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Studies of Marine Sulfated Polymannuroguluronate on Endothelial Cell Proliferation and Endothelial Immunity and Related Mechanisms

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Abstract. Anti-proliferation action and enhancement of endothelial cell immunity and related mechanisms by marine sulfated polymannuroguluronate (SPMG) were investigated in the present studies. Endothelial cell proliferation was evaluated by MTT assay. Intercellular adhesion molecule-1 (ICAM-1) expression was analyzed by flow cytometry. The interaction of SPMG with basic fibroblast growth factor (bFGF) was evaluated by surface plasmon resonance. Results showed that SPMG exhibited a significant inhibitory effect against proliferation in both normal human umbilical vein endothelial cells (HUVEC) and bFGF-treated HUVEC, the action of which was completely abrogated by bFGF antibody. SPMG exerted high affinity to bFGF in a multivalent pattern, characterized by one molecule SPMG binding to 3 – 4 molecules of bFGF. Moreover, SPMG enhanced ICAM-1 expression in HUVEC and prevented and restored bFGF-treated downregulation of ICAM-1 expression in HUVEC, the expression of which was not counteracted by bFGF antibody. In conclusion, this is the first report demonstrating that SPMG exerted an anti-proliferation effect dependent on the bFGF-regulated pathway and afforded upregulatory activity on ICAM-1 expression regardless of the involvement of bFGF.

Keywords: sulfated polymannuroguluronate, basic fibroblast growth factor, intercellular adhesion molecule-1, endothelial cell, angiogenesis

Introducmtion

The endothelial cell is the main component of new capillary vessels. There is abundant data showing that endothelial proliferation is one of the key steps involved in angiogenesis, suppression of which may inhibit angiogenesis, and angiogenesis-dependent solid tumor growth and metastasis. Regulation of endothelial cell proliferation depends on various angiogenic molecules, and the most important one is basic fibroblast growth factor (bFGF) (1, 2). Much evidence has shown that bFGF is a heparin-binding protein and plays an important role in regulating endothelial cell proliferation (3 – 5). Furthermore, it has been confirmed that tumor angiogenesis can remarkably suppress endothelial cell immunity against tumors, which may serve as a tumor-protecting mechanism (6). Mounting evidence has highlighted the pivotal role of bFGF involved in the down-regulation of endothelial intercellular adhesion molecular-1 (ICAM-1) during the angiogenesis process, which plays a crucial role in the infiltration of cytolytic effector leukocytes into tumors (7, 8).

Marine sulfated polymannuroguluronate (SPMG), a kind of sulfated polysaccharide extracted from brown algae with specific means of fractionation and chemical modification, bears a 1,4-linked β-D-mannuronic block with a modified sulfate group. Our previous experiments showed that it exerted marked inhibition on the growth and metastasis of tumors (data not published, submitted to Chinese Journal of Marine Drugs), and enhanced the immune function in vivo by SPMG (9). Most importantly, SPMG inhibited angiogenesis of rat aorta rings in a serum-free matrix culture (data not published, sub-

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mitted to Chinese Journal of Marine Drugs). Thus, in order to further elucidate the mechanism underlying its effect on angiogenesis, its inhibition on endothelial cell proliferation and enhancement on endothelial cell immunity and related mechanisms of action were investigated in this paper.

Materials and Methods

Reagents

SPMG, a kind of sulfated polysaccharide extracted from brown algae with specific means of fractionation and chemical modification, was obtained from Marine Drug and Food Institute, Ocean University of China, Qingdao, Shandong, China. Collagen I, trypsin, gelatin, basic fibroblast growth factor (bFGF), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), leukocyte separation solution, Sulfo-NHS-biotin, and streptavidin were purchased from Sigma (St. Louis, MO, USA). Anti-basic fibroblast growth factor antibody (anti-bFGF Ab) was purchased from PeproTech EC, Ltd. (London, UK). Endothelial cell growth factor (ECGF) and Rose Begal were purchased from Roche Diagnostics Gmbh (Roche Molecular Biochemicals, Mannheim, Germany). Medium 199 (M199) and fetal bovine serum were purchased from Hyclone (Logan, UT, USA). The carboxymethylated dextran sensor chip (CM5) was purchased from Biacore (Uppsala, Sweden). Anti-ICAM-1 monoclonal antibody and mouse IgG1 were purchased from Immunotech (Marseille, France).

