CD44 StimulationInduces Integrin-mediated Adhesion of Colon Cancer Cell Lines to Endothelial Cells by Up-Regulation of Integrins and c-Met and Activation of Integrins

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ABSTRACT

For cancer metastasis, tumor cells present in the circulation must first adhere to the endothelium. Integrins lymphocyte function-associated antigen (LFA) 1 and very late antigen 4 play a central role in leukocyte adhesion to the endothelium and subsequent migration into tissues. The majority of tumor cells derived from solid cancers including colorectal cancer do not express suitable adhesion receptors, LFA-1 and very late antigen 4. We investigated the mechanisms of adhesion and transendothelial migration of cancer cells using colorectal carcinoma cell lines. Our results showed the following novel features of CD44 on the cells: (a) colon cancer cells express high levels of CD44; (b) stimulation of cancer cells by CD44 cross-linking or fragmented hyaluronan markedly induces the expression of LFA-1, some of which reveal an activation epitope on the cells; (c) CD44 cross-linking induces F-actin polymerization in the cell cortex; (d) fragmented hyaluronan induces up-regulation of the activation epitope of LFA-1, which is mediated through protein kinase C; (e) stimulation of CD44 augments the LFA-1-mediated adhesion of cancer cells to endothelial cells and intercellular adhesion molecule 1-transfected cells and facilitates transendothelial migration; (f) stimulation of CD44 also induces expression of the hepatocyte growth factor (HGF) receptor c-Met on cancer cells; and (g) HGF further amplifies the LFA-1-mediated adhesion of cancer cells prestimulated by CD44-derived signaling. Our results indicated that stimulation by CD44 induces “outside-in signaling,” which consists of a direct pathway via CD44 and an alternate pathway through the induction of c-Met expression via HGF. Such stimuli augment the expression and trigger the function of integrins via “inside-out signaling” in colon cancer cells, which leads to amplification of integrin-mediated adhesion to the vessel wall and subsequent transendothelial migration.

INTRODUCTION

The role of adhesion molecules in the interaction between tumor cells and host cells has been the focus of intense investigation in recent years to understand the process of tumor metastasis. In the metastatic cascade involving coordinated cellular responses, tumor cell spread through the blood is the most important step in metastasis. In this process, tumor cells must make contact and adhere to endothelial cells lining the vessel wall within the target organ using certain adhesion molecules and then transmigrate to the tissue (1–4). With regard to the extravasation of tumor cells, several studies have identified the mechanisms responsible for leukocyte recruitment into tissues (5–8). The latter process is regulated by a sequence of interactions between leukocytes and endothelial cells. In the initial step of this process, selectin mediates a transient interaction termed tethering, which brings the cell into contact with the vessel wall, allowing it to sample the endothelium for the presence of activating factors that can trigger a strong, integrin-mediated adhesion. This results in stabilizing the cell on the vessel wall. We and others (9–12) have reported that this mechanism also appears to be relevant to the tissue infiltration of malignant cells in leukemia, lymphoma, and myeloma. For example, T-cell leukemia cells overexpress certain integrins, including LFA-1 and VLA-4, which are necessary for tight adhesion to endothelial ligands. Such molecules involved in leukocyte/leukemic cell adhesion to the endothelium have been implicated in tumor cell adhesion to the endothelium. Recent studies have indicated that CD44 and its variant form play a pivotal role in the hematogenic spread of tumor cells (13, 14). However, the majority of tumor cells derived from solid carcinomas in the lung, breast, prostate, and colon do not express suitable adhesion receptors, LFA-1 and VLA-4, to ICAM-1 and VCAM-1 present on endothelial cells, respectively. These features suggest that such tumor cells cannot firmly adhere to endothelial cells through integrins.

The adhesive capacity of integrins expressed on peripheral leukocytes/leukemic cells is tightly regulated (15). Although integrins expressed on resting cells do not mediate firm adhesion to endothelial ligands, stimulation of these cells results in a rapid increase in integrin function. Thus, activation of integrin is essential for integrin-mediated adhesion in which a signal transduced to the circulating cells converts the functionally inactive integrin to an active adhesive configuration (8, 16–18). In this regard, we and others have previously reported that chemokines produced in large amounts at the site of inflammation activate integrins on leukocytes and result in their accumulation in the tissues (9, 11, 19–22). We also proposed that chemokines produced by leukemic cells are involved in triggering integrin LFA-1 through cytoskeletal rearrangement in an autocrine manner (11). However, the exact mechanisms that enhance the adherence of circulating tumor cells to the endothelium and their subsequent infiltration into the tissues or those regulating integrin adhesiveness to the endothelium are not very clear at present.

