A New Endothelial Molecule Involved in Melanoma Cell Binding to Human Dermal Microvascular Endothelial Cells

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***Background*: We have previously demonstrated that phorbol myristate acetate(PMA)-induced increases in melanoma cell binding to endothelial cells derived from human dermis (HDMEC) are not mediated via known cell adhesion molecules and may be affected through microvessel-specific novel proteins not previously described on endothelial cells.**

***Objective*: This study was performed to identify new molecules which may play a role in HDMEC-melanoma cells binding.**

***Methods*: We have generated a monoclonal antibody(Mab) against PMA-stimulated HDMEC. A Mab was evaluated functionally through melanoma cell-endothelial cell adherence assay and characterized by Western immunoblot.**

***Results*: Mab EM-71 recognized a molecule with expression levels *in vitro* that could be upregulated by PMA(EM-71 molecule). The expression of EM-71 molecule on HDMEC was increased in a dose-dependent manner by PMA only, but not affected by interleukin 1 alpha(IL-1α) or tumor necrosis factor alpha(TNFα). PMA augmented melanoma cell adherence to HDMEC, which is coincident with an increase in EM-71 molecule expression on HDMEC by PMA. Mab EM-71 partially inhibited up to 59% of the increased melanoma cell binding to PMA-stimulated HDMEC and failed to block melanoma cell binding to IL-1α or TNFα-stimulated HDMEC. Western immunoblots of lysates of HDMEC demonstrated a 200 kDa protein on HDMEC.**

***Conclusion*: This study demonstrates that EM-71 molecule may play a partial role in melanoma binding to PMA-stimulated HDMEC. (Ann Dermatol 6:(1) 9~16, 1994)**

Key Words: Cell adhesion molecule, Human dermal microvascular endothelium, Melanoma

The adhesion of tumor cells to vascular endothelial cells(EC) is an essential process in the development of hematogenous metastasis[1], and the limitation of the localization of tumor cells within selective sites may be dependent on the specificity of vascular EC in different vascular beds[2-4]. Recent evidence suggests that *in vitro* stimulation of cultured human umbilical vein endothelial cells(HUVEC) with cytokine increases the adhesion of tumor cells[5-7]. This interaction is mediated by cell adhesion molecules(CAM) on EC and tumor cells with each other[8]. We have previously demonstrated that the adherence of melanoma cells to microvascular endothelial cells derived from human dermis(HDMEC) was enhanced in a dose-and time-dependent manner by

Received April 23, 1993.
Accepted for publication July 19, 1993.
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This work was supported by the Research Grant of Yonsei University College of Medicine, 1992.

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the treatment of HDMEC with interleukin 1 alpha(IL-1α), tumor necrosis factor alpha(TNFα), and phorbol myristate acetate(PMA), and that PMA-induced increases in melanoma cell binding to HDMEC are not mediated via known CAM and may be affected through microvessel-specific novel proteins not previously described on endothelial cells[9].

In this study, we produced monoclonal antibodies(Mab) against PMA-stimulated HDMEC to identify new molecules which may play a role in HDMEC-melanoma cell binding and we characterized a new molecule on HDMEC involved in HDMEC-melanoma adhesion by Western immunoblot analysis.

MATERIALS AND METHODS

Endothelial cell culture

HDMEC were isolated from human neonatal foreskins as described previously[10]. Briefly, the foreskins were cut into small pieces, treated with 0.03% trypsin(Sigma Chemical Co., St. Louis, U.S.A.), and 1% EDTA(Sigma) and individual segments were compressed with a scalpel blade to express microvascular fragments. The microvascular segments were layered onto a 35% Percoll(Pharmacia AB, Sweden) gradient in Hank's balanced salt soultion(HBSS) and spun at 40×g for 15min at room temperature. The fraction with a density less than 1.048 g per ml was applied to gelatin (Sigma)-precoated tissue-culture dishes and cultured in endothelial basal media(Clonetics Corp., San Diego, U.S.A.) with epidermal growth factor 5ng/ml(Clonetics), hydrocortisone acetate 1μg/ml (Sigma), dibutyryl cyclic AMP 5×10^{-5} M(Sigma), and 30% human serum(Irvine Scientific, Santa Ana, U.S.A.). The resulting cell cultures were consistently pure, as assessed by morphologic and immunochemical criteria. Experiments were conducted with endothelial cells at passages[2-8].