Cells culture

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh human umbilical vein, as reported by Jaffe EA et al. previously (10); and then they were grown on 25 cm^2 culture flasks (Coastar, New York, NY, USA) coated with 0.2% gelatin in M199 supplemented with 20% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 μg/ml ECGF in a humidified atmosphere with 5% CO_2 at 37°C. Cultures were dissociated with 0.1% trypsin. Cells between passages 2 and 3 were used for experiments.

Cell proliferation assay (11)

HUVEC were harvested in 0.1% trypsin solution, collected by centrifugation and resuspended with M199 containing 10% fetal bovine serum, and then seeded in 96-well plates (5×10^3/well): 24 h later, the medium was removed and replaced by fresh M199 (200 μl/well) with 10% fetal bovine serum. SPMG at concentrations of 1, 10, 50, 100 μg/ml; bFGF at a concentration of 10 ng/ml; or anti-bFGF Ab at a concentration of 5 μg /ml was added accordingly. The cell number was estimated by MTT assay (570 nm) after 72-h culture.

Proliferation% = $A_{\text{treated group}} / A_{\text{control}} \times 100$ and thus inhibition rate% = $[1 - A_{\text{treated group}} / A_{\text{control}}] \times 100$.

Leukocyte adhesion assay (12)

HUVEC were seeded in 96-well plates with M199 containing 10% fetal bovine serum (1.5×10^4/well). Twenty-four hours later, the medium was removed and replaced by fresh medium (200 μl/well) with 10% fetal bovine serum. SPMG at 100 μg/ml or bFGF at 10 ng/ml was added accordingly. Then, 72 h later, peripheral blood mononuclear cells (PBMC), prepared by density gradient centrifugation using leukocyte separation solution from healthy human peripheral blood, were added to each well (5×10^5/well) except for the blank control wells; and the cells were cultivated in a 95% O_2 – 5% CO_2 humidified incubator. One hour later, each well was gently washed with prewarmed PBS several times to remove the unadhered cells. Then 0.25% Rose Begal in PBS (100 μl/well) was added, allowed to react for 10 min at room temperature, and then the dissociated Rose Begal was gently removed with PBS. Then PBS-ethanol (1:1, 200 μl /well) was added to each well, and the samples were allowed to stand for 1 h at room temperature. Absorbance (A) was estimated by a spectrophotometer (570 nm) (Spectra Rinbow; Tecan Austria GmbH, Grodig, Austria).

Adhesion% = $(A_{\text{treated group}} - A_{\text{blank control}}) / (A_{\text{control}} - A_{\text{blank control}}) \times 100$

ICAM-1 expression analysis

HUVEC (4×10^5/25 cm^2 flask) were incubated with 10 ng/ml bFGF, 5 μg/ml anti-bFGF, or 100 μg/ml SPMG for 72 h. After cells were collected, washed, and counted, they were incubated for 30 min on ice in 20 μl of PBS with 2% BSA and 0.1% sodium azide containing either 5 μg of anti-ICAM-1 monoclonal antibody or mouse IgG1 as the isotype control. A sample of 1.0×10^4 cells was collected for analysis by FCM (FACS Vantage™; Becton Dickinson, San Jose, CA, USA).

Surface plasmon resonance assay (SPR) (13)

Biosensor measurements based on SPR was used to evaluate the interaction between SPMG and bFGF. In this assay, SPMG was immobilized on the surface of a carboxymethylated dextran sensor chip (CM5) via a biotin-streptavidin capture procedure. A solution containing bFGF at concentrations of 16, 31, 62, 125, and 250 nM was passed over the sensor chip surface accordingly, and the changes in mass due to the binding response was measured.

Statistics

Student's *t*-test and analysis of variance were performed by Statview, ANOVA. $P<0.05$ was regarded as statistically significant and $P<0.01$ was regarded as remarkable statistical significance. Data were expressed as the means ± S.D. of triplicate experiments.