CD44, a widely expressed cell surface glycoprotein, serves as an adhesion molecule in cell-substrate and cell-cell interactions, including lymphocyte homing, hemopoiesis, and cell migration (23–25). Several isoforms and variants of CD44, particularly those generated by splicing, have been identified and reported to be causally involved in the formation of tumor metastases (14, 24, 26). However, the functional relevance of CD44 on tumor cells to metastasis remains to be explored.

These insights have prompted us to investigate the stimulatory molecules that induce integrins, especially LFA-1, in the present study. Based on a survey of cross-linking of multiple stimulations

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3 The abbreviations used are: LFA, lymphocyte function-associated antigen; HGF, hepatocyte growth factor; HUVEC, human umbilical vein-derived endothelial cell; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; VLA, very late antigen; mAb, monoclonal antibody; HSA, human serum albumin; Ab, antibody; IL, interleukin; rHGF, recombinant HGF.
4427
using colorectal carcinoma cell lines, we found that CD44 was unique in its induction of LFA-1, especially the activation epitope of LFA-1, on the cells. We propose that CD44 stimulation can induce integrin expression as well as integrin activation on tumor cells and can augment integrin-mediated tumor cell adhesion to endothelial cell. This, in turn, leads to the transendothelial migration of tumor cells.

MATERIALS AND METHODS

Colorectal Carcinoma Cell Lines. A variety of human colorectal carcinoma cell lines were used in the present study: (a) LS174T (well to moderately differentiated); (b) HT-29 (moderately differentiated); (c) HCT-15 (moderately differentiated); (d) Lovo (moderately differentiated; derived from metastasized lymph nodes); (e) DLD-1 (moderately to poorly differentiated); (f) RKO (poorly differentiated); (g) HCT-116 (poorly differentiated); and (h) SW-620 (poorly differentiated; derived from metastasized lymph nodes). All cell lines were grown in DMEM (Life Technologies, Inc., Grand Island, NY) including 10% FCS (Bio-Pro, Karlsruhe, Germany).

Reagents and mAbs. The following mAbs were used as purified immunoglobulin in cell surface analyses and functional assays: (a) CD54 (ICAM-1) mAb 84H10 [kindly provided by Dr. S. Shaw (NIH, Bethesda, MD); Ref. 27]; (b) CD106 (VCAM-1) mAb 2G7 [kindly provided by Dr. W. Newman (Osuka America, Rockville, MD)]; (c) CD11a (LFA-1a) mAb TSI/22 (American Type Culture Collection, Manassas, VA); (d) antiaactivation epitope of LFA-1α mAb NKI-L16 [kindly provided by Dr. C. Fidgor (University Hospital, Nijmegen, the Netherlands); Refs. 28 and 29]; (e) CD44 mAb NIH44-1 (kindly provided by Dr. S. Shaw; Ref. 25); (f) CD44v3 mAb BBA11 and CD44v6 mAb BBA13 (both are from Cosmo-Bio, Tokyo, Japan); (g) CD49d (VLA-4) mAb NIH49d-1 (from Dr. S. Shaw); (h) c-Met mAb (Seikagaku, Tokyo, Japan); and (i) control mAb thy1.2 and anti-glycophorin mAb 10F7 (both are from American Type Culture Collection). Fragmented and native hyaluronan were kindly donated by the Tokyo Research Institute of Seikagakuen (Tokyo, Japan), transfected COS cells and mock-transfected COS cells were also prepared as reported previously (9). Multiple inhibitors of cytoplasmic signaling were applied to each assay system, and all reagents were used at the indicated concentrations. At these concentrations, none of these inhibitors produced cytotoxic effects on LS174T cells, as confirmed by trypan blue staining. We used wortmannin (Wako Pure Chemical, Osaka, Japan), a tyrosine kinase inhibitor, 3’-kinase inhibitor, H89, an A-kinase inhibitor, H7 and staurosporine (Seikagaku), C-kinase inhibitors, herbimycin A (Sigma, St. Louis, MO), and genistein (Calbiochem, San Diego, CA), a tyrosine kinase inhibitor.

