VEGF-C, VEGFR-3, and COX-2 enhances growth and metastasis of human cervical carcinoma cell lines *in vitro*

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Abstract. The study was conducted to clarify the expression and biological significance of VEGF-C and VEGFR-3 in human cervical cancer cell lines and to investigate the correlation between VEGF-C and cyclooxygenase-2 (COX-2) expression in these cells. Flow cytometry, Western blotting, RT-PCR, cell immunochemistry assay, cell proliferation assay, *in vitro* invasion assay and cell immunochemistry assay were used to detect the gene expression and to evaluate the biological features in test cell lines. VEGF-C expression was low while VEGFR-3 was quiet high in all cell lines detected. COX-2 expression coincided with that of VEGF-C. Recombinant human VEFG-C-treated HeLa cells showed increased proliferation and invasion in a dose-depended manner while NS398, a specific inhibitor of COX-2, blocked the invasive ability of HeLa. VEGF-C and its VEGFR-3 played a crucial role in the regulation of tumor growth and metastasis in cervical cell lines, and COX-2 might be a regulator of VEGF-C expression.

Introduction

Invasion and metastasis are the most important characteristics of malignant tumors. Formation of lymphatic metastasis is the initial step of generalized spreading of tumor cells and predicts poor clinical prognosis, therefore, it plays an important role in tumor staging, especially in gynecological and breast cancers (1). The mechanism by which tumor cells detach from the primary tumor, invade lymphatic vessels, and metastasize to regional lymph nodes, however, is complex and remains unclear. Studies have suggested that the vascular endothelial growth factor (VEGF)/VEGF receptor (VEGF-R) system is important in tumor growth and metastasis (2). VEGF-C and VEGF-D, members of the VEGF family, and the ligands for VEGFR-3 which is predominantly expressed on lymphatic endothelium in adults, have been reported as lymphatic-specific growth factors (3,4). Both can stimulate the lymphatic vessel growth (lymphangiogenesis) and enhance lymphatic metastasis in animal models (5-11). When recombinant VEGF-C was placed on the chick chorio-allantoic membrane, lymphangiogenesis was stimulated (5-11). Furthermore, metastases to regional lymph nodes and distant organs which associated with the overexpression of VEGF-C have been identified in several cancer types such as bladder cancer, pancreatic endocrine tumors, cervical cancers, colon cancer, gastric cancer, and prostate cancer (12-18). Evidence showed that VEGF-C was the paracrine factor essential for lymphangiogenesis and that both VEGF-C alleles were required for normal lymphatic vessel sprouting (11,19). Moreover, the serum levels of VEGF-C have a potential role as a biologic marker of the squamous carcinoma of the cervix (19,20).

VEGF-C requires proteolytic processing to generate several higher affinity forms that are better able to bind and activate VEGFR-3. Partially processed forms of VEGF-C can specifically activate VEGFR-3, whereas the fully processed short ones can stimulate VEGFR-2 (21,22). The fully processed short forms are predominantly expressed in the activated endothelia of blood vessels, although they are less potent activators of VEGFR-2 than of VEGFR-3 (21-23). By binding to VEGFR-3, VEGF-C is capable of triggering the proliferation of VEGFR-3-expressing cells *in vitro* and *in vivo* (6,24-26). In transgenic mice expressing VEGF-C or a VEGF-C mutant (VEGF-C-156S) that specifically binds VEGFR-3 but not VEGFR-2, VEGFR-3 signaling was shown to be sufficient to mediate selective lymphangio-genesis without affecting angiogenesis (6,26). Transgenic mice expressing soluble VEGFR-3-Ig or mice carrying a spontaneous mutation in VEGFR-3 displayed features of lymphedema, which could be reversed by VEGF-C or a VEGF-C mutant (VEGF-C-156S) (26-28). Pytowski B, *et al* (29) reported that complete blocking of VEGFR-3 may specifically prevent both physiologically normal and neoplastic VEGF-C-enhanced lymphangio-genesis in adult mouse, but had no effect on either blood angiogenesis or the survival and function of existing lymphatic vessels. The data demonstrate the importance of these ligand-receptor interactions for lymphatic activation.

