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Adhesive Force of Human Hepatoma HepG2 Cells to Endothelial Cells and Expression of E-Selectin

Guanbin Song*,†, Toshiro Ohashi†, Naoya Sakamoto† and Masaaki Sato†

Abstract: Expression of adhesion molecules may play an important role in the interaction of tumor cells with vascular endothelial cells during tumor invasion and metastasis. In this study, the adhesive force of human hepatoma HepG2 cells to human umbilical vein endothelial cells (HUVECs) was investigated using a micropipette aspiration technique. Expression of an adhesion molecule, E-selectin, was also observed by immunofluorescence microscopy. In particular, the adhesive force after stimulation of HUVECs with recombinant human interleukin-1β (rhIL-1β) was examined. The results demonstrated that the adhesive force of HepG2 cells to stimulated HUVECs is significantly higher than that of unstimulated control cells, and that immunofluorescence of E-selectin in stimulated HUVECs showed a higher fluorescent intensity compared to control cells. Moreover, addition of monoclonal anti-human E-selectin decreased the adhesive force of HepG2 cells to stimulated HUVECs by 50%. These results suggest that endothelial E-selectin may be a main mediator of carcinoma metastasis of malignant tumor through blood circulation, possibly increasing the adhesive force of human hepatoma HepG2 cells to HUVECs in the early stage of metastases.

keyword: Hepatoma cells, Endothelial cells, Adhesive force, E-selectin, Micropipette aspiration

1 Introduction

Cancer is a malignant disease characterized by disorganization of the cell cycle and the resulting uncontrolled proliferation, which can be very harmful to human health (1). Tumor invasion and metastasis are the major causes of treatment failure and death in gastrointestinal cancer patients. About 30% of patients with newly diagnosed tumors already have detectable metastases. Of the remaining 70%, who are free of detectable metastasis, about half will develop metastatic spread after potentially radical treatment of the primary tumor (2, 3). Therefore, at least 60% of cancer patients have microscopic or clinically evident metastases at the time of primary tumor treatment. The process of metastasis involves multiple tumor-host interactions. To metastasize, tumor cells must shed into the bloodstream (intravasation) either directly, by invasion into the tumor-derived vasculature, or indirectly, by lymphatic drainage. The cells must then survive in the circulation, experience a circulation slowdown and rolling on endothelial cells and adhesion to a vascular endothelium, and finally, migrate through the vessel barrier (extravasation) and proliferate in the target organs (4, Fig. 1). In particular, adhesion of tumor cells to endothelial cells (ECs) plays a key role in the early stages of metastasis. Therefore, expression of adhesion molecules may be an important factor in determining the adhesion behavior of tumor cells (5).

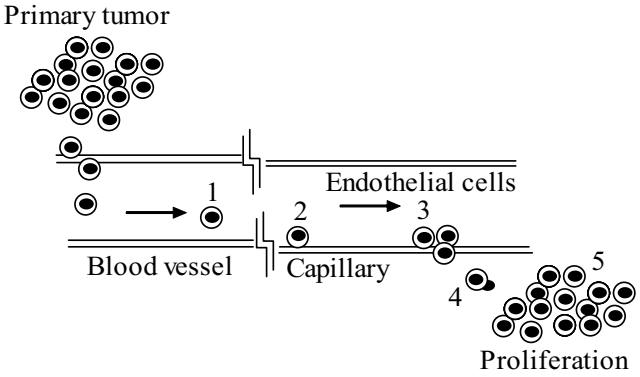

Figure 1 : Metastasis of tumor cells through blood circulation. 1. Tumor cell rolling on endothelial cells; 2. Adhesion to endothelial cells; 3. Crossing the blood vessel wall; 4. Amoeba-like movement to target organ(s); 5. Proliferation and formation at a new tumor site.

