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Possible Inhibition of Cancer Cell Adhesion to the Extracellular Matrix in NK4-induced Suppression of Peritoneal Implantation

MAMORU YOSHIMURA[1*], HITOSHI FUJIWARA[1*], TAKESHI KUBOTA[1*], HISASHI AMAIKE[1], KAZUHIRO TAKASHIMA[1], SATOSHI INADA[1], KIYOTO ATSUJI[1], YASUNOBU ARAKI[1], KUNIO MATSUMOTO[2], TOSHIKAZU NAKAMURA[2] and HISAKAZU YAMAGISHI[1]

[1]*Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kawaramachi-hirokoji, Kamigyo-ku, Kyoto, 602-0841;*
[2]*Division of Molecular Regenerative Medicine, Course of Advanced Medicine, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka, 565-0871, Japan*

Abstract. *Milky spots (MS), peritoneal lymphoid tissues, expose the extracellular matrix (ECM) due to a defect of mesothelial cells on their surface, which may explain why peritoneal implantation of cancer cells preferentially takes place at MS. We recently reported that adenovirus vector-mediated intraperitoneal production of NK4 strongly suppressed MS-selective implantation of cancer cells and subsequent peritoneal dissemination, without histological evidence of angiogenesis inhibition. The present study was conducted to clarify the mechanisms underlying the suppressive effects of NK4 on peritoneal implantation. In mice intraperitoneally injected with CT26 cells that were genetically modified to produce NK4 (CT26-NK4), peritoneal dissemination was significantly suppressed with survival prolongation. A decreased cell implantation to omental MS was also detected and evaluated by green fluorescence protein (GFP) imaging. In an in vitro adhesion assay, hepatocyte growth factor-stimulated adhesion to ECM components, such as fibronectin and collagen, was inhibited in CT26-NK4 compared to control cells. These results strongly suggest an inhibition of cancer cell adhesion to the ECM in the suppression of peritoneal implantation by NK4.*

Hepatocyte growth factor (HGF), a mediator of tumor-stromal interactions, stimulates invasive and metastatic growth of various cancers through HGF/c-Met receptor binding (1-3). NK4, which was produced as an internal fragment of HGF, exerts potent antitumor effects by acting not only as an HGF antagonist, but also as an angiogenesis inhibitor (4-8). Recent studies have demonstrated that NK4 suppresses subcutaneous tumor growth, hepatic metastases, lung metastases and peritoneal dissemination of various types of cancer in mice (8-13), and it was shown that angiogenesis inhibition is a predominant *in vivo* antitumor mechanism of NK4 (8).

We previously reported that murine colon cancer CT26 cells, genetically modified to produce NK4, strongly suppressed subcutaneous tumor growth *in vivo* with histological evidence of angiogenesis inhibition (9). In peritoneal dissemination, intraperitoneally (*i.p.*)-injected cancer cells showed selective implantation to the peritoneal lymphoid tissues called milky spots (MS) (14, 15). We recently reported that adenovirus vector-mediated *i.p.* expression of NK4 strongly suppressed MS-selective implantation of MKN45 human gastric cancer cells, thereby resulting in suppression of peritoneal dissemination and prolongation of survival (12). However, no definite histological evidence of angiogenesis inhibition was found in the disseminated tumor. Peritoneal implantation, an initial step in peritoneal dissemination, consists mainly of adhesion and invasion of cancer cells to the peritoneal surface and is, therefore, considered to be angiogenesis-independent. This strongly suggests that NK4 may act on and suppress peritoneal implantation *via* mechanisms other than angiogenesis inhibition. MS show a defect of mesothelial cells on their surface (16) and, therefore, peritoneal free cancer cells may adhere to and invade the extracellular matrix (ECM) around MS in an MS-selective implantation.

In the present study, the suppression of peritoneal dissemination of CT26 cells, genetically modified to produce NK4, was demonstrated and the suppression of peritoneal

*These authors contributed equally to this work.