Results

SPMG inhibited normal HUVEC proliferation

Endothelial cells are the main component of new capillary vessels, and endothelial cell proliferation is well known to play a critical role in the formation of new capillaries. We first examined the effect of SPMG on normal HUVEC proliferation. Results showed that SPMG at concentrations of 10, 50, and 100 μg/ml inhibited the proliferation of HUVEC remarkably in a dose-dependent manner, with the inhibition rate of 17.3%, 24.5%, and 44.9% respectively. However, SPMG at a concentration of 1 μg/ml had no obvious effect on the HUVEC proliferation (Fig. 1).

SPMG inhibited bFGF-treated HUVEC proliferation

BFGF, a strong endothelial cell mitogen, is well known as one of most important angiogenic factors, and bFGF-treated endothelial cell proliferation exerts an important role in angiogenesis. In our experiment, bFGF at the final concentration of 10 ng/ml increased HUVEC proliferation by nearly 56.1%, compared with that of the control. Results indicated that SPMG at concentrations of 50 and 100 μg/ml inhibited markedly the proliferation of bFGF treated HUVEC in a dose-dependent fashion, with the inhibition rate of 23.4% and 33.6%, respectively, while SPMG at concentrations of 1 and 10 μg/ml exerted no obvious effects on the bFGF-treated HUVEC proliferation (Fig. 1).

SPMG interacted with bFGF by SPR

Much evidence has shown that bFGF is a heparin-binding protein, and the binding of bFGF with heparin plays a crucial role in proliferation-promoting activity. The fact that SPMG bears similar structures to heparin suggested that there might be a possible interaction of SPMG with bFGF. In our experiments, binding of SPMG to bFGF was analyzed by SPR. Results showed that SPMG and bFGF had a remarkable association and the binding response increased with the enhancement of the concentration of bFGF (Fig. 2).

Kinetic studies were performed by Biacore evaluation 3.0 software. The association constant and dissociation constant were 1.2E+08 (1/M) and 8.4E-09 (M), respectively, suggestive of high avidity of SPMG for bFGF.

Stoichiometry was calculated according to the following formulation:

$$\text{Stoichiometry} = \frac{R_{max(bFGF)}}{R_{immboSPMG}} \times \frac{MW_{SPMG}}{MW_{bFGF}}$$

, where $R_{max(bFGF)} = 1083$ RU, $R_{immboSPMG} = 162.2$ RU, $MW_{SPMG} = 8$ kDa, $MW_{bFGF} = 16.4$ kDa.

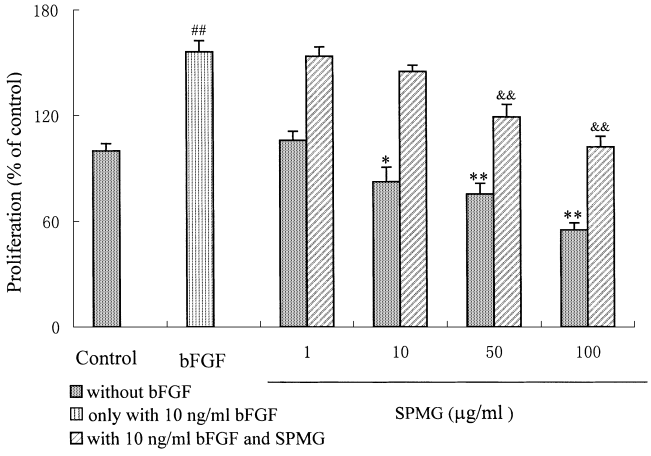

Fig. 1. SPMG Inhibited normal and bFGF-treated HUVEC proliferation. HUVEC (5.0×10^3/well) were plated in 96-well plates, and SPMG at a concentrations of 1, 10, 50, and 100 μg/ml or 10 ng/ml bFGF was added respectively to the cultures on the next day. After 72 h, the cells were trypsinized and the cell numbers were estimated by MTT assay. Data represent means ± S.D. of triplicate experiments, $^*P<0.05$, $^{**}P<0.01$, $^{\#\#}P<0.01$ *vs* control, $^{\&\&}P<0.01$ *vs* bFGF.