Interleukin-1(IL-1), an inflammatory mediator, consists

* Corresponding author. College of Bioengineering, Chongqing University, Chongqing 400044, P. R. China. Email: song@cqu.edu.cn

† Department of Bioengineering and Robotics, Graduate School of Engineering, Tohoku University, Sendai 980-8579, Japan

of two separate but related proteins, IL-1α and IL-1β. IL-1β is a pluripotent cytokine that promotes angiogenesis, tumor growth, and metastasis. In various experimental models, IL-1β increases tumor invasiveness and metastasis. For example, IL-1β expression at the site of tumor development enhances the expression of adhesion molecules on endothelial and malignant cells and facilitates the invasion of malignant cells into the circulation and their dissemination to remote tissues. The endothelium is involved in several homeostatic mechanisms, such as the maintenance of a non-thrombotic surface, the metabolism of lipoproteins and in immune response. Several conditions may induce activation of endothelial cells, which leads to the appearance of adhesion molecules on the cell surface. Adhesive molecules, such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), mediate the attachment of certain leukocytes to the endothelial surface and may be important in controlling the extravasation of leukocytes from the circulation to sites of inflammation. The interaction between tumor cells and endothelial cells is similar to the adhesion of leukocyte to endothelial cells in inflammation. Integrins and selectins, are expressed in tumor cells and endothelial cells, are the main mediating adhesion molecules in metastasis (6, 7). However, the detailed mechanism of invasion and metastasis through blood circulation has not fully been understood.

Both cell-matrix and cell-cell adhesions play important roles in many physiologically process including tumor development and wound healing (8). Formation of cell-cell adhesions is believed to be a key stage during tumor invasion and metastasis. At this stage, cell mechanical properties including deformation, adhesion and migration, and several growth factors are believed to play a key role (9, 10). Micropipette aspiration technique is an especially widely used method to study cell mechanical properties because it does not require complicated experimental setup. Recently, the micropipette aspiration technique has been developed to study E-selectin/ligand interactions on the surface of red cells and HL-60 or Colo-205 cells (11), and single-cell adhesions between lymphatic cells and endothelial cells (12).

In this study, we used the micropipette aspiration technique to quantify adhesive force of hepatoma cells to endothelial cells and to investigate the contribution of the adhesion molecule E-selection in this process.

2 Materials and Methods

2.1 Materials

Medium 199 (M199) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Invitrogen Corporation (USA). Fetal bovine serum (FBS) was from Biowest (Japan), recombinant human basic fibroblast growth factor (bFGF) and recombinant human interleukin-1β (rhIL-1β) were from Austral Biologicals (USA). Monoclonal mouse anti-human E-selectin/FITC and monoclonal mouse IgG1/FITC were purchased from Ancell Corporation (USA).

2.2 Cell isolation and culture

Human hepatoma cell line (HepG2) was bought from Health Science Research Resources Bank (Japan). HepG2 cells were routinely cultured in suspension of DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 units/ml streptomycin in a humidified atmosphere of 5% CO_2/95% air at 37 °C. HepG2 cells were detached by a brief exposure to 0.05% trypsin/0.02% EDTA for experiments.

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords with an enzymatic digestion method (13). HUVECs were cultured with M199 supplemented with 20% FBS, 100 units/ml penicillin, 100 units/ml streptomycin and 1ng/ml bFGF in a standard incubator. HUVECs from passages 3-8 were used for experiments.

2.3 Micropipette aspiration system

The adhesive force of HepG2 cells to HUVECs was determined using a micropipette aspiration system (Fig. 2). This system is composed of an inverted microscope (IX71S8F-2, Olympus, Japan), a micromanipulator (MMO-203, Narishige, Tokyo, Japan), an image recording system (DVR-77H, Pioneer, Japan), an image process system (Slidebook 4.1, Japan), a video monitor (12M310, Tokyo Electronic Industry, Japan), a pressure controlling system (self-assembled by Biomechanics Laboratory, Tohoku University, Japan) and a glass micropipette. The micropipette was made from a glass tube (G-1, Narishige, Japan) using a micropipette puller (Micro Forge, Narishige, Japan). The inner radius of micropipettes used here was approximately 2.5-3.0 μm.

