using stereo-microscope SZX16 (Olympus, Tokyo, Japan). Hence, the ocular scaling was calibrated by a stage micrometer. Animal experiments were done in accordance with the guidelines of the Regierung von Oberbayern (Germany).

**Reverse papain zymography and ELISAs.** Reverse papain zymography of supernatants of AdCysC-transduced cells and of serum of AdCysC-transduced mice was done as described previously (18). Each sample tested was loaded on a reverse papain zymogram and, as control, on a SDS gel without substrate. Prestained molecular weight marker (PageRuler, Fermentas, St. Leon-Rot, Germany) served as standard. Quantification of transgenic cystatin C protein in the supernatants of transduced cells and in the serum of transduced animals was done with a specific human cystatin C ELISA system, which does not show cross-reactivity with related cystatins (Krika, Ljubljana, Slovenia). For evaluation of cathepsin B or L in tissues, protein from liver and lung was isolated and total protein was determined as described previously (19). Determination of the amount of the respective antigen Protein was done with cathepsin B or cathepsin L ELISA (Krika). Both ELISAs recognize total protein (proenzyme and mature enzyme) of the human and murine species of the respective cathepsin (manufacturer’s information). All ELISAs were done in duplicates.

**Isolation of total RNA and reverse transcription.** Total tissue RNA was isolated from snap-frozen liver samples using RNeasy Midi Kits (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Reverse transcription was done as described previously (19).

**Design of primers and probes and quantitative real-time PCR (TaqMan).** Primers and probes for synthetic cystatin C and for lacZ cDNA were designed and obtained from Applied Biosystems (Darmstadt, Germany; cystatin C, forward primer, 5′-GCCGGGCTCTTGGTGTG-3′; reverse primer, 5′-GACGGCAACACCTCTTCTT-3′; probe, 5′-FAM-TCCGATGACGC-CTTCT-nFQ-3′; lacZ, forward primer, 5′-CAACGGTGTCGTTAGCAGA-3′; reverse primer, 5′-GCTATCTAGCCTGACCAT-3′; probe, 5′-FAM-ACCGT-ACGAGCAGCAT-nFQ-3′). qRT-PCR was done in 96-well optical plates in triplicates as described previously (19).

**Statistical analysis.** Data were analyzed using Mann-Whitney rank sum test. For statistical analysis of frequency distribution, the Mantel-Haenszel test was used. Pearson correlation coefficient (R) and P value were used to describe correlation of cathepsin B protein levels and number of metastases. P value <0.05 was considered significant.

**Results and Discussion.**

To test the influence of elevated host cystatin C levels on extravasation, a crucial step of the metastatic cascade leading to formation of tumor colonies in distant organs, we used an experimental metastasis model with lacZ-tagged human fibrosarcoma cells (HT1080lacZ-K15). Examination of tumor- and host-derived cathepsin B and L expression revealed that formation of lung colonies closely correlated with the amount of cathepsin B protein (correlation coefficient, R = 0.928; P < 0.008; Fig. 1). In contrast, upon metastasis to the liver, no such correlation was observed.
found. Cathepsin L was also expressed in lung and liver, but in neither organ was its expression correlated with metastasis burden (Fig. 1). It was shown that cathepsin B levels in the tumor-host environment could be associated with a metastasis-promoting increase of proteolytic activity (1). Here, we tested whether overexpression of the natural cathepsin B inhibitor cystatin C by the host tissue can efficiently inhibit formation of experimental metastasis in the fibrosarcoma model. We decided to transduce the host rather than the tumor cells in order to focus on extracellular proteolytic events. This way, we also minimized the possibility of antiapoptotic effects of cystatin C in the fibrosarcoma cells, because cathepsin B was shown to be able to exert proapoptotic features on tumor cells (10). To achieve high systemic levels of cystatin C in the host, we constructed an adenoviral vector system, which is known to efficiently transduce the liver, which, in turn, leads to highly elevated levels of the secreted transgene product in the blood. To avoid inhibition of virus production by cystatin C accumulation in the producer cells (13), we optimized secretion of cystatin C by adding the Igk-leader sequence, resulting in efficient secretion of the transgene (data not shown).

Only for the purpose of testing secretion and inhibitory activity of the human cystatin C encoded by our novel adenovirus (AdCysC), we infected HT1080 cells. As control, HT1080 cells were infected with the control adenovirus Addl70-3. Secretion of cystatin C closely correlated with the dosage (multiplicity of infection) of AdCysC, and reached up to 970 ng of cystatin C protein per 1 x 10^4 cells in the supernatant at a multiplicity of infection of 200 (data not shown). The inhibitory activity of the secreted cystatin C was dose-dependent as shown by reverse papain-zymography (data not shown).

