Original Article

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Effect of mild hypothermia on breast cancer cells adhesion and migration

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Summary **To explore the effect of mild hypothermia (35ºC) on breast cancer cells adhesion to vascular endothelial cells, a parallel plant flow chamber was used to observe the adhesion of human breast cancer cells MDA-MB-231 to endothelial cells Eahy926 under physiological flow at 35ºC and 37ºC, as well as the role of intercellular adhesion molecule 1 (ICAM-1) in this process. Further, the effect of mild hypothermia (35ºC) on migration of MDA-MB-231 was also studied. Our results show that mild hypothermia can inhibit the adhesion of tumor cells to endothelial cells and ICAM-1 plays an important role in this process. However, mild hypothermia inhibits breast cancer cell adhesion in a way independent on the change of ICAM-1 expression under our experimental conditions. Mild hypothermia can weaken the chemotaxis of breast cancer cells while it has no obvious effect on unidirectonal migration capacity. These results suggest that mild hypothermia could be used as a potentially adjunct treatment combined with surgery to decrease tumor cell adhesion and migration.**

Keywords: Mild hypothermia, breast cancer, cell adhesion, cell migration, intercellular adhesion molecule 1, flow chamber

1. Introduction

The metastasis of tumors is responsible for most cancer related deaths, which occurs through a series of complex processes, involving tumor cells detachment from the primary tumor, intravasation and survival in the circulation, and extravasation in the vasculature to invade the target tissue, followed by proliferation and angiogenesis at the metastasis focus (*1*). A potentially rate-limiting step in metastasis would be the extravasation process that involves tumor cells arrest or adhesion to endothelium ("docking"), transition to more established cell contacts and numerous focal adhesions ("locking") and transmigration through the endothelial cell monolayer and basement membranes (*2*). Various endothelial adhesion molecules (including E-selection, P-selection, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), β1 integrin, N-cadherin, *etc*.) contribute to tumor cells extravasation (*2-4*). ICAM-1 is widely expressed at a low basal level and is upregulated by inflammatory cytokines (TNF-α, IL-1, IFN-γ) in endothelial cells. In various cancers, such as non-small cell lung cancer (*5*), breast cancer (*6*), gastric cancer (*7*), colorectal cancer (*8*), and bladder cancer (*9*), ICAM-1 expression is at an elevated level and is associated with the malignant potential of cancer. Increasing evidence suggest that ICAM-1 plays an important role in the adhesion of tumor cells to endothelial cell monolayers and subsequent transendothelial migration (*10-13*). Adherence of human small-cell lung carcinoma to cultured vascular endothelial cells in stasis and flow depends on the expression of endothelial ICAM-1 (*10*). In addition, endothelial ICAM-1 supports breast cancer cells adhesion *via* the ligand mucin-1 (MUC-

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1), a transmembrane glycoprotein expressed by normal breast epithelium and virtually all breast cancers. Tumor binding to endothelial ICAM-1 *via* MUC-1 initiates a calcium signal and triggers Src family kinase-mediated cell motility, and eventually promotes transendothelial migration (*11-13*). Furthermore, leukocytes act as bridge-cells and facilitate the contact between tumor cells and the endothelium in which ICAM-1 plays an important role (*14-16*). *In vivo*, total body irradiation triggers tumor cell extravasation and lung metastasis in Balb/c mice accompanied by upregulation of both E-selectin and ICAM-1 mRNA levels and activation of nuclear factor-κB (NF-κB) in large lung blood vessels (*17*). Lovastatin impairs irradiation induced upregulation of both E-selectin and ICAM-1 and attenuates irradiation induced metastasis (*17*). All of these data imply that ICAM-1 plays a vital role in tumor adhesion to endothelial cells and extravasation.

Although many prevention methods such as chemoprevention, risk-reduction surgery, molecular genetics, epidemiology, and imaging have been used to reduce the incidence of cancer, few have paid attention to mild hypothermia (32-35ºC) as a strategy to prevent tumor cell adhesion and extravasation although mild hypothermia (32-35ºC) has been studied to improve outcome from cardiac arrest, brain trauma, stroke, hypoxic encephalopathy of neonates, spinal cord injury, open cardiac surgery, and other ischemic tissue injury for a long time (*18*). One of the important mechanisms is that mild hypothermia inhibits the inflammation response which contributes significantly to secondary injury after ischemia (*19,20*). Mild hypothermia suppresses ICAM-1 overexpression and neutrophil accumulation following acid-induced lung injury (*21*) and experimental stroke (*20,22*). Suppression of ICAM-1 induction by mild hypothermia is mediated by enhanced ERK1/2 activation and subsequent attenuation of STAT3 action (*23*). In addition, mild hypothermia can protect against TNF-α-induced endothelial barrier dysfunction and apoptosis through an MKP-1-dependent mechanism (*24*). Besides the effect on the expression of ICAM-1, mild hypothermia may also influence the binding thermodynamics of tumor cells to endothelial cells. In all cases of adhesion of cells to other cells or to extracellular matrix (ECM), noncovalent receptor-ligand interactions are a prerequisite for firm adhesion. The receptor-ligand binding mainly depends on the size, structure, formational dynamics and environmental temperature of their binding sites. In many cases, receptor-ligand binding can be treated as a reversible reaction. Both the association rate and dissociation rate are affected by temperature (*25-28*).

All these findings described above identify that mild hypothermia can be a potential method to reduce cancer adhesion and extravasation and then can prevent cancer metastases. The objective of the present study is to test the hypothesis that mild hypothermia can reduce breast tumor cells adhesion to endothelial cells and to try to investigate the potential mechanism. To reduce hypothermia-associated adverse side effects in the clinic and reduce the difficulty to achieve mild hypothermia, only 35ºC was chosen as the mild hypothermia to test this hypothesis in our study.

2. Materials and Methods

2.1. *Cell culture*

Human breast cancer cells MDA-MB-231 (from the Kunming Institute of Zoology, the Chinese Academy of Sciences, Kunming, China) were cultured in high-glucose DMEM medium (Life Technologies, Carlsbad, CA, USA) with 10% FBS (TBD, Tianjin, China), 100 UI/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 26 mM $NaHCO_3$ in a humidified 95% air/5% CO_2 environment at 37ºC. Human breast cancer cells MCF-7 (from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) were cultured in low-glucose DMEM medium (Life Technologies) with 10% FBS. Endothelial cells (ECs) Eahy926 (from ATCC, USA) were grown in RPMI1640 medium (Fisher Scientific, Beijing, China) containing 10% FBS in the same conditions. At 80-90% confluence, cells were trypsinized (0.25% trypsin and 0.02% EDTA) and passaged at a 1:5 ratio.