HUVEC were isolated from fresh umbilical cords by collagenase(Worthington Biochemical Co., Freehold, U.S.A.) treatment and maintained in tissue culture as described previously[11]. Isolated HUVEC were cultured in growth media consisting of medium 199(Gibco Laboratories, Grand Island, U.S.A.), supplemented with endothelial cell growth supplement 50μg/ml(Biomedical Technologies, Stoughton, U.S.A.), glutamine 2×10^{-9} M (Sigma), penicillin 100 U/ml, streptomycin 100 μg/ml, and amphotericin B 250μg/ml(Sigma), and 20% fetal bovine serum(FBS)(Gibco). HUVEC were used between passages 2-8. In order to normalize any differences in culture conditions, HUVEC were routinely cultured in HDMEC media for 3-5 days prior to use in experiments.

Melanoma cell lines

Human melanoma cell line SK-MEL-2 was obtained from the American Type Culture Collection(ATCC, Rockville, U.S.A.). The cell line was culltured on tissue culture dishes in Dulbecco's modified Eagle's medium(DMEM)(Gibco) and supplemented with glutamine 2×10^{-9} M (Sigma), and 10% FBS(Gibco). Cells were passaged with trypsin-versene(Irvine Scientific).

Hybridoma production

Confluent HDMEC, which was stimulated with PMA(20 ng/ml×24hours) were removed with 5mM EDTA(Sigma) in PBS with 1% bovine serum albumin(BSA), washed, resuspended in PBS and used to immunize mice. Female BALB/C mice at 6 weeks of age were injected intraperitoneally with 200μl of the mixture of HDMEC suspension and the same amount of Freund's complete adjuvant. After 4 weeks and 5 weeks, the mice received a booster of HDMEC suspension in 200μl PBS. Three days after the last immunization, splenocytes were obtained by removing and mincing the spleen of an immunized animal. One week before fusion, V653 myeloma cell line were expanded in RPMI 1640, supplemented with 20% fetal calf serum, 1 ml glutamine, 1 mM pyruvate. Hybridomas were generated by fusing splenocytes with V653 fusion partner, and the resulting hybridomas were grown in HAT medium. Tissue culture supernatants were screened on unstimulated and PMA-stimulated HDMEC by an enzyme-linked immunosorbent assay(ELISA). Hybridomas showing a differential reaction pattern were further analyzed. Clones producing antibody reacting more strongly with PMA-stimulated HDMEC than unstimulated HDMEC were expanded and tested for their ability to inhibit melanoma cell binding to HDMEC monolayers. High-titer monoclonal antibody preparations were obtained from the ascites fluid of mice inoculated intraperitoneally with monoclonal antibody-producing hy-

bridoma cells. The ascites fluid was collected several times after injection of the cells. It was heat inactivated, titered, and stored.

Melanoma cell-endothelial cell adherence assay

HDMEC and HUVEC were plated in gelatin-coated 96-well flat-bottomed culture plates. They were preincubated with either cell culture media alone or with different biological response modifiers(BRM) at appropriate concentrations and times: IL-1α(gift of Dr. I. Green, NIH, Bethesda, MD), TNFα(Genetech Corporation, San Francisco, U.S.A.), and PMA(Sigma). Tumor cell lines were labeled with ^{51}Cr by incubating 300 μCi per 8×10^6 cells for 18h at 37°C. They were then removed from tissue culture plates with 5mM EDTA(Sigma) in PBS with 1% bovine serum albumin(BSA)(Sigma), washed, resuspended to 8×10^5 cells per ml in RPMI with 10% FBS and 100 μl of cell suspension was added to each well containing HDMEC or HUVEC and incubated for 30 min. After incubation at 37°C, the plates were washed and filled with HBSS with 0.5% BSA. The plates were then covered with thick filter paper and lids, sealed with parafilm, inverted, and centrifuged (600×g, 10 min) to remove nonadherent cells. Remaining adherent cells were then lysed with 1% triton-X(Sigma)and harvested supernatants were read in a gamma counter. The percentage of bound melanoma cells was calculated as follows:

