Abstract. Background: Matrix metalloproteinases (MMP) are strongly associated with cancer progression. Broad-spectrum MMP inhibition is rarely beneficial clinically due to adverse effects. Of all MMPs, the gelatinases are associated with the spread of several types of cancer, including oral carcinoma. We have developed gelatinase-specific peptides, as well as their fusion with green fluorescent protein (GFP), capable of effectively targeting carcinomas.

Materials and Methods: Effects on tumor growth and lymphatic micrometastatic spread in vivo was studied by use of HSC-3-cell xenografted athymic nude mice. Antigelatinolytic mono- vs. polytherapies, as well as biological activity of peptide-GFP fusion, were also analyzed in vivo.

Results: Antigelatinolytic therapy effectively inhibited growth of xenografted tumors in mice but the proportion of enlarged lymph nodes remained the same; antigelatinolytic polytherapy seemed not to potentiate the antitumor effects. The peptide–GFP chimera sustained its activity in vivo and effectively homed to the primary tumors.

Conclusion: Peptide gelatinase inhibitors are effective in inhibiting primary tumor growth but alone do not prevent the spread of carcinoma cells; however, their bioactive GFP fusion is a candidate for tumor characterization and imaging.

In carcinomas, the epithelial cells escape from the control of normal regulatory mechanisms and not only recklessly divide at their original site, but also induce vasculature growth, invade to adjacent structures and eventually escape from their origin and home to secondary sites forming metastase. In carcinoma expansion, malignant cells need to protrude through the underlying basement membrane to compensate for their spatial needs. This process includes the proteolytic processing of basement membrane and extracellular matrix, combined with the enhanced cell motility and reduced cohesion (1). In carcinoma progression, the metalloproteinases (MMPs) in particular are considered to play a
significant role and interestingly they are not only produced by cancer cells but also by stromal and inflammatory cells (2, 3). Full-spectrum MMP inhibition has yielded disappointing results in clinical trials, mainly because of the need for reduced dosage due to unwanted side-effects such as muscle or joint pain (4). So far, nonselective MMP inhibitors have been demonstrated to have clinical benefits only in the treatment of end-stage Kaposi’s sarcoma and prostate cancer (5, 6). For that reason, development of more targeted and limited-spectrum MMP inhibitors has recently been intense. However, no such clinical trials of MMP inhibitors exist to date. Proteolytic patterns between different types of cancer may vary greatly but still only a few enzymes dominate in carcinoma invasion in vitro and in vivo, namely the gelatinases (MMP-2 and MMP-9) (1, 3, 7).

We have developed a gelatinase-inhibiting peptide CTTHWGFTLC (CTT) which prevented osteosarcoma and melanoma growth in vivo and can also target tumor microvasculature (8, 9). We have further demonstrated the biological efficacy of CTT variants and another gelatinase inhibitor in vitro and in vivo (10, 11). Limited-spectrum MMP inhibitors are promising anticancer agents, but thus far little is known about their effects against carcinoma metastasis formation. Another aspect regarding elevated tumor gelatinase levels is that they can be utilized in tumor imaging and perisurgical detection of tumor spread. Conventional toluidine blue and radiolabeling methods target only adjacent lymphatic nodes but do not give information about local tumor spread peripherally and their sensitivity is often compromised. In vivo phage display or monoclonal antibodies would be one strategy but they are time- and resource-consuming since they need to be developed individually for each cancer patient (12). For this reason, we have recently developed a fusion of CTT and green fluorescent protein (GFP) capable of binding to cancer cells in vitro (13). Since CTT has been shown to target tumor microvasculature, tumor-targeting with CTT–GFP would be an interesting option in tumor imaging pre- and peripherally. However, to date its bioefficacy has not been demonstrated.

Materials and Methods

Antigelatolytic peptides. Antigelatolytic CTTHWGFTLC (CTT) peptide and its control peptide CERGLETSC (C) were custom made (Neosystem, Strasbourg, France). ProMMP-9-targeting CGYGRFSPPC (PPC) and CRVYGPYLLC (CRV) peptides were synthesized, purified and verified as described in (10) and were a generous gift from Dr. Erkki Koivunen (MD Anderson Cancer Center, Houston, TX, USA). The lyophilized peptides were prediluted (20 mg/ml in dimethyl sulfoxide or 1 mg/ml in saline) stock solutions prior to use. Peptide solutions were microfiltered before injection to animals.