2.2. *Preparation of parallel-plate flow chamber*

The structure and working principle of the flow chamber have been described in detail (*29*). In brief, the parallel plate flow chamber consisted of a parallel plate flow chamber, silicone tubes, a peristaltic pump, and a reservoir. The parallel plate flow chamber is composed of two silicone shims (B_1 and B_2), three plexiglass plates (C_1, C_2 and C_3), and a glass slide coated with cells, with the depth (d) of the chamber (A) being 300 μm, the width (b) 2.5 cm, and the length (l) 7.5 cm (Figure 1). Both the liquid inlet D and the outlet G were linked to the tubes (H_1 and H_2) on the first plexiglass plate C_1. The triangle narrow slit E in silicone insole B_1 can distribute the liquid uniformly and reduce pulsation. Then, the liquid goes into the chamber through rectangular slit F. The glass slide coated with the endothelial cells is placed into the chamber and perfused with medium containing tumor cells. Under fully developed laminar flow, the shear stress (τ) on the cell monolayer can be calculated by $\tau = 6\mu Q/bd^2$ dyn/cm^2, where Q is the volumetric flow rate (cm^3/sec), μ is the viscosity of the circulating medium (dyn•sec/cm^2), b is the width of chamber and d is the depth of the chamber. In our experiments, the viscosity of the medium μ was chosen as 0.012 dyn•sec/cm^2 (*30*) and the shear stress τ was set at 1.5 dyn/cm^2 by changing the volumetric flow rate Q (*31*).

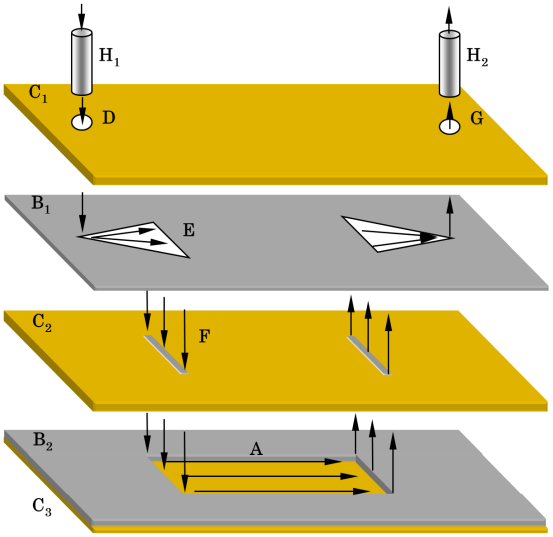

Figure 1. Sketch of parallel-plate flow chamber. A is the flow chamber (l = 7.5 cm, b = 2.5 cm, d = 0.3 mm), B_1 and B_2 are the two silicone shims with 0.5 mm and 1.5 mm depths, respectively. C_1, C_2 and C_3 are the plexiglass plates with 4 mm, 4 mm and 2 mm depths. D is the liquid inlet and G is the liquid outlet. E is the triangle narrow slit, F is the rectangular slit. H_1 and H_2 are the tubes made from polytetrafluoroethylene.

2.3. *Flow chamber adhesion assays*

At 80% confluence, the ECs were trypsinized (0.25% trypsin and 0.02% EDTA), and seeded on a glass microscope slide (75 mm × 25 mm) at a cell concentration of 10^5 cells/mL. The ECs could be confluent in two days after seeding. The slide was attached to the bottom of the flow chamber before the flow experiment. The MDA-MB-231 cells were trypsinized and stained with 2 μM Calcein-AM (C3099, Molecular Probes, Life Technologies) for 15 min at 37ºC. After washing twice with PBS, the MDA-MB-231 cells were resuspended at 5×10^5 cells/mL in high-glucose DMEM medium with 1% FBS, and incubated at 37ºC for 1 h before perfusion. First, the incubator was adjusted to 35ºC or 37ºC. The chamber was circulated with cell-free DMEM medium at 1.5 dyn/cm^2 for 20 min to balance the ECs and sweep the loose ECs away and to keep the parallel-plate flow chamber at 35ºC or 37ºC. Then medium with 5×10^5 cells/mL MDA-MB-231 cells was circulated at 1.5 dyn/cm^2 for 20 min. Finally, the chamber was perfused at 1.5 dyn/cm^2 for 5 min with cell-free medium to remove nonadherent cancer cells. In these assays, perfusions were done both at 37ºC and 35ºC and repeated three times at each temperature. Adherent MDA-MB-231 cells were counted with a fluorescence microscope (IX71, Olympus, Tokyo, Japan) (more than 10 fields for each slide at ×40).

Cell death rate of MDA-MB-231 cells was verified using trypan blue (Sigma-Aldrich, St. Louis, MO, USA) before and after the experiment. The live cell rate was about 97% prior to the experiment while it was about 96% after 20 min perfusion. This implies that the MDA-MB-231 cells can maintain a high survival rate in the parallel-plate flow chamber under flow condition for 20 min.

For antibody blocking assays, an endothelial monolayer on a glass slide was incubated at 37ºC for 30 min with an anti-ICAM-1 antibody (bs-0608R, Bioss, Beijing, China) (final concentration: 10 μg/mL) before perfusion at 37ºC.

Our experimental results showed that MCF-7 hardly adhered to the Eahy926 monolayer under the same conditions used for MDA-MB-231 cells flow chamber adhesion assays described above (data not shown). Thus, a detachment assay (*32*), which was a little different from the MDA-MB-231 cells adhesion assay, was carried out inside the parallel plate flow chamber to investigate the effect of mild hypothermia on adhesion of MCF-7 cells to ECs. In brief, the chamber was circulated with cell-free DMEM medium at 1.5 dyn/cm^2 for 20 min to balance the ECs, to sweep the loose ECs away, and to keep the parallel-plate flow chamber at 35ºC or 37ºC. Medium with 5×10^5 cells/mL MCF-7 cells stained by Calcein-AM was introduced and circulated at 1.5 dyn/cm^2 for 10 min. Then, the flow was stopped and MCF-7 cells were allowed to settle and adhere to the endothelial monolayer for a period of 10 min. Finally, the chamber was perfused with cell-free medium at 1.5 dyn/cm^2 for 5 min to remove nonadherent cancer cells. The adherent MCF-7 cells were also counted under a fluorescence microscope.