Correspondence to: Hitoshi Fujiwara, MD, Ph.D., Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kawaramachi-hirokoji, Kamigyo-ku, Kyoto, 602-0841, Japan. e-mail: hfuji@koto.kpu-m.ac.jp

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implantation was evaluated quantitatively using GFP imaging. Furthermore, an *in vitro* adhesion assay was conducted to clarify the possible involvement of NK4-mediated inhibition of cancer cell adhesion to the ECM in the NK4 suppression of peritoneal implantation.

Materials and Methods

Reagents. Human recombinant HGF was purified from the conditioned medium of Chinese hamster ovary cells transfected with human HGF cDNA (17, 18).

Cells and culture. CT26 is an undifferentiated colon adenocarcinoma cell line originally derived from a cancer produced by intrarectal injections of N-nitroso-N-methylurethamine into a female BALB/c mouse (19). CT26 cells, that stably produced large amounts of NK4 (CT26-NK4), were established by NK4 gene transfection, as described previously (9). Cells transfected with the neomycin-resistance gene (CT26-NEO) were used as a control. Cells were maintained in RPMI 1640 (Nacalai Tesque, Kyoto, Japan) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA) and 10% heat-inactivated fetal bovine serum (FBS; JRH Bioscience, Lenexa, KS, USA).

Adenovirus vector. The replication-deficient adenovirus vectors used in this study are based on the human Ad5 genome with E1 and E3 deletions. The AdCAG-EGFP (AdGFP) vector, that expresses enhanced green fluorescence protein (GFP) under the control of the CAG early promoter/enhancer, was kindly provided by Dr Nishioka (Department of Internal Medicine, Tokushima University, Tokushima, Japan). The vectors were amplified in human embryonic kidney 293 cells (Takara, Otsu, Japan). The viral particle concentration (expressed as plaque-forming units (pfu)/ml) was determined by a plaque formation assay using 293 cells. There was no detectable replication-competent adenovirus or E1+ virus in the preparation.

In vivo animal study. The investigation protocol was approved by the Ethics Committee of Kyoto Prefectural University of Medicine, Japan. Eight- to 10-week-old BALB/c female mice (Shimizu Laboratory Animal Center, Kyoto, Japan) were maintained free of specific pathogens. For the peritoneal cancer dissemination model, $5x10^5$ cells of CT26-NK4 or CT26-NEO in 1.0 ml of phosphate-buffered saline (PBS) were injected into the peritoneal cavity of mice. The mice were sacrificed on day 28, and the number and weights of disseminated nodules were measured. Survival was analyzed by the Kaplan-Meier method and compared between groups by the log-rank test.

Immunohistochemical analysis for microvessels. Disseminated nodules in the omentum, removed 28 days after injection of CT26-NK4 or CT26-NEO, were fixed in 70% ethanol for 1 day and then embedded in paraffin. Tissue sections were pretreated with 0.1% trypsin at room temperature for 20 min and incubated overnight at 4˚C with rat anti-mouse CD31 antibody (directed against platelet-endothelial cell adhesion molecule, or PECAM-1; PharMingen, San Diego, CA, USA). The sections were then incubated with biotinylated rabbit anti-rat IgG antibody (DAKO, Glostrup, Denmark), followed by streptavidin-biotin-peroxidase complex.

Evaluation of peritoneal implantation by GFP imaging using fluorescent microscopy and flow cytometry. CT26-NK4 and CT26-NEO cells were infected *in vitro* with AdGFP at 50 moi for 1 h in adhesion and maintained in RPMI1640 with 10% FCS for 48 h. The cells were washed twice with PBS and resuspended in PBS at $1x10^6$ cells/ml. The cell viability, determined by the trypan blue exclusion test, was more than 95% and the efficacy of transgene by AdGFP was routinely more than 85%, as assessed by flow cytometry. Then, $1x10^6$ cells of each transfectant infected with AdGFP were injected into the peritoneal cavity of mice that were preinjected with 0.5 mg of activated carbon particles (Nacalai Tesque) to identify MS as black-stained spots. The mice were sacrificed 24 h after injection; fresh omentum was resected and observed by fluorescence microscopy, and a cell suspension prepared from the omentum by mechanical and enzymatic treatment was analyzed by flow cytometry for GFP expression. The *in vitro* cultured CT26-NEO cells and a cell suspension from omentum of tumor-free mice were used as negative controls.