Fluorescence-activated Cell-sorting Analysis. Staining and flow cyto- metric analysis of colon cancer cell lines were carried out with a FACSscan (Becton Dickinson, Mountain View, CA) using standard procedures, as described previously (8, 10). Briefly, cells (2 × 105) were incubated with negative control mAb thy1.2, ICAM-1 mAb 84H10, VCAM-1 mAb 2G7, LFA-1α mAb TSI/22, activation epitope of LFA-1α mAb NKI-L16, CD44 mAb NIH44-1, CD44v3 mAb, CD44v6 mAb, CD49d mAb NIH49d-1, and c-Met mAb in fluorescence-activated cell-sorting media consisting of HBSS (Nissui, Tokyo, Japan), 0.5% HSA (Green-Cross, Osaka, Japan), and 0.2% NaN3 (Sigma Aldrich) for 30 min at 4°C. After washing, the cells were further incubated with FITC-conjugated goat antioimune IgG Ab for 30 min at 4°C. Amplification of mAb binding was provided by a three-decade logarithmic amplifier. Quantification of the cell surface antigens on each cell was performed using QIFKIT beads (Dako Japan, Kyoto, Japan) as reported previously (11, 30, 31).

F-Antigen Polymerization Assay. For microscopic analysis, LS174T cells were allowed to settle for 30 min at 4°C on fibronectin-coated slides. After incubation for 1 min at 37°C, the cells were fixed with 1% formaldehde. F-actin was stained with rhodamine-phalloidin (1 unit/slide; Molecular Probes, Inc., Eugene, OR) and analyzed later with a confocal laser microscope system (model LSM 410UV; LD Achroplan 20 objective lens; Carl Zeiss, Juna, Germany).

Adhesion Assay. The adhesion assay of colon cancer cell lines to HUVECs was performed as described previously (9). HUVECs were applied to 8-well culture plates (Costar Corp, Cambridge, MA) coated with 2% gelatin and cultured to confluence in DMEM containing 100 units/ml penicillin G, 100 units/ml streptomycin, 20% heat-inactivated FCS, 20 μg/ml endothelial mito-
ilar to those of the other cells suggests that the expression of a variant form of CD44 is not relevant to the induction of LFA-1 and c-Met by CD44 stimulation.

Low Molecular Weight Fragments of Hyaluronan Induced the Expression of Activation Epitope of LFA-1 and c-Met. Hyaluronic acids are major ligands for CD44 on the cell surface (34). LFA-1 requires an active configuration to bind to its ligand, a process that can be induced by a variety of stimuli. Furthermore, NKI-L16 mAb reacts with a Ca²⁺-dependent activation epitope located on the ectodomain of the α-chain of LFA-1 (11, 28, 30). We next assessed the biological activities of hyaluronan on the expression of the activation epitope of LFA-1 and c-Met on LS174T cells. Expression of the activation epitope of LFA-1, as recognized by the NKI-L16 mAb, was significantly induced by the addition of the Mr 6,900 fragment of hyaluronan (1 nM). In contrast, native hyaluronan, a Mr 1,700 fragment of hyaluronan, and a Mr 40,000 fragment of hyaluronan (all 1 nM) failed to induce its expression (Fig. 3A). Furthermore, the expression of c-Met was also increased by the Mr 6,900 fragment of hyaluronan, but not by others, including native hyaluronan (Fig. 3B). These data suggest that hyaluronan, especially when fragmented, is a possible ligand involved in CD44-mediated LFA-1 expression and/or activation and c-Met expression on LS174T cells.

Cross-Linking of CD44 Induces F-Actin Polymerization. It has been proposed that conformational changes of the ectodomain and/or the multimerization of integrins are mediated by actin polymerization that is physiologically and functionally associated with the endodomain of integrins (15). LS174T cells seeded on fibronectin spread slightly, and their F-actin content and distribution remained constant, as observed by confocal microscopy. In contrast, cells that were cross-linked with CD44 mAb and a second Ab for 1 min showed increased expression, marked spreading, and polymerization of F-actin in the cell cortex (Fig. 4). These results indicated that CD44 induced an immediate polymerization and rearrangement of F-actin, which might lead to active configuration of LFA-1.