2.4. *Western blotting for ICAM-1*

ECs plated on a glass slip were perfused for 45 min in the flow chamber with cancer cells-free medium at 37ºC and 35ºC at 1.5 dyn/cm^2. Then, treated cells were lysed with RIPA buffer containing a protease inhibitor cocktail. Equal amounts of cell lysates were separated using 5-10% SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. The transferred membrane was blocked with blocking buffer at room temperature (RT) for 1 h followed by being immunoblotted with anti-human ICAM-1 antibody (dilution 1:250, sc-8439, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4ºC overnight, washed in TBST for 5 min each time and repeated twice. The same membrane was reprobed with monoclonal anti-β-actin antibody (dilution 1:500, sc-47778, Santa Cruz Biotechnology). The membrane was immunoblotted with appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at RT. The membrane was subsequently washed in washing buffer three times, 5 min for each time, and then visualized with a DAB kit (ST033, Beyotime Biotech, Haimen, China). A densitometric measurement was made from the film using a GS-800

imaging densitometer (Bio-Rad Laboratories, Hercules, CA, USA). To quantify the relative protein expression, the optical density of the protein band of ICAM-1 was quantified by Quantity One software (Bio-Rad Laboratories) and normalized to the optical density of β-actin on the same membrane.

2.5. *Cancer cells adhesion to ICAM-1-coated glass slide*

To improve protein adsorption, glass slips for ICAM-1 mobilization were silanized with 3-aminopropyltrimethoxysilane (Sigma-Aldrich) as described (*33*). Briefly, glass slips were cleaned by overnight immersion in 20% sulfuric acid, washed with copious amounts of running tap water for 20 min and with distilled water three times, then rinsed with 95% ethanol and dried overnight. The cleaned slips were treated with 0.1 M sodium hydroxide and were blotted dry. 3-Aminopropyltrimethoxysilane (4% v/v in anhydrous acetone) was layered onto one side of each slip ensuring complete coverage. After air drying, the silane-treated slips were washed three times with PBS. Recombinant human ICAM-1 (Cat#150-05, PeproTech, Rocky Hill, NJ, USA) in PBS (5 μg/mL, 0.5 mL) was pipetted onto the treated surfaces of the slips which were allowed to incubate overnight at 4ºC. The slips were washed using PBS and further incubated with 1% BSA for 1 h at RT, then stored at 4ºC under PBS until flow chamber assays. An ICAM-1-coated slip was placed into the flow chamber and perfused with medium containing with 5×10^5 cells/mL MDA-MB-231 cells for 20 min at 37ºC and 35ºC. The following steps were the same as the assay of MDA-MB-231 cells adhesion to endothelial cells described in Section 2.3.

2.6. *Cell migration*

Wound healing assays (non-oriented migration): cancer cell monolayers in 6 wells were disrupted to generate a cross wound with a pipette tip (1,000 μL). The cultures were washed three times with PBS to remove floating cells and then incubated at 37ºC or 35ºC in DMEM medium with 10% FBS and mitomycin C (1 μg/mL) (Sigma-Aldrich). To evaluate 'wound closure' under different temperature conditions, four selected areas close to each cross wound were first photographed under phase contrast microscopy at 0 h. Cells were stained with DAPI after 36 h and followed by photography under the fluorescence microscope to count cells invading the artificial wound.

Transwell migration assays (oriented migration): 1.5 mL DMEM medium within 3×10^5 MDA-MB-231 cells stained with Calcein-AM (Life Technologies) were plated in the top chamber (6-well insert; pore size, 8 μm; Millipore, Billerica, MA, USA). Medium supplemented with 10% FBS was used as a chemoattractant in the lower chamber. The cells were incubated for 6 h at 37ºC or 35ºC and cells that did not migrate through the pores were removed using a cotton swab. Cells on the lower surface of the membrane were counted in 5 fields (×40) per well under a fluorescence microscope. Stromal cell-derived factor-1 (SDF-1) plays an important role in chemotaxis of cancer cells and in tumor metastasis (*34*). To investigate the effect of mild hypothermia on MDA-MB-231 cells migrating to a specific chemoattractant, 40 ng/mL SDF-1 in DMEM medium with 1% FBS was also added in the lower chamber and cancer cells in DMEM medium with 1% FBS were plated in the top chamber, then incubated for 6 h at 37ºC or 35ºC.

2.7. *Statistical analysis*

In our experiments, adherent cell numbers were measured at 20 min and expressed as cellnumber per cm^2 or mm^2 plane area of a calculated segment. Each experiment was performed at least three times. All values in the text are means ± S.E. (standard error). Comparisons between groups were made by one-way ANOVA. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. *Effect of mild hypothermia on tumor cell adhesion to ECs*

Figure 2 illustrates the adhesion of MDA-MB-231 tumor cells to ECs monolayer under flow condition (1.5 dyn/cm^2) at normothermia (37ºC), mild hypothermia (35ºC) and pretreated ECs with anti-ICAM-1 antibody at 37ºC. Figures 2A-C show the photomicrographs of adhesion of MDA-MB-231 tumor cells to ECs at 20 min in the flow chamber at normothermia (37ºC), at normothermia (37ºC) after pretreated ECs with anti-ICAM-1 antibody and at mild hypothermia (35ºC). The adherent MDA-MB-231 cells were the white points under the fluorescence microscope after perfusion with cell-free medium for 5 min. Under normothermia (37ºC), 493.92 (± 72.70 S.E., $n = 3$) cells/cm^2 adhered firmly to the ECs under 1.5 dyn/cm^2 shear stress. After 20 min, mild hypothermia (35ºC) significantly decreased the adherent MDA-MB-231 cells from 493.92 (± 72.70 S.E., $n = 3$) to 50.22 (± 4.01 S.E., $n = 3$) cells/cm^2 ($p < 0.01$) (Figure 2D). The results of cell adhesion suggested that mild hypothermia (35ºC) can attenuate MDA-MB-231 cell adhesion to ECs. To study whether the adhesion of MDA-MB-231 tumor cells to ECs was mediated by ICAM-1, adhesion experiments were further performed after incubating ECs with mAb against ICAM-1. Pretreatment of the ECs with anti-ICAM-1 antibody significantly prevented the adhesion of tumor cells under experimental shear stress