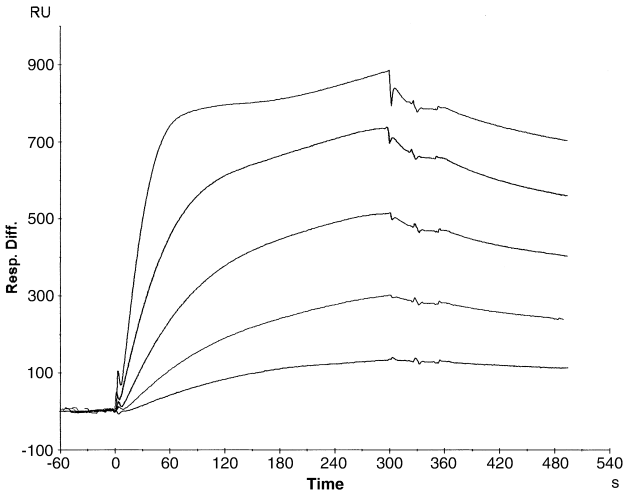

Fig. 2. SPMG interacted with bFGF by SPR. The biotin-SPMG was immobilized to the streptavidin-sensor chip surface, and different concentrations of bFGF were flowed over the sensor chip surface to test for its interaction with SPMG. The concentrations of bFGF from the bottom to the top were 16, 31, 62, 125, and 250 nM, respectively. The X-axis stands for the flow time, and the Y-axis indicates the binding resonance units. The experiment was carried out at 25°C, with a flow rate of 5 μl/min HBS-EP buffer.

Results indicated that one SPMG molecule bound to 3 – 4 bFGF molecules.

Anti-bFGF Ab abrogated the inhibitory effect of SPMG on HUVEC proliferation

The above experiment showed that SPMG could interact with bFGF markably; therefore, we hypothesized that the interaction between SPMG and bFGF might interfere with the binding of bFGF to its receptor and thus led to blocking of the promoting effect of bFGF on endothelial cell proliferation. In our experiment, 100 μg/ml SPMG inhibited the HUVEC proliferation significantly. However, 5 μg/ml anti-bFGF Ab obviously blocked the inhibitory effect of SPMG, indicating that the inhibitory activity of SPMG on endothelial cell proliferation was bFGF-mediated (Fig. 3).

SPMG enhanced ICAM-1 expression of HUVEC

ICAM-1, one of the most important adhesion molecules of endothelial cells involved in the recirculation of blood leukocytes and in the infiltration of cytolytic effector leukocytes into tumors, plays a crucial role in the endothelial cell immunity against tumors. In our study, results showed that the expression of ICAM-1 on HUVEC was enhanced dramatically after they were preincubated with SPMG at a concentration of 100 μg /ml (Fig. 4).

SPMG enhanced ICAM-1 expression of bFGF-treated HUVEC

Suppression of cell immunity by tumor angiogenesis is evidenced by the low expression of ICAM-1. Increasing evidence demonstrated that bFGF is also involved in this process. Our findings demonstrated that a 72-h incubation of HUVEC with 10 ng/ml bFGF induced a remarkable downregulation of ICAM-1 as compared with that of normal cells, whereas 100 μg/ml SPMG abrogated this downregulation (Fig. 5).

SPMG overcame bFGF-induced ICAM-1 downregulation

Furthermore, the ability of SPMG to re-induce ICAM-1 expression following pre-incubation with bFGF was tested. After a 72 h pretreatment with 10 ng/ml bFGF, fresh culture medium was added and HUVEC were treated for another 72 h with 100 μg/ml SPMG and newly added 10 ng/ml bFGF. It was showed that the incubation with 100 μg/ml SPMG resulted in a remarkable re-induction of ICAM-1 expression even in the continuously concomitant presence of bFGF (Fig. 6).