CTT–GFP Fusion. CTT–GFP chimera and its controls were manufactured and purified as described elsewhere (13). Briefly, plasmid pLEB633 encoding for CTT–GFP and plasmid pLEB634 with coding sequence for the negative control chimera, Ala–CTT–GFP, and plasmid pLEB726 encoding for histidine-tagged GFP were transformed into the Escherichia coli expression strain BL21Star(DE3)pLysS. The transformed cells were grown to logarithmic growth phase at 37˚C with shaking, and protein production was induced by isopropyl β-D-1-thiogalactopyranosid addition. After 3 hours’ cultivation, the cells were harvested by centrifugation, followed by cell disruption with lysozyme and sonication. The solubilized CTT–GFP and Ala–CTT–GFP chimeras were then separated from cell debris by centrifugation and purified using HisBind Quick 900 cartriges (Novagen, Darmstadt, Germany).

Cell culture. Human HSC-3 tongue carcinoma cells (JCRB Cell Bank 0623; National Institute of Health Sciences, Japan) for in vitro and in vivo experiments were cultured in 1:1 of Dulbecco's modified Eagle’s medium (calcium-free) and Ham's F12 culture medium supplemented with 10% fetal bovine serum, 0.65 mg/ml G418, 100 U/ml penicillin and 1 mM CaCl₂ as described earlier (14, 15).

Conditioned media preparation and cytotoxicity. Effects of peptide therapy on cell viability were assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described in (11). For the in vitro assays, the cells were precultured as above but the analysis was performed using serum-free medium.

Animal experiments. The animal experiments were performed as approved by the Ethics Committee for the Animal Experiments at the University of Helsinki, and by the Ethics Committee for Animal Experimentation in the State Provincial Office of Southern Finland. The HSC-3 tongue carcinoma tumour xenografts were initiated as described elsewhere (20) by subcutaneous injection of 2×10⁶ HSC-3 cells in 200 μl of serum-free medium to both flanks of 6- to 8-week-old athymic nude female mice (Harlan, the Netherlands). In the combined peptide-therapy study, on days 4, 8, mice (n=8/group) were treated daily with peptides (either with CTT alone at 2 mg/ml, or with combination of CTT, CRV and PPC, all at 1 mg/ml). Solutions were injected in 100 μl of 0.9% NaCl/H₂O i.v. to the tail. Criteria for euthanasia were: significant weight loss observed or a tumor diameter >10 mm. Tumor volumes were calculated with formula (Π/6)×A×B×B, where A is the length and the B is the width of the tumor. In the lymph node spread study, the mice (n=10/group) were treated with CTT or controls, and tumor formation and size was monitored as described above. The mice were euthanized as a cohort when the first tumor reached the level for euthanasia level, and enlarged lymph nodes were analyzed as described in (21). In the CTT–GFP fusion tumor-targeting study, HSC-3 tumors were initiated as described above. The tumors were allowed to grow for 18 days. The mice (n=5/group) were injected either with saline, gelatinase targeting CTT–GFP, or non functional Ala–CTT–GFP or His–GFP (all at 1 mg/ml in 100 μl of PBS). The fusion peptides were allowed to redistribute 1 h after injection and then the mice were euthanized for tumor fluorescence studies.

Tumor fluorescence analysis. The tumors were embedded in Tissuetek-OCT (Sakura, Japan) and frozen. Frozen sections (10 μm) were cut with a microtome. Tumor tissue was confirmed by histological analysis (11) and sample fluorescence detected with an Axiovert 200M microscope (Zeiss, Germany) using filters for GFP.
Fluorescence of each sample was quantified from the three most intensive areas (magnification ×400) with Quantity-One program (Bio-Rad, CA, USA).

Statistical analysis. Differences between the groups in tumor metastasis, size and sample fluorescence were compared with Mann-Whitney and Student’s t-test. Results of \( p<0.05 \) were considered statistically significant. Data are expressed as the mean±SD.

Results

Antigelatinolytic peptides exert no cytotoxic potential. Antigelatinolytic and proMMP-9 activation inhibiting properties of CRV and PPC peptides have been demonstrated (10). Similarly, CTTHWGFTLC peptide (CTT) and its hydrophilic derivate GRENYHGCTTHWGHTLC (CTT2) have also been shown to exert antigelatinolytic potential, but their effect seems to more likely be anticatalytic than inhibitory of proMMP activation (10, 11). In the MTT assay, the used peptide concentrations did not affect HSC-3 tongue carcinoma cell viability in vitro indicating that the effects demonstrated below are due to their antigelatinolytic and antitumor properties (data not shown).