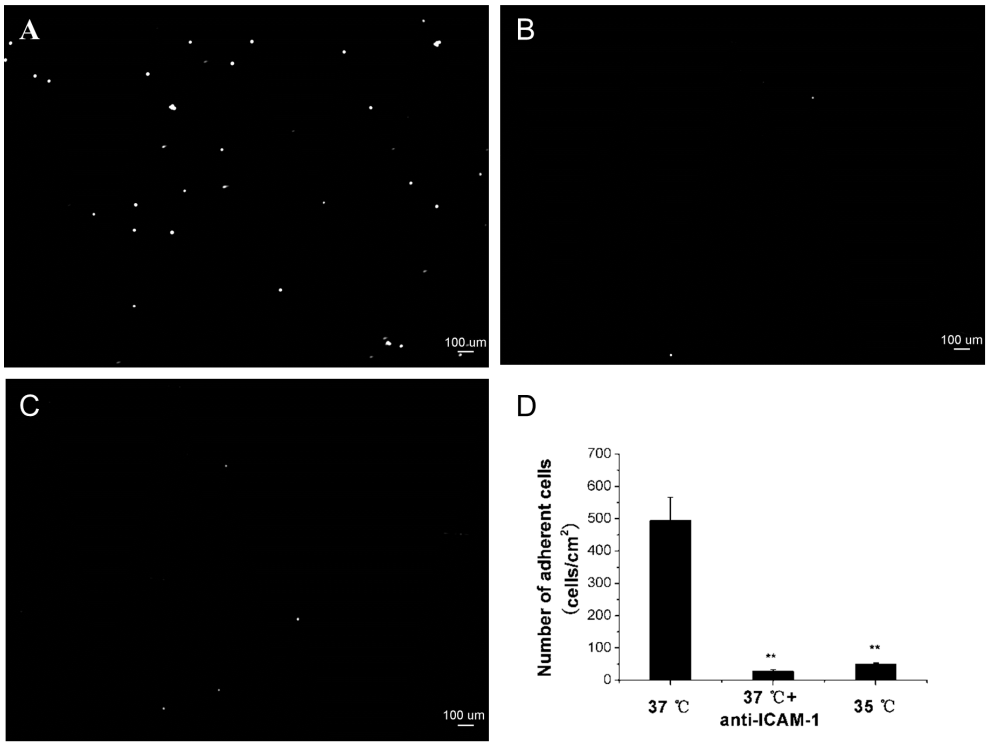

Figure 2. Effect of the mild hypothermia (35ºC) and anti-ICAM-1 on the adhesion of MDA-MB-231 tumor cells to Eahy926 endothelial cells under flow (1.5 dyn/cm^2). (A-C), Photomicrographs of adhesion of MDA-MB-231 tumor cells to Eahy926 endothelial cells under flow (1.5 dyn/cm^2) at normothermia (37ºC) **(A)**, pretreatment of the endothelium with anti-ICAM-1 antibody at 37ºC **(B),** and at mild hypothermia (35ºC) **(C)**. The white points were adherent tumor cells. The images were taken by with a fluorescence microscope (×40). **(D)** Adherent MDA-MB-231 cells were counted with a fluorescence microscope (more than 10 fields for each slide at ×40). Values are the mean of three experiments (n = 3) and are means ± S.E. ** $p < 0.01$ Comparison of adhesion at mild hypothermia (35ºC) or anti-ICAM-1treatments with the control treatment (37ºC).

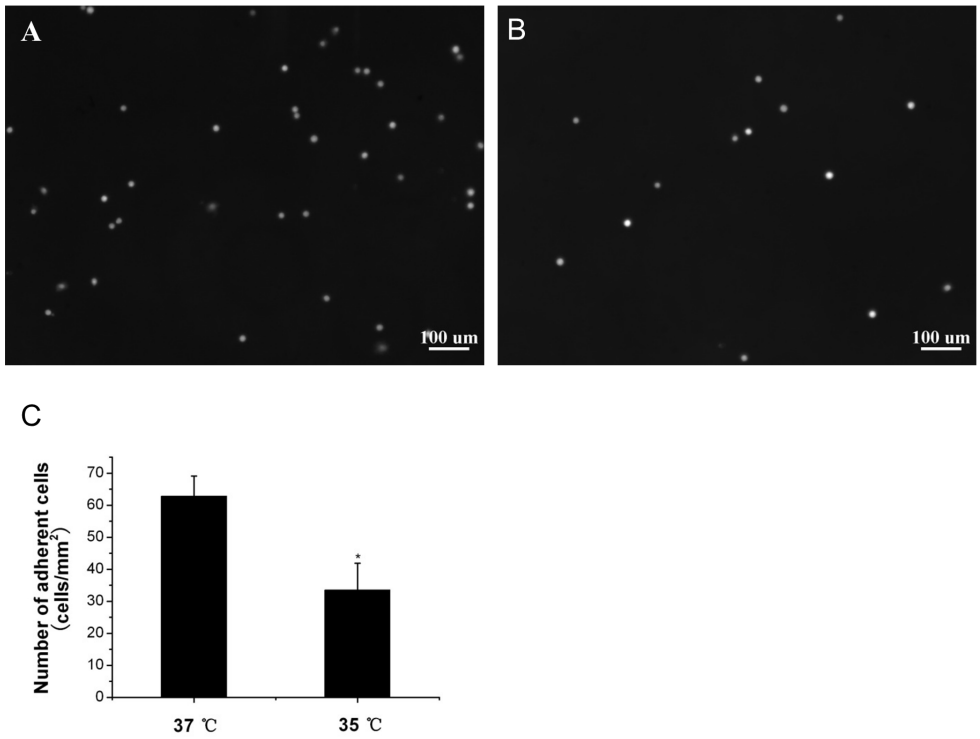

Figure 3. Effect of the mild hypothermia (35ºC) on the adhesion of MCF-7 tumor cells to Eahy926 endothelial cells under flow (1,5 dyn/cm^2). (A, B) Photomicrographs of adhesion of MCF-7 tumor cells to Eahy926 endothelial cells under flow (1.5 dyn/cm^2) at normothermia (37ºC) **(A)** and mild hypothermia (35ºC) **(B)**. The white points were adherent tumor cells. The images were taken with a fluorescence microscope (×100). **(C)** Adherent MCF-7 cells were counted with a fluorescence microscope (more than 10 fields for each slide at ×100). Values are the mean of three experiments and are means ± S.E. * $p < 0.05$ Comparison of adhesion at mild hypothermia (35ºC) with the control treatment (37ºC).

levels and only 28.35 (± 4.03 S.E., n = 3) cells/cm^2 firmly adhered to the endothelium at 37ºC ($p < 0.01$) (Figure 2D). This indicated that ICAM-1 might play an important role in the adhesion of MDA-MB-231 tumor cells to ECs. Because the number of adherent MDA-MB-231 cells in the field under the fluorescence microscope at ×40 was very few after pretreatment with mild hypothermia (35ºC), the effect of anti-ICAM-1 antibody on tumor cell adhesion under mild hypothermia (35ºC) was not further observed. Our results indicated that both mild hypothermia and anti-ICAM-1 antibody treatments significantly decreased adhesion of MDA-MB-231 tumor cells to ECs under flow conditions (1.5 dyn/cm^2).

