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Recombinant CBD–HepII polypeptide of fibronectin inhibits αvβ3 signaling and hematogenous metastasis of tumor ☆

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Abstract

The interaction of integrin αvβ3 and its ligands are crucial for tumor metastasis. Recombinant CBD–HepII polypeptide of fibronectin, designated as CH50, suppressed the binding of tumor cells to ECM molecules, and abolished the promoting effect of soluble fibronectin and fibrinogen on tumor cell adhesion to ECM molecules. The underlying mechanisms involve the blockade and downregulation of αvβ3 and its co-receptor syndecan 1 by CH50. The activation of FAK, upregulation of cdc2, the production and activation of MMP-2 and MMP-9 by ECM molecules-stimulated tumor cells were inhibited by CH50. CH50 reduced the tumor cell arrest during blood flow, and also inhibited the invasive ability of tumor cells. The *in vivo* expressed CH50 suppressed the lung metastasis of circulating tumor cells, and prolonged the survival of mice after tumor cell inoculation. These findings suggest a prospective utility of CH50 in the gene therapy for prevention of tumor metastasis.

Keywords: CBD–HepII polypeptide; Fibronectin; Integrin αvβ3; Tumor metastasis

Tumor cell arrest within the vasculature and the following migration are the prerequisite for metastasis from the blood stream [1]. αvβ3 is the key integrin mediating tumor cell arrest during flow [1,2]. The ligands of αvβ3 serve as adhesive matrices when they are immobilized at the surface of endothelial cells, or as constituents of subendothelial matrix [2]. αvβ3-matrix interaction can break the flow of a tumor cell and immediately stabilize firm arrest [2]. αvβ3-mediated tumor cell binding to platelets requires multivalent plasma protein ligands as cross-linking bridges [1]. Plasma proteins such as fibrinogen and fibronectin have been shown to contribute to hematogenous metastasis by supporting tumor cell cohesion with matrix-attached platelets [2–4]. Therefore, interfering with the interaction between integrins and their ligands provides a potent means to inhibit tumor invasion and metastasis.

As an important ligand for integrins, fibronectin (FN) is involved in various aspects of tumor biology, including tumor invasion and metastasis [5], whereas the proteolytic fragments or recombinant polypeptides containing some domains of FN can function negatively on tumor [6–9]. The central cell-binding domain (CBD, also CellI) and C-terminal heparin-binding domain (HepII) in the intact FN contribute to tumor cell adhesion and migration [10,11]. In contrast, CBD–HepII recombinant polypeptide (designated as CH50) inhibits the growth and the invasiveness of tumor [12–14]. In this study, we investigated the inhibitory effect of CH50 on the metastasis of circulating tumor cells to target organ and the underlying mechanisms. We found that CH50 suppressed the interaction of tumor cells with different integrin ligands, and inhibited the activation of tumor cells by extracellular matrix (ECM)

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molecules. Moreover, the *in vivo* expressed CH50 effectively inhibited the invasiveness of tumor cells from blood to lung.

Materials and methods

Animals and cell lines. Female C57BL/6 mice, 6- to 8-week-old, were purchased from Center of Experimental Animals of Chinese Academy of Medical Science (Beijing, China). B16F1 cell line was purchased from China Center for Type Culture Collection (Wuhan, China), and cultured according to their guidelines.

Reagents. CBD–HepII recombinant polypeptide of human fibronectin, designated as CH50, is composed of Pro1239–Ser1515 (CBD) linked with Ala1690–Thr1960 (HepII) through Met. CH50 was prepared and purified from the engineered bacteria harboring a prokaryotic expression vector carrying the recombinant cDNA encoding CH50 [14]. Eukaryotic expression vector pCH510 was constructed by insertion of the recombinant cDNA into plasmid pcDNA3.1 (Invitrogen). Matrigel, mouse vitronectin, fibronectin, fibrinogen, and laminin were purchased from BD Biosciences (Mountain View, CA) and Sigma (St. Louis, MO).

Adhesion assay. B16 cells were cultured in the presence or absence of 20 μg/ml CH50 for 24 h, and then added (1×10^5/well) to 96-well plates pre-coated with matrigel or individual integrin ligand. CH50, fibronectin, or fibrinogen was added to the wells as indicated. After 2-h incubation at 37 °C and the removal of non-adherent cells, the adherent cells were detected by LDH assay [15] and the results were expressed as A_{570} values.