Signaling Pathways Involved in the Induction of the Activation Epitope of LFA-1 by Low Molecular Weight Fragments of Hyaluronan. We analyzed the ability of several signaling inhibitors to block the induction of the activation epitope of LFA-1 on LS174T cells. Pretreatment of the cells with H7 and staurosporine, C-kinase inhibitors, blocked the up-regulation of the activation epitope of LFA-1 by hyaluronan stimulation. However, H89 (an A-kinase inhibitor), herbimycin A and genistein (tyrosine kinase inhibitors), and wortmannin (a phosphatidylinositol 3'-kinase inhibitor) only slightly decreased the expression of the activation epitope of LFA-1 (Fig. 5).
Thus, these data indicate that although the up-regulation of the activation epitope of LFA-1 induced by hyaluronan was mediated by a number of signaling pathways, it depended mainly on the activation of protein kinase C.

The Activation Epitope of LFA-1 Was Most Efficiently Induced by CD44 Cross-Linking and HGF. CD44 cross-linking also highly induced the expression of the activation epitope of LFA-1 on LS174T cells. Although HGF caused a low induction of expression of the activation epitope of LFA-1, the expression was further augmented when the cells were prestimulated by CD44 cross-linking, but not by ICAM-1 cross-linking (Fig. 6). These results suggest that CD44 cross-linking induces not only the expression of LFA-1 but also the active configuration of LFA-1. Furthermore, HGF is also involved in the expression of the acti-

Fig. 2. Up-regulation of LFA-1 and c-Met by CD44 cross-linking on colon cancer cell lines. A, LS174T cells were cross-linked with anti-ICAM-1 mAb 84H10 (a and d), anti-VCAM-1 mAb 2G7 (b and e), or anti-CD44 mAb NIH44–1 (c and f) and a second antimouse immunoglobulin for 6 h, and the expression of the indicated molecules was analyzed before (thin line) and after (thick line) stimulation by FACScan. B, expression of LFA-1 and c-Met was compared before (thin line) and after (thick line) cross-linking with anti-CD44 mAb on HCT-15 (a and d), DLD-1 (b and e), and HC-T116 (c and f).

Fig. 3. Fragmented hyaluronan induces the activation epitope of LFA-1 as well as c-Met on LS174T cells. LS174T cells were incubated with the native or fragmented form of hyaluronan (1 nM) for 6 h. The molecular weights of hyaluronan fragments were M_r 1,700, M_r 6,900, and M_r 40,000. Expression of the activation epitope of LFA-1 (A) and c-Met (B) was determined by NKI-L16 mAb and anti-c-Met mAb using a FACScan. Each bar represents the number of molecules expressed per cell, calculated using standard QIKFIT beads in one representative experiment of five similar experiments.
vation epitope of LFA-1 through the c-Met receptor induced by CD44 cross-linking as an alternate pathway.

**CD44 Cross-Linking or Fragmented Hyaluronan Induced Integrin-mediated Adhesion of LS174T Cells to ICAM-1-transfected COS Cells and HUVECs.** We next investigated whether CD44-stimulated or hyaluronan-induced activation of colon cancer cells was involved in their adhesion to integrin-ligand ICAM-1-transfected cells or endothelial cells. Colon cancer cell line LS174T showed minimal adhesion to ICAM-1-transfected COS cells in the absence of any stimulus. However, stimulation of LS174T cells by CD44 cross-linking or by the Mr 6,900 hyaluronan fragment resulted in a 3–5-fold increase in their adhesion to ICAM-1-transfected COS cells, but not to control mock-transfected COS cells, as compared with the control or the second Ab only. The adhesion of LS174T cells stimulated as described above was significantly inhibited by anti-LFA-1 mAb (Fig. 7A). The adhesion of LS174T cells to IL-1-activated HUVECs was also markedly augmented after stimulation by CD44 cross-linking or by the Mr 6,900 hyaluronan fragment. Furthermore, fragmented hyaluronan-induced adhesion of LS174T was inhibited by the addition of anti-LFA-1 and VLA-4 mAbs (Fig. 7B). These results indicate that CD44 stimulation by mAbs or fragmented hyaluronan induces integrin expression as well as integrin activation, which results in an enhancement of integrin-mediated adhesion to their ligands on endothelial cells.