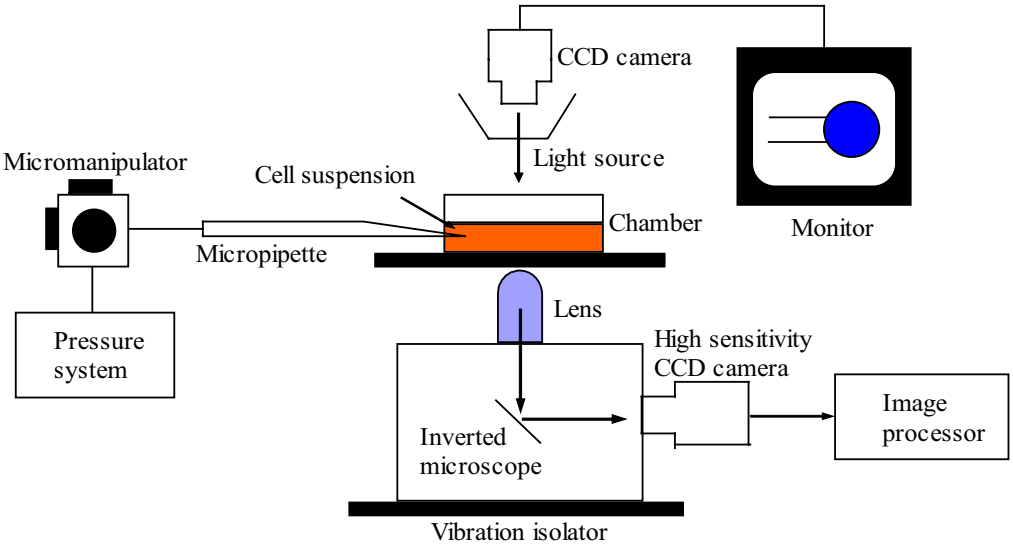

Figure 2 : Schematic of the micropipette aspiration system.

2.4 Measurement of adhesive force

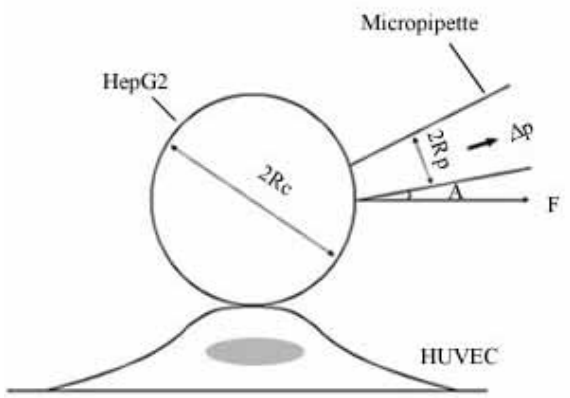

Figure 3 : Pipette aspiration technique to measure the adhesive force of HepG2 cell to HUVECs. Where, *Rp* is the inner radius of micropipette, *Rc* the radius of HepG2 cell, ΔP the critical aspirated negative pressure, *A* the angle between micropipette and plane of monolayer HUVECs, *F* adhesive force.

HUVEC monolayers were cultivated in a special chamber. 0.5ml HepG2 cell suspension, containing 1×10^5 cells/ml, was then added to the chamber and the chamber was placed in a 37 ° C incubator for 30 min prior to the experiments. A schematic diagram of the micropipette aspiration system used to measure adhesive force is shown in Fig. 3. Regulated pressure was set to zero (zero-pressure state) through the pressure controlling system, a HepG2 cell was selected under the microscope. The tip of the micropipette was then positioned close to the surface of the HepG2 cell using the micromanipulator and a small portion of the HepG2 cell was aspirated into the micropipette by application of negative pressure (ΔP). Next, the micropipette was carefully pulled away from the cell. If the HepG2 cell was not detached from the surface of the adhered HUVEC, the negative pressure was increased by 5 mmH_2O. This process continued until the HepG2 cell was finally detached from the adhered HUVEC, thus giving the critical negative pressure for detachment. The experimental process was continuously recorded by the DVD recorder. The experiments were carried out on between 12 and 26 HepG2 cells per HUVEC monolayer at room temperature and the procedure was completed within 2 h. During the experiments, detachment of HUVECs from the glass substrate was never observed.

The formula for calculating adhesive force was defined as $F = \pi \times R_P^2 \times \Delta P \times \cos A$ (14-16). Where, R_p is the inner radius of the micropipette, ΔP the critical negative pressure, and *A* the angle between the micropipette and the plane of monolayer HUVECs. As the angle, *A*, was fixed at 10^0, the formula could be simplified to $F = \pi \times R_P^2 \times \Delta P$.