SPMG enhanced adhesion of leukocyte to HUVEC

ICAM-1 exerts a chief function in the adhesion of leukocytes to HUVEC, as previously described. To confirm this action, HUVEC were cultured with or without 10 ng/ml bFGF, accompanied by simultaneous addition of 100 μg/ml SPMG or medium. Data demonstrated that bFGF, at the same concentration that

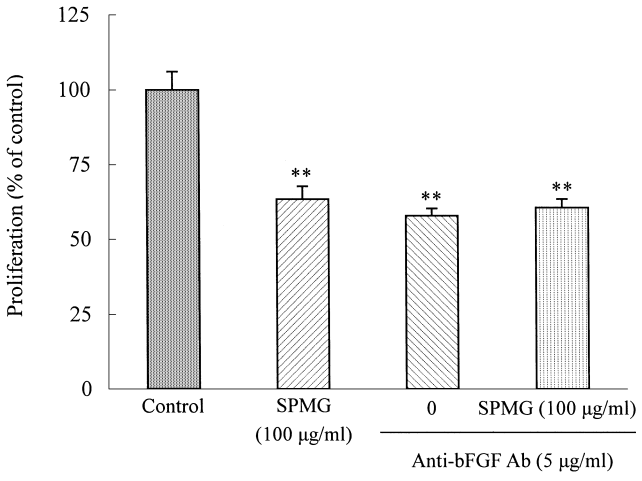

Fig. 3. Anti-bFGF Ab abrogated the proliferation inhibition of HUVEC by SPMG. HUVEC were plated in 96-well plates, and 5 μg/ml anti-bFGF Ab with or without SPMG (100 μg/ml) was added to the cultures on the next day. Then 72 h later, cell numbers were evaluated by MTT. Data represent means ± S.D. of triplicate experiments, $^{**}P<0.01$ *vs* control.

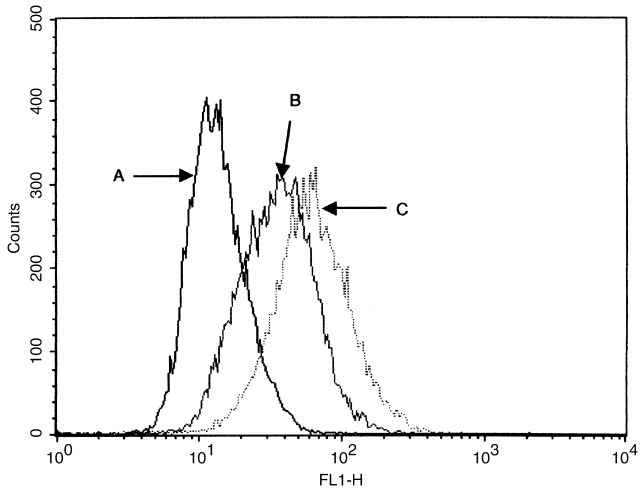

Fig. 4. SPMG enhanced ICAM-1 expression of HUVEC. HUVEC (4.0×10^5/25 cm^2 flask) were cultured for 72 h in the presence of SPMG at a concentration of 100 μg/ml. After the cells were collected, washed, and counted, they were incubated for 30 min on ice in 20 μl of PBS with 2% BSA and 0.1% sodium azide containing either 5 μg of anti-ICAM-1 monoclonal antibody or mouse IgG1 as the isotype control. A sample of 1.0×10^4 cells was collected for analysis by FCM. A, isotype control; B, normal cells; C, cells with 100 μg/ml SPMG; fluorescent intensities of which were 15.37, 41.86 ± 4.61, and 74.98 ± 5.08 au, respectively.