**HGF Further Augmented the Adhesion to HUVECs and Transendothelial Migration of LS174T Stimulated by CD44 Cross-Linking.** Finally, we examined the significance of the induction of HGF receptor c-Met by CD44 cross-linking on the adhesion of stimulated LS174T cells to HUVECs and their migration. HGF augmented adhesion to HUVECs and the migration of LS174T cells in Transwell chambers. However, when LS174T cells were prestimulated with CD44 cross-linking, HGF further amplified their adhesion to HUVECs and migration (Fig. 8, A and B). These results suggest that c-Met induction on colon cancer cells by CD44 cross-linking results in a further enhancement of their adhesion and migration in response to HGF.

**DISCUSSION**

The main findings of the present study were the following: (a) CD44 cross-linking on colon cancer cells up-regulated LFA-1 expression; (b) fragmented hyaluronan, a major ligand for CD44, also induced the expression of LFA-1; (c) CD44 cross-linking and fragmented hyaluronan induced the activation epitope of LFA-1 and integrin-mediated adhesion of tumor cell lines to endothelial cells and ICAM-1-transfected COS cells; (d) CD44 cross-linking induced F-actin polymerization in the cell cortex; (e) the up-regulation of the activation epitope of LFA-1 induced by fragmented hyaluronan was mediated by the signaling pathway of protein kinase C; (f) CD44 and hyaluronan also induced HGF receptor c-Met expression on tumor cells.

**Fig. 4.** Confocal microscopy analysis of poly-merized F-actin on LS174T cells. LS174T cells stimulated without (A) or with (B) CD44 mAb and a subsequent second Ab were incubated on fibronectin-coated slides for 1 min. F-actin in these cells was stained with rhodamine-phalloidin and was observed by confocal microscopy (×1000).

**Fig. 5.** Effects of multiple signaling inhibitors on the up-regulation of the activation epitope of LFA-1 by hyaluronan stimulation. LS174T cells were pretreated with or without the indicated concentrations of multiple inhibitors of intracytoplasmic signaling, and flow cytometric analyses were performed as described in the Fig. 3 legend.

**Fig. 6.** LFA-1 activation is most efficiently induced by both cross-linking of CD44 and rHGF. LS174T cells were cross-linked with anti-CD44 mAb or anti-ICAM-1 mAb for 6 h, and then rHGF (20 ng/ml) was added to the culture for 30 min. Expression of the activation epitope of LFA-1 was determined by N6J-L16 mAb using a FACSscan. Each bar represents the number of molecules expressed per cell, calculated using standard QIFKIT beads in one representative experiment of five similar experiments.
A minute using confocal microscopy.

polymerization and rearrangement of F-actin in the cell cortex within LFA-1. Here we observed that CD44 cross-linking resulted in the grins, is involved in the induction of the active configuration of F-actin filaments, which are associated with the endodomain of inte-

timerization of LFA-1 on the cell surface, and that polymerization of avidity of LFA-1 are induced by conformational change and/or mul-

and others (11, 15, 30, 32) have proposed that higher affinity and/or was induced by the addition of the fragmented hyaluronan, which was of the activation epitope of LFA-1, as recognized by NKI-L16 mAb, 

It is well known that the expression and function of adhesion molecules are regulated through cytoplasmic signaling induced by several cellular stimuli, a process known as “inside-out signaling” (15). Among these stimuli, cytokines including IL-1β or tumor necrosis factor α up-regulate the immunoglobulin superfamily of adhesion molecules, such as ICAM-1 and VCAM-1 (35, 36). The present study is the first to show that one particular adhesion molecule, CD44, is involved in the up-regulation of expression of LFA-1. In this regard, the function of LFA-1 is tightly regulated through the inside-out signaling stimulated by several other cellular stimuli; namely, the activation of integrin is essential for integrin-mediated adhesion in which a signal converts the functionally inactive integrin to an active adhesive configuration. It is noteworthy in the current study that CD44 cross-linking or fragmented hyaluronan appears to induce not only a quantitative change of LFA-1 expression but also induces a qualitative change of LFA-1 on the colon cancer cells and amplified integrin-mediated adhesion of the cells. We observed that expression of the activation epitope of LFA-1, as recognized by NKI-L16 mAb, was induced by the addition of the fragmented hyaluronan, which was decreased by staurosporin or H7, protein kinase C inhibitors. Also, we and others (11, 15, 30, 32) have proposed that higher affinity and/or avidity of LFA-1 are induced by conformational change and/or multimerization of LFA-1 on the cell surface, and that polymerization of F-actin filaments, which are associated with the endodomain of inte-