2.5 Stimulation of HUVECs with rhIL -1β

HUVECs were cultured in M199/20% FBS until confluence, then the medium was replaced with M199 contain-

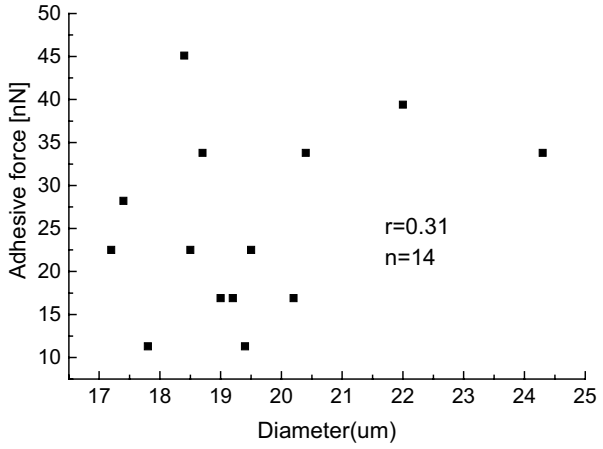

Figure 4 : Relationship between adhesive force and the diameter of HepG2 cells. Where, n: number of cells measured, r: correlative coefficient, r=0.31<$r_{0.05}$=0.532.

ing rhIL-1β. In order to study the effects of both loading period and concentration of rhIL-1β, HUVECs were stimulated for different period of time from 1 h to 6 h at a concentration of 200 units/ml, and at different concentrations of rhIL-1β from 50 units/ml to 400 units/ml for 4 h. After this the medium was replaced with M199/20% FBS, the cells were used for micropipette pipette experiments as described previously.

2.6 E-selectin blocking antibodies

Monolayer HUVECs were incubated with monoclonal mouse anti-human E-selectin/FITC at a concentration of 10μg/ml in M199 at 37 ˚C for 30 min. The suspension was then discarded and HUVECs were used for adhesion assay. Non-specific binding was determined by incubation with monoclonal mouse IgG1/FITC, a nonspecific antibody of the same isotype, under identical conditions.

2.7 Expression of E-selectin in HUVECs

Immunochemistry was employed to examine expression of E-selectin in HUVECs after stimulation by rhIL-β. Briefly, HUVECs were stimulated with 200 units/ml rhIL-β for 4 h (see Results), fixed with 10% formalin for 15-20 min and rinsed 3 times with phosphate buffer solution (PBS). HUVECs were then stained with monoclonal mouse anti-human CD62E (E-selectin)/FITC for 30 min at 4 ˚C and rinsed 3 times with PBS. Fluorescent intensity of E-selectin was observed under a fluorescent microscope (IX71S8F-2, Olympus, Japan).

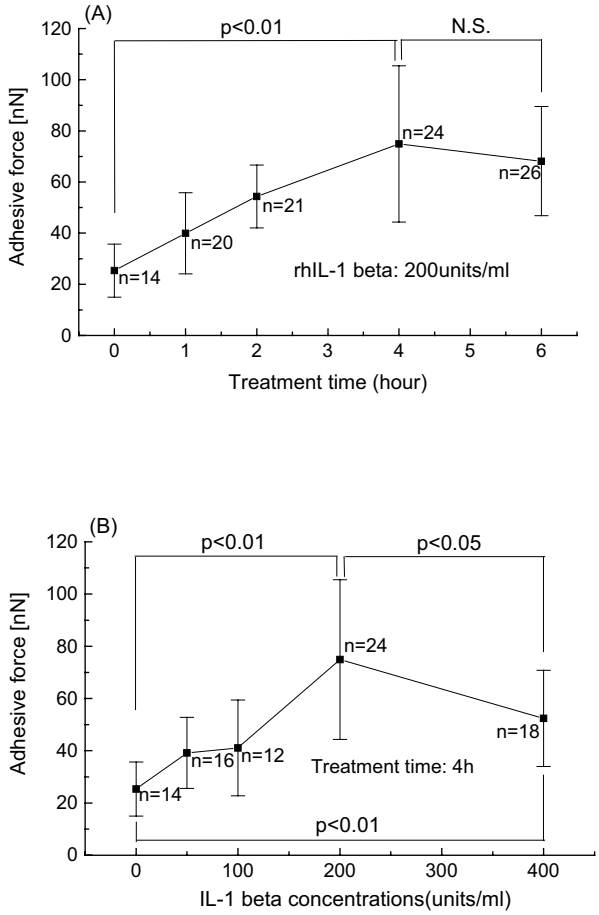

Figure 5 : Effect of stimulating period and concentrations of rhIL-1β on adhesion of HepG2 cells to HUVECs. The relationship (A) between adhesive force and stimulating period of time and (B) between adhesive force and concentrations of rhIL-1β. n: number of cells measured.