Adhesion assay. Plastic 96-well plates were coated with type I collagen (Nacalai Tesque) or fibronectin (Nacalai Tesque) overnight at 4˚C. The wells were rinsed with Hank's buffer and blocked with 0.2% BSA for 30 min at room temperature. Subconfluent cells were harvested and resuspended in Hank's buffer with 1% BSA. Cells ($1x10^5$cells/well) in 100 μl of Hank's buffer with 1% BSA, supplemented with 2 mM of $MgCl_2$ or $CaCl_2$, were added to the wells and incubated in the absence/presence of HGF (0, 10, 30 ng/ml) at 37˚C for 1-2 h. After non-adherent cells had been removed by rinsing the wells with the same buffer, adherent cells were quantified using the WST-8 assay (Nacalai Tesque) (20), according to the manufacturer's instructions. The data are shown as the mean±S.E. of three independent experiments.

Statistical analysis. The two-tailed Student's *t*-test was used to statistically evaluate the difference between two values. The level of statistical significance was set at $p<0.05$.

Results

NK4 expression suppressed peritoneal dissemination and prolonged survival. We have previously demonstrated that c-Met expression levels were almost equal between CT26-NEO and CT26-NK4. However, the *in vitro* HGF-stimulated invasion and migration of CT26-NEO were completely inhibited in CT26-NK4, and *in vivo* subcutaneous tumor growth was also strongly suppressed in CT26-NK4 compared to CT26-NEO (9). In the present study, we determined whether NK4 gene transfer could suppress peritoneal dissemination and prolong survival in a peritoneal dissemination model using CT26-NEO and CT26-NK4. As a result, potent suppression of peritoneal dissemination with survival prolongation was observed in CT26-NK4 mice compared to CT26-NEO mice. In a macroscopic view, the number of disseminated nodules, that were mainly formed in the greater omentum and mesentery, was clearly lower in the mice injected with CT26-NK4 than in those injected with CT26-NEO (Figure 1A (a, b)). As