Second, certain adhesion molecules not only function as a glue but also transduce extracelluar information to the cytoplasmic organelle through “outside-in signaling,” resulting in cell activation and cyto-

tume including IL-1, VLA-4, CD2, CD28, and CD154 are the best known molecules that induce costimulatory signals in T-cell-antigen-presenting cell binding, which are essential to T-cell activa-

tion and cytokine production (27, 37–39). Studies from our laborato-

ries have also shown that ICAM-1 on rheumatoid synoviocytes induces cytokine gene transcription in synoviocytes through the activ-

ation of nuclear factor activating protein (AP)-1 (33). Recent studies have also identified CD44 as a signaling molecule (23, 25, 40). Stimulation of CD44 transmits the signal into the cells, which leads to the activation of T cells and cytokine release from monocytes or chondrocytes (41–43). Although CD44 is up-regulated in colon can-

cers, and its expression as well as that of its variant form is thought to be relevant to cancer metastasis (14, 24, 26, 44, 45), the precise functional role of CD44 in metastasis is still unclear. Our results showed that CD44 cross-linking or fragmented hyaluronan induced LFA-1 expression and activation. It is of interest that CD44 stimulation failed to induce LFA-1 and c-Met expression on one cell line, HCT-15, which bears little CD44 on the surface. Furthermore, another cell line, HCT-116, bearing higher levels of CD44v6, expressed adhesive properties similar to those of other cell lines lacking CD44v6 after CD44 cross-linking. These results suggest that a variant form of CD44 is not relevant to the induction of LFA-1 and c-Met by CD44 stimulation and that the amount of CD44 on the cell surface appears to be important for signaling. Furthermore, it is noteworthy that integrin activation through outside-in signaling induced by CD44 stimulation possesses two differential pathways. Namely, CD44 stimulation alone satisfactorily induced the activation epitope of LFA-1

Fig. 7. Induction of adhesion of LS174T cells by CD44 stimulation to ICAM-1 transfectants or HUVECs. The adhesion assay of 3H-labeled LS174T cells that were pretreated with or without CD44 mAb cross-linking or M, 6,900 fragments of hyaluronan to (A) ICAM-1-transfected COS cells (ANA, mock-transfected COS cells; A, ICAM-1-transfected COS cells) or (B) IL-1β-activated HUVECs was carried out by a 30-min incubation in the presence or absence of the indicated adhesion-blocking mAbs (in 10 

μg/ml). γ-Emission of lysates of adherent cells was determined. Data represent the mean percentage of binding of the added LS174T cells from a representative experiment.

cells, and, in turn, HGF further augmented the expression of the activation epitope of LFA-1 and LFA-1-mediated adhesion; and (g) CD44 or fragmented hyaluronan induced transendothelial migration of cancer cells, and HGF further increased their migration. Based on these findings, we postulate that stimulation of CD44 is involved in the integrin-mediated adhesion of tumor cells to endothelial cells, which subsequently results in the transendothelial migration of tumor cells.

It is well known that the expression and function of adhesion molecules are regulated through cytoplasmic signaling induced by several cellular stimuli, a process known as “inside-out signaling” (15). Among these stimuli, cytokines including IL-1β or tumor necrosis factor α up-regulate the immunoglobulin superfamily of adhesion molecules, such as ICAM-1 and VCAM-1 (35, 36). The present study is the first to show that one particular adhesion molecule, CD44, is involved in the up-regulation of expression of LFA-1. In this regard, the function of LFA-1 is tightly regulated through the inside-out signaling stimulated by several other cellular stimuli; namely, the activation of integrin is essential for integrin-mediated adhesion in which a signal converts the functionally inactive integrin to an active adhesive configuration. It is noteworthy in the current study that CD44 cross-linking or fragmented hyaluronan appears to induce not only a quantitative change of LFA-1 expression but also induces a qualitative change of LFA-1 on the colon cancer cells and amplified integrin-mediated adhesion of the cells. We observed that expression of the activation epitope of LFA-1, as recognized by NKI-L16 mAb, was induced by the addition of the fragmented hyaluronan, which was decreased by staurosporin or H7, protein kinase C inhibitors. Also, we and others (11, 15, 30, 32) have proposed that higher affinity and/or avidity of LFA-1 are induced by conformational change and/or multimerization of LFA-1 on the cell surface, and that polymerization of F-actin filaments, which are associated with the endodomain of inte-

Grins, F-actin, and others (11, 15, 30, 32) have proposed that higher affinity and/or avidity of LFA-1 are induced by conformational change and/or multimerization of LFA-1 on the cell surface, and that polymerization of F-actin filaments, which are associated with the endodomain of integrins, is involved in the induction of the active configuration of LFA-1. Here we observed that CD44 cross-linking resulted in the polymerization and rearrangement of F-actin in the cell cortex within a minute using confocal microscopy.