$$\%\ \text{melanoma cell binding} = \frac{\text{adherent counts-background counts}}{\text{counts added per well-background counts}} \times 100$$

In some experiments, HDMEC monolayers, melanoma cells, or both were preincubated with 100 μl of Mab for 45min. One hundred microliters of 1:100 diluted ascites were used. After preincubation with antibodies, the adherence assay was performed as described above in the continuous presence of antibodies. Statistical analyses were performed using an independent Student *t* test.

Elisa

Endothelial cells were plated into 96-well flat-bottomed microtiter plates at a concentration of 4×10^4 cells per well and were preincubated with either cell culture media alone or with IL-1α, TNFα, or PMA for 1-10 μg/ml was added to each well and the plates were incubated at 37°C for 1h. After washing, 100 μl of peroxidase-conjugated goat anti-mouse IgG(Biorad, Richmond, U.S.A.), diluted 1:500, was added to each well and plates were incubated for 1 h. The plates were again washed and the binding of antibody was quantitated colorimetrically by the addition of tetramethylbenzidene(TMB, 1 mg/ml, Sigma). One ml of a 100 mg/ml stock solution of TMB in acetone was added to 100 ml of distilled water. Ten microliters of 30% H_2O_2 was added immediately prior to use. The chromogenic reaction was stopped with 25 μl 8N H_2SO_4 and the plates were read spectrophotometrically at 450 nm on ELISA reader.

Flow cytomertic analysis

HUVEC or HDMEC, either untreated or treated with cytokines, were removed from tissue culture plates by incubation with 2 mM EDTA(Sigma) and 1% BSA(Sigma). Cells were then washed twice and then aliquoted into tubes for antibody staining. Unconjugated Mab was incubated for 30 min on ice with a 1 : 20 dilution of FITC conjugated sheep anti-mouse IgG(Sigma). The cells were washed and fluorescence analyzed on a FACStar flow cytometer(Becton Dickinson, Mountainview, U.S.A.) Non-viable cells were gated by propidium iodide staining.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS-PAGE) and Immunoblotting

HDMEC were removed from tissued culture flasks with EDTA and BSA, washed and solubilized by incubation for 30 min on ice with buffer containing 1mM PMSF, 1 mM EDTA, and 1% Triton-X(Sigma). The mixture was centrifuged at 4°C at 10,000×g for 30 min and the supernatant was mixed with equal amount of SDS-PAGE sample buffer proteins in the supernatant were separated by SDS-PAGE, and then electrophoretically transferred to nitrocellulose. Unbound sites were blocked by preincubation with a blocking buffer containing 5% nonfat dried milk. Blots were then probed by overnight incubation with Mab EM-71. After washing, they were incubated with peroxidase conjugated goat anti-mouse IgG(Big-Rad), followed by visualization of the reacting proteins

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with diaminobenzidine(Sigma).

RESULTS

Isolation of a Mab EM-71 to an endothelial cell associated antigen upregulated by PMA

Mab were generated against PMA-stimulated HDMEC. Hybridoma supernatants were screened to detect clones producing antibodies reacting more strongly with PMA-stimulated HDMEC than with untreated HDMEC using an ELISA. One Mab EM-71 was selected because it showed an increased reaction with PMA-stimulated HDMEC compared with untreated cells. Figure 1 shows binding of Mab EM-71 to resting and PMA-stimulated HDMEC when examined by flow cytometric analysis. PMA caused a twofold increase in the expression of EM-71 molecule on HDMEC after 24 h(Fig. 2).