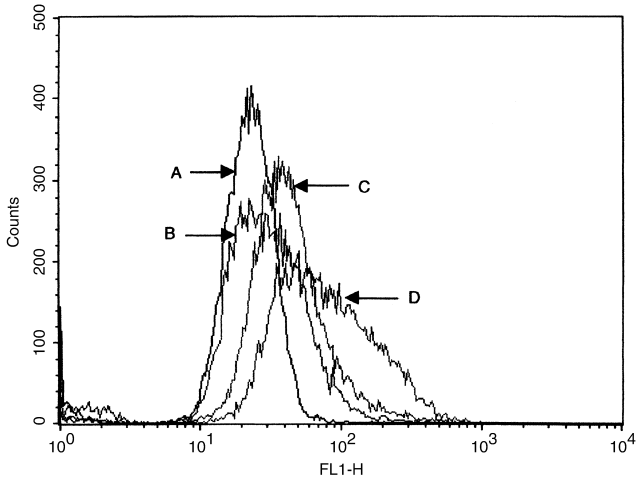

Fig. 5. SPMG enhanced ICAM-1 expression of bFGF-treated HUVEC. HUVEC (4.0×10^5/25 cm^2 flask) were cultured with or without 10 ng/ml bFGF, with simultaneous addition of 100 μg/ml SPMG for 72 h. After the cells were collected, washed, and counted, they were incubated for 30 min on ice in 20 μl of PBS with 2% BSA and 0.1% sodium azide containing either 5 μg of anti-ICAM-1 monoclonal antibody or mouse IgG1 as the isotype control. A sample of 1.0×10^4 cells was collected for analysis by FCM. A, isotype control; B, cells with bFGF; C, cells with bFGF and SPMG; D, normal cells; fluorescent intensities of which were 24.21, 36.64 ± 3.58, 51.87 ± 3.43, and 101.07 ± 5.39 au, respectively.

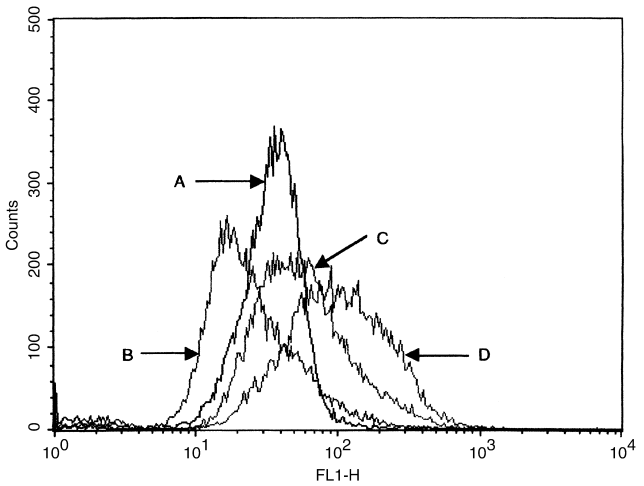

Fig. 6. SPMG overcame bFGF-induced ICAM-1 downregulation. After pretreatment with 10 ng/ml bFGF for 72 h, fresh culture medium was added and HUVEC (4.0×10^5/25 cm^2 flask) were treated with 100 μg/ml SPMG and newly added 10 ng/ml bFGF for another 72 h. After the cells were collected, washed, and counted, they were incubated for 30 min on ice in 20 μl of PBS with 2% BSA and 0.1% sodium azide containing either 5 μg of anti-ICAM-1 monoclonal antibody or mouse IgG1 as the isotype control. A sample of 1.0×10^4 cells was collected for analysis by FCM. A, isotype control; B, cells with bFGF; C, cells with SPMG and bFGF; D, normal cells; fluorescent intensities of which were 37.70, 32.41 ± 2.32, 73.82 ± 5.11, and 132.49 ± 4.22 au, respectively.

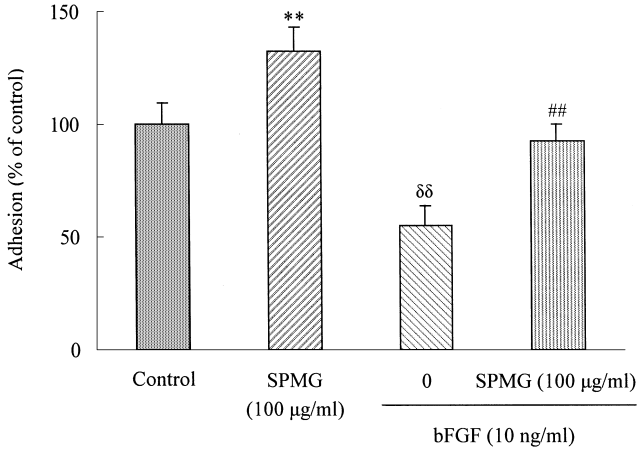

Fig. 7. SPMG enhanced adhesion of leukocytes to HUVEC. HUVEC were cultured with or without 10 ng/ml bFGF, with simultaneous addition of 100 μg/ml SPMG. After 72 h, adhesion experiments were performed. Data represent means ± S.D. of triplicate experiments, $^{**}P<0.01$, $^{\delta\delta}P<0.01$ *vs* control, $^{\#\#}P<0.01$ *vs* bFGF-treated group.