Second, certain adhesion molecules not only function as a glue but also transduce extracellular information to the cytoplasmic organelle through “outside-in signaling,” resulting in cell activation and cytokine production (15). LFA-1, VLA-4, CD2, CD28, and CD154 are the best known molecules that induce costimulatory signals in T-cell-antigen-presenting cell binding, which are essential to T-cell activation and cytokine production (27, 37–39). Studies from our laboratories have also shown that ICAM-1 on rheumatoid synoviocytes induces cytokine gene transcription in synoviocytes through the activation of nuclear factor activating protein (AP)-1 (33). Recent studies have also identified CD44 as a signaling molecule (23, 25, 40). Stimulation of CD44 transmits the signal into the cells, which leads to the activation of T cells and cytokine release from monocytes or chondrocytes (41–43). Although CD44 is up-regulated in colon cancers, and its expression as well as that of its variant form is thought to be relevant to cancer metastasis (14, 24, 26, 44, 45), the precise functional role of CD44 in metastasis is still unclear. Our results showed that CD44 cross-linking or fragmented hyaluronan induced LFA-1 expression and activation. It is of interest that CD44 stimulation failed to induce LFA-1 and c-Met expression on one cell line, HCT-15, which bears little CD44 on the surface. Furthermore, another cell line, HCT-116, bearing higher levels of CD44v6, expressed adhesive properties similar to those of other cell lines lacking CD44v6 after CD44 cross-linking. These results suggest that a variant form of CD44 is not relevant to the induction of LFA-1 and c-Met by CD44 stimulation and that the amount of CD44 on the cell surface appears to be important for signaling. Furthermore, it is noteworthy that integrin activation through outside-in signaling induced by CD44 stimulation possesses two differential pathways. Namely, CD44 stimulation alone satisfactorily induced the activation epitope of LFA-1

Fig. 8. Effects of HGF on the adhesion and transendothelial migration of CD44 mAb-stimulated LS174T cells to HUVECs. IL-1β-activated HUVECs were incubated on the filters of a modified Transwell chamber for 3 days. HGF (20 ng/ml) was added in the upper chamber, and 51Cr-labeled LS174T cells, which were pretreated with or without cross-linking by the CD44 mAb, were placed in the upper chamber. The γ-emission of the lysates of adherent cells on the upper side of the filter was determined after a 30-min incubation (adhering cells, A), and the γ-emissions of the lysates of cells beneath the filter and in the lower chamber were determined after a 24-h incubation (migrating cells; B). Data are expressed as the mean percentage of binding or migrating LS174T cells among added cells from a representative experiment.

4432
and its mediated adhesion. In addition, our results also indicated that CD44 stimulation further induced the expression of c-Met, a major receptor for HGF. CD44 also stimulated responsiveness to HGF, which in turn augmented LFA-1-mediated adhesion induced by CD44 stimulation.

The principal ligand of CD44 is hyaluronan. Hyaluronan is a high molecular weight digosacchahide bearing linear repeats of disaccharide-β-D-glucuronyl-β-D-N-acetylglucosamine (46). Several studies have examined the biological activities of hyaluronan and shown that its high molecular weight fragments inhibit the proliferation of endothelial cells, fibrocytes, and mitogen-stimulated lymphocytes, whereas the low molecular weight fragments exert a stimulatory effect (47). The functional role of hyaluronan in metastasis is virtually unknown. Our results showed that fragmented hyaluronan up-regulated LFA-1, activated LFA-1, and induced the integrin-mediated adhesion of colon cancer cells to endothelial cells. In this regard, several studies have shown that the stroma of various tumors is hyaluronan-rich compared to the supporting connective tissue of normal parenchyma (48). Recent studies have also shown that certain malignant cells secrete or present membrane-bound hyaluronan in adjacent fibroblasts and that high concentrations of hyaluronan in turn stimulate cancer cell motility (48, 49). Hyaluronan also stimulates angiogenesis, a vital process for tumor growth, and low molecular weight hyaluronan fragments strongly stimulate tumor growth (48). For human colon carcinoma, tumor cells show hyaluronidase activity, thus enhancing the generation of fragmented hyaluronan (50, 51). Based on the above findings, both the synthesis and degradation of hyaluronan seem to support tumor invasion and growth. Furthermore, the present results indicate that fragmented hyaluronan is more effective in up-regulating LFA-1 than native HA. These results demonstrate that fragmented hyaluronan, far from being an inert space filler, plays an important biological role, including qualitative and quantitative regulation of adhesion molecules in cancer cells.