Expression and modulation of EM-71 molecule on endothelial cells

To examine whether the expression of EM-71 molecule could be regulated by the proinflammatory cytokines, we exposed HDMEC and HUVEC to IL-1α, TNFα, and PMA. Incubation of HDMEC with PMA(20 ng/ml×24h) led to a dose-dependent increase in the cell surface expression of EM-71 molecule on HDMEC. Doses of PMA as low as 5ng/ml induced increases in expression of EM-71 molecule on HDMEC when examined after 24 h of stimulation(Fig.3). In contrast, HUVEC showed constitutively higher expression than HDMEC, and PMA did not affect the expression of EM-71 molecule on HUVEC. Further more, incubation of either HDMEC or HUVEC with IL-1α(100U/ml×24h), TNFα(100U/ml×24h) had no effect on expression of EM-71 molecule on both cells(Fig. 4).

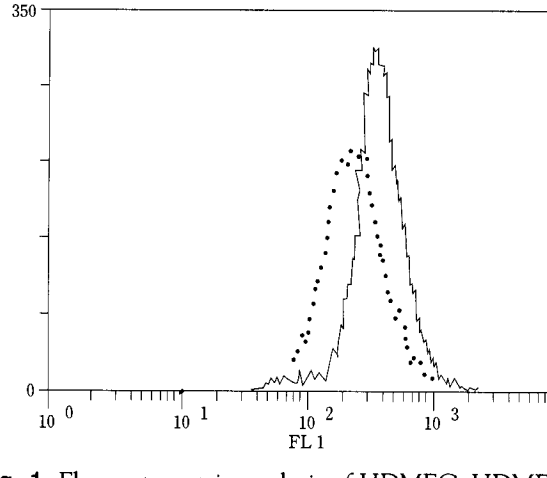

Fig. 1. Flow cytometric analysis of HDMEC. HDMEC were incubated with Mab EM-71 and evaluated for EM-71 molecule expression on unstimulated HDMEC (dotted curve) and PMA-stimulated HDMEC(solid curve) by flow cytometric analysis.

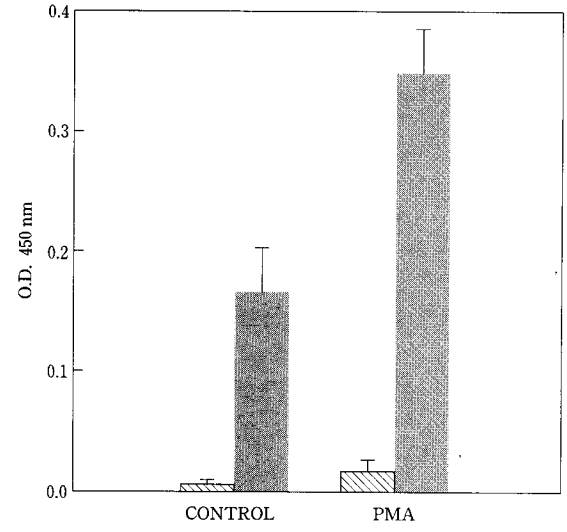

Fig. 2. Effect of PMA on expression of EM-71 molecule on HDMEC. HDMEC were stimulated with PMA(20 ng/ml) for 24 h and then assayed for cellsurface EM-71 molecule expression by ELISA. ▨ : irrelevant Mab, ▩ :EM-71 Mab