Antigelatinolytic CTT peptide inhibited primary tongue carcinoma growth but did not affect tumor cell spread. The mice (n=10/group) bearing human HSC-3 tongue squamous cell carcinoma tumors were monitored and tumor size measured three times a week. All mice in the control as well as in the CTT-treated group were euthanized when the first individual reached end point criteria, tumor diameter 10 mm, in day 21 postinoculation. Tumors were significantly smaller in CTT–treated group as compared to saline-treated controls (\( p=0.006 \)) as can be seen in Figure 1A. After resection of the primary tumors, the mice were also dissected to search for lymphatic spread. Approximately one half of the six major auxiliary lymph nodes were affected in both CTT–(mean 2.8±1.229) and saline (mean 3.3±1.567)–treated groups, and there was no statistically significant difference between the two groups (\( p=0.199 \)).

Combined antigelatinolytic and proMMP-9 targeting therapy increased HSC-3 tumor growth in vivo. The mice (n=7/group) were inoculated with HSC-3 cells as described above and then treated either with antigelatinolytic CTT alone or with a combination of CTT/CRV/PPC cocktail which targets both activation and activity of gelatinases. Tumors in mice of the CTT group grew slightly slower as compared to those of the CTT/CRV/PPC group. On day 45 post inoculation, the tumors had an average volume of 28.4 mm\(^3\)±62.5 in CTT group and 162.3 mm\(^3\)±349 in the CTT/CRV/PPC group, however the difference was not statistically significant (\( p=0.17 \)). Clearly, there was no evident benefit from combinatory therapy as revealed by tumor size or survival functions (Figure 1B) and the experiment was terminated. In the dissection of CTT/PPC/CRV-treated mice, two abdominal metastases were also found.

Chimera between CTTHWGFTLC and green fluorescent protein (CTT–GFP) homed to tongue carcinoma cell tumors in vivo. The HSC-3 tumors were initiated as described above, and allowed to grow for 18 days. CTT–GFP or the non-functional Ala–CTT–GFP and His–CTT controls were injected into the tail vein and allowed to distribute for 1 h, after which the mice were euthanized. Tumor fluorescence (Figure 2A and B) was analyzed from the frozen sectioned samples by densitometry. The measured fluorescence in the CTT–GFP group (4.69±0.29 U/mm\(^2\)) was significantly higher as compared to the that of non functional Ala–CTT–GFP (3.18±1.23 U/mm\(^2\)) and His–GFP (3.42±0.09 U/mm\(^2\)) (\( p=0.025 \)) (Figure 2C).

Figure 1. CTTHWGFTLC (CTT) therapy effectively targets HSC-3 tumors in vivo but multilevel gelatinase inhibition does not enhance antitumor effects. Athymic nude female mice (n=10/group) were inoculated with HSC-3 tumors and then treated either with (CTT) or saline (CTRL). All mice were euthanized as a cohort and tumor size analyzed. Growth of CTT-treated tumors was significantly lower as compared to controls (A). To evaluate whether it is possible to promote antitumor effects of antigelatinolytic therapy in vivo by affecting both MMP activity and proMMP activation, the HSC-3 cells were again implanted into athymic nude mice (n=7/group). The mice were treated either with CTT alone or with wide range antigelatinolytic CTT/CRV/PPC mix. There is no statistical difference between the groups as can be seen by Kaplan-Meier survival analysis (B).
Discussion

Prognosis of head and neck carcinoma patients is rather poor, and carcinomas of the tongue and floor of the mouth are often especially problematic since they are commonly treated surgically, and as a result, the postoperative quality of life is often compromised despite facial reconstruction. For this reason, alternatives and adjuvant therapies are warranted for more efficient treatment and earlier diagnostics. We have here demonstrated that gelatinase-inhibiting and tumor vasculature-targeting CTT is capable of interfering with the growth of xenografted human tongue squamous cell carcinomas in mice. However, CTT therapy affected only the growth of the primary tumors and there was no significant difference in tumor spread. It is evident that MMPs, especially gelatinases (MMP-2 and -9), have often been linked to tumor growth and spread. MMP-2 and MMP-9 knockout mouse models suggest that these gelatinases may also actively participate in the carcinogenic process; Bergers et al. reported that only MMP-9 seems to affect the number of developing tumors, whereas both of the gelatinases seem to affect tumor progression (22). MMP-9 also seems to be especially associated with tumor vascularization since MMP-9-deficient mice have been shown to fail to grow tumor vasculature, whereas this ability was gained after transplantation of MMP-9 producing wild-type stroma (23). However, it seems that the nature of MMP-9 in some cases is dualistic since it has also been reported to be able to release angiostatin and other bioactive molecules from the carcinoma stroma, which results in diminished neovascularization (24, 25).

