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Engagement of CD44 modulates cyclooxygenase induction, VEGF generation, and cell proliferation in human vascular endothelial cells

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ABSTRACT

CD44 is a receptor for hyaluronic acid and is found on the surface of hematopoetic cells and in mesenchymal tissue. It is also expressed on endothelial cells (EC). Cyclooxygenase (COX) is the rate-limiting enzyme in the production of prostaglandins in EC. Here we show that engagement of CD44 with signaling monoclonal antibodies (mAbs) or its natural ligand hyaluronic acid induces COX-2 and prostacyclin (PGI_2) formation in human EC. This induction was blocked by mAbs that have been shown to inhibit CD44-mediated intracellular signaling. COX-1 induction was not observed after CD44 ligation. CD44-stimulated COX-2 activation/PGI_2 production was accompanied by the production of the potent endothelial mitogen, vascular endothelial growth factor (VEGF) and was inhibited by a neutralizing VEGF antibody. Moreover, this COX-2 induction was also associated with an increase in EC proliferation that was inhibited by the blocking anti-CD44 mAbs and a COX-2-specific inhibitor. This is the first study to show that engagement of CD44 with mAbs or its natural ligand induces COX-2, generates VEGF, and thus leads to an increase in EC proliferation. Results from this study may have important and widespread implications for the development of novel therapeutic agents for modulating blood vessel growth during ischemic heart disease, during inflammation, or around solid tumors.

Key words: vascular endothelial growth factor • prostacyclin • COX

The cell adhesion molecule CD44 is involved in a variety of important biological events such as embryogenesis, hematopoiesis, lymphocyte homing and activation, inflammatory reactions, and tumor dissemination (1–4). CD44 represents a large protein family, which includes the standard form or CD44 with a molecular mass of 85-90 kDa, and a multiplicity of isoforms generated by alternative splicing of transcripts and subsequent variable glycosylation (reviewed in ref 5). These high molecular mass variants are rarely expressed on normal cells. CD44 is the principal cell surface receptor for extracellular matrix glycosaminoglycan hyaluronan (HA). CD44-HA-mediated cell adhesion is important in several pathophysiological

processes such as inflammation and metastatic spread of cancer cells. In this context, it has been recognized that CD44 can function as a signaling receptor in a variety of cell types (6). Cell stimulation by monoclonal anti-CD44 antibody or natural CD44 ligands activate signaling pathways that culminate in cell proliferation, cytokine secretion, chemokine gene expression, and cytolytic effector functions. Normal endothelial cells (EC) express low levels of CD44, but expression is up-regulated by activation with, for example, cytokines, and by culturing of these cells (7). Expression is also increased on the vasculature of solid tumors (7, 8). Expression of CD44 on EC is associated with homing and migration of leukocytes (i.e., inflammation and migration). In addition, it has been demonstrated that CD44 plays some role in new blood vessel formation (angiogenesis), although its precise role in this process is not clear (7, 9, 10).

EC proliferation is driven by the growth factor vascular endothelial growth factor (VEGF) that enhances vascular permeability (11) and stimulates angiogenesis in vivo (12). Several VEGF isoforms, including $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{199}$, and $VEGF_{206}$, have been identified (13, 14), which are products of a single gene. VEGF acts through two high affinity tyrosine kinase receptors expressed by EC and hematopoietic cells, flt-1 (VEGFR-1) and KDR (VEGFR-2) (15, 16). Moreover, Soker et al. (17) identified a novel VEGF receptor that binds to $VEGF_{165}$ but not $VEGF_{121}$. Among several signals, VEGF can induce prostacyclin (PGI_2) generation by EC (18, 19). PGI_2 is a member of a family of potent biological mediators termed prostaglandins (PGs), which are implicated in the pathogenesis of cardiovascular disease, inflammation, and cancer. The formation of PGs is highly regulated at a number of points in their biosynthetic pathway. One of these is the enzyme cyclooxygenase (COX) of which there are two isoforms, COX-1 and COX-2, that are the products of distinct genes. COX-1 is constitutively expressed in most tissues and cell types (20) and is induced during cell differentiation and angiogenesis (21, 22). COX-2 is rarely detected but is induced by several stimuli, including cytokines, hormones, and growth factors (23, 24). Recently, we have shown that both isoforms are induced after exposure to VEGF (25). Moreover, we have demonstrated that signaling through the integrin $\alpha_v\beta_3$, which is also expressed on EC, results in PGI_2 formation and COX-1 and -2 induction (26).