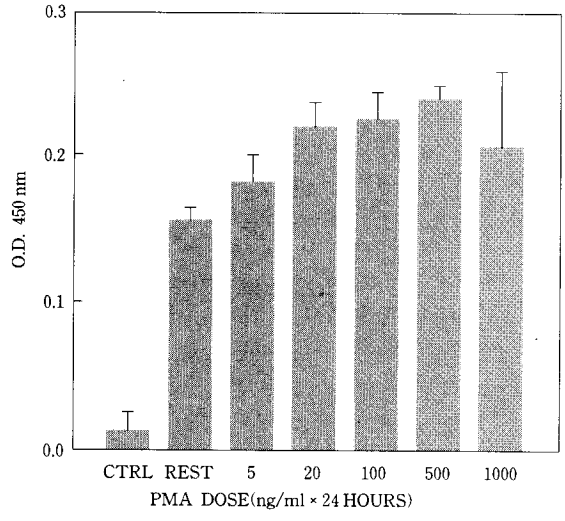

Fig. 3. Dose response of PMA-induced increases in expression of EM-71 molecule on HDMEC. HDMEC were incubated with doses of PMA from 5-1000 ng/ml for 24 h.

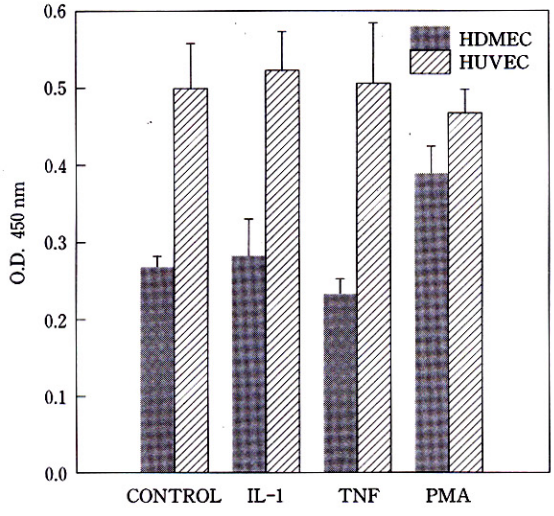

Fig. 4. Regulation of expression of EM-71 molecule on HDMEC and HUVEC by IL-1α, TNFα and PMA. HDMEC and HUVEC were stimulated with IL-1α (100 U/ml), TNFα(100 U/ml), or PMA(20 ng/ml) for 24 h and assayed for expression of EM-71 molecule by ELISA.

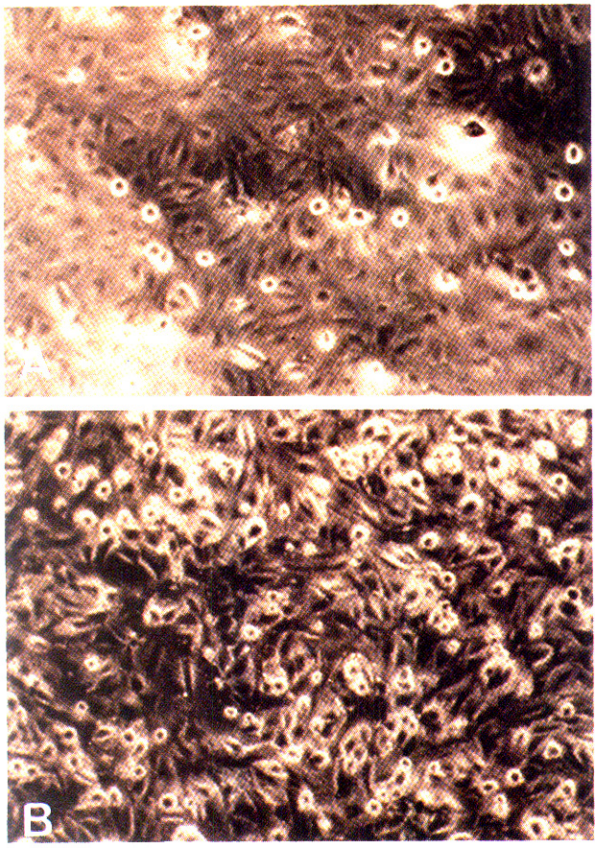

Fig. 5. Phase-contrast photomicrographs of the adhesion of SK-MEL-2 melanoma cells to unstimulated HDMEC monolayer(A), and PMA-stimulated HDMEC monolayer(B).

