Inhibitors of 5-lipoxygenase inhibit expression of intercellular adhesion molecule-1 in human melanoma cells[1]

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ABSTRACT

AIM: To study the effect of 5-lipoxygenase inhibitors on the expression of intercellular adhesion molecule-1 (ICAM-1) in melanoma cells. **METHODS:** ICAM-1 protein of human melanoma cell a375 was detected by enzyme-linked immunosorbent, flow cytometry and Western blot analysis. Level of ICAM-1 mRNA in a375 was evaluated by Northern blot analysis. Adhesion of a375 to endothelial cell EC304 was analyzed by isotopic tracing. **RESULTS:** 5-Lipoxygenase inhibitors nordihydroguaiaretic acid, AA861 and MK886, could suppress the expression of ICAM-1 protein as well as of its mRNA in a375 cells and reduce the adhesion of a375 to EC304. **CONCLUSION:** 5-Lipoxygenase inhibitors can inhibit the expression of ICAM-1 in human melanoma cells and may be valuable for treatment of melanoma metastasis.

INTRODUCTION

Although malignant melanoma accounts for less than 5 % of all cancers, it is among the most lethal because of its extremely high metastatic propensity[1-3]. Metastasis develops after melanoma cancer cells complete a complex sequence of events which include dissociation from the primary cancer, invasion and survival in the circulation and extravasation and proliferation at a second site[4]. At each step during the cascade, melanoma cancer cells interact with various host cells. These interactions influence the efficiency of the metastatic process and are mediated by a number of adhesion molecules[5,6].

ICAM-1, a member of the immunoglobulin superfamily of adhesion molecules, has been proposed to be associated with the development of metastatic behavior in melanoma[7]. ICAM-1 is expressed at high levels on advanced primary melanoma (predominantly vertical growth phase), suggesting an important role for ICAM-1 in melanoma progression and metastasis[8-11].

Most epidemiological investigations support an association between dietary fats and melanoma[12]. The level of dietary fat intake has been shown to influence the growth and metastatic behavior of melanoma[13,14]. Arachidonic acid, an ω-6 polyunsaturated essential fatty acid, is the mainly ingredients of dietary fatty acid and released from cellular membrane phospholipids through phospholipase A_2[15]. Free arachidonic acid is metabolized in mammalian cells by a free radical reaction to cyclic endoperoxides via the cyclooxygenase system and to cyclic hydroperoxides via the lipoxygenase system[16]. Arachidonic acid and some specific metabolites were found to affect melanoma metastasis[17,18].

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Lipoxygenase inhibitors has been utilized in a number of studies for their anti-metastatic effects[19,20]. Treatment of melanoma cells with antisense 5-lipoxygenase oligonucleotides inhibits metastasis by 39 % in Lipofectin-treated cells[21]. This provides a rationale for investigating possible inhibition of melanoma metastasis among patients as a result of 5-lipoxygenase inhibitors used. Previous reports predominantly focused on the effects of these inhibitors on integrins expression or matrix metalloproteinase production. There are few studies dealing with the effect of 5-lipoxygenase inhibitors on ICAM-1 expression of melanoma cancer.

As one approach to seek an understanding of the effect of the various 5-lipoxygenase inhibitors on metastasis of melanoma cancer, we studied their effects on the expression of ICAM-1 in human melanoma cancer cell a375. Since formation of heterotypic emboli significantly increase melanoma cancer matastasis, we also tested the effects of such inhibitors on melanoma cell-endothelial cell adhesion.

MATERIALS AND METHODS

Cells and cell culture Human melanoma cancer cell a375 and human umbilical vein endothelial cell EC304 were obtained from the Cell Bank in Institute of Cell Biology (Shanghai, China) and routinely grown in Iscove's modified Dulbecco's medium (GIBCO, Grand Island, NY) with 10 % heat-inactivated fetal bovine serum, 100 mg penicillin/L and 100 mg streptomycin/L, in a humidified atmosphere of 5 % CO_2/95 % air at 37 ºC.