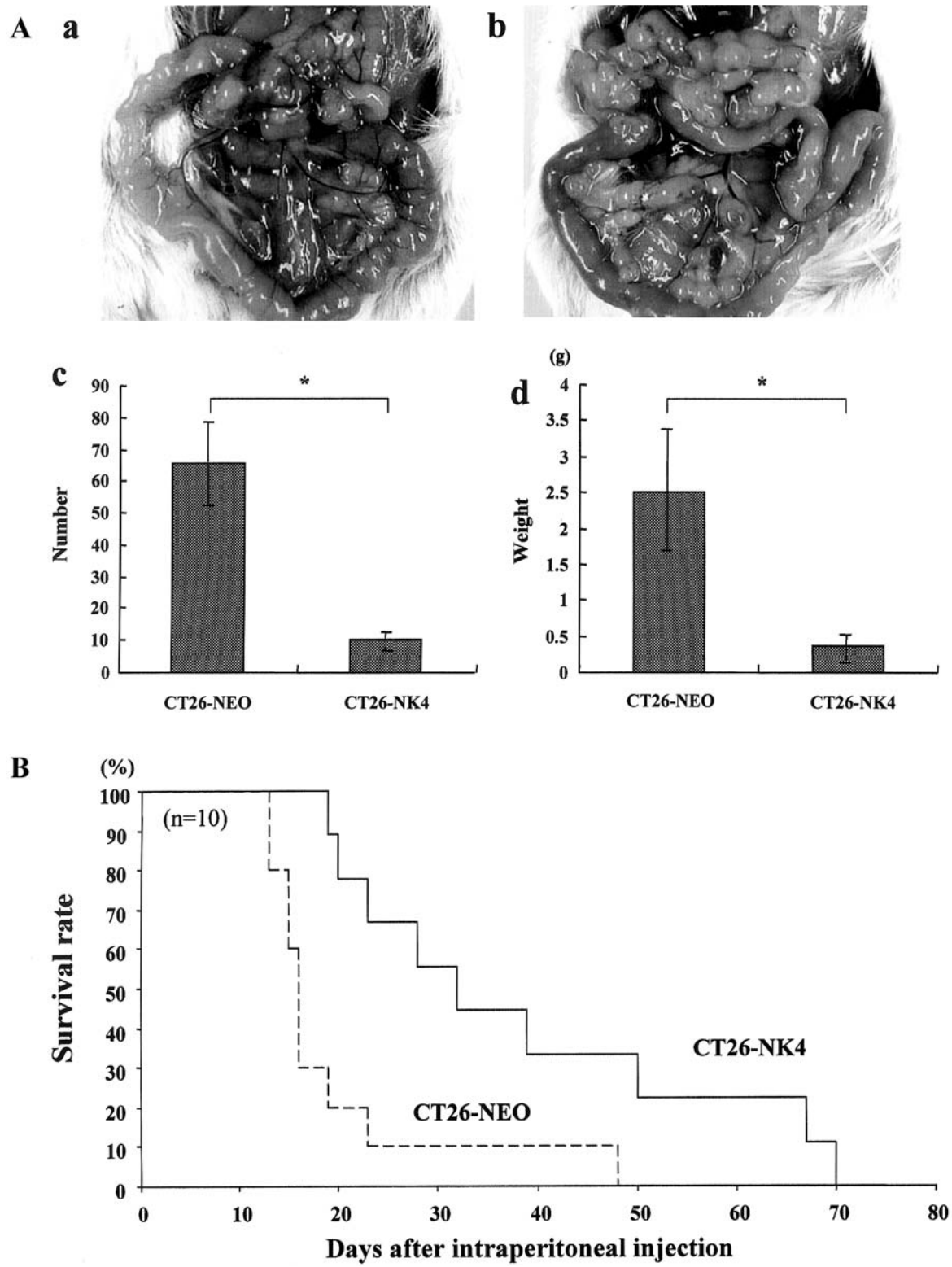

Figure 1. *Suppressive effects of NK4-expressing cancer cells on peritoneal dissemination. (A) 5x10^5 cells of CT26-NK4 or CT26-NEO were injected into the peritoneal cavity of mice and, 28 days later, the mice were sacrificed and the development of peritoneal dissemination was evaluated. Laparoscopic findings in mice injected with CT26-NK4 or CT26-NEO are shown in (a) and (b), respectively. The number (c) and weights (d) of disseminated nodules in the peritoneal cavity were measured and compared between the mice. Each error bar denotes the standard deviation (*$p<0.05$). (B) Survival was analyzed by the Kaplan-Meier method and compared between the two groups by the log-rank test.*

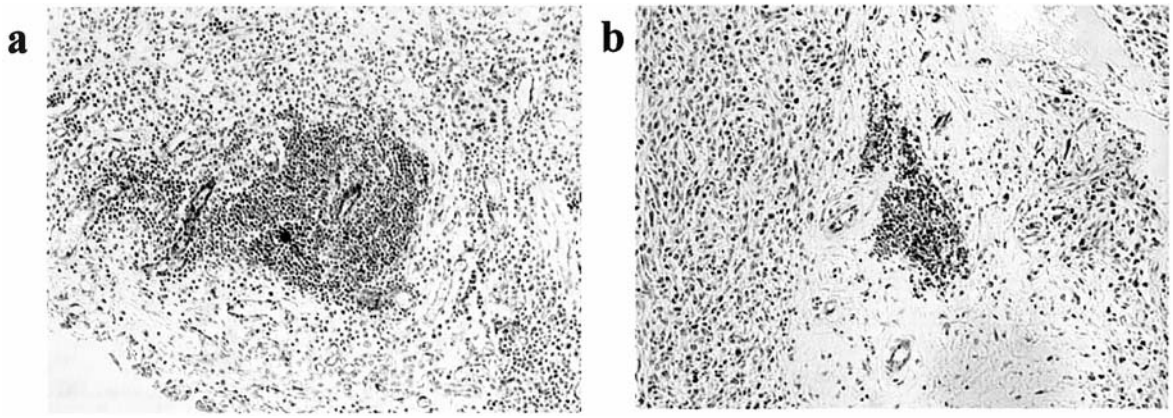

Figure 2. *Immunohistological findings of the disseminated tumor in the omentum. Disseminated tumor nodules in the omentum were resected 28 days after injection of CT26-NK4 or CT26-NEO and were then subjected to CD31 immunostaining ((a): CT26-NK4, (b): CT26-NEO).*