2.8 Statistical analysis of data

Data from experiments were expressed as mean ± SD and statistically analyzed using Student's *t*–test and one-way ANOVA. A value of $p< 0.05$ was considered significant.

3 Results

3.1 Relationship between adhesion force and diameter of HepG2 cells

The relationship between adhesive force and the diameter of HepG2 cells was examined, as illustrated in Fig. 4. No significant correlation was observed between adhesive force and cell diameter.

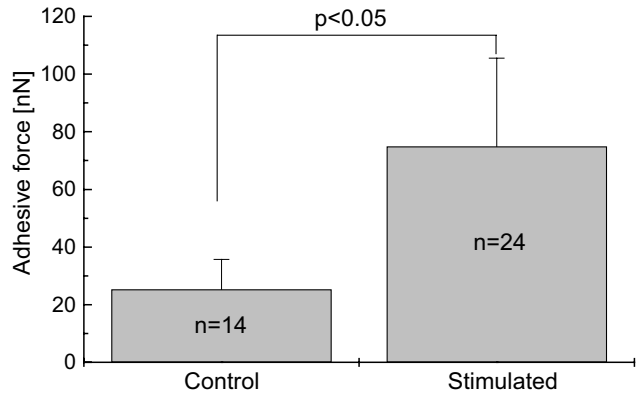

Figure 6 : Adhesive force of HepG2 cells to HUVECs after treatment with 200 units/ml rhIL-1β for 4 h. n: number of cells measured.

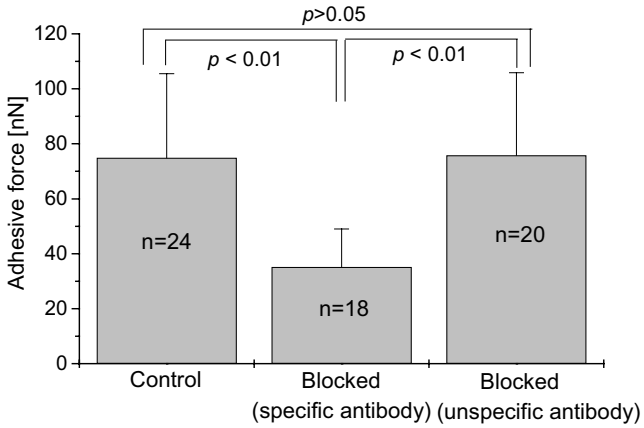

Figure 7 : Inhibition of human hepatoma HepG2 cell adhesion to HUVECs by monoclonal anti-human E-selectin. HUVECs were stimulated with 200 units/ml rhIL-1β for 4h. The adhesive force of HepG2 cells to HUVECs without antibody incubation (control), HUVECs incubated with monoclonal anti-human E-selectin/FITC (specific antibody) and HUVECs incubated with monoclonal mouse IgG1/FITC of the same isotype (nonsperific antibody) was evaluated (one-way ANOVA). n: number of cells measured.

3.2 Treatment time and concentration of rhIL − 1β

The effects of treatment time and concentration of rhIL-1β on the adhesive force of HepG2 cells to HUVECs are illustrated in Fig. 5A and 5B, respectively. In Fig. 5A, the adhesive force significantly increases with increasing loading period and reaches at a peak value of 74.9±30.6 nN at 4 h followed by an equilibrium state at 6 h. Figure 5B demonstrates that as concentration of rhIL-1β increased, the adhesive force significantly increased, reaching a peak at 200 units/ml. Therefore, subsequent experiments, HUVECs were stimulated with 200 units/ml rhIL-β for 4 h.

3.3 The effect of rhIL − 1β *on the Adhesive force of HepG2 cells to HUVECs*

The adhesive force of hepatoma HepG2 cells to HUVECs after treatment with 200 units/ml rhIL-1β for 4 h is shown in Fig. 6. The adhesive force showed a significant increase when compared with unstimulated control HUVECs.

3.4 Inhibition of adhesive force by anti-human E-selectin

Figure 7 shows inhibition of adhesive force of HepG2 cells to HUVECs treated with monoclonal anti-human E-selectin. The adhesive force was decreased by 50% by incubation with the specific antibody. This value was significantly less than the adhesive force of HepG2 cells to untreated control HUVECs and HUVECs incubated with the nonspecific antibody. In contrast, adhesive force was not significantly affected by incubation with a nonspecific antibody of the same isotype.