downregulated ICAM-1 expression, also remarkably decreased adhesion of leukocytes to HUVEC. SPMG at 100 μg/ml, a concentration that upregulated ICAM-1 expression, enhanced adhesion of leukocytes to HUVEC as well (Fig. 7).

Anti-bFGF Ab failed to abrogate ICAM-1 upregulation of HUVEC by SPMG

As bFGF was demonstrated to be involved in the inhibitory effect on HUVEC proliferation by SPMG, together with the fact that bFGF was associated with the downregulation of ICAM-1 expression, here we assumed that the upregulatory effect on ICAM-1 expression by SPMG might be closely related to the involvement of bFGF. Thus, in our present experiment, we introduced anti-bFGF Ab to the experimental system. HUVEC were cultured with or without 5 μg/ml anti-bFGF Ab, in co-existence with SPMG at a concentration of 100 μg/ml, for 72 h. The results demonstrated that 5 μg/ml Anti-bFGF Ab exerted no obviously effect on HUVEC ICAM-1 expression, while 100 μg/ml SPMG enhanced HUVEC ICAM-1 expression significantly. Most importantly, 5 μg/ml anti-bFGF Ab failed to abrogate the effect of SPMG on HUVEC ICAM-1 expression (Fig. 8).

Discussion

Endothelial cell proliferation is well known to play a critical role in the formation of new capillaries. In fact, endothelial cell proliferation is regulated by many factors. Under normal conditions, these factors are in a

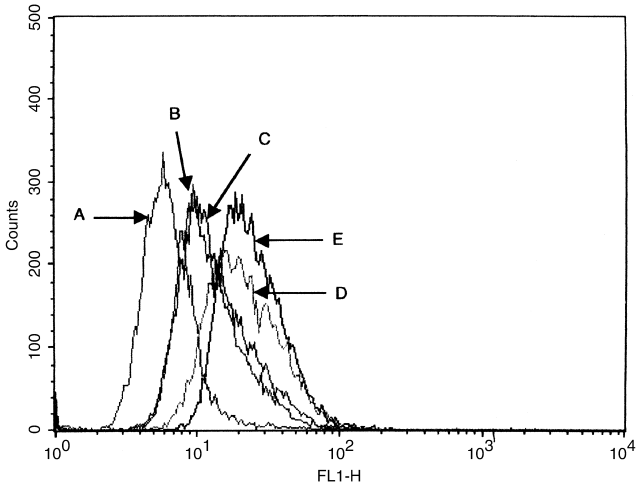

Fig. 8. Anti-bFGF Ab failed to abrogate ICAM-1 upregulation of HUVEC by SPMG. HUVEC (4.0×10^5/25 cm^2 flask) were cultured with or without 5 μg/ml anti-bFGF Ab and 100 μg/ml SPMG for 72 h. After the cells were collected, washed, and counted, they were incubated for 30 min on ice in 20 μl of PBS with 2% BSA and 0.1% sodium azide containing either 5 μg of anti-ICAM-1 monoclonal antibody or mouse IgG1 as the isotype control. A sample of 1.0×10^4 cells was collected for analysis by FCM. A, isotype control; B, normal cells; C, cells with anti-bFGF Ab; D, cells with anti-bFGF Ab and SPMG; E, cells with SPMG; fluorescent intensities were 6.87, 14.32 ± 2.01, 15.72 ± 1.53, 24.38 ± 3.34, and 27.56 ± 1.87 au, respectively.