shown in Figure 1A (c, d), the number and weight of disseminated nodules in CT26-NK4-injected mice were significantly lower than in those injected with CT26-NEO (number: 10.3±2.0 in CT26-NK4 *versus* 65.5±12.5 in CT26-NEO; $p<0.05$; weight: 0.355±0.150 g in CT26-NK4 *versus* 2.495±0.865 g in control; $p<0.05$). The survival of the mice injected with CT26-NK4 was significantly longer than that of those injected with CT26-NEO (Figure 1B; $p<0.05$).

Angiogenesis inhibition was not histologically evident in disseminated tumor. NK4 suppresses *in vivo* tumor growth and metastasis by its bifunctional properties as an HGF antagonist and angiogenesis inhibitor (4-8). To confirm the mechanisms by which NK4 expression strongly suppressed peritoneal dissemination *in vivo*, the microvessel density in disseminated tumor tissues was examined. It was found that the microvessel density was not decreased, but appeared to be slightly increased in CT26-NK4 mice compared to CT26-NEO mice, thus showing no histological evidence of angiogenesis inhibition (Figure 2). In addition, a much higher accumulation of mononuclear cells around the microvessels was observed in CT26-NK4 mice. The first of these findings conflicts with a number of previous studies that histologically demonstrated angiogenesis inhibition as a predominant mechanism of *in vivo* antitumor actions by NK4.

NK4-induced suppression of peritoneal implantation evaluated by GFP imaging. To clarify the involvement of antitumor mechanisms by NK4 other than angiogenesis inhibition, we focused on peritoneal implantation and evaluated it using GFP imaging. Peritoneal implantation is an initial event in peritoneal dissemination, that mainly consists of adhesion and invasion of cancer cells to the peritoneal surface and, therefore, is considered to be angiogenesis-independent. Microscopic findings in the resected omentum are indicated in Figure 3A. Omental MS, that were stained black by activated carbon particles, were detected by light microscopy (a, c), and GFP-expressing cells accumulated in the omental MS were detected by fluorescent microscopy (b, d). A decreased cell accumulation in the omental MS was detected in mice injected with CT26-NK4 (b) compared to those injected with CT26-NEO (d), and was also confirmed quantitatively by flow cytometry (Figure 3B; 6.81% in CT26-NK4 (d) *versus* 13.97% in CT26-NEO (c)).

NK4 expression inhibited in vitro adhesion of cancer cells to ECM. In MS-selective accumulation of cancer cells in peritoneal implantation, cancer cells are considered to adhere to and invade the ECM around MS because of a lack of mesothelial cells on the MS (13, 14). Accordingly, NK4 expression may inhibit adhesion of CT26 cells to the ECM at MS. To confirm this, *in vitro* cell adhesion to the ECM was analyzed. It was found that CT26 cells adhered to fibronectin and type I collagen in an HGF dose-dependent manner in the presence of Mg^{2+} and Ca^{2+}, however, the HGF-stimulated cell adhesion to the ECM in CT26-NEO was blocked in CT26-NK4 (Figure 4).

Discussion

In the present study, we demonstrated, using GFP imaging, that peritoneal implantation of cancer cells was strongly suppressed by genetic modification of CT26 cells to produce NK4 (CT26-NK4), resulting in the suppression of peritoneal dissemination and prolongation of survival. A decreased cell accumulation at omental MS was detected by fluorescent microscopy in mice injected with CT26-NK4 compared to those injected with CT26-NEO and was also quantitated by flow cytometry.