Chemicals and reagents Indomethacin, nordihydroguaiaretic acid (NDGA) and AA861 were purchased from Sigma Chemical company (St Louis, MO). MK886 were purchased from Merck company (Darmstadt, Germany). Monoclonal antibody 84H10 to ICAM-1 and its cDNA probe are the kind gifts from Dr C Frank BENNETT (ISIS pharmaceuticals, Carlsbad, California 92008).

Enzyme-linked immunosorbent assay Melanoma cells were plated on 96-well tissue culture plate and incubated at 37 ºC overnight. The cells were treated with the inhibitors for 24 h. Control cells received 0.05 % ethanol only. Following treatment, a375 cells were washed once with PBS. Cells were then fixed with 1 % formaldehyde/PBS for 15 min. Fixed cells were washed with PBS for 3 times and incubated with 2 % BSA solution for 1 h at 37 ºC. This solution was aspirated and replaced with anti-ICAM-1 monoclonal antibody 84H10 dilution 1:1000 incubation for 1 h at 37 ºC. Solution was aspirated and the fixed cells were washed three times with PBS. A 1:1000 dilution of anti-mouse IgG/horseradish peroxidase conjugate was added to the fixed cells and incubated for 1 h at 37 ºC. Conjugated solution was aspirated and cells were washed three times. OPD solution was then added into the cells and incubated for 30 min at 37 ºC. An equal volume of 1 mol/L sulfuric acid was added to stop the reaction and absorbance at 492 nm was measured.

Flow cytometry a375 cells were trypsinized and resuspended in PBS containing 1 % BSA. A total of 1×10^5 cells were incubated at 4 ºC with anti-ICAM-1 antibody for 1 h, washed 3 times with PBS and stained with fluorescein-conjugated goat anti-mouse IgG. Stained cells were fixed with 1 % formaldehyde and analyzed on a flow cytometer.

Western blot analysis Cytosolic fractions were prepared from normal or inhibitor-treated a375 cells. Aliquots were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 8 % acrylamide gels before electrophoresis transfer to nitrocellulose membrane and membranes were incubated with 3 % BSA for 1 h at room temperature. Proteins were probed with a 1:1000 final dilution of mouse anti-human monoclonal antibody 84H10 for 2 h at room temperature. Membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody and detected with enhanced chemiluminescence method.

Northern blot analysis Total cellular RNA (10 μg) isolated from normal or inhibitor-treated a375 cells was separated by gel electrophoresis in 2 % agarose formadehyde gel and transferred to nitrocellulose membrane. Membranes were hybridized with a ICAM-1 cDNA probe fragment labeled by [α-^{32}P]dCTP using Random Prime Labeling Kit (Takara). Membranes were then exposed to phosphor screen and developed with Cyclone scanner.

Adhesion assay Adhesion studies were done as previously described[21]. Briefly, a375 cells were labeled overnight with [^{125}I]UDR and then were incubated with inhibitor prior to adhesion assays. To measure adhesion, labeled cells were detached from culture plates and suspended in adhesion medium. About 1×10^5 cells were added to 24-well plates containing a confluent monolayer of EC304 cells. After incubation, the medium was aspirated and the well was washed with PBS to remove nonadherent cells. The remaining cells were

lysed with bleach and an aliquot was taken for liquid scintillation counting to determine the percentage of attached cells.

Statistical analysis The data are presented as mean±SD. ANOVA procedures were used to assess the significance of differences among experimental groups.

RESULTS

Inhibition of ICAM-1 expression of a375 cells by 5-lipoxygenase inhibitors There existed basal expression of ICAM-1 on the surface of human melanoma cancer a375. Treatment of a375 cells with nonspecific 5-lipoxygenase inhibitor NDGA, specific 5-lipoxygenase inhibitor AA861 and 5-lipoxygenas activated-protein inhibitor MK886, ranging from 5 to 20 μmol/L, resulted in a concentration-dependent inhibition of ICAM-1 expression. However, cyclooxygenase inhibitor indomethacin did not affect cell surface ICAM-1 expression.