Analysis of gene expression by real-time RT-PCR. Total RNA was extracted from cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The mRNA level was determined by real-time RT-PCR using procedures and primers for αv, β3, cdc2, MMP-9, and GAPDH genes as described previously [14]. Other primer sequences were as follows: syndecan 1, sense 5′-ACACCGAGACTGCTTTTACC-3′, antisense 5′-CGACAACCTCTTTGATGAC-3′; MMP-2, sense 5′-TTCTTCG CAGGGAATGAG-3′, antisense 5′-GCGATGAAGATGATAGGG-3′.

Western blot analysis. Western blot assay was done as described previously [14]. Antibodies were purchased from Chemicon (Temecula, CA), Upstate (Charlottesville VA), Santa Cruz (Santa Cruz, CA), R&D systems (Minneapolis, CA), and Cell Signaling (Beverly, MA), respectively.

MMP assay by gelatin zymography. B16 cells, untreated or protreated with CH50 for 24 h as above, were cultured for 48 h in DMEM medium containing 1% FCS in matrigel-coated or uncoated plates. The assay of MMP-2 and MMP-9 in supernatants was performed as described previously [14].

In vivo gene transfection. Plasmid DNA was prepared and analyzed as described previously [16]. The *in vivo* transfection with plasmid pCH510 or control plasmid pcDNA3.1 was performed by i.v. injection. Briefly, naked plasmid DNA (100 μg in 2 ml of saline) was injected to mouse via tail vein within 10–15 s. The expressed CH50 in serum was monitored by Western blot as described previously [14].

Assay of tumor cell arrest in lung. B16 cells were pretreated with CH50 as above and then labeled with CFSE, or mixed with 30 μg of CH50 after labeling with CFSE without pretreatment. About 5×10^5 CFSE-labeled B16 cells were injected into mice via tail vein. Or, untreated CFSE-labeled B16 cells were injected into mice via tail vein 2 days after the i.v. injection of pCH510, pcDNA3.1, or saline. Lungs were harvested from mice 5 h and 24 h after tumor cell injection. Frozen sections were prepared and analyzed by fluorescence microscopy. Fluorescent spots were counted from 20 randomly chosen fields in the sections of each mouse.

Matrigel invasion assay. The matrigel invasion assay was performed using modified Boyden chambers (Transwell, Corning, Inc., Corning, NY). The transwell filter inserts (Costar) were coated with matrigel. B16 cells, either pretreated with CH50 for 24 h and then washed or directly mixed with CH50 (20 μg/ml), were placed (2.5×10^5 cells) in the upper compartment. After 48-h incubation, B16 cells that had migrated to the lower surface of the membrane were counted, and the migrated cell percentage was calculated.

Animal experiments and treatment protocol. B16 cells (5×10^5) were injected into mice via tail vein on day 0 (d0). The mice ($n = 8$ per group) were sacrificed on d15, and the tumor nodes on the surface of lung were counted. To evaluate the direct effect of CH50, B16 cells were either pretreated with CH50 (20 μg/ml) for 24 h or directly mixed with 30 μg of CH50 before i.v. injection. To evaluate the inhibitory effect of *in vivo* expressed CH50, the mice received the i.v. injection of pCH510, pcDNA3.1, or saline on −d2, d1, d4, d7, and d10.

In survival rate follow-up experiments, the mice inoculated with B16 cells were first treated by i.v. injection of plasmids as above, and then once a week for 5–10 weeks depending on the survival time of each mouse. The number of living mice was recorded. The living mice were continuously fed until d110, and then sacrificed for the analysis of tumor nodes.

Data analysis. Results were expressed as mean values ± SD and interpreted by ANOVA-repeated measures test. Differences were considered to be statistically significant when $P < 0.05$.

Results

CH50 polypeptide inhibits tumor cell adhesion to integrin ligands

To clarify the effect of CH50 on tumor cell adhesion, we first analyzed the adhesion of tumor cells to matrigel. The amount of adherent cells was decreased in the presence of CH50 (Fig. 1A). More importantly, the pretreatment with CH50 also inhibited tumor cell adhesion (Fig. 1A), and the inhibitory effect lasted for several days after the removal of CH50 (Fig. 1B), suggesting that CH50 not only blocks integrins but also reduces the activity of integrins in a relatively long period of time. The adhesion of tumor cells to individual ligands was also inhibited by pretreatment with CH50 (Fig. 1C) or directly mixing with CH50 (data not shown). Soluble fibronectin and fibrinogen promoted the adhesion of B16 cells to matrigel, whereas this promoting effect was abolished by pretreatment of tumor cells with CH50 (Fig. 1D). When B16 cells were pretreated with CH50 together with soluble FN, the inhibitory effect of CH50 was the predominant effect on tumor cells (Fig. 1E).