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Reports on the relationship between cyclooxygenase (COX)-2 and VEGF-C have increased. The presumption that COX-2 may be a regulator of VEGF-C expression in malignancies have been proposed. However, there is no previous report on the production of VEGFR family or their roles in growth and metastasis of human cervical carcinoma cells; but, there are a few studies of VEGF-C and COX-2 production in human cervical carcinoma.

In this study, the expression of VEGF-C and VEGFR-3 of human cervical carcinoma cells as well as the potential roles of this system in cancer invasion and proliferation were investigated. Another aim of the study was to investigate the relationship between VEGF-C and COX-2.

Materials and methods

Reagents. Rabbit polyclonal antibody to VEGF-C (H-190), Rabbit polyclonal antibody to VEGFR-3 (C-20) and the blocking peptide of VEGFR-3 were purchased from Santa-Cruz Biotechnology (Santa-Cruz, CA). Recombinant human VEGF-C (rhVEGF-C, 2179-VC/CF) and mouse monoclonal antibody to VEGFR-2 (MAB3572) were obtained from R&D Systems (Minneapolis, MN). Rabbit polyclonal antibody to cyclooxygenase-2 (COX-2) and its specific inhibitor NS398 (70590), were purchased from Cayman Chemical (Ann Arbor, MI). Transwell plate (24-well) and the PET membrane with 8-μM pores were purchased from Costar UK, Ltd. (High Wycombe, UK). Matrigel matrix was purchased from BD Biosciences (Bedford, MA).

Cell culture. Four cell lines, HeLa, Siha, C33A, and Caski were used. All cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in RPMI-1640 culture medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 50 U/ml penicillin and 50 U/ml streptomycin. Cells were incubated at 37˚C under 95% air and 5% CO_2.

Detection of the VEGF-C and VEGFR-3 expression by flow cytometry. The harvested cells ($1x10^6$) were fixed overnight with iced ethanol (75%) and preserved at -20˚C. Then the fixed cells were washed 2 times with PBS, resuspensed in 200-μl PBS and equally added into two tubes. To one tube was added the rabbit polyclonal antibody to VEGF-C or VEGFR-3 (1:200) and to the other the rabbit anti-human IgG as a matched control, then incubated overnight at 4˚C. The next day, after culture was washed with PBS, it was added to secondary antibody conjugated with fluorescence (FITC) in the dark at 37˚C, incubated for 2 h. The flow cytometer, FASCalibur (B&D, USA), was used for immunofluorescent assay. The CV value of flow cytometry before detection was adjusted to ≤5%. The VEGF-C and VEGFR-3 expression was analyzed with Cellguest software. No less than 10,000 cells were analyzed in each sample.

Protein extraction and Western blot analysis. Cells were washed 2 times with ice-cold phosphate-buffered saline and lysed in buffer [50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin] for 15 min on ice. The lysates were centrifuged in an Eppendorf tube at 4˚C for 15 min. The equal amounts of protein from the cell lysates were resuspended in gel sample buffer, resolved by 10% SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Millipore). The membrane was blocked with 5% non-fat milk in PBS/0.1% Tween-20 for 2 h at 37˚C, and then the primary antibodies as indicated were incubated with membranes for 2 h. After washing with PBS/0.1% Tween-20, the membranes were incubated with alkaline phosphatase-conjugated secondary antibody for 2 h and analysed using an NBT/BCIP reagent (Amersham).

Western blotting for ß-actin was also performed as an internal (sample loading) control using a monoclonal antibody (1:2000; Sigma, St. Louis, MO). The level of expression in the cell line samples was assessed using Imagerfluorence 5500 imaging densitometer (Bio-Rad). To exclude possible densitometric signal errors due to background and sample loading, the intensity of VEGF-C or VEGFR-3 protein expression was normalized with ß-actin expression. VEGF-C or VEGFR-3 protein level was normalized to protein amount and expressed as mean ± standard deviation of three independent experiments.