The peritoneal surface, which consists of a mesothelial cell monolayer, has been reported to prevent infiltration of cancer cells into the peritoneum (21, 22). Therefore, a lack

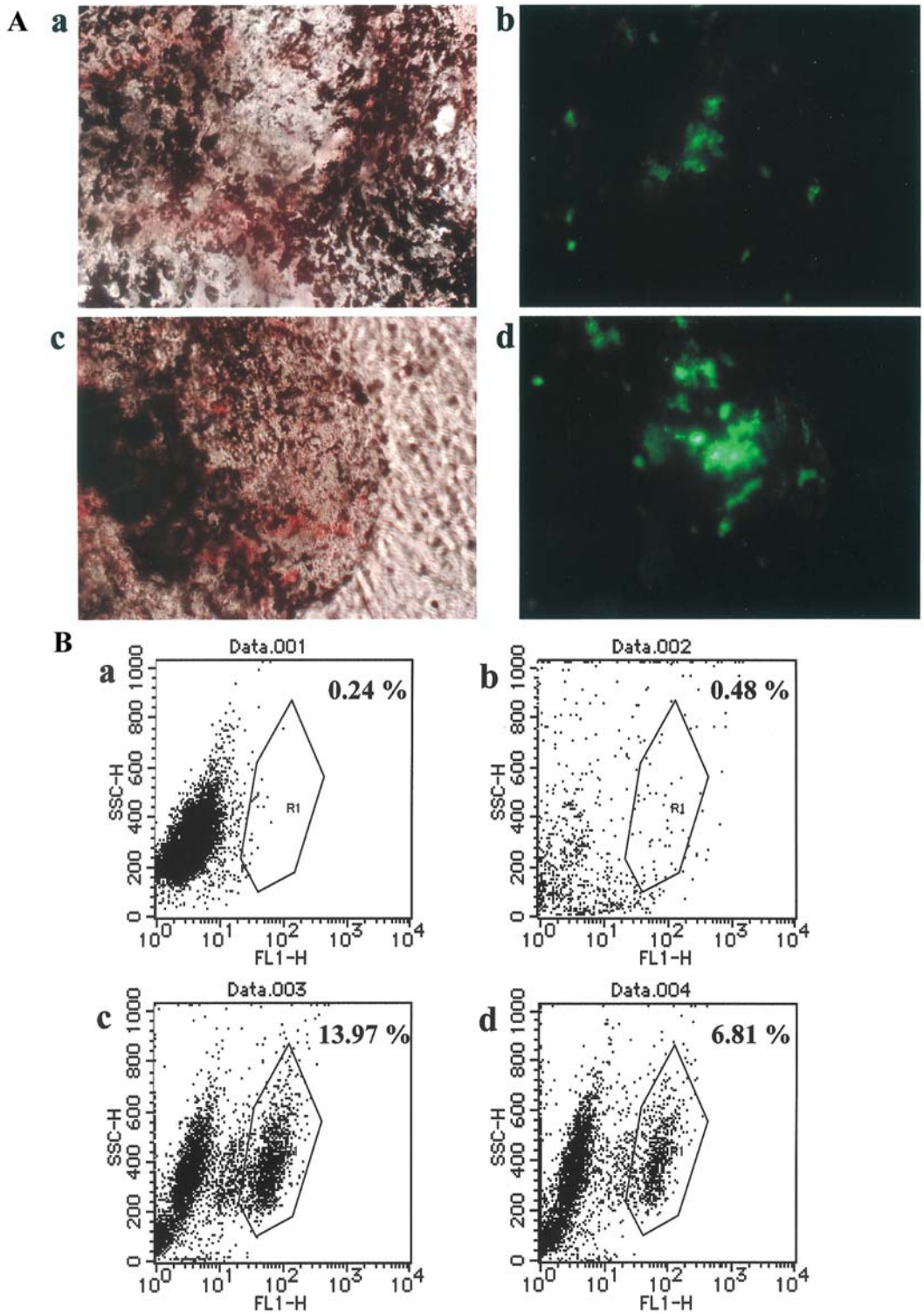

Figure 3. *Suppression of peritoneal implantation by NK4-expressing cancer cells as evaluated by GFP imaging. (A) 1x10^6 cells of CT26-NK4 or CT26-NEO infected with AdGFP were injected into the peritoneal cavity of mice that were preinjected with activated carbon particles to identify MS as black-stained spots. The mice were sacrificed 24 h after injection and the fresh omentum of mice injected with CT26-NK4 (a, b) or CT26-NEO (c, d) was resected and observed under light microscopy (a, c) and fluorescence microscopy (b, d). The pairs (a and b) and (c and d), respectively, were cut from the same sections. (B) A cell suspension prepared from the omentum by mechanical and enzymatic treatment was analyzed for GFP expression by flow cytometry. Results from cells of mice injected with CT26-NEO or CT26-NK4 are shown in c and d, respectively. The in vitro cultured CT26-NEO (a) and a cell suspension from the omentum of tumor-free mice (b) were used as negative controls. GFP-expressing cells were gated in R1 and represented as a percentage of the total number of cells.*