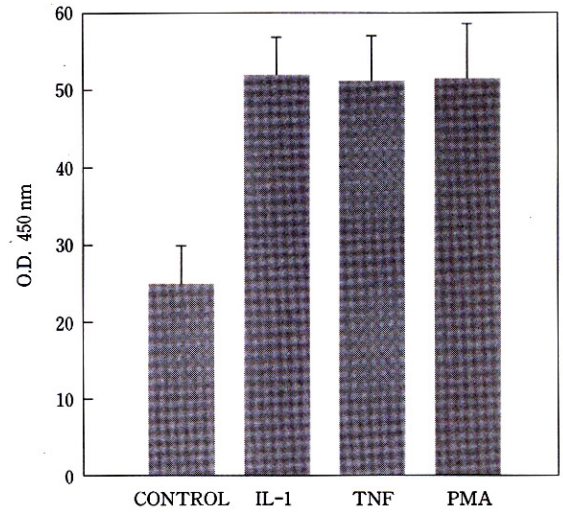

Fig. 6. Cytokines induce increase in melanoma cell binding to HDMEC. HDMEC were incubated with IL-1α(100 U/ml) or TNFα(100 U/ml) for 24 h and then coincubated with radiolabeled SK-MEL-2 melanoma cells as described in Materials and Methods.

Blockade of melanoma cell-HDMEC adhesion with mab EM-71

SK-MEL-2 melanoma cells bound more avidly to PMA-induced HDMEC than resting HDMEC when observed under phase contrast microscope (Fig. 5). Stimulaltion of HDMEC with IL-1α(100 U/ml), TNFα(100 U/ml) or PMA(20 ng/ml) for 24h resulted in a significant increase in SK-MEL-2 binding. Binding of SK-MEL-2 melanoma cells increased from baseline of 24.5% to 51.8% after IL-1α treatment, 50.7% after TNFα treatment, and 51.5% after PMA treatment(Fig.6.) In order to elucidate whether EM-71 molecule mediate melanoma cell-HDMEC adhesion, we examined the effect of Mab EM-71 on HDMEC-melanoma cell adherence. Mab EM-71 did not block IL-1α-induced increases(Fig. 7) or TNFα-induced increases (Fig. 8) of melanoma cell binding to HDMEC. In contrast, increased binding of SK-MEL-2 melanoma cells to PMA-induced HDMEC was interestingly inhibited by Mab EM-71 by 58.7%(Fig. 9).

Characterization of EM-71 molecule on HDMEC by immunoblotting

To further characterize the protein identified by Mab EM-71 on HDMEC, we examined the lysates of HDMEC by Western immunoblot. Mab EM-71 recognized 200 kDa protein from the lysates of HDMEC(Fig. 10).

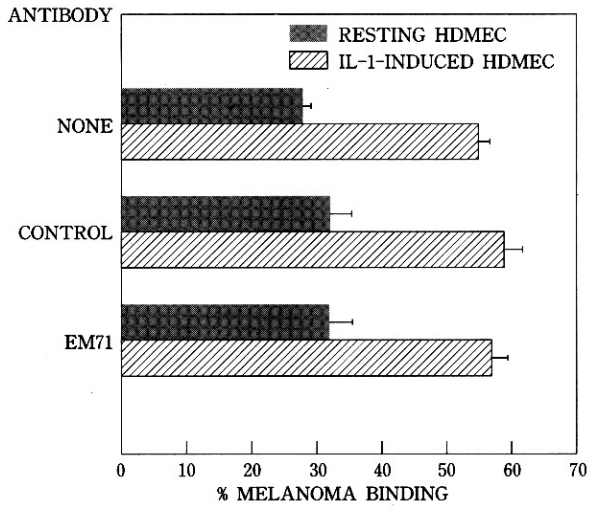

Fig. 7. Mab EM-71 did not significantly inhibit IL-1α (100U/ml×24 h) induced increased binding of melanoma cells to HDMEC. HDMEC monolayers were pretreated for 30min with Mab EM-71 and melanoma adherence assay were conducted in the continuous presence of antibody.