RNA extraction and reverse transcriptase PCR (RT-PCR). RNA extraction total RNA was extracted by Trizol one-step procedure, and suspended in DEPC-treated reverse osmosis-H_2O, and conserved at -80˚C for reverse transcription. RNA yield and purity were determined by a standard UV spectrophotometric assay. The ratio of A260/A280 was ~1.80.

First strand cDNA synthesis. The total RNA (4 μg) was dissolved in 25 μl of mixture containing 5 μl 5M-MLV reaction buffer, 200 U M-MLV reverse transcriptase, 5 μl dNTPs, 25 U RNAsin, 500 ng Oligo$(dT)_{15}$, and DEPC-treated reverse osmosis-H_2O. The reaction conditions were: at 42˚C for 60 min, and at 95˚C for 5 min. The first strand cDNA was stored at -20˚C until use.

PCR amplification. The primers of VEGF-C, VEGFR-3 and GAPHD were synthesized according to the primer design principles; all primers span an intron to control against amplification of genomic DNA sequences. First strand cDNA (4 μl) was amplified in 50 μl volume. The primers of VEGF-C yielded a 398-bp product as follows: 5'-end primer: 5'-CGG CTT ATG CAA GCA AAG AT-3'; 3'-end primer: 5'-GAG GTA GCT CGT GCT GGT GT-3'. The primers of GAPDH, which was amplified with VEGF-C as an internal control, yielded a 230-bp product as follows: 5'-end primer: 5'-ACG GAT TTG GTC GTA TTG GG-3'; 3'-end primer: 5'-TGA TTT TGG AGG GAT CTC GC-3'. Both VEGF-C and GAPDH were following an initial denaturation at 95˚C for 4 min, the samples were amplified by 8 cycles of denaturation at 94˚C for 50 sec, annealing at 42˚C for 50 sec, extension at 72˚C for 50 sec, and then were amplified for the second time by 30 cycles of denaturation at 94˚C for 50 sec, annealing at 53˚C for 50 sec, extension at 72˚C for 50 sec, and ended by extension at 72˚C for 10 min. The primers of VEGFR-3 yielded a 298-bp product as follows: 5'-end primer: 5'-AGC CAT TCA TCA ACA AGC CT-3'; 3'-end primer: 5'-GGC AAC AGC TGG ATG TCA TA-3'. The primers of GAPDH,

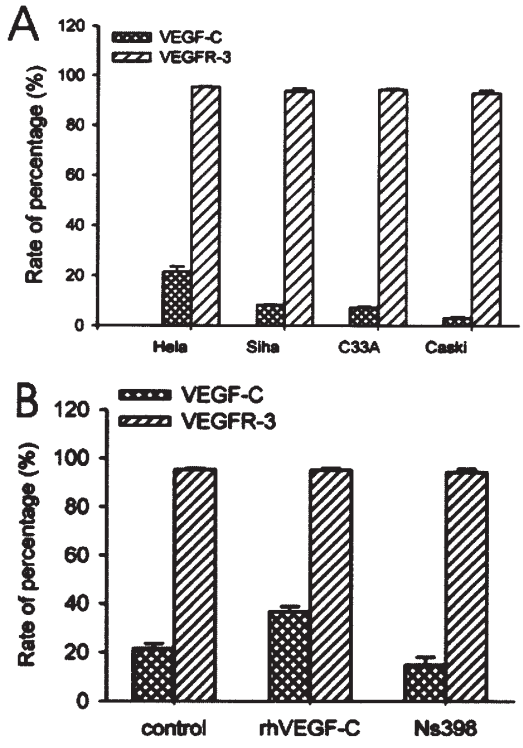

Figure 1. (A) The expression rates of VEGF-C and VEGFR-3 in four human cervical carcinoma cell lines. The harvested cells ($1x10^6$) were fixed and incubated with rabbit polyclonal antibody to VEGF-C or VEGFR-3, the expression rates of VEGF-C and VEGFR-3 were detected by flow cytometer. No less than 10,000 cells were analyzed in each sample. (B) The expression rates of VEGF-C and VEGFR-3 in HeLa cells treated with rhVEGF-C or NS398 compared with untreated cells. Cells were treated with 375 ng/ml of rhVEGF-C for 24 h or 60 μg/ml of NS398 for 48 h, or kept untreated, then the expression rates of VEGF-C and VEGFR-3 were obtained.