To verify whether the mild hypothermia function is a general phenomenon in breast cancer cell lines, a detachment assay was used to investigate the effect of mild hypothermia on MCF-7 cells adhesion to ECs. The number of adhesion MCF-7 cells was decreased from 62.88 (± 6.23 S.E., n = 3) to 33.53 (± 8.32 S.E., n = 3) cells/mm^2 ($p < 0.05$) (Figure 3), which implied that mild hypothermia also has a similar effect on MCF-7 cells adhesion to ECs.

3.2. *Effect of mild hypothermia on the expression of ICAM-1 on Eahy926 endothelial cells*

As shown in Figures 2 and 3, mild hypothermia (35ºC) could reduce the MDA-MB-231 and MCF-7 tumor cells adhesion to ECs and ICAM-1 might play an important role in this process. Quantitative measurement of ICAM-1 expression on ECs was performed using Western blot analysis to examine the effect of the mild hypothermia on the expression of ICAM-1. Pretreatment of the ECs with mild hypothermia (35ºC) for 45 min in the flow chamber did not change the expression of ICAM-1 significantly (p = 0.325) (Figure 4). These results imply that mild hypothermia may decrease tumor cell adhesion by other mechanisms but not by decreasing the expression of ICAM-1 on ECs.

3.3. *Effect of mild hypothermia on tumor cells migration*

To determine whether mild hypothermia can affect the migration ability of tumor cells *in vitro*, wound healing assay (non-oriented migration) and transwell migration assays (oriented migration) were performed. In standard wound healing assays, the numbers of migrating MDA-MB-231 cells in a 100 μm long wound line were 25.57 (± 3.98 S.E., n = 3) and 23.62 (± 4.18 S.E., n = 3) cells under normothermia (37ºC) and mild hypothermia (35ºC), respectively (Figure 5). However, there is no obvious difference between the migrating cell numbers at normothermia (37ºC) and mild hypothermia (35ºC) ($p > 0.05$) (Figure 5B). Without mitomycin C treatment, the number of tumor cells in the 100 μm long wound

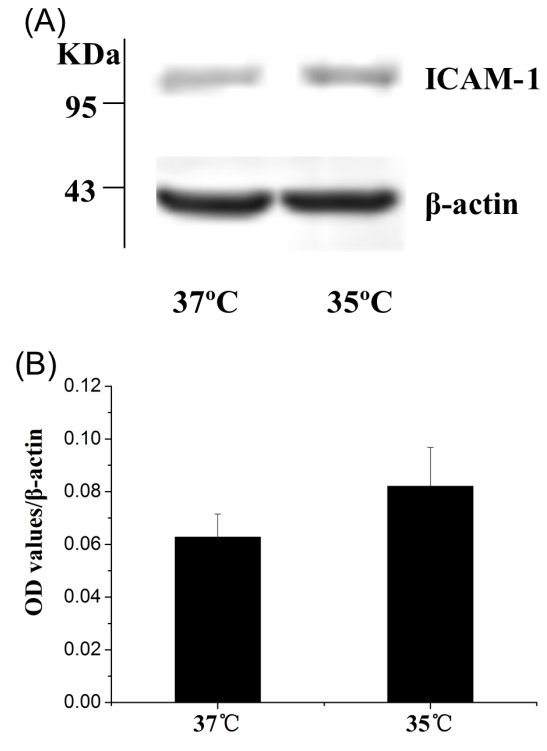

Figure 4. Effect of mild hypothermia on the expression of ICAM-1 on Eahy926 endothelial cells in the flow chamber. (A) Results of Western blotting. **(B)** Results of relative optical density values. The Eahy926 endothelial cells were perfused for 45 min in the flow chamber at 1.5 dyn/cm^2 with cancer cell-free medium at 37ºC and 35ºC. The expression of ICAM-1 showed no significant difference between 37ºC and 35ºC. Relative optical density values are the mean of three experiments (n = 3) and are means ± S.E.

line at normothermia (37ºC) were more than that at mild hypothermia (35ºC) for 36 h (data not shown). Temperature affects the growth of tumor cells without mitomycin C treatment.

In transwell migration assays, 6 h mild hypothermia treatment decreased the MDA-MB-231 cells oriented migration from 37.26 (± 2.85 S.E., n = 3) to 18.67 (± 3.37 S.E., n = 3) cells/visual field significantly ($p < 0.05$) and from 122.17 (± 11.65 S.E., n = 3) to 64.08 (± 11.03 S.E., n = 4) cells/visual field significantly ($p < 0.05$), using 10 % FBS and 40 ng/mL SDF-1 as chemoattractant, respectively (Figure 6). These results suggested that the effects of mild hypothermia on non-oriented migration and oriented migration of MDA-MB-231 cells were different. Mild hypothermia (35ºC) was able to weaken the oriented migration of MDA-MB-231 cells, but was not able to weaken the non-oriented migration.

3.4. *Effect of mild hypothermia on tumor cell adhesion to ICAM-1-coated substrate*

The above experimental results showed that mild hypothermia could reduce adhesion of tumor cells to the ECs, but could not reduce the expression of ICAM-