3.5 Expression of E-selectin in HUVECs

Fluorescent images of E-selectin distribution in HUVECs are shown in Fig. 8. Expression of E-selectin, visualized with monoclonal anti-human E-selectin/FITC, was greater after stimulation with rhIL-1β (Fig. 8A) than in unstimulated controls (Fig. 8C). Fluorescence of stimulated HUVECs after incubation with the nonspecific IgG1/FITC (Fig. 8B) was reduced compared to those incubated with anti-human E-selectin/FITC.

4 Discussion

Tumor cell invasion and metastasis requires a close interaction between cancerous cells and the normal surrounding tissue. To successfully establish a metastatic colony, circulating tumor cells must survive immunologic surveillance, be arrested at a distant vascular step, and extravasate. To initiate the metastatic process, tumor cells will first penetrate the basement membrane and then invade the interstitial stroma by active proteolysis. Finally, tumor cells must invade and proliferate in the sec-

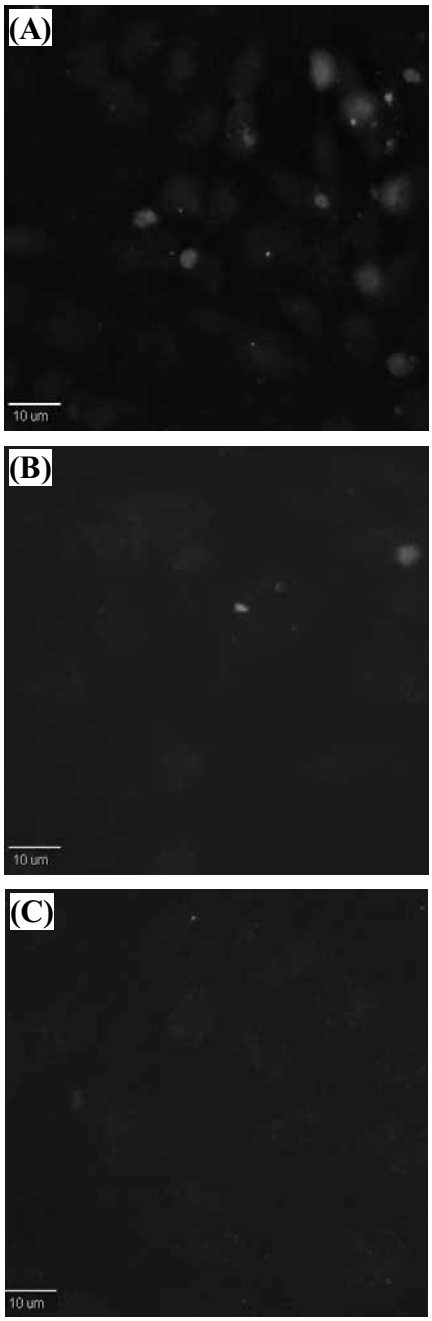

Figure 8 : Fluorescent images of E-selectin distribution in HUVECs. (A) HUVECs stimulated with 200 units/ml rhIL-1β for 4h and stained with monoclonal anti-human E-selectin/FITC, (B) HUVECs stimulated with 200 units/ml rhIL-1β for 4h and stained with monoclonal mouse IgG1/FITC, and (C) HUVECs without stimulation and stained with monoclonal anti-human E-selectin /FITC.

ondary organ. In this study, we used micropipette technique to quantify the adhesive force of human hepatoma cells to HUVECs and to investigate the contribution of the adhesion molecule E-selectin in this process.

The relationship between adhesive force and the diameter of HepG2 cells was initially examined to assess the effect of the variation in contact area between HepG2 cells and HUVECs on adhesive force. The results showed no significant correlation between adhesive force and the diameter of HepG2 cells. Accordingly, the effect of the variation in contact area on adhesive force may be negligible.

In a preliminary experiment, we investigated the change in adhesive force of HepG2 cells to HUVECs pre-stimulated with rhIL-1β for different periods of time (0-6 h, 0 referred to as control) and at different concentrations (0-400 units/ml, 0 referred to as control). The results indicate that adhesive force increased in both a time-dependent and a concentration-dependent manner with adhesive force peaks at 4 h and 200 units/ml, respectively. Adhesive force seemed to reach an equilibrium state after 4 h incubation with 200 units/ml, since there was not statistically significant between at 4 h and at 6 h. In contrast, the adhesive force after 4 h incubation with 400 units/ml significantly decreased when compared to 4 h with 200 units/ml ($p < 0.05$). Earlier studies have demonstrated that interactions between tumor cells and endothelial cells are mediated by multifarious adhesion molecules including selectins, integrins, IgG superfamily, cadherins and others (17, 18). Thus, different stimulating conditions may change the expression of a variety of adhesion molecules, possibly leading to changes in adhesive force.