which was amplified with VEGFR-3 as an internal control, yielded a 788-bp product as follows: 5'-end primer: 5'-GGT CGG AGT CAA CGG ATT TGG TGG-3'; 3'-end primer: 5'-CTT CCG ACG CCT GCT TCA CCAC-3'. Both VEGFR-3 and GAPDH were following an initial denaturation at 94˚C for 5 min, the samples were amplified by 30 cycles of denaturation at 94˚C for 30 sec, annealing at 58˚C for 1 min, extension at 72˚C for 1 min, and ended by extension at 72˚C for 10 min. The PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide. The ratios of VEGF-C/GAPDH and VEGFR-3/GAPDH were used to semi-quantify the expression levels of VEGF-C and VEGFR-3. The method of image analysis was the same as the proteins.

Cell proliferation assay. Cell proliferation was measured using a nonradioactive cell proliferation assay system (MTT assay; Boehringer Mannheim, Indianapolis, IN). HeLa cells ($5x10^3$) were plated in 96-well plates in RPMI-1640 medium with 10% FBS for 4 h. Then the cells were cultured in 100 μl serum-free RPMI-1640 with or without various concentrations of rhVEGF-C or NS398. After 24 or 48 h of culture at 37˚C, 10 μl of MTT (5 mg/ml) were added to each well. The reaction was stopped after 4 h of incubation by adding 150 μl of dimethyl sulphoxide (DMSO). The optical density (OD) value was obtained by measuring absorbance at the wavelength of 570 nm. The growth assay was done in triplicate, and repeated 3 times.

Cell in vitro invasion assay. *In vitro* invasive ability was evaluated using a 24-well Matrigel invasion chamber with 8-μm pore polycarbonate membranes (Transwell; Costar UK, Ltd.). Briefly, the upper surface of the membranes was coated with matrigel matrix (120 μg/well), incubated at 37˚C for 3 h and left to dry at room temperature overnight. The matrigel matrix was rehydrated with 100 μl of pre-warmed DMEM for 2 h at room temperature. Before invasion assay, HeLa cells were cultured in serum-free RPMI-1640 in the presence or absence of 375 ng/ml rhVEGF-C or 60 μg/ml NS398 for 24 h based on the MTT assay. Also other cells were cultured in serum-free RPMI-1640 and treated with monoclonal anti-human VEGFR-2 antibody (50 ng/ml) for 2 h, after which the blocking peptide of VEGFR-3 (1 μg/ml) was added to the culture medium. Then the cells were harvested, washed and resuspended in the same culture medium and added to the upper chamber ($2.5x10^4$/well). The lower chamber was filled with serum-free NIH-3T3 conditioned media as the chemo-attractant. The plates of inserts were placed over the lower chamber wells. Each cell group was plated in 3 duplicate wells. Noncoated membrane inserts served as controls. The invasion chamber was incubated for 24 h at 37˚C and 5% CO_2 after which noninvading cells on the upper surface of the membrane were removed by gentle scrubbing with a cotton swab. Membranes were fixed in 70% cold methanol and stained with hematoxylin. Then the cells having migrated to the lower side of the PET membrane in 5 random visual fields (x200) were counted under a light microscope. Cells in RPMI-1640 absence of rhVEGF-C or NS398 were used as negative control experiments. The assay was repeated 3 times.

Statistical analysis. Paired t-test were used to compare data from two groups. One-way ANOVA was used to compare data from multiple groups. Correlation between proliferation activity and dose of rhVEGF-C or Ns398 was analyzed using Pearson's correlation. Statistical analyses were made with SPSS software (version 10.0). $P<0.05$ was considered statistically significant.