The expression of ICAM-1 on the surface of melanoma cancer cells were further demonstrated by flow cytometry. A375 cells were treated with 20 μmol/L of indomethacin, NDGA, AA861 or MK886 and stained with ICAM-1 antibody. Consistent with data obtained by ELISA analysis, basal a375 cells expressed ICAM-1. Treatment of a375 cell with lipoxygenase inhibitors reduced cell surface ICAM-1 expression by nearly 44.2 %, 42.7 %, or 39.6 %, while cyclooxygenase inhibitor did not change the level of ICAM-1 expressed on the cell surface.

To investigate the effects of 5-lipoxygenase inhibitors on ICAM-1 protein expression in a375 cells, Western blot were performed on a375 cells extracts using an ICAM-1 specific antibody. As shown in Fig 2, ICAM-1 expression is reduced significantly after treatment with NDGA, AA861, or MK886. At 20 μmol/L these inhibitors suppressed the ICAM-1 expression by 44.1 %, 74.7 %, and 64.8 %, respectively. Indomethacin did not influence ICAM-1 protein expression at the same concentration.

The effects of inhibitors on ICAM-1 mRNA were analyzed by Northern blot. A375 cells expressed relatively high basal levels of ICAM-1 mRNA. 5-Lipoxygenase inhibitors treatment reduced expression of ICAM-1 mRNA in a375 cells. However, treatment of a375 cells with indomethacin did not change ICAM-1 mRNA expression, which the level was similar to basal level. None of inhibitors had a significant effect on expression of the constitutive gene product β-actin.

5-Lipoxygenase inhibitors suppressed melanoma cell adhesion to endothelial cell Because ICAM-1 is involved in numerous cell-cell interactions, we postulated that reduced ICAM-1 expression on melanoma cells would inhibit metastasis by influencing adhesion of cancer cells to endothelium. 5-Lipoxygenase inhibitors-treated a375 cell did exhibit depressed adherence to human umbilical vein endothelial cells.

A375 cells were incubated with 20 μmol/L of indomethacin, NDGA, AA861, MK886 or 0.05 % ethanol prior to the adhesion assay. Nonherent cells were removed by washing the wells and the percentage of melanoma cells adherent to the endothelium monolayer was determined by liquid scintillation counting after lysing the cells with 2 % Triton X-100.

Treatment of a375 cells with 5-lipoxygenase inhibitors all resulted in significant decrease in adhesion to endothelial cell monolayer. However, indomethacin did not reduce basal adhesion of a375 cell to endothelium.

DISCUSSION

Despite continuous improvements in early diagnosis, surgical techniques, local and systemic adjuvant

Tab 1. ICAM-1 expression in melanoma cancer cell a375 (results expressed as optical density). *n*=5. Mean±SD. $^{b}P<0.05$ *vs* control group.

Group	0	5 μmol/L	10 μmol/L	20 μmol/L
Control	0.687±0.084	-	-	-
Indomethacin	-	0.672±0.102	0.651±0.098	0.645±0.111
NDGA	-	0.604±0.109	0.534±0.089	0.359±0.087^{b}
AA861	-	0.591±0.092	0.478±0.154	0.342±0.086^{b}
MK886	-	0.634±0.114	0.588±0.101	0.412±0.095^{b}