Next, CD1 nu/nu mice received 2 x 10^9 pfu of the cystatin C-encoding adenovirus AdCysC or the control virus (Addl70-3) i.v. Three days after gene transfer, mice were sacrificed and lungs, livers, and blood collected. qRT-PCR from total RNA isolated from livers and lungs showed that cystatin C was expressed in both organs, in the latter to a much lesser degree (Fig. 2A). Efficient expression of cystatin C in host tissue, especially the liver, was likely responsible for the accumulation of cystatin C protein in the blood (60 ng cystatin C/mL serum) 3 days after adenoaviral transfer (Fig. 2B), which led to an increase of papain-inhibitory activity by cystatin C in the sera of AdCysC-transduced animals as compared with the sera of controls (Fig. 2C).

At the time point of elevated cystatin C levels in the different compartments of the host (blood, liver, and lung), lacZ-tagged human fibrosarcoma cells (HT1080lacZ-K15) were iv. inoculated into mice to evaluate their metastatic potential. Therefore, in this experimental setting, the tumor cells encounter high levels of cystatin C in the bloodstream as well as at the tumor- and host-cell interface during extravasation into the target organs. Screening of lungs at day 21 after tumor cell inoculation revealed a significantly reduced number of established metastatic foci in cystatin C overexpressing mice (reduction by 92% versus control; Fig. 3A, left and middle). This reduction of lung metastasis was also evident from the significantly higher proportion of metastasis-free lungs in the cystatin C overexpressing group (75%) as compared with the control (18%; Fig. 3A, right). The findings that cystatin C overexpression suppressed colony formation in the lung, and that cathepsin B protein levels correlated with the number of metastases in this organ, both indicate the important role of cathepsin B in successful extravasation and early colonization events. In addition to cathepsin B, yet other proteases, like urokinase-type plasminogen activator, were shown to be effective targets in the latter steps of metastasis in this fibrosarcoma model (16). Also, urokinase-type plasminogen activator itself can be activated by cathepsin B (2). This reveals the complex interplay of different proteolytic systems in metastasis and their downstream proteolytic (e.g., matrix metalloproteinases) or nonproteolytic (growth factors) effectors. Gelatinase and plasminogen activator expression was not modified by overexpression of cystatin C, neither in lung nor in liver (data not shown). In contrast to studies showing the antimetastatic effects of cystatin overexpression by the tumor cells (20, 21), the present study shows for the first time the ability of host cell–secreted cystatin C to inhibit either the cysteine-cathepsins secreted by the tumor cells or host-derived intracellular or secreted cysteine-cathepsins. In contrast to the lungs, livers revealed no correlation between cathepsin B or L protein levels and metastasis. The differences of metastasis-induced expression of proteolytic factors in lung or liver, respectively, indicate organ-specific proteolytic responses to metastasis of tumor cells of the same origin. Consequently, prophylactic overexpression of cystatin C led to significant inhibition of metastasis to the lung but not to the liver (Fig. 3B, left). Likewise, cystatin C overexpression by the host was sufficient to significantly increase the number of metastasis-free lungs but not livers (Fig. 3B, right), although the highest expression of cystatin C was found in the liver (Fig. 2A). In a recent study, the antimetastatic effects of cystatin M overexpression by the tumor cells, and not by the host, were described: the tumor cell burden

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**Figure 2.** Transgenic cystatin C expression in the host after adenoviral gene transfer. Three mice were inoculated with 2 x 10^9 pfu of the cystatin C encoding adenovirus AdCysC. As control, three mice were transduced with control adenovirus (2 x 10^9 pfu Addl70-3 per mouse). Three days after adenoviral gene transfer, mice were sacrificed and livers, lungs, and blood were collected. A, cystatin C versus 18S mRNA levels in liver and lung. Columns, mean; bars, SE of the relative amounts of human cystatin C mRNA to 18S RNA as detected by qRT-PCR (Addl70-3: liver, 0.00 ± 0.00; lung, 0.00 ± 0.00; AdCysC: liver, 2.56 ± 1.41; lung, 0.027 ± 0.015). B, ELISA-detection of cystatin C levels in serum. Columns, mean; bars, SE per treatment group (Addl70-3, 0.00 ± 0.00; AdCysC, 59.62 ± 17.71 ng/mL serum). C, detection of antipapain activity ex vivo. Equal amounts of serum samples from three animals per group, either transduced with Addl70-3 (control) or AdCysC, were pooled and 10 µL of each serum pool (diluted 1:10 in PBS) were loaded on a reverse papain zymogram. Increased inhibitory activity of cystatin C in the sera of cystatin C–overexpressing animals was detectable by the increased band with antipapain activity.
on spontaneous metastasis of human breast cancer cells in lungs and livers of severe combined immunodeficiency mice was shown to be reduced (20). However, the incidence of metastatic colonies in the target organs of metastasis was only slightly decreased, without reaching statistical significance, indicating that expression of cystatin M in tumor cells did not affect extravasation of these cells, which was confirmed by an experimental metastasis assay (20). Therefore, expression of cystatin M in the tumor cells primarily affects growth and survival of the breast cancer cells and not extravasation. In contrast, we show in the present study that overexpression of cystatin C by the host reduced extravasation as well as tumor cell growth in the lung, as we found metastases >0.4 mm in the control and not in the cystatin C group (Table 1).