CH50 polypeptide reduces the response of tumor cells to ECM molecules

Since CH50 inhibited the adhesion of tumor cells to fibrinogen, a ligand for integrin αvβ3 but not other integrins, we then detected αvβ3 expression in melanoma cells. After 24 h-treatment with CH50, both αvβ3 and its co-receptor syndecan 1 were downregulated in B16 cells (Fig. 2A). We then analyzed the expression of cdc2 gene, a down-stream gene of αvβ3 signaling pathway. The pretreatment of B16 cells with CH50 suppressed both cdc2 expression and the upregulation of cdc2 expression in response to ECM molecules (matrigel) (Fig. 2B). Consistently, the activation of FAK by ECM molecules was also inhibited by the pretreatment of tumor cells with CH50 (Fig. 2C). Finally, we analyzed the production of MMPs. B16 cells produced both latent form and active form of MMP-2 and MMP-9 after stimulation with matrigel. However, they were significantly inhibited by the pretreatment of tumor cells with CH50 (Fig. 2D).

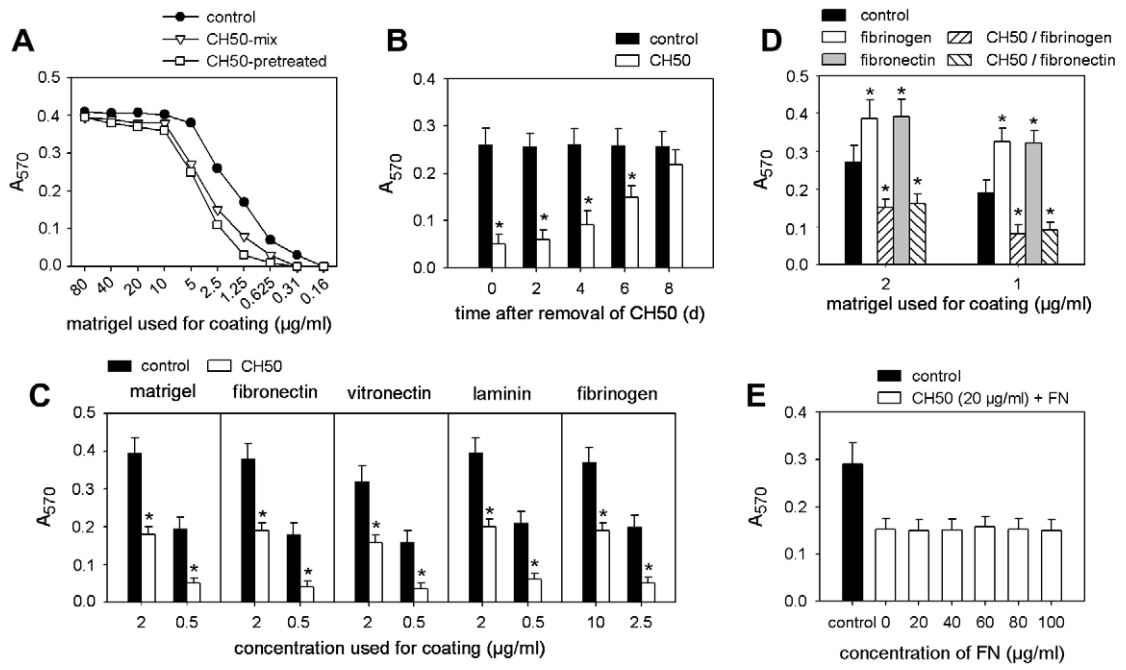

Fig. 1. CH50 inhibits the adhesion of B16 cells to ECM molecules. (A) CH50 inhibits tumor cell adhesion to matrigel. B16 cells were pretreated or directly mixed with CH50, and used for the adhesion to matrigel-coated plate. (B) The slow recovery of adhesive ability of CH50-pretreated tumor cells. B16 cells were used for adhesion to matrigel after treated with CH50 for 24 h and then cultured for the indicated time. (C) CH50 inhibits tumor cell adhesion to individual ligands. B16 cells were pretreated with CH50 and used for the adhesion to the plate coated with different ligands. (D) CH50 suppresses the promoting effect of fibrinogen and FN on tumor cell adhesion to matrigel. Untreated or CH50-pretreated B16 cells were used for adhesion assay in the presence or absence of soluble fibrinogen and soluble FN. $^{*}P < 0.05$, compared with control group. (E) Soluble FN does not influence the effect of CH50. B16 cells were pretreated with CH50 and soluble FN, and then used for the adhesion assay.