kinetic balance and thus inhibit the occurrence of angiogenesis. However, in tumor angiogenesis, tumors not only produce angiogenic factors, but also induce other cells to secrete angiogenic factors, subsequently stimulating endothelial proliferation remarkably (14, 15). Since 1983, several factors responsible for angiogenesis have been identified, and the most commonly known ones are bFGF and vascular endothelial growth factor (VEGF) (16). bFGF is a strong endothelial cell mitogen in stimulating endothelial proliferation. Clinically, high bFGF levels have been correlated with certain tumors and progressing hemangiomas (17, 18). In vitro, bFGF can activate endothelial cells to allow capillary cord formation (19). Furthermore, bFGF-induced endothelial cell proliferation has long been confirmed to be related to the involvement of heparin or heparan sulfate. Much evidence has highlighted the paramount role of heparin binding action in endothelial cell proliferation. The binding of bFGF with heparin can protect bFGF against heat, acid denaturation, or protease cleavage. Most importantly, binding of bFGF with heparin induces a conformational change in bFGF that is required for a biologically active interaction with its high affinity receptor (20 – 22). The addition of exogenous heparin or heparan sulfate or sulfated polysaccharide can replace the function of cell-associated heparin by enhancing bFGF receptor binding under some conditions, or inhibiting under others, due to different molecular backbone or different molecular size by interfering with the binding of bFGF to its cell surface receptor. Additionally, the alteration in bFGF-heparin interactions by soluble heparin and analogs as sulfated polysaccharide dramatically enhanced bFGF release from the extracellular matrix (23 – 26). SPMG inhibited the proliferation of bFGF-induced endothelial cell remarkably in a dose-dependent manner. The possible mechanism underlying the inhibitory action of SPMG on proliferation of HUVEC, we supposed, might contribute to the fact that SPMG blocked binding of heparin to bFGF. This might be better supported by the kinetic study indicating that one molecule SPMG bound to 3 – 4 molecules of bFGF in a multivalent manner, suggesting that this multivalent binding abolished binding of bFGF to its receptor and thus not favoring the oligomerization of the bFGF receptor (5). Most interestingly, SPMG also exerted an inhibitory effect on proliferation of normal cells. These findings, we supposed, might also benefit from the involvement of bFGF, as HUVEC can synthesize bFGF in normal cell culture and also the presence of bFGF in serum medium cannot be excluded. In fact, this supposition was further supported by the fact that anti-bFGF antibody abrogated the inhibitory effect of SPMG on HUVEC proliferation (27).

In addition, suppression of cell immunity by tumor angiogenesis is evidenced by the decrease in the adhesion of leukocytes to endothelial cells, accompanied by the low expression of ICAM-1. Actually, ICAM-1 is the main adhesion molecule of endothelial cells and exerts a pivotal role in the adhesion of leukocytes to endothelial cells. Increasing evidence demonstrated that bFGF was also involved in this process (6). In vitro, bFGF decreased the expression of ICAM-1 both at the protein and RNA level, thus leading to the decrement in adhesion of leukocytes to HUVEC (7, 8, 28). Griffioen found that many angiogenic inhibitors, such as platelet factor-4 (PF-4), thrombospondin-1 (TSP-1), and interferon-γ inducible protein-10 (IP-10), could prevent and restore bFGF-induced ICAM-1 downregulation, accompanied by the decrease in adhesion of leukocytes to endothelial cells (29). Interestingly, SPMG, at the same concentration that inhibited bFGF-induced HUVEC proliferation, also enhanced ICAM-1 expression, and thus led to enhancement of leukocyte adhesion to HUVEC. Although a better understanding of the effect of SPMG on ICAM-1 needs further elucidation, one possible mechanism underlying this mode of action might be, at least in part, explained by the speculation that SPMG-induced upregulation of ICAM-1 was due to a pathway other than the bFGF-regulated one, because

anti-bFGF antibody failed to counteract the upregulating effect of SPMG on ICAM-1 expression.

In conclusion, we are the first to report that SPMG exerted its anti-angiogenic effect in a bFGF-dependent pathway, whereas SPMG afforded its upregulatory effect on ICAM-1 expression regardless of the involvement of bFGF.

Acknowledgments

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