As CD44 expression has previously been associated with inflammatory processes, cancer metastasis, and angiogenesis, we investigated whether signaling through CD44 in EC could induce PGI_2 formation, COX-2 induction, and VEGF secretion. We found that both cross-linking CD44 using antibodies and engagement with the physiological ligand HA resulted in up-regulation of COX-2 expression and PGI_2 formation with no significant COX-1 induction. Signaling through CD44 also resulted in stimulation of VEGF secretion and EC proliferation. Neutralizing antibodies to VEGF significantly inhibited CD44-stimulated COX-2 induction/PGI_2 formation. Furthermore, CD44-induced EC proliferation was inhibited by a COX-2-selective inhibitor. These results implicate CD44 as having a key role, not just as an adhesion molecule, but also as a signaling molecule the engagement of which results in the production of molecules central to processes such as inflammation, metastasis, and angiogenesis.

MATERIALS AND METHODS

All laboratory reagents were from Sigma Chemical Co., (St. Louis, MO) unless otherwise stated. Culture medium (M199), fetal calf serum (FBS), antibiotics, Hanks' balanced salt solution (HBSS), phosphate buffered saline (PBS), and HEPES were from Life Technologies Inc. (Paisley, UK). NS398 was from Cayman Chemical Co. (Ann Arbor, MI). SC 560 was a kind gift

from Dr. Peter Isakson at Searle (Skokie, IL). Hylauronic acid fragments (200 kDa) were from ICN Biomedicals.

Monoclonal antibodies (mAbs)

For the anti-CD44 antibodies, D2.1 monoclonal antibody was produced by immunizing mice with HUT 78 (T cell lymphoma) cells using standard hybridoma technology (27, 28, unpublished observations). L3D1 was a kind gift from Dr. Bishr Omary (Stanford University), and Bric 238 was from Dr. P. A. Judson (Bristol, UK) (29); KM201 was a kind gift from Dr. Katherine Fitzgerald (Trinity College, Dublin), and IM7.8.1 and Anti-IE (HB 179) were from the American Type Culture Collection (ATCC).

Culture of human umbilical vein endothelial cells (HUVEC)

EC were isolated and grown in culture as described by Jaffe et al. (30). Briefly, cells were isolated from human umbilical vein cords by enzymatic digestion using collagenase type II (Worthington Chemical Co., Freehold, NJ) and grown in medium 199 supplemented with 10-20% FBS and penicillin/streptomycin (100 U). EC mitogen (Biogenesis, Poole, England) was solubilized with heparin (1000 U) and added at a final concentration of 100 μg/ml. Cells were grown to confluence on gelatin-coated 75 cm^2 flasks (COSTAR, Cambridge, MA) in a humidified atmosphere at 37°C in 5% CO_2. Confluent primary cultures were routinely passaged by trypsin/EDTA digestion and expanded through passages 2-3.

Detection of CD44 on HUVEC

CD44 expression on HUVEC was determined by Western blot analysis. Cells were isolated and cultured as described above. Detergent-soluble lysates were prepared by incubating the cells in a TBS containing 1% Triton X-100, 1 mM EDTA, and protease inhibitors, and the lysates were separated by 10% PAGE. Resolved proteins were transferred to a PVDF membrane (for 1 h using a semidry system), which was blocked using notfat dried milk (in PBS) and then probed with an anti-CD44 specific antibody (Bric 238) followed by a secondary HRP-labeled, secondary rabbit anti-mouse antibody. Blots were stripped by incubating them in 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7 for 30 min at 60°C, and were reprobed with an anti-actin antibody (Polyclonal from Sigma) as a loading control. Immunoreactive bands were visualized by enhanced chemical luminescence (ECL) and autoradiography.

Estimation of COX isozyme induction

EC to be assayed for induction of COX isoforms 1 and 2 were grown to confluence on either 6- or 24-well plates and serum starved in 2.5% FBS the night before. Cells were then treated with aspirin at a final concentration of 200 μM for 45 min to destroy all COX activity and washed twice with 50 ml warm PBS. In experiments where a neutralizing antibody to VEGF was used, anti-VEGF (0.1 μg/ml; R&D Systems, Minneapolis, MN) was added 30 min before addition of stimulus. Cells were then incubated in M199/2.5% FBS alone or in medium containing phorbol myristate acetate (PMA; 1 μM), VEGF (50 ng/ml), mAbs (5-10 μg/ml), HA, or HA fragments (100 μg/ml) for 3-6 h. The cells were again washed twice in PBS and treated with arachidonic acid (150 μM) in HBSS/CaCl for 10 min. The supernatant was analyzed for 6-keto $PGF_{1\alpha}$ by