Results

Expression of VEGF-C and VEGFR-3 in living cells. VEGF-C and VEGFR-3 were all assayed in the four human cervical carcinoma cell lines by flow cytometry. The expressions were different among the four lines. The level of VEGF-C expression was low (<30%) while VEGFR-3 was quite high (≥90%) (Fig. 1A). The study examined whether the expression of VEGF-C and VEGFR-3 changed under rhVEGF-C- or NS398-treated conditions. The results showed that the expression of VEGF-C significantly increased ($P<0.01$) when stimulated with rhVEGF-C or decreased when treated with NS398 ($P<0.05$) in HeLa cells. However, VEGFR-3 expression did not change under the same conditions ($P>0.05$) (Fig. 1B).

Expression of VEGF-C and VEGFR-3 proteins and mRNA in cell lyses. VEGF-C and VEGFR-3 proteins were all detected

Table I. Expression of protein and mRNA of VEGF-C and VEGFR-3 before or after treatment with rhVEGF-C or NS398 in HeLa cells (x±s).

Group	VEGF-C protein	VEGF-C mRNA	VEGFR-3 protein	VEGFR-3 mRNA
A	0.437±0.0165[a]	0.819±0.08209[a]	0.858±0.0706[c]	0.775±0.0173[c]
B	0.191±0.0258[a]	0.307±0.0159[b]	0.854±0.0642[c]	0.751±0.0353[c]
C	0.288±0.0899	0.494±0.0216	0.858±0.0334	0.777±0.0324

The expression of VEGF-C and VEGFR-3 in HeLa cells treated with rhVEGF-C or NS398 compared with untreated cells detected by Western blotting (protein) and RT-PCR (mRNA). Cells were treated with 375 ng/ml of rhVEGF-C for 24 h or 60 μg/ml of NS398 for 48 h, or kept untreated, then the cells were collected for analysis. Group A, rhVEGF-C; B, NS398; and C, control. Compared with group C, [a]$p<0.05$; [b]$p<0.01$; [c]$p>0.05$.

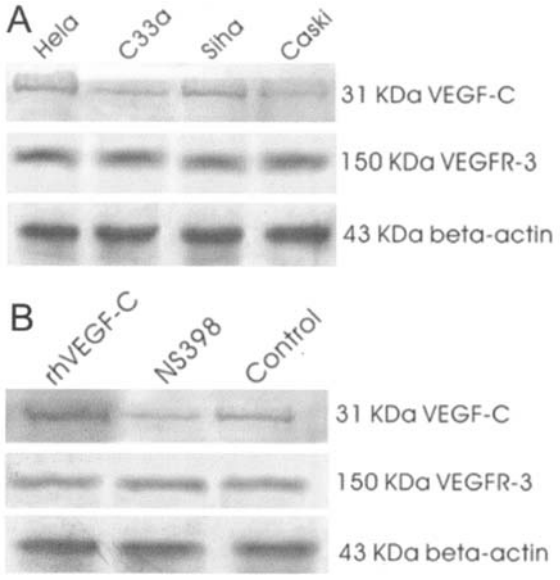

Figure 2. (A) The expression of VEGF-C and VEGFR-3 in the four human cervical carcinoma cell lines detected by Western blotting. Cellular protein extract (50 μg) was electrophoresed on an SDS-10% polyacrylamide gel, transferred to nitrocellulose, probed with the corresponding antibody for each lane. (B) The expression of VEGF-C and VEGFR-3 in HeLa cells treated with rhVEGF-C or NS398 compared with untreated cells detected by Western blotting. Cells were treated with 375 ng/ml of rhVEGF-C for 24 h or 60 μg/ml of NS398 for 48 h, or kept untreated, then the cellular protein extracts were loaded for Western blotting.