The observed antimetastatic efficacy of cystatin C seems to contradict the clinical findings that expression levels of cystatin C are associated with a poor prognosis in different cancers (4). However, it is possible that the elevated levels of cystatin C found in blood of cancer patients are not directly associated with the invasion of tumor cells into target organs but represent a secondary effect due to a reduced elimination rate of cystatin C by glomeruli caused by treatment-related or disease-related damage of the kidneys (22). Alternatively, elevated cystatin C levels may be a reflection of increased cathepsin activity in tumors or the stromal host cells, respectively, as it is also discussed for other natural protease inhibitors. Interestingly, in cystatin C knock-out mice, experimental metastasis of murine melanoma cells is promoted (23). However, as to whether the absence of cystatin C could lead to counter-regulation of metastasis-related effector molecules during the development of these mice has not been described. To circumvent such long-term adaptation of the mouse to transgene expression, we used adenoviral gene transfer with subsequent challenge from tumor cells. Thus, the present study is the first demonstration of the antimetastatic efficacy of prophylactic overexpression of cystatin C by the host cells. This may lead to a novel antimetastatic therapeutic approach by

![Figure 3](image3.png)

**Figure 3.** Reduction of metastasis by overexpression of cystatin C by host tissue. CD1 nu/nu mice received 2 × 10^6 pfu of AdCysC or Addl70-3 (Addl, control virus), respectively, i.v. 3 days prior inoculation of 1 × 10^6 lacZ-tagged human fibrosarcoma cells (HT1080lacZ-K15) into the tail vein. A, reduction of human fibrosarcoma metastasis to the lung by prophylactic overexpression of cystatin C. X-gal stained lungs of each group, arrow points at metastasis (left). All metastases on the lung surface were counted (middle). Number of metastases for both treatment groups; columns, 25th and 75th percentiles; bars, 10th and 90th percentiles, respectively. Thick bar, median value. Lungs from both treatment groups were screened for the presence of metastases (right). Proportion of metastasis-free lungs per group (Addl70-3, 18.8%, n = 16; AdCysC, 75.0%, n = 16; P = 0.0046; Mantel-Haenszel test), Bars. 2.5 mm. B, human fibrosarcoma metastasis in livers overexpressing cystatin C. Scattered metastasis burden of livers was quantified by lacZ qRT-PCR (left). Columns, mean; bars, SE of the relative amount of lacZ mRNA versus 18S RNA for three mice per treatment group (Addl70-3, 0.016 ± 0.002; AdCysC, 0.0015 ± 0.003; P > 0.05; Mann-Whitney rank sum test). Livers from both treatment groups were screened for the absence of metastases (right). Proportion of metastasis-free livers per group (Addl70-3, 18.8%, n = 16; AdCysC, 37.5%, n = 16; P > 0.05; Mantel-Haenszel test; n.s., not significant).

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<tr>
<th>Treatment group</th>
<th>Proportion of metastases of respective size within the group</th>
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<td>&lt;0.1 mm (%)</td>
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<td>Addl70-3 (control)</td>
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<td>AdCysC</td>
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**Table 1.** Reduced size of metastases by overexpression of cystatin C in the host

NOTE: Size of metastases was determined as described in Materials and Methods. Seventy-three metastatic foci were measured in the control group (Addl70-3) and 25 metastatic foci in the AdCysC treatment group.
reconstitution of the proteolytic balance in the stroma between cathepsins and their natural inhibitors. However, we point at the necessity to be aware of organ-specific protease expression during tumor-host cell interactions and its consequence for the efficacies of antiproteolytic intervention.

Acknowledgments


References

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