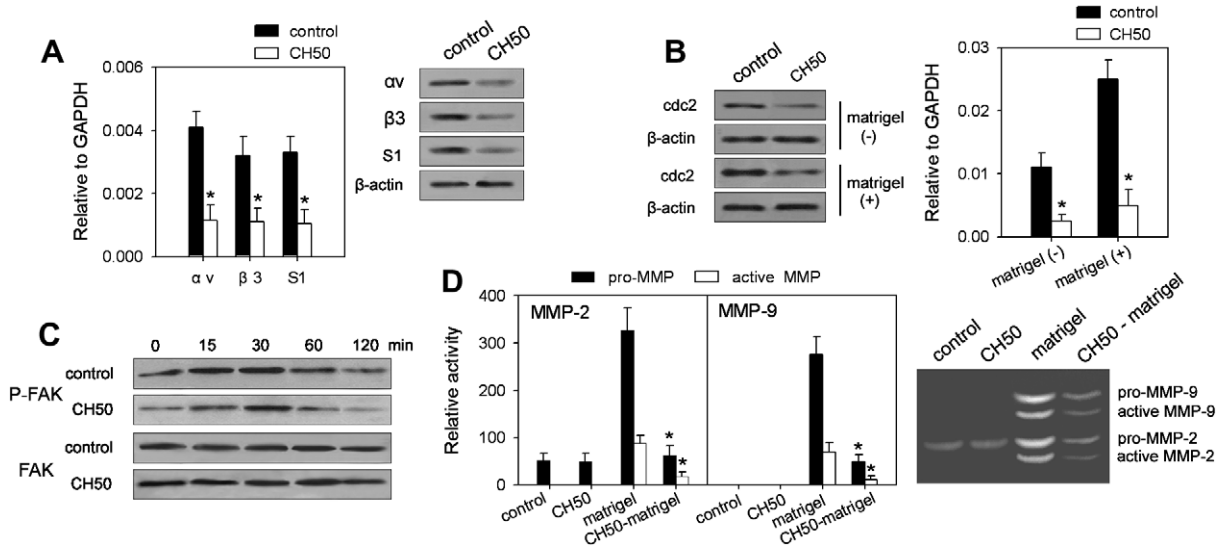

Fig. 2. CH50 suppresses the expression and activity of αvβ3. (A) Assay of αv, β3, and syndecan 1 (S1) expression. B16 cells were treated with CH50 for 24 h *in vitro*. The relative mRNA levels and proteins of αv, β3, and syndecan 1 were detected by real-time RT-PCR and Western blot, respectively. (B–D) Assay of cdc2 expression, the activation of FAK, and the production of MMP-2 and MMP-9. Untreated and CH50-pretreated B16 cells were cultured in the presence or absence of ECM molecules (matrigel). The relative mRNA level and protein of cdc2 were detected by real-time RT-PCR and Western blot, respectively (B). The phosphorylated FAK and total FAK in matrigel-stimulated cells were analyzed by Western blot (D). MMP-2 and MMP-9 in supernatants were detected by zymography assay (D). $^{*}P < 0.05$, compared with matrigel group.

CH50 polypeptide inhibits the metastatic ability of melanoma cells

We next investigated the direct effect of CH50 on the invasiveness of circulating tumor cells to target organ. Both 5 h and 24 h after i.v. injection of CFSE-labeled B16 cells, the fluorescent spots in lung tissues were significantly reduced by directly mixing or pretreating tumor cells with CH50 (Fig. 3A), suggesting that CH50 inhibited both melanoma cell arrest in lung and the following invasion into