ELISA (Assay Designs Inc., Ann Arbour, MI). The cell pellets were lysed (1% Triton X-100 in PBS, 1 mM EDTA, and protease inhibitors, Complete™, Boehringer-Mannheim) for 30 min on ice before electrophoresis and Western blot analysis. Lysate samples (20-40 μg/lane) were applied to a 7.5% SDS polyacrylamide minigel, electrophoresed for 2 h and then transferred to nitrocellulose membranes overnight at 30 V constant voltage at 4°C. Nitrocellulose was checked for the presence of protein using Ponceau staining (0.1% w/v, 0.1% acetic acid v/v in dH_2O), washed three times in NT buffer (170 mM NaCl, 0.2% igepal detergent CA-630 v/v, 50 mM Tris, pH 7.4) containing 5% bovine serum albumin (BSA), and blocked for a further 30 min. Immunostaining for COX-1 and -2 expression was performed with either a mouse anti-human COX-1 or COX-2 monoclonal antibody (Caymen Co.). Immunoreactive bands were detected as described above.

VEGF detection and cell proliferation assays

After COX induction experiments described above, supernatant (100 μl) was removed and assayed for VEGF using a Quantikine human immunoassay (R&D Systems). The assay uses a standard recombinant human $VEGF_{165}$ and recognizes native human VEGF and $VEGF_{121}$. The ELISA was performed according to the manufacturer's instructions. For cell proliferation, EC were grown to 50-60% confluence in 96-well tissue culture plates (COSTAR). mAbs, ligands, or control agents were added and incubated at 37°C for 8-9 h followed by addition of (3-[4, 5-dimethylthiazol-2-y]-2, 5-diphenyltetrazolium bromide (MTT; R&D Systems) for a further 1 h. MTT is reduced by viable, metabolically active cells to insoluble purple formazan dye crystals. Detergent was added to the wells solubilizing the crystals, and the absorbance was read by a spectrophotometer at 570 nm. The rate of tetrazolium reduction is directly proportional to the rate of cell proliferation. In assays where the COX-1 (SC 560, 1 μM)- and COX-2 (NS398, 1 μM)-specific inhibitors were used, inhibitors were added 30 min before addition of CD44 mAbs or ligands.

Statistics

Each experiment was carried out in replicate as described, and the data are means ± SE for the indicated number of experiments. Statistical analysis was carried out using the Student's *t*-test.

RESULTS

HUVEC express CD44

We investigated the status of CD44 expression on cultured HUVEC. Cells were seeded at a low density [1.5×10^5 cells per flask (T75)], and CD44 expression was determined by Western blot analysis 24, 48, and 72 h later. It was found that levels of this molecule increased as the confluency of cells increased (Fig. 1).

Anti-CD44 mAbs induce COX-2 in EC

As CD44 is expressed on the surface of EC, we investigated whether engagement with anti-CD44 mAbs would regulate COX activity and induction. mAbs or control agents were added to EC for 3-4 h and assayed for 6-keto-$PGF_{1\alpha}$ generation and COX protein expression by ELISA

and Western blot analysis, respectively. PMA was used as positive control, as we have previously shown it to induce COX-2 and 6-keto-$PGF_{1\alpha}$ formation (25). Figure 2*A* shows that anti-CD44 mAbs L3D1 and D2.1 significantly up-regulated the amount of 6-keto-$PGF_{1\alpha}$ generated by these cells. COX-2 protein expression was also induced by both mAbs (Fig. 2*B*). A nonsignaling IgM anti-CD44 antibody, Bric238, did not affect either 6-keto-$PGF_{1\alpha}$ generation or COX-2 protein expression. COX-1 is constitutively expressed in HUVEC (Fig. 2*B*), and none of the CD44 mAbs induced COX-1 above basal levels.

Native, high molecular mass HA induces COX-2 in EC

Having observed that the two CD44-specific signaling mAbs induced COX-2 expression in EC, we then investigated whether HA, the natural ligand for CD44, could stimulate COX-2 induction. HA induced 6-keto-$PGF_{1\alpha}$ in a concentration-dependent manner (Fig. 2*C*). As there was no increase in 6-keto generation above 100 μg/ml, this was the concentration used in all subsequent experiments. HA-stimulated 6-keto-$PGF_{1\alpha}$ generation was inhibited by CD44 antibodies IM7.8.1 and KM201 (Fig. 2*D*). These antibodies have previously been shown to block HA signaling through CD44 (31, 32). IM7.8.1 stimulates shedding of CD44 while KM201 blocks HA binding (32). 6-keto-$PGF_{1\alpha}$ generation in response to HA was completely inhibited by the COX-2 inhibitor NS398 confirming that HA-driven PGI_2 formation is via COX-2 (data not shown). HA also stimulated COX-2 protein expression as demonstrated by Western blot analysis (Fig. 2*E*). Fragments of HA, previously shown to have signaling capacity (31, 33), did not significantly induce 6-keto-$PGF_{1\alpha}$ formation at 100 μg/ml (Fig. 2*D*). 6-keto-$PGF_{1\alpha}$ was not detected at any other HA fragment concentration (50-200 μg/ml) tested (data not shown). Treatment of cells with PMA or ligation of CD44 with native HA or HA fragments for 3 or 6 h did not induce COX-1 above resting levels.