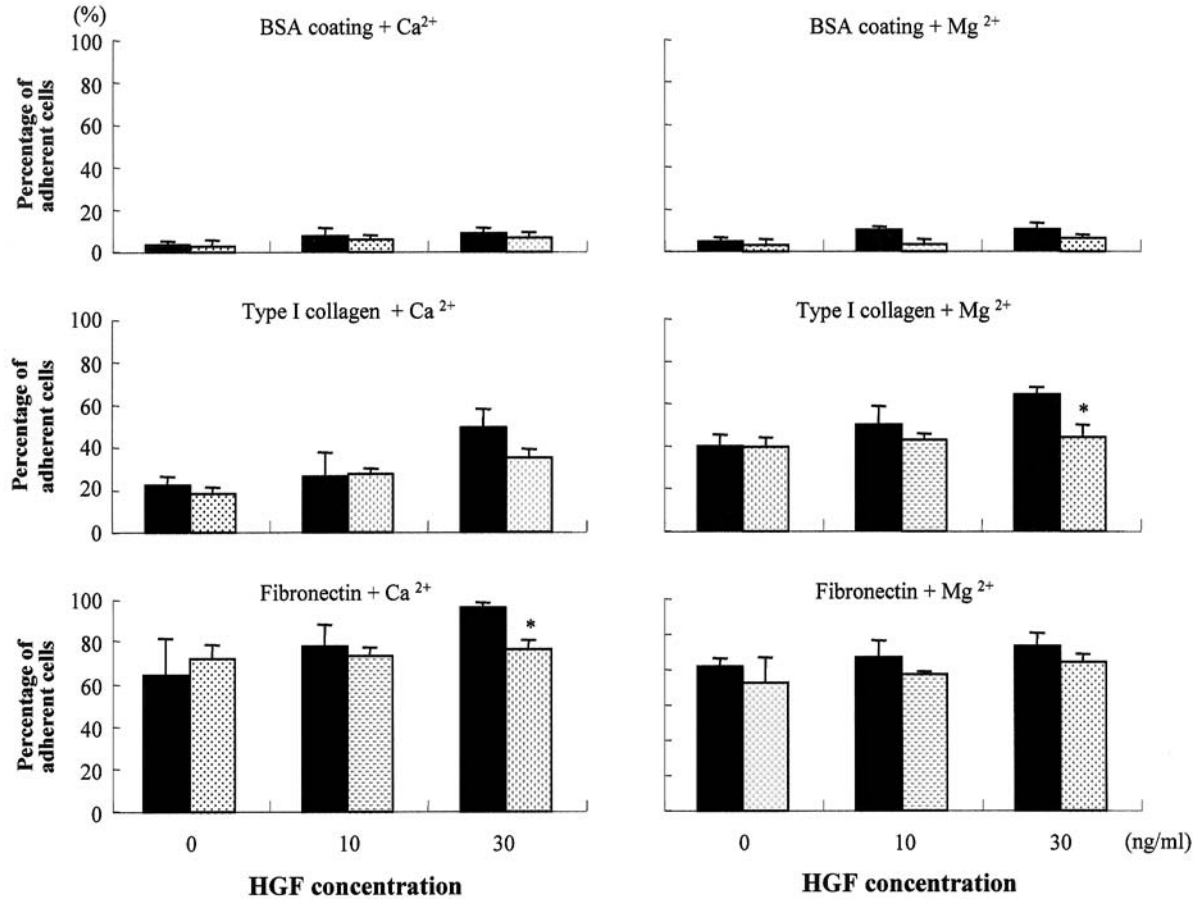

Figure 4. *In vitro adhesion of CT26-NK4 and CT26-NEO to extracellular matrix. 1x10^5 cells of CT26-NK4 or CT26-NEO, suspended in 100 μl of medium containing 2 mM of Mg^{2+} or Ca^{2+}, were added to wells coated with BSA, type I collagen or fibronectin in a 96-well plate and incubated in the absence/presence of HGF (0, 10, 30 ng/ml), as described in Materials and Methods. After rinsing the wells to remove non-adherent cells, adherent cells were quantified using the WST-8 assay. The data are shown as the mean±S.E. of three independent experiments (*p<0.05). Values for CT26-NEO and CT26-NK4 are shown as black and dotted bars, respectively.*