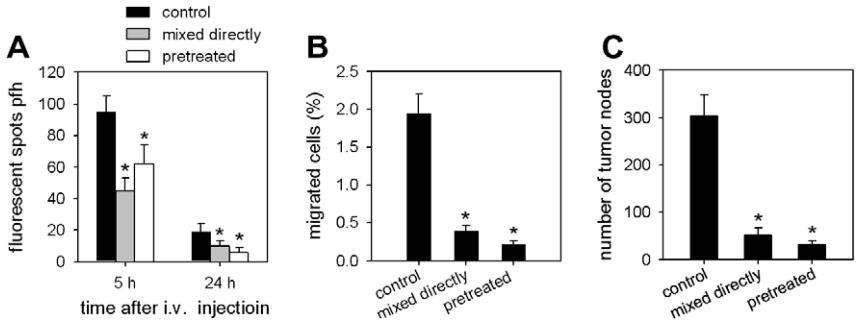

Fig. 3. CH50 inhibits the metastatic ability of tumor cells. (A) B16 cells were either directly mixed or pretreated with CH50 and labeled with CFSE before i.v. injection as described in Materials and methods. Mice were sacrificed 5 h ($n = 8$ per group) and 24 h ($n = 8$ per group) after tumor injection. Fluorescent spots in the frozen sections of lung tissues were counted. (B) Migratory activity of B16 cells. B16 cells, directly mixed or pretreated with CH50, were used for matrigel invasion assay. (C) Mice ($n = 8$ per group) were inoculated by i.v. injection with B16 cells either directly mixed or pretreated with CH50. The tumor nodes on the surface of lung were counted 15 days later. $^*P < 0.05$, compared with control group.

lung tissue. The results from matrigel invasion assay also revealed a reduced invasive ability of B16 cells which were mixed or pretreated with CH50 (Fig. 3B). When B16 cells were directly mixed or pretreated *in vitro* with CH50, and then injected into mice via tail vein, the tumor metastatic nodes in mice were significantly reduced by CH50 (Fig. 3C).

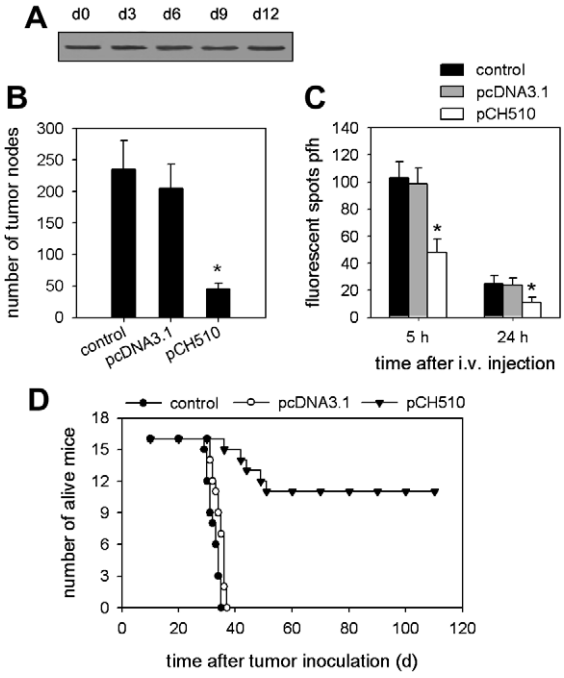

Fig. 4. *In vivo* expressed CH50 inhibits the metastasis of circulating melanoma cells. (A) Gene delivery was performed as described in Materials and methods. CH50 in serum was detected by Western blot at the indicated time. (B) Mice were inoculated with B16 cells and received *in vivo* transfection of pCH510. 15 days later, the mice were sacrificed and the tumor nodes on the surface of lung were counted. $^*P < 0.05$, compared with control group. (C) The delivery of pCH510 was performed 2 days before tumor cell injection. Mice were sacrificed 5 h ($n = 8$ per group) and 24 h ($n = 8$ per group) after the i.v. injection of CFSE-labeled B16 cells. Fluorescent spots in the frozen sections of lung tissues were counted. (D) The survival rate follow-up after gene therapy with CH50. Mice were inoculated with B16 cells and treated as described in Materials and methods. Mice were monitored for the survival. The data were the combination of two independent experiments ($n = 8$ in each experiment), in which the same results were obtained.