Interactions between tumor cells and endothelial cells during tumor invasion and metastasis may require the cooperative action of several receptor-ligand systems. In a process, similar to leukocyte rolling during the inflammatory response (19), tumor cells present rolling on the vascular endothelium and then adhere to endothelial cells (Fig. 1). The hypothesis of "docking and locking" was put forward to describe this adhesive behavior between tumor cells and endothelial cells (20). Integrins and selectins expressed in tumor cells and endothelial cells, and are the main adhesion molecules mediating "docking and locking" phase. Brodt et al (21) found that an anti E-selectin monoclonal antibody could inhibit tumor cell adhesion to hepatic endothelial cells and demonstrated that endothelial E-selectin was a mediator of carcinoma

metastasis to liver. In a preliminary study of lung carcinoma H-59 cells, they also found that a polyclonal antibody to integrin subunit β_1 partially blocked the basal adhesion (35% inhibition). In the current study, we examined the adhesion properties of human hepatoma HepG2 cells to HUVECs using a micropipette aspiration technique, and in a previous study, performed a similar experiment with human hepatoma SMMC-7721 cells (15). These results show similar values of basal adhesive force to HUVECs. The adhesive force of HepG2 cells to HUVECs post-stimulated with rhIL-βghowed a significant increase when compared to unstimulated control HUVECs. Moreover, the adhesive force could be blocked by monoclonal anti-human E-selectin resulting in a decrease of 50%. In our earlier study, we assessed the role of integrin β_1 in the adhesion of human hepatoma SMMC-7721 cells to HUVECs and found that adhesive force could also be significantly blocked by monoclonal anti-human integrin β_1(15). These results agree well with the findings by Brodt et al.(21), providing direct quantitative evidence that E-selectin and integrin $\beta_1$may be major adhesive molecules mediating the adhesion of hepatoma cells to endothelial cells during tumor invasion and metastasis.

E-selectin is a cytokine-inducible endothelial cell adhesion receptor, which includes an N-terminal lectin-like domain, an epidermal growth factor repeat and variable numbers of modules with sequence homology to complement-binding proteins. Sialyl-Lewis^x (s-Le^x) and sialy-Lewis^a (s-Le^a) have been identified as the selentin ligands. Since several studies have implicated these receptors in carcinoma cell adhesion to the endothelium, it has been suggested that this interaction may be required for tumor extravasation during metastasis (21, 22). Our results showed that fluorescent immunolabelling of E-selectin in control HUVECs was very low, but the intensity significantly increased after stimulation with rhIL-1β. These results are in good agreement with previous reports (21, 23, 24) which have shown that nascent E-selectin levels in vascular endothelial cells were found to be constitutively low but could be up-regulated by cytokine stimulation.

There is a limitation in this study that should be noted. The adhesive force measured may depend on mechanical properties of both HUVEC and HepG2 cells. As the formula for calculating adhesive force used here does not consider the effect of mechanical properties of cells, development of the formula will be required for future study. It is possible to measure cell compliance using atomic force microscopy (AFM). Thus combination of the AFM with the micropipette aspiration technique may be effective to obtain more accurate results.

Biological therapy designed to block molecular mediators of metastasis may provide an effective alternative to conventional treatments. Kobayashi et al. (25) demonstrated that cimetidine may block the adhesion of a colorectal tumor cell line to the endothelial cell monolayer and thus prevent metastasis. They also demonstrated that these antimetastasis effects might occur through down-regulation of E-selectin expression on endothelial cells. Many studies have shown that advanced metastatic colorectal carcinomas express increased levels of E-selectin ligands and have an increased adhesion to HUVECs stimulated with rhIL-1β or tumor necrosis factor α (TNFα) (26, 27). Taking these and other findings (28) together with our results, it can be suggested that reagents to block E-selectin-mediated adhesion may have potential clinical benefits in the prevention of metastases of malignant tumor.

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