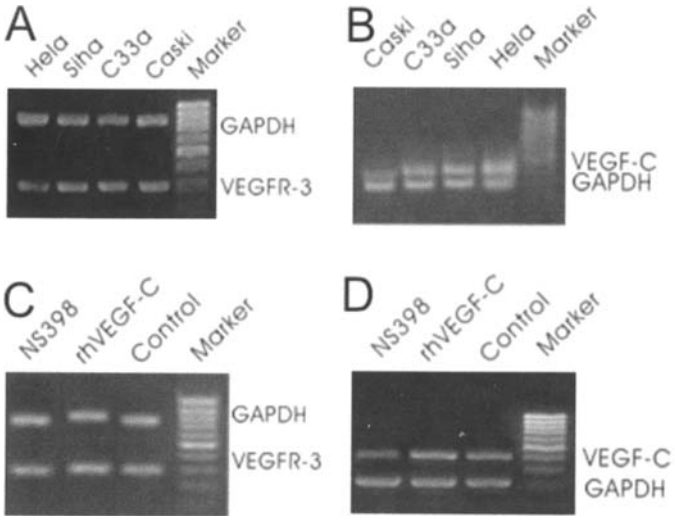

Figure 3. (A and B) The expression of VEGF-C and VEGFR-3 mRNA in the four human cervical carcinoma cell lines was detected by RT-PCR. Total RNA was extracted from cells following reverse transcription as described in Materials and methods. Following PCR using VEGF-C-, VEGFR-3-, or GAPDH-specific primers, PCR products were electrophoresed on a 1.5% agarose gel. (C and D) The expression of VEGF-C and VEGFR-3 mRNA in HeLa cells treated with rhVEGF-C or NS398 was compared with untreated cells detected by RT-PCR. Cells were treated with 375 ng/ml of rhVEGF-C for 24 h or 60 μg/ml of NS398 for 48 h, or kept untreated, then total RNA was extracted from cells for RT-PCR assay.

in HeLa, Siha, C33A and Caski cell lines by Western blotting. In detail, VEGF-C was detected in its 31-kDa form. A Mt150.00 band corresponding to VEGFR-3 protein was detected. However, the expression of VEGF-C protein was very weak in caski cells (Fig. 2A). The mRNA of VEGF-C and VEGFR-3 were also detected in the four cell lines by RT-PCR (Fig. 3A and B). Furthermore, VEGF-C proteins and mRNA of HeLa cells significantly increased when stimulated with rhVEGF-C (375 ng/ml) or decreased when treated with NS398 (60 μg/ml). However, VEGFR-3 expression did not change under the same conditions (Table I, Figs. 2B and 3C and D).

Cell proliferation assay. In the present study, HeLa cells were chosen to test whether rhVEGF-C or NS398 have any effect on the proliferation of human cervical carcinoma cells. HeLa cells were incubated with a range of concentrations of rhVEGF-C or NS398. After culture medium was changed to serum-free medium, HeLa cells still proliferated at 24 h. As shown in Fig. 4A, rhVEGF-C induced a significant increase of the proliferative activity of HeLa cells in a dose-dependent manner ($r=0.857$, $P=0.002$, $P<0.01$). The optimal concentration of rhVEGF-C was 375 ng/ml. On the contrary, NS398 induced a decrease of the proliferate activity of HeLa cells at 48 h also in a dose-dependent manner ($r=-0.968$, $P=0$, $P<0.01$) and the optimal concentration of NS398 was 60 μg/ml (Fig. 4B). However, there was no visible change when incubated with the same concentrations of NS398 at 24 h.

Cell invasive ability assay. Transwell assay showed that the number of cells that penetrated the artificial basement membrane in rhVEGF-C (55.76±2.16, Fig. 5B) was more than that of control (40.38±0.72, Fig. 5A) ($P=0.002$, $P<0.01$). However, fewer penetrated cells in NS398 (25.45±3.67,