In vivo expressed CH50 inhibits the metastasis of circulating melanoma cells

We then inoculated mice with B16 cells and performed *in vivo* transfection of pCH510. The *in vivo* expressed CH50 was identified (Fig. 4A). The metastatic tumor nodes were significantly reduced in pCH510 group (Fig. 4B). The inhibitory effect of CH50 in blood on circulating tumor cells was further proved by injection of CFSE-labeled B16 cells 2 days after a single i.v. injection of pCH510. The fluorescent spots in lung tissues, both 5 h and 24 h after tumor cell injection, were significantly reduced in pCH510 group (Fig. 4C). Then, survival rate follow-up experiment was performed to evaluate the therapeutic effect of CH50 on hematogenous metastasis of tumor cells. All of the mice in control and pcDNA3.1 groups died within 37 days, whereas 75% of mice in pCH510 group survived up to 110 days (Fig. 4D). The average size of tumor nodes carried by these living mice, sacrificed on d110, was less than 0.5 mm. The number of tumor nodes was 23.4 ± 9.1, whereas the lungs in a parallel control group had already been full of metastatic tumor nodes (>500 nodes in each mouse) on d25 after tumor inoculation.

Discussion

Recombinant polypeptide CH50 contains two domains from fibronectin. CBD binds integrins [17,18] and HepII binds syndecans which cooperate with integrins for signal transduction [10,19]. Unlike the intact FN, the binding of CH50 to αvβ3 blocks this integrin instead of producing active signaling, because it does not upregulate cdc2 expression, and can directly inhibit the binding of tumor cells to fibrinogen, a specific ligand for αvβ3 [2,20]. On the other hand, CH50 may also bind other integrins to disturb the expression and function of αvβ3, since the binding of one integrin by a ligand can produce an inhibitory effect

on other integrins [21]. The intact FN can not interfere with the function of CH50, suggesting that CH50 may bind to some integrins which FN can not compete because of the spatial conformation restriction in intact FN and higher freedom of CH50 which has only two domains. Furthermore, αvβ3 is dependent on syndecan 1 to become activated and to mediate signals for tumor metastasis [19,22]. The downregulation of syndecan 1 expression by CH50 may further reduce the activity of αvβ3. Although CH50 may block integrins and/or syndecans to produce a quick inhibitory effect, the downregulation of these receptors may reduce the adhesive ability of tumor cells for a relatively longer time, which might be the main mechanism through which CH50 suppresses tumor metastasis.

αvβ3 is the key integrin mediating tumor cell arrest during flow [1,2]. Downregulation of αvβ3 and syndecan 1 by CH50 can suppress tumor cell arrest, which was demonstrated by the reduced accumulation of tumor cells in lung 5 h after the injection of tumor cells via tail vein. In addition, CH50 can abolish the promoting effect of fibrinogen and fibronectin on tumor cell adhesion, which can further suppress tumor cell arrest, since fibrinogen and fibronectin promote the adhesion of circulating tumor cells to matrix-attached platelets and matrix-attached tumor cells so to enhance tumor cell arrest under dynamic flow conditions [2–4].

The subsequent invasive behavior after the adhesion of an invasive tumor cell is regulated and promoted by the proteins in basement membrane and ECM such as fibronectin, laminin, and vitronectin [20,23,24]. The interaction of integrins with these molecules can alter cellular behavior through the recruitment and activation of signaling protein FAK which functions to promote cell motility by generating signals leading to tumor cell migration and invasion [25,26], and upregulation of cdc2 which takes part in the modulation of tumor cell migration [27]. Metastatic spread also depends critically upon the ability of tumor cells to breach basement membrane by elaborating and secreting matrix metalloproteinases (MMPs) [28–30]. Vitronectin, laminin, and fibronectin can trigger or promote the production of active MMP-2 and MMP-9 by tumor cells [23–25,29]. The appearance of active MMP-2 and MMP-9 is closely correlated with invasiveness of metastatic tumor cells. CH50 suppressed the binding of tumor cells to fibronectin, laminin, and vitronectin, and also inhibited the activation of FAK, the expression of cdc2, and the production of active MMP-2 and MMP-9, indicating that CH50 inhibits the invasiveness of tumor cells by suppressing multiple effects mediated by integrin–matrix interaction.

Tumor metastasis may occur anytime in a long period of time after diagnosis and treatment of tumor. Gene therapy with CH50 is suitable for the application in this situation. In addition, the efficacy of drugs or peptides which inhibit tumor metastasis by simply blocking integrins will be influenced by fluctuation of their concentration in blood, whereas the long-lasting inhibitory effect of CH50 on tumor cells will reduce the influence of its efficacy by fluctuation of its concentration in blood. Therefore, the gene therapy with CH50 can effectively suppress tumor metastasis in a relatively long period of time, which implicates a potent benefit for prevention of tumor metastasis after surgical removal of tumor or tumor chemotherapy.

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