Here we present new findings that antigelatinolytic therapy alone cannot prevent tongue carcinoma cell spread from the primary site, which suggests that gelatinase inhibitors may not be functional as a means of preventing the major cause of cancer death, metastasis. Similar conclusions can also be drawn based on in vivo findings where antigelatinolytic polytherapy with CTT/CRV/PPC combination resulted in an even slightly poorer outcome as compared to CTT alone. These data support the present findings that other mechanisms in invasion-associated extracellular matrix remodelling, such as force-mediated invasion, can often be equally important as proteolytic mechanisms (18). This may also at least partly explain the poor results in the use of MMP inhibitors in clinical trials. In fact, the disappointing results in such trials have often been explained by the fact that they were performed on end-stage cancer patients already having metastases; these new findings suggest that MMPs do not always have the key role in the spread of carcinoma cells, making the discussion irrelevant. It has also been demonstrated that protease profiles of micrometastases and primary tumors may differ greatly which makes development of anticancer strategies even more challenging (26). Protease–cell surface receptor interactions makes the whole process even more interesting and challenging to control by therapeutic means. An example of this is the ability of MMP-14 to both activate and inhibit MMP-2, depending on its concentration (27-29).

Prognosis of a carcinoma patient depends on the phenotype and the spread of the tumor. Conventional pre- and perioperative tumor spread evaluations are based on x-ray, positron-emission tomography or magnetic resonance tomography, and/or preoperative lymphatic mapping by cyan blue or radioisotopes. However, these methods have certain limitations: cyan blue and radioisotopes map only adjacent lymph nodes and do not reflect actual peritumoral spread, whereas imaging techniques are not useful perioperatively. Custom made peptide display or tumor specific antigens fused to light-emitting dye for each patient individually would be ideal but it is highly time- and resource-consuming (30). For this reason, discovery of new constructs recognizing different tissues is a highly important area of research that would benefit therapeutic as well as diagnostic goals. In fact, very recently, promising results have been reported of using fusion proteins as target-seeking therapy in wound healing (31). We have also earlier demonstrated that antigelatinolytic CTT peptide is capable of targeting tumor vasculature in vivo.

Figure 2. CTT−GFP fusion targets tongue carcinomas in vivo. CTT−GFP chimera or its non-functional counterparts Ala−CTT−GFP and His−GFP were injected into veins of HSC-3 tumor-bearing athymic nude mice and were allowed to reorientate for 1 h. The mice were then euthanized and tumor fluorescence was analyzed from 10 μm frozen sections. CTT−GFP-treated tumors (A) had significantly stronger fluorescence as compared to these treated with Ala−CTT−GFP (B). The difference in tumor fluorescence between CTT−GFP and controls was statistically significant (C).
Therefore the recently developed chimera of CTT and GFP is a promising construct for developing new tools for cancer diagnostics (13). We have here demonstrated that high molecular weight CTT-GFP can still target carcinomas in vivo and has future potential in tumor imaging. However, the results presented here are limited to post mortem findings and therefore real-time in vivo imaging setups are needed for sensitivity and potential clinical evaluations.

In summary, peptide gelatinase inhibitors, such as CRV, CTT and its derivates, are effective in reducing the growth of the primary tumors in vivo but it seems that they do not affect tumor spread. This suggests that MMP inhibitors should be used as adjuvant therapy in the future clinical studies. However, gelatinase-targeting CTT peptide is an interesting option when developing future tools for pre- or perioperative diagnostics, since it maintains its tumor targeting activity in vivo even when fused to a 30 kDa green fluorescent protein. This observation emphasizes the highly flexible nature of the CTT peptide as a tumor-homing agent which can be linked to a large-sized molecules without disturbing its tumor-targeting capabilities.

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