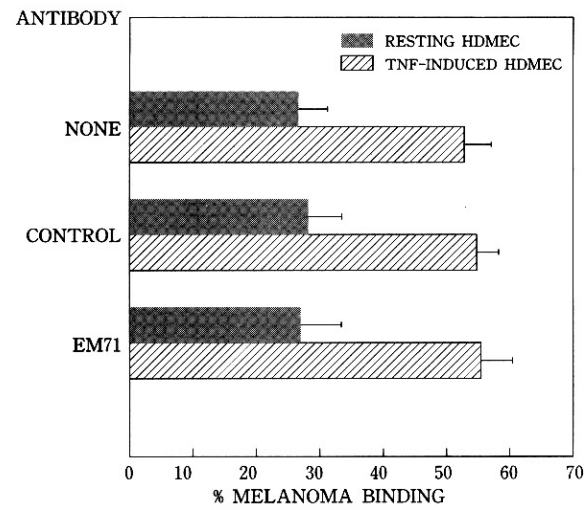

Fig. 8. Effect of Mab EM-71 on melanoma cell binding to TNFα-induced HDMEC. Mab EM-71 did not significantly inhibit TNFα(100 U/ml×24 h)-induced increased binding of melanoma cells to HDMEC.

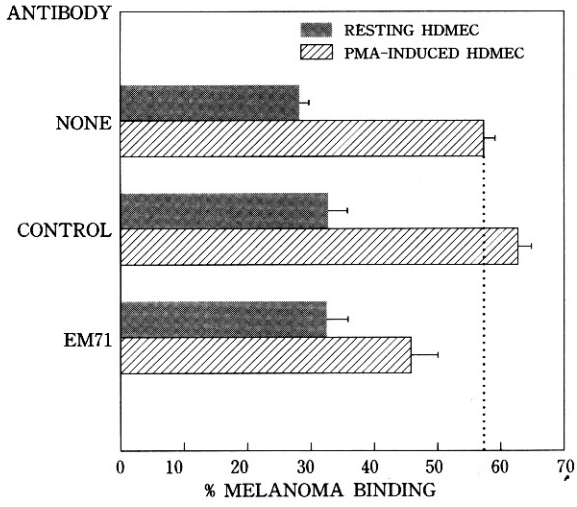

Fig. 9. Effect of Mab EM-71 on melanoma cell binding to PMA-induced HDMEC. Mab EM-71 significantly inhibited PMA(20 ng/ml×24 h)-induced increased binding of melanoma cells to HDMEC.

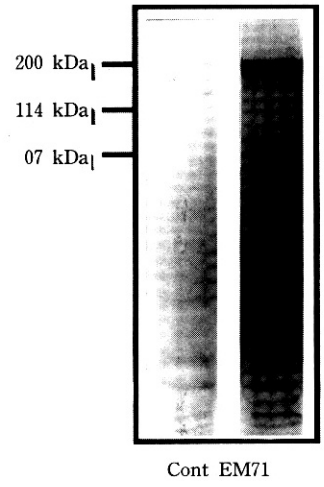

Fig. 10. Western immunoblot of HDMEC for EM-71 molecule. EM-71 demonstrate a 200 kDa protein identified by Mab EM-71.

DISCUSSION

Tumor cells must first adhere to and traverse across microvascular endothelial cells in order to invade distant tissues. Various tumor cells have been shown to adhere directly to endothelial monolayers *in vitro*[12], and highly metastatic tumor cell strains have a higher binding ability for vascular endothelial cells than nonmalignant ones[2,12]. It has been suggested that the adherence of tumor cells to endothelial cells may be different depending on whether endothelial cells were derived from different organs and different sized vessels[3,13,14]. Recent studies have also demonstrated that activation of large vessel vascular endothelial cells by cytokines can alter the adhesion of human

melanoma and carcinoma cells *in vitro* via endothelial leukocyte adhesion molecule 1(ELAM-1), inducible cell adhesion molecule 110(INCAM-110), and intercellular cell adhesion molecule 1(I-CAM-1)[5-7,15].