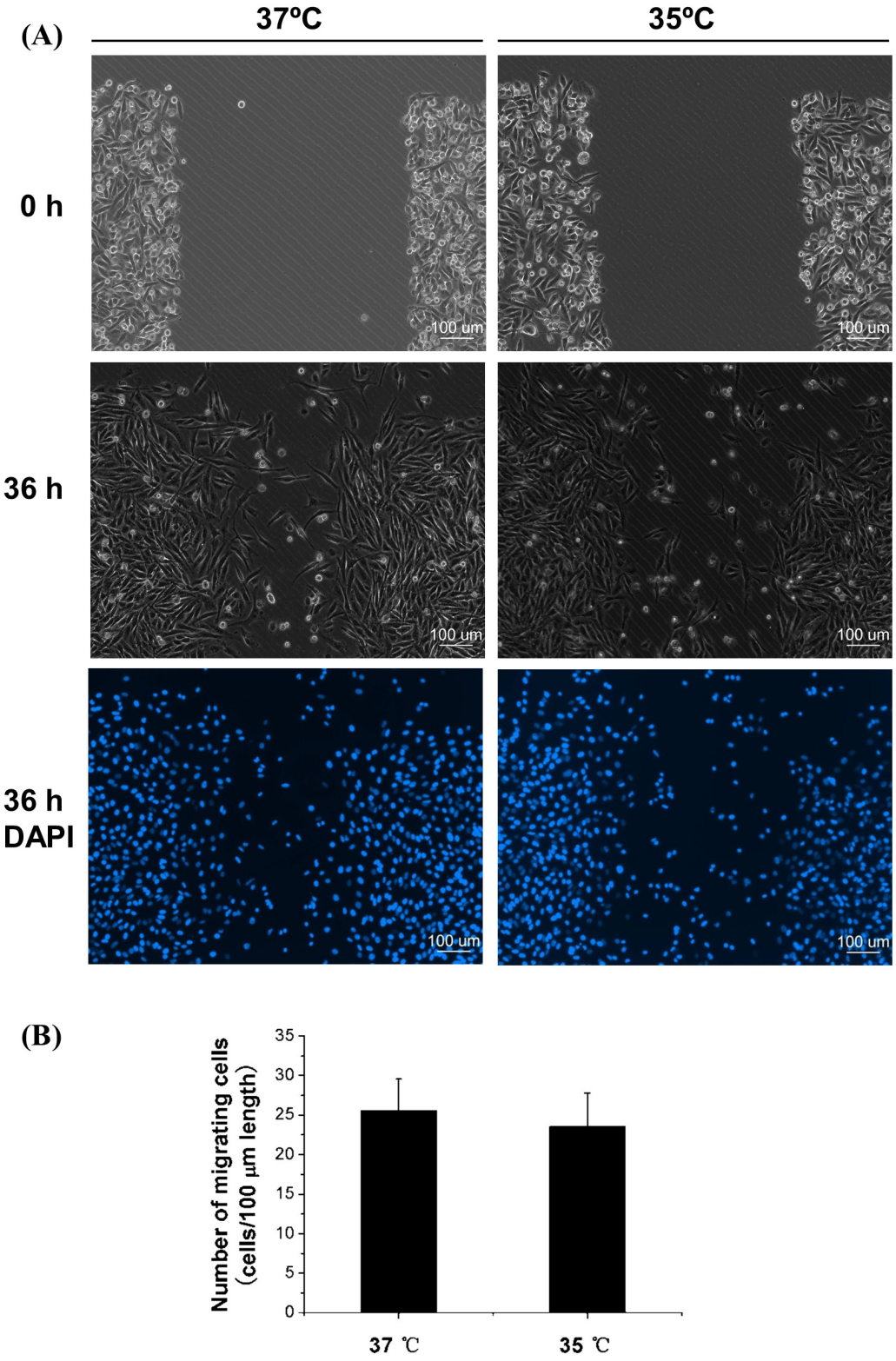

Figure 5. Effects of mild hypothermia on MDA-MB-231 tumor cells non-oriented migration in wound healing assay. (A) The images at 0 h and 36 h (the first and second rows) were taken using phase contrast microscopy (×100). The images at the bottom row were the same fields as those at 36 h and the cells were stained with DAPI, which were taken using a fluorescence microscope (×100). **(B)** Number of non-oriented migrating MDA-MB-231 tumor cells at 36 h in wound healing assay at normothermia (37ºC) and mild hypothermia (35ºC). The number of migrating cells was counted in 100 μm long wound line. Values are the mean of three experiments (n = 3) and are means ± S.E.