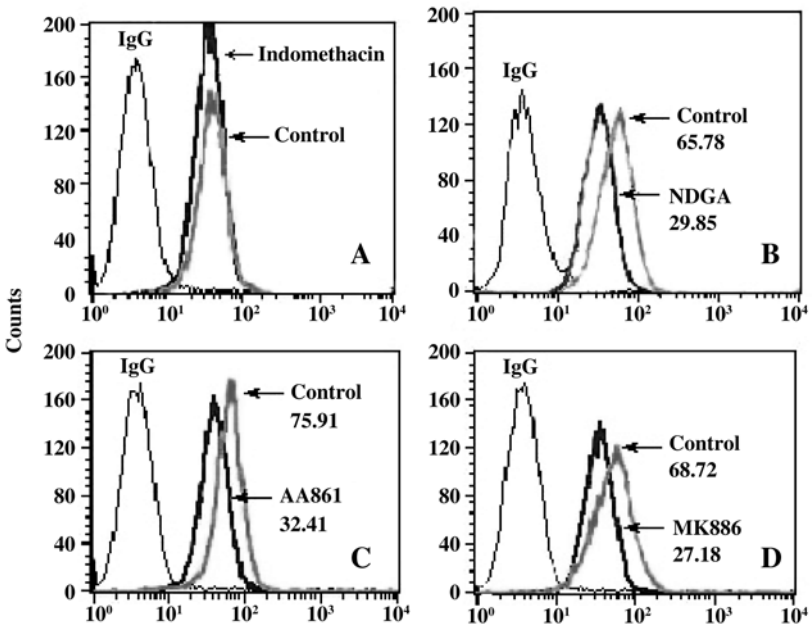

Fig 1. Flow cytometry analysis of ICAM-1 expression in melanoma cancer cell a375 in the basal state and after treated by 20 μmol/L of indomethacin (A), NDGA (B), AA861 (C), and MK886 (D). Suspended cells were labeled by indirect immunofluorescence. Ordinates show the number of cells by channel and abscissas represent the relative fluorescence intensity ion arbitrary units (lg scale). Control mouse IgG was used as a control.

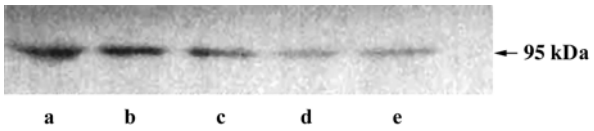

Fig 2. Western blot analysis of expression of ICAM-1 protein in a375 cells. a) 0.05 % ethanol; b) indomethacin; c) NDGA; d) AA861; e) MK886.

Tab 2. Adhesion of melanoma cancer cell a375 to endothelial cell EC304. *n*=5. Mean±SD. $^{b}P<0.05$ *vs* control group.

Group	cpm
Control	1854±319
Indomethacin	1798±276
NDGA	1069±298[b]
AA861	896±239[b]
MK886	775±221[b]

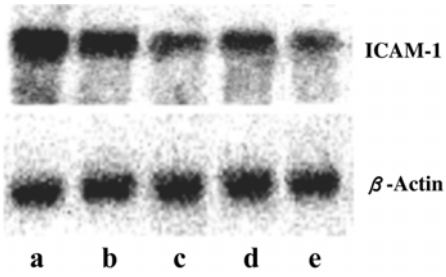

Fig 3. Northern blot of ICAM-1 mRNA in melanoma a375 cell. Cells were treated with a) the solvent (0.05 % ethanol), b) indomethacin (20 μmol/L), c) NDGA (20 μmol/L), d) AA861 (20 μmol/L) or e) MK886 (20 μmol/L). Total RNA was isolated from a375 cells, size fractionated, and transferred to a nitrocellulose membrane. The blot was hybridized with ICAM-1 or β-actin cDNA probe labeled with [α-^{32}P]dCTP by random prime labeling. ICAM-1 transcript were at 1.0 kb.

therapies, the metastasis of cancer cells is a major cause of morbidity in individuals with cancer[22].

Cancer metastasis process are that the cells detach from the primary site, enter the vasculature, survive sheer forces and immune cell killing during transit, attach to and extravasate through the endothelium and basement membrane, and proliferate at a second site. Endothelium provide a structure support for epithelial tissues or blood vessels, form a molecular filter preventing the passage of protein and also act as a physical barrier to the passage of the cells[23].