Previous studies reported that specific tumors clearly localize preferentially to the microvasculature[3,14], and this specificity may be related to unique surface characteristics and phenotypes of microvascular endothelial cells. We have previously demonstrated that HDMEC are phenotypically distinct from HUVEC[16-19] and therefore we have examined tumor cell adherence to HDMEC using melanoma cells as a model[9]. Our data have demonstrated that cytokine or PMA stimulation of HDMEC results in increases in melanoma cell binding *in vitro*. Our previous study has suggested that the binding of melanoma cells to cultured microvascular endothelial cells can be modulated *in vitro* and this binding appears to be mediated via novel, microvascular endothelial cell specific CAM.

In this study, we describe a new molecule, recognized by the EM-71 Mab, of which the expression on HDMEC is upregulated by protein kinase C(PKC) agonist. This molecule is expressed constitutively on both resting HDMEC and HUVEC, although its expression on resting HUVEC is higher than on HDMEC. However, this molecule is upregulated consistently two or three fold on HDMEC only by PMA but not by IL-1α or TNFα treatment. This data provides further evidence of distinct phenotypic differences between HDMEC and large vessel endothelial cells. Previous studies have demonstrated that PKC agonists treatment of EC results in the modulation of some of endothelial CAM. These include ICAM-1, CD36, and vitronectin receptor(VnR) integrin[17-19]. ICAM -1 and CD36 are *in vitro* downregulated by PMA treatment, in contrast to the EM-71 molecule. The expression of VnR integrin is upregulated by PMA treatment, not by IL-1α as same as EM-71 molecule, but molecular weight of VnR is different from EM-71 molecule and is a dimer of 150/95 kDa. In addition, Mab against VnR did not inhibit the melanoma binding to HDMEC in the previous study(data shown).

We examined melanoma binding to HDMEC, after 24 h of biological response modifiers, because melanoma cell adherence was maximal after 24h of BRM stimulation in the previous study. The binding of SK-MEL-2 melanoma cells to HDMEC was increased about twofold after treatment of HDMEC with either IL-1α, TNFα, or PMA, same as the previous observation. PMA treatment of HDMEC also results in an increase in their melanoma cell binding, which is coincident with a marked increase in EM-71 molecule expression. The increases in melanoma-cell HDMEC binding after treatment of IL-1α or TNFα were not affected by Mab EM-71. In contrast, increased binding induced by PMA was effectively(10-60%) blocked by Mab EM-71. This suggests that these BRM may induce changes in adherence via different mechanisms and EM-71 molecule may play a partial role in melanoma cell-HDMEC binding stimulated only by PMA.

Characterization of the protein identified by Mab EM-71 on HDMEC demonstrated an 200 kDa protein on immunoblot. The molecular weight of EM-71 molecule is similar to that of the laminin receptor which is an integrin to laminin. But laminin receptor integrin of HDMEC is not modulated by PMA(KH Lee: unpublished observation). Molecular weight, expression and regulation patterns *in vitro* on HDMEC after incubation with biological response modifiers can exclude most of the known endothelial cell adhesion molecules.

In summary, we have produced Mab EM-71, which recognizes 200kDa protein on HDMEC. The expression of this molecule on HDMEC is upregulated by PMA, but not by IL-1α or TNFα. Furthermore, we have also shown that this Mab is able to assess EM-71 molecule expression *in vivo* immunohistochemically. In addition, crosslinking experiment will have to be performed to determine the melanoma cell ligand to which EM-71 molecule on HDMEC bind. Mab EM-71 may contribute to providing clues as to the mechanism of hematogenous metastasis of tumor cells. Also, identification of this epitope may lead to the ability to better evaluate the biologic behavior of malignant melanoma and eventually to help to prevent the development of metastasis.

ACKNOWLEDGEMENTS

We wish to thank Mr. Nam Soo Jang for his technical assistance.

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