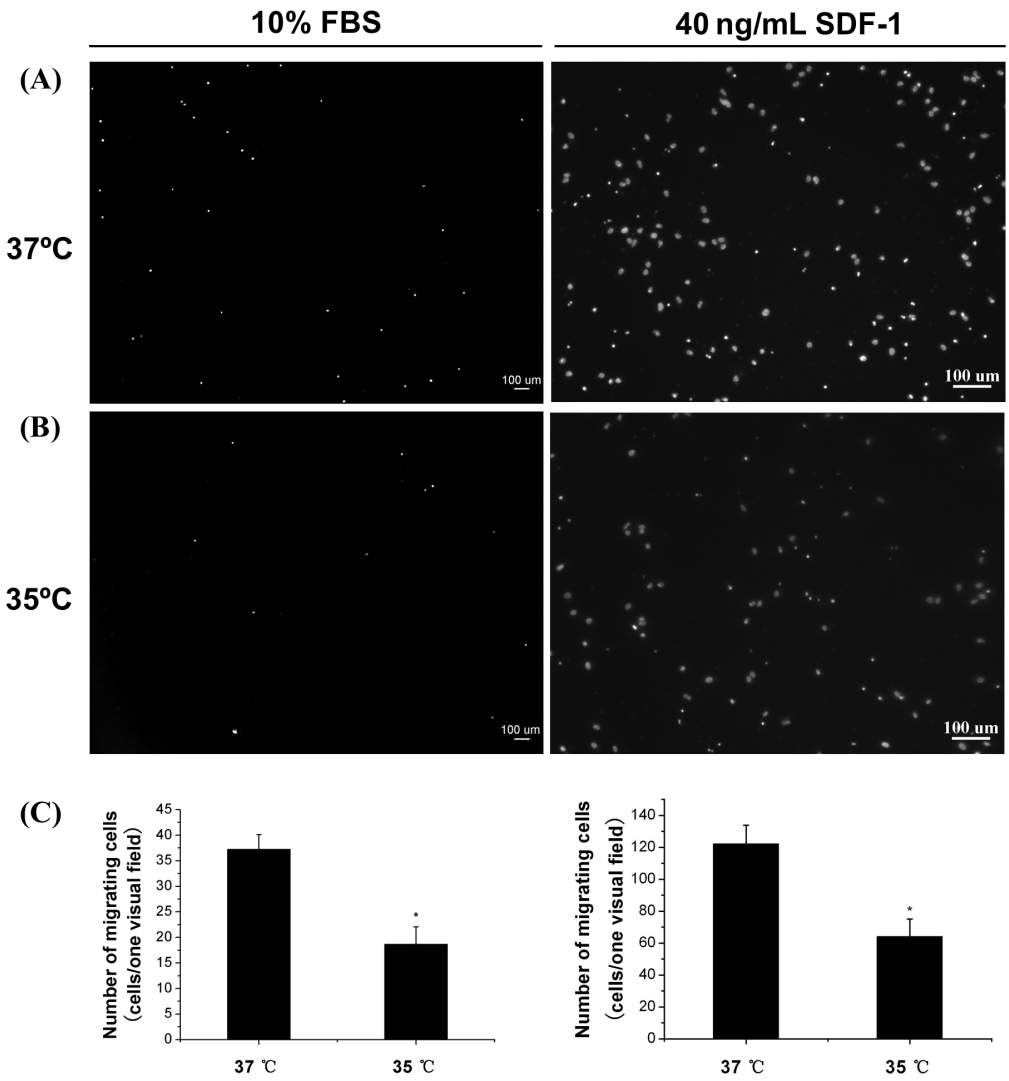

Figure 6. Effects of mild hypothermia on MDA-MB-231 tumor cells oriented migration in transwells migration using 10% FBS and 40 ng/mL SDF-1 as chemoattractant. (A, B) Photomicrographs of MDA-MB-231 tumor cells oriented migration in transwells migration assay at normothermia (37ºC) **(A)** and mild hypothermia (35ºC) **(B)**. The white points were adherent tumor cells. The images were taken using a fluorescence microscope (×40 for left and ×100 for right). **(C)** Number of oriented migrating MDA-MB-231 tumor cells in transwell migration assay at normothermia (37ºC) and mild hypothermia (35ºC). The number of migrating cells was counted in one visual field under a fluorescence microscope (×40 for FBS and ×100 for SDF-1). Values are the mean of at least three experiments and are means ± S.E. * $p < 0.05$ Comparison of migrating cells in transwells migration assays at normothermia (37ºC) and mild hypothermia (35ºC).

1 on ECs. It is indicated that the decreasing of tumor cells adhesion to ECs under mild hypothermia was not due to decreased expression of ICAM-1 on ECs, although ICAM-1 played an important role in the adhesive process. Tumor cells adhesion to the ICAM-1-coated substrate under flow conditions (1.5 dyn/cm^2) was further performed at normothermia (37ºC) and mild hypothermia (35ºC). 138.01 (± 19.18 S.E., $n = 3$) cells/mm^2 MDA-MB-231 cells adhered firmly to the ICAM-1-coated substrate under flow at 37ºC, while the adhesion was significantly reduced and only an average of 21.50 (± 8.15 S.E., $n = 3$) cells/mm^2 MDA-MB-231 cells adhered at 35ºC ($p < 0.01$) (Figure 7). These results further confirmed that mild hypothermia (35ºC) was able to reduce the adhesion of MDA-MB-231 tumor cells to ECs by a way independent of the change of ICAM-1 expression.

4. Discussion

The above results proved that minimal mild hypothermia (35ºC) was able to significantly reduce adhesion of MDA-MB-231 tumor cells to ECs Eahy926 under physiological flow *in vitro*. This finding implies that mild hypothermia can effectively reduce the adhesion of tumor cells to endothelium while the effective temperature is slightly lower than physiological temperature, 35ºC *versus* 37ºC. A deeper level of mild hypothermia such as 33ºC was widely used to study protection of mild hypothermia in ischemic tissue injury

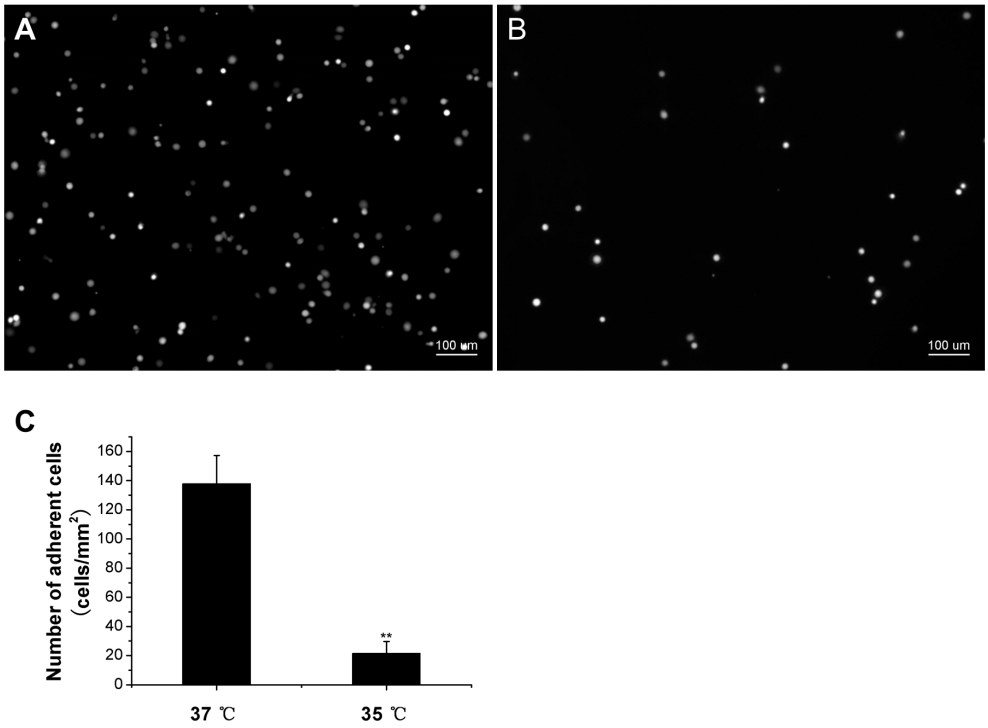

Figure 7. Effects of mild hypothermia on MDA-MB-231 tumor cells adhesion to ICAM-1-coated substrate under flow (1.5 dyn/cm^2). (A, B) Photomicrographs of MDA-MB-231 tumor cells adhesion to ICAM-1-coated substrate under flow (1.5 dyn/cm^2) at normothermia (37ºC) **(A)** and mild hypothermia (35ºC) **(B)**. The white points were adherent tumor cells. The images were taken using a fluorescence microscope (×100). **(B)** Number of adherent of MDA-MB-231 tumor cells to ICAM-1-coated substrate at 20 min at normothermia (37ºC) and mild hypothermia (35ºC). The number of migrating cells was counted in mm^2. Values are the mean of three experiments (n = 3) and are means ± S.E. ** $p < 0.01$ Comparison of adhesion at mild hypothermia (35ºC) with the control treatment (37ºC).

models and in clinical trials (*35,36)*. In comparison to 33ºC, 35ºC is easier to achieve in the clinic and better to tolerate. In addition, the hypothermia-associated adverse side effects might be less.