of mesothelium on the surface of MS and the resulting exposure of the ECM to the peritoneal cavity may contribute to MS-selectivity for peritoneal implantation, where adhesion and invasion of cancer cells to the ECM are considered to play an essential role. On the other hand, it has been demonstrated, by *in vitro* studies, that HGF produced by peritoneal fibroblasts can induce loss of cell-cell adhesion in mesothelial cells (23); HGF promotes adhesion of cancer cells to the ECM by affecting their adhesion molecules such as integrins (24-26). These observations strongly suggested that HGF would promote peritoneal implantation by stimulating adhesion of cancer cells to the ECM. Our *in vitro* adhesion assay demonstrated that HGF stimulated adhesion of CT26-NEO to fibronectin and type I collagen, and that HGF-stimulated adhesion was inhibited in CT26-NK4. In addition, we have previously demonstrated, using an *in vitro* invasion assay, that HGF-stimulated invasion of CT26-NEO *via* HGF/c-Met binding was completely inhibited in CT26-NK4 (9). These results show the possible involvement of the suppression of HGF-stimulated motility (adhesion and subsequent invasion) of cancer cells in NK4-induced suppression of peritoneal implantation at MS.

Integrins are important transmembrane cell receptors that mediate cell interactions with the ECM (27), and HGF promotes adhesion of cancer cells to the ECM by stimulating integrin-initiated signaling pathways (24-26, 28). The effects of HGF on surface expression of several integrin subunits (α2, α3, α4, α5, αV, β1, β3) were examined by flow cytometry on CT26-NEO and CT26-NK4, however, their surface expression levels were similar in both transfectants, and were not altered by HGF (data not shown). It has been reported that HGF can increase cancer-matrix adhesion without affecting the surface expression levels of integrins, by

activating focal adhesion kinase (FAK), which plays a central role in integrin-initiated signaling pathways, or by increasing integrin avidity (27-29). The detailed mechanism of *in vitro* inhibition of HGF-stimulated adhesion of CT26 cells to the ECM by NK4 is a matter for further investigation.

Angiogenesis inhibition plays a predominant role in *in vivo* antitumor effects of NK4 (8). However, there was no histological evidence of angiogenesis inhibition by NK4 in the disseminated tumor tissues in the present study using NK4-expresssing tumor cells, or in the previous study using NK4-expressing adenovirus vector. On the other hand, we previously demonstrated a significant decrease in microvessel density in CT26-NK4 tumors compared to CT26-NEO tumors in a subcutaneous tumor model (9). This difference may be explained by the site-specificity of tumor development. MS are tissues with an abundant blood supply and potent angiogenic activity (16, 30). During peritoneal dissemination, the MS were destroyed and completely replaced by infiltrating cancer cells in CT26-NEO, while they were conserved in CT26-NK4, suggesting that immunostaining for microvessel density in a disseminated tumor formed at MS may detect not only tumor-derived angiogenesis, but also MS-derived angiogenesis. NK4-induced angiogenesis inhibition may have been masked by the potent angiogenic activity of MS.

Finally, our present study revealed *in vivo* potent suppressive effects on peritoneal implantation and subsequent peritoneal dissemination of NK4 continuously produced by cancer cells, and also revealed that NK4 could inhibit *in vitro* HGF/c-Met-stimulated adhesion of cancer cells to the ECM. These results strongly suggest that NK4 could suppress peritoneal dissemination, not only by inhibiting HGF/c-Met-stimulated adhesion/invasion of cancer cells at the step of peritoneal implantation, but also by inhibiting angiogenesis at the successive steps.

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