A crucial step in metastasis process is the attachment of cancer cells to the endothelium[23]. Although potential cellular interactions are numerous, adhesion

of cancer cell to host cells appears to increase metastatic potential[24,25]. Direct adherence to vessel walls or formation of multicellular emboli are more readily trapped in microvessel and enhance metastatic efficiency[21,26]. Identifying molecules that mediate the cell-cell interactions involved in metastasis is essential if therapies blocking specific steps in the metastatic process are to be developed.

A number of evidences showed that there existed a closely correlation between melanoma progression and ICAM-1 expression[5,8,27]. Higher ICAM-1 expression in melanoma metastasis compared to nevi suggests that this molecule may be responsible for metastasis formation. Increased expression of ICAM-1 may facilitate metastasis by affecting formation of heterotypic emboli which may be more efficiently trapped in microvessel and could also be involved in cancer cell-endothelial adhesion[21,26].

When ICAM-1 expression on cancer cells was down-regulated by treatment with 5-lipoxygenase inhibitor, inhibitors-treated a375 cells that adhered to monolayers of endothelial cells significantly decreased more than untreated a375 cells. Since emboli form metastasis more efficiently than single cells, we proposed that decreased ICAM-1 expression could mediate metastasis formation.

5-Lipoxygenase is a key enzyme in the metabolism of arachidonic acid. It catalyzes both the oxygenation of arachidonic acid to (5*S*)-hydroperoxy-6,8,11, 14-eicosatetraenoic acid and its subsequent conversion to leukotrienes. The synthesis of leukotrienes also required the membrane bound 5-lipoxygenase activated-protein, which activates 5-lipoxygenase by specific binding arachidonic acid and by transferring this substrate to the enzyme[28]. Leukotrinese are potent mediators of numerous biological process, including chemotaxis, vascular permeability and smooth muscle contraction. Inhibitors of arachidonic acid metabolism can directly or indirectly induce biochemical and morphological changes in culture cells.

Our work for the first time demonstrated that 5-lipoxygenase inhibitors could suppress the expression of ICAM-1 protein as well as its mRNA in human melanoma cancer cell a375. In addition, inhibition of 5-lipoxygenase resulted in the reduction of melanoma cell-endothelial cell adhesion. Furthermore, ICAM-1 expression of melanoma cancer could selectively be inhibited *in vitro* by 5-lipoxygenase inhibitors NDGA, AA861, and MK886. Although the specificity of the 5-lipoxygenase inhibitors is not complete, the similar results with the three different types of 5-lipoxygenase inhibitors are consistent with the lipoxygenase pathway playing a significantly role in melanoma metastasis. Since the adhesion of melanoma cell to endothelial cell could be inhibited by 5-lipoxygenase inhibitor, further consideration of this approach in advanced stage and early cancer is merited. Furthermore, the effective drug concentrations needed to inhibit melanoma cancer metastasis with the compounds used in our study are achievable in human beings[29].

A number of antagonists for this pathway are being used in specific clinical settings. Inhibitor of 5-lipoxygenase metabolism have shown promise in the treatment of asthma and shock with limited side effects in preclinical and clinical trials[30,31]. In contrast to the usual toxic chemotherapeutic agents, the modest clinical toxicity of the downstream arachidonic acid inhibitors make them appropriate for consideration not only for metastasis treatment but also for chemopreventive applications.

This work establishes an association between 5-lipoxygenase inhibitors and their inhibitory effect on adhesion molecule expression, a crucial step in metastasis progression. That 5-lipoxygenase inhibitors could inhibit the expression of ICAM-1 of a375 cell as well as melanoma cell-endothelial cell adhesion, although the mechanism of action remains to be clarified, suggest that these inhibitors might be potential implication in regulating of melanoma metastasis.

A more detailed understanding of the mechanism by which these drugs affect the expression of adhesion molecule as well as test to determine whether melanoma cancer cell metastasis *in vivo* can be inhibited if animals received such inhibitors pre- and post–cancer formation, may shed more light on the potential use of 5-lipoxygenase inhibitors as anti-metastasis therapeutics for melanoma cancer.

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