In blocking assays, treatment of ECs with anti-ICAM-1 antibody significantly prevented adhesion of tumor cells under experimental shear stress level. The result indicated that ICAM-1 might play an important role in the adhesion of MDA-MB-231 tumor cells to ECs Eahy926 which was consistent with previous studies (*10,37*). But Western blotting assays showed that mild hypothermia did not immediately reduce endothelial ICAM-1 expression under our experimental conditions. Thus, the reduced adhesion of MDA-MB-231 tumor cells to ECs Eahy926 under mild hypothermia (35ºC) is not due to a decrease of ICAM-1 expression by mild hypothermia (35ºC) in our study. ICAM-1 is expressed constitutively on endothelial cells and induced overexpression is caused by inflammatory cytokines (TNF-α, IL-1, IFN-γ). It is reported that mild hypothermia (33ºC) treatment inhibited the overexpression of ICAM-1 after acid-induced lung injury in rats, whereas it did not significantly affect express of ICAM-1 in normal rats (*21*). Sutcliffe and his colleagues (*38*) reported that ICAM-1 expression has no significant difference when human cerebral endothelial cells were exposed to IL-1β at 37ºC and 32ºC. However, a significant reduction in IL-8 and IL-1β mRNA expression was seen in human cerebral endothelial cells exposed to IL-1β at 32ºC. In contrast to IL-1β, TNF-α is increased after application of hypothermia (32ºC) 8 h after the insult in an endothelin-1-induced transient focal cerebral ischemia model (*19*). Therefore, it is likely that the inhibition of ICAM-1 overexpression by mild hypothermia in injury models is a cumulative outcome due to different effects of mild hypothermia on individual inflammatory factors (such as TNF-α and IL-1β), transcription factors involved in inflammation (such as NF-κB and signal transducer and activator of transcription-3 (STAT3)). A role of inflammation in tumorigenesis is now generally accepted. There are several types of inflammation associated with cancer, including chronic inflammation associated with infections or autoimmune disease, inflammation caused by environmental and dietary exposure, tumor-associated inflammation, and therapy-induced inflammation (*39*). The targeting of inflammatory mediators (chemokines and cytokines, such as TNF-α and IL-1β), key transcription factors involved in inflammation (such as NF-κB and STAT3) or inflammatory cells decreases the incidence and spread of cancer (*40*). In various cancers, ICAM-

1 expression is at an elevated level and is associated with the malignant potential of cancer (*5-9*). To some extent, ICAM-1 overexpression is associated with the inflammatory environment in tumors. There is no report about the effect of mild hypothermia on cancer-related inflammation and the effect of mild hypothermia on the incidence and spread of cancer after radiotherapy, chemotherapy or surgery. In this study, we just investigated the effect of mild hypothermia on tumor cells adhesion to ECs *in vitro* and have not obtained information about the intricate systemic response *in vivo*. Therefore, it is worthwhile to find out whether mild hypothermia treatment will promote the outcome of animals with tumors after traditional therapy.

To investigate the potential mechanisms of the effect of mild hypothermia on tumor cells adhesion to ECs under our experimental conditions, tumor cell adhesion to immobilized ICAM-1 under flow at 35ºC and 37ºC was further conducted. The results showed that mild hypothermia could inhibit cells adhesion in flow when the density of adhesion molecules was kept constant. First, Rico and coworkers (*41*) observed that the work required to detach an atomic force microscope (AFM) cantilever tip functionalized with human ICAM-1-Fc from the cells' surface decreased dramatically from 37ºC to 16ºC. They further found it was due to two main factors. (1), reduced cell stiffness at higher temperatures would enhance the number of bonds formed by increasing the area of contact between the surfaces. (2), a lower linkage between the plasma membrane and cytoskeleton at higher temperatures would favor the extraction of long membrane tethers, which would prolong the time the cells remain in contact with the surface, favoring bond reformation. In addition, at the high experimental temperature, the membrane fluidity is increased and integrins realign themselves more effectively to bind with receptors (*42*). Second, it is worth mentioning the effect of temperature on functional conformation of adhesion molecules. It is well known that conformation of proteins is sensitive to temperature (*43*). Salas *et al*. (*44*) reported that LFA-1 (αLβ2) affinity for ICAM-1 was regulated by the conformation of the αL domain. The open or high affinity I domain conformation supports strong adhesion, whereas the closed, low affinity conformation mediates weak interactions or rolling. It was reported that LFA-1-dependent adhesion required divalent cations and was temperature-sensitive (*45,46*). Thus, it is likely that mild hypothermia may affect protein folding, which would be unfavorable to the formation of the high affinity conformation. Finally, decreased temperature may decrease the association rate constant of bonds, prolonging the time needed for cell attachment, as a consequence, cells detach from substrate before firm adhesion in the presence of shear force. The potential mechanisms of the effect of mild hypothermia on tumor cells adhesion to ECs should be further examined in detail, including the functional characteristics of ICAM-1.

Cell migration plays a central role in a wide variety of biological phenomena in both normal physiology and pathophysiology. Particularly in a tumor setting, cell migration is critical to metastasis. To test whether mild hypothermia could affect tumor cells migration, wound healing assays (non-oriented migration) and transwell migration assays (oriented migration) were performed. Because temperature affects cell proliferation (*47*), mitomycin C was used in the wound healing assay to avoid cell migration diversity due to cell proliferation differences affected by temperature. It is interesting that mild hypothermia can effectively weaken tumor cells oriented migration (chemotaxis to serum and SDF-1), but has no obvious effect on non-oriented migration. It is significant because a growing body of evidence shows that some tumor cells expressed chemokine receptors responding to chemokine gradients *in vitro*, and certain chemokines could serve as tissue-specific attractant molecules for tumor cells, promoting tumor-cell migration to particular sites *in vivo* (*48,49*). These results suggested that mild hypothermia may weaken chemokine-mediated metastatic spread of tumor cells, which is worthwhile to be investigated further.

In conclusion, results of the present study show that minimal mild hypothermia (35ºC) could reduce tumor cells adhesion to endothelium under physiological flow *in vitro* and weakened tumor cells oriented migration (chemotaxis). ICAM-1 was important for adhesion of human breast cancer cells MDA-MB-231 to ECs Eahy926, but mild hypothermia could not affect endothelial ICAM-1 expression. Reduced adhesion of tumor cells to endothelium may be caused by other mechanisms. These results suggest that mild hypothermia could be used as a potentially adjunct treatment combined with surgery to decrease tumor cell adhesion and migration. Further studies need to explore the accurate mechanism that mild hypothermia modulates adhesion and migration of tumor cells to endothelial cells.

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