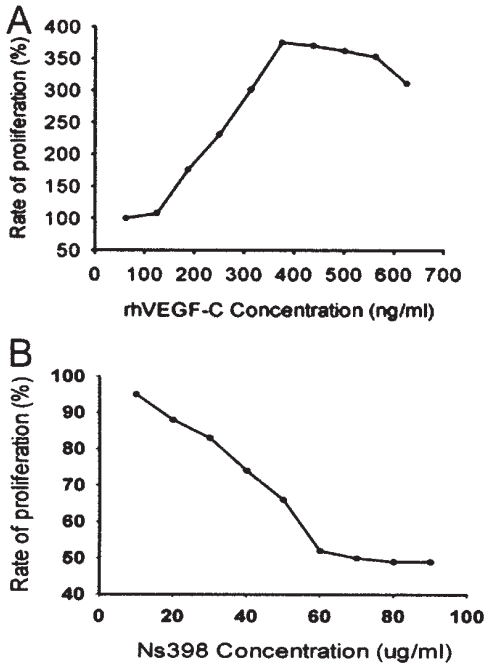

Figure 4. Proliferation rate of HeLa cells treated with different concentrations of rhVEGF-C for 24 h (A) or NS398 for 48 h (B). HeLa cells ($5x10^3$) were plated in 96-well plates in RPMI-1640 medium with 10% FBS for 4 h. Then the cells were cultured in 100 μl serum-free RPMI-1640 with or without various concentrations of rhVEGF-C or NS398. After 24 or 48 h of culture at 37˚C, MTT assay was carried out as described in Materials and methods. The optical density (OD) value was obtained by measuring absorbance at the wavelength of 570 nm. The growth assay was done in triplicate, and repeated 3 times.

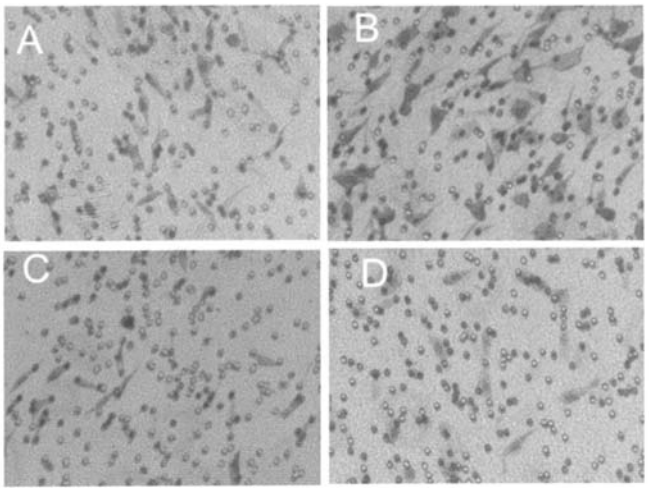

Figure 5. HeLa cells were treated as described in Materials and methods. The cells that penetrated the artificial basement membrane in untreated (A), rhVEGF-C treated (B), NS398 treated (C) and monoclonal VEGFR-2 antibody and the blocking peptide of VEGFR-3 treated (D) were photographed and counted.

Fig. 5C) were noted as compared with those in control (P=0.016, P<0.05). When treated with monoclonal anti-human VEGFR-2 antibody and the blocking peptide of VEGFR-3, HeLa cells that penetrated the artificial basement membrane (27.15±3.99, Fig. 5D) were also fewer than that of in control (P=0.031, P<0.05).

Discussion

VEGF-C expression has been detected in multiple adult tissues including most of the important human cancers analyzed and its expression level correlated with lymph node metastases (5-11,15,18,30-33). Usually, VEGF-C and VEGFR-3 are co-expressed at sites with lymphatic vessel sprouting, in the embryo and in various pathological conditions. Clinical and experimental findings strongly suggest a potential role for VEGF-C-mediated lymphangiogenesis in human cancer metastasis.