HGF is a pleiotropic growth factor with various biological activities, including mitogenic, motogenic, and/or morphogenic properties, in a variety of epithelial tissues, and its ligand for a tyrosine kinase cell surface receptor is encoded by the Met proto-oncogene c-Met (52, 53). Recent studies have shown the importance of mutual interactions between carcinoma cells and these cells (as the inducer of HGF within the supporting stroma of tumors) in the invasion and metastasis of carcinoma cells. Thus, HGF derived from these cells stimulates the motility and invasiveness of various carcinoma cell types in vitro (54, 55). Furthermore, overexpression of c-Met in gastric and hepatocellular carcinoma is associated with poor prognosis (56, 57). Recent studies have also shown that NIH3T3 fibroblasts transfected with both HGF and c-Met genes exhibit tumorigenic capacity in nude mice (58). Furthermore, HGF/c-Met has been shown to be involved in the adhesion of tumor cells; specifically, HGF follows the initial adhesion of cancer cells via selectins and increases the adhesion of c-Met-positive cancer cells to the extracellular matrix via integrins (59, 60). Our early findings that HGF activates integrins and induces integrin-mediated adhesion of lymphocytes and leukocytes, as chemokinases do (32, 61), were followed by several studies that demonstrated activation of VLA-4 and VLA-5 of lymphoma cells by HGF (59, 62). Based on this background work, we investigated in the present study whether HGF is involved in the activation of integrin LFA-1 and adhesion of colon carcinoma cells. Our results showed that HGF further augmented the expression of the activation epitope of LFA-1 and its dependent adhesion as well as the transendothelial migration of colorectal carcinoma cells that was mediated by c-Met induced by CD44 stimulation.

Although colorectal carcinoma cells did not express sufficient LFA-1 and VLA-4, taken together, our results showed that stimulation of these cells by CD44 cross-linking or fragmented hyaluronan resulted in the expression of LFA-1 and the activation epitope of LFA-1. This allows the adhesion of cancer cells to endothelial cells, followed by transendothelial migration. Furthermore, CD44 not only acted directly via integrins but induced c-Met expression and enhanced the response to HGF, and HGF further augmented the integrin-mediated adhesion and migration of CD44-stimulated colon cancer cells. Based on the findings presented here, we postulate that cellular functions are regulated by cross-talk between two distinctive adhesion molecules on the same cell. Thus, stimulation by CD44 cross-linking or ligation with fragmented hyaluronan induces outside-in signaling, which augments integrin expression and triggers integrin function by inside-out signaling. Furthermore, the outside-in signaling in colon cancer cells appears to consist of at least two pathways, a direct pathway through CD44 and an alternate pathway through the induction of c-Met expression involved by HGF, both of which cooperatively play a pivotal role in the integrin-mediated adhesion of colon cancer cells to the vascular wall. We believe that the concept of the adhesion cascade described in leukocyte adhesion to endothelial cells can also be applied to tumor cell extravasation and metastasis. Induction of expression and activation of integrins on the cells induced by CD44 stimulation could explain the transendothelial migration of tumor cells, especially with regard to colon cancer cells. Recent studies have suggested that CD44 receptor globulin, anti-CD44 mAb, native hyaluronan, and hyaluronan oligomers efficiently inhibit tumor growth, metastasis, and invasion in vivo and vitro (63, 64). Our findings using colorectal carcinoma cells warrant additional studies to unravel the mechanisms of integrin activation in other types of cells and the pathogenesis of tumor metastasis. Such studies may potentially lead to the design of novel pharmacological approaches to control these diseases.

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CD44 Stimulation Induces Integrin-mediated Adhesion of Colon Cancer Cell Lines to Endothelial Cells by Up-Regulation of Integrins and c-Met and Activation of Integrins

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