In this study, both VEGF-C and VEGFR-3 proteins were detectable by flow cytometry and Western blotting in all of the four cervical carcinoma cell lines investigated, but the expressions were different among them. The levels of VEGF-C expression were usually low (≤30%) while the levels of VEGFR-3 expression were high (≥90%). These results indicated that the signal of VEGFR-3 in human cervical carcinoma cell lines was redundant. Among the four cervical carcinoma cell lines, HeLa expressed the highest level of VEGF-C (21.51%) and Caski expressed the lowest (3.26%). Furthermore, VEGF-C mRNA and VEGFR-3 mRNA were also detected in these cell lines by RT-PCR, and the expression levels coincided with that of the proteins. These suggested that VEGF-C and VEGFR-3 were simultaneously produced in human cervical carcinoma cell lines. Generally, simultaneous production of a growth factor and its receptor in tumor cells indicates the possible existence of an autocrine system (34). Therefore, rhVEGF-C or monoclonal anti-human VEGFR-2 antibody and the blocking peptide of VEGFR-3 were added to the culture medium to investigate the role of VEGF-C/VEGFRs in cell migration and metastasis. As a result, the invasive ability of rhVEGF-C-incubated cells was significantly increased compared to that of the untreated ones (P<0.01), and decreased in cells incubated with monoclonal VEGFR-2 antibody plus the blocking peptide of VEGFR-3 (P<0.05). These results indicated that VEGFR-2 and VEGFR-3 might be biologically activated in the human cervical carcinoma cell lines, and the function of VEGF-C could only be realized when it was bound to the receptors.

Recently, a significant positive correlation between the expressions of COX-2 and VEGF-C in several human tumors was reported (35-37). In the present study, the expression of COX-2 in human cervical carcinoma cell lines was detected by immunochemistry and the result showed that the level of COX-2 expression coincided with the expression of VEGF-C. These results indicated a relationship between the expression of COX-2 and VEGF-C that might exist in human cervical carcinoma cell lines. Lee *et al* (38) suggested that COX-2 expression in cervical adenocarcinomas may contribute to tumor progression by increasing angiogenesis and cell proliferation. However, treatment of tumor cells with NS398 (or rofecoxib), a COX-2-specific inhibitor, significantly attenuated the endogenous VEGF-C expression level (39). Su *et al* (36) used the cDNA microarray technique to discover that VEGF-C was one of the major downstream genes of COX-2 in human lung adenocarcinoma cells. Furthermore, the up-regulation of VEGF-C by COX-2 through the EP1/Src/HER-2/Neu signaling pathway was demonstrated in their studies. The above suggests that COX-2 may be a regulator of VEGF-C expression in malignancies.

In the present study, whether the expression of VEGF-C and VEGFR-3 changed in cervical cancer cells when treated with rhVEGF-C or NS398 were examined. The results showed that the expression of VEGF-C significantly increased when stimulated with rhVEGF-C or decreased when treated with NS398. However, VEGFR-3 expression did not change under the same conditions suggesting that rhVEGF-C and NS398 had an effect only on VEGF-C expression but not on VEGFR-3 expression in human cervical carcinoma cell lines.

After being incubated *in vitro* with recombinant human VEGF-C, HeLa cells showed a significantly increasing invasive ability and proliferative activity. Moreover, the increase of proliferate activity was dose-dependent. By using NS398 at an appropriate concentration, a different result was obtained demonstrating that VEGF-C protein could promote tumor cell invasion and proliferation and suggested that the expression of VEGF-C in human cervical carcinoma cells might be regulated by the COX-2 gene, and that VEGF-C-mediated tumor proliferation and metastases might be used in therapeutics by targeting COX-2.

In summary, this study showed that both VEGF-C and VEGFR-3 were expressed in human cervical carcinoma cell lines, and the expression of COX-2 coincided with that of VEGF-C. Several biological functions of HeLa cells such as invasive ability, proliferate activity and VEGF-C expression were significantly increased when stimulated with recombinant human VEGF-C. However, a different result was obtained when treated with a COX-2-specific inhibitor NS398. The results indicated that VEGF-C and its receptors played a crucial role in the regulation of tumor growth and metastasis. COX-2 may be a regulator of VEGF-C expression in human cervical carcinoma cell lines. Moreover, the studies also provided a possible therapeutic method for the inhibition of VEGF-C-mediated tumor growth and metastases by using a COX-2-specific inhibitor.

Acknowledgements

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