CD44 engagement induces VEGF

As COX and its by-products have been shown to be involved in EC proliferation and to regulate angiogenesis (34, 35), we next sought to determine whether ligand-mediated signaling through CD44 would affect generation of VEGF. VEGF is produced under hypoxic conditions but is also released during angiogenic blood vessel growth (36). VEGF was not detected in supernatant from resting cells whereas ligation of CD44 with L3D1 or D2.1 antibodies leads to a significant increase in VEGF generation (Fig. 3*A*). PMA was used as positive control. The nonsignaling (IgM) Bric 238 or the IgG isotype control anti-IE did not affect VEGF generation. HA also lead to a very significant increase in VEGF production that was completely inhibited by the two blocking mAbs IM7.8.1 and KM201 (Fig. 3*B*). HA fragments did not stimulate significant VEGF production by EC (Fig. 3*B*).

CD44-mediated 6-keto-$PGF_{1\alpha}$ generation is dependent on VEGF

To determine whether 6-keto-$PGF_{1\alpha}$ production was dependent on the generation of VEGF, we carried out experiments similar to those above in the presence of neutralizing anti-VEGF antibody. Results show that CD44-induced 6-keto-$PGF_{1\alpha}$ by both the anti-CD44 mAb D2.1 and HA was significantly inhibited in the presence of this antibody (Fig. 4*A* and *B*). These results

indicate that CD44 mediated COX-2 induction and 6-keto-$PGF_{1\alpha}$ generation is dependent on VEGF.

CD44 engagement leads to EC proliferation

Based on these findings, we then looked at the effect of CD44 ligation on EC proliferation. EC were incubated with mAbs for a period of 8-9 h and then assayed for induction of cell proliferation as described in Materials and Methods. Again, both mAbs D2.1 and L3D1 significantly up-regulated EC proliferation, whereas Bric 238 and the anti-IE mAb had no effect (Fig. 5*A*). HA (100 μg/ml) also significantly increased EC proliferation that was blocked by the blocking mAbs IM7.8.1 and KM201 (Fig. 5*B*). HA fragments did not have a significant effect on the rate of cellular proliferation over the 8-9 h period. EC proliferation stimulated by HA was significantly attenuated by the COX-2 inhibitor NS398 and not by the COX-1-selective inhibitor SC 560 (Fig. 5*C*). Taken together, our results strongly suggest that EC proliferation can be regulated by ligation of CD44, and this proliferative response is related to COX-2 induction and VEGF generation.

DISCUSSION

This study has demonstrated that signaling through the adhesion molecule CD44 on EC results in the induction of COX-2 gene expression and PGI_2 production, generation of VEGF, and subsequent stimulation of EC proliferation. These biological effects are both mediated via stimulating CD44 antibodies or the natural CD44 ligand HA. Although CD44 has been well characterized as a molecule that mediates cellular adhesion and motility (37), its role in cellular activation is less well described. Antibodies to CD44 have been shown to be costimulatory for T cell activation and thymocyte apoptosis (38), while HA fragment induced signaling through CD44 results in the transcription of proinflammatory genes, such as chemokines, cytokines, cell adhesion molecules, and inducible NO synthase (39, 33, 40)

Our previous results demonstrating COX induction by the vitronectin receptor $\alpha_v\beta_3$ show that receptor/ligand interactions mediate COX induction and subsequent downstream effects (26). In the present study, high molecular mass HA, the physiological ligand of the CD44 receptor, was a potent inducer of COX-2 and PGI_2 production in EC. Stimulation of cells with fragments of HA did not result in stimulation of COX-2 expression. These differences between the role of native HA and HA fragments in COX-2 activation and PGI_2 production correlate with their effect on VEGF production and induction of EC proliferation. In terms of signaling, this would suggest that the COX-2 induction, PGI_2 formation, VEGF production and cellular proliferation induced by engagement of CD44 is dependent on a high avidity interaction that can only be achieved with high molecular mass HA and also by antibody cross-linking. However, fragments of HA have been shown to have potent signaling capacity mediated through CD44 in many cellular systems (31, 33, 40) resulting in the activation of NFκB and transcription of proinflammatory genes. HA degradation products or "angiogenic oligosaccharides of hyaluronan" have also been shown to stimulate EC proliferation and transient up-regulation of immediate early genes including c-fos, c-jun and jun-B. (41). It has become clear, however, that the term "HA fragment" is heterogeneic and that it covers a multitude of sizes. In the study of Deed et al. (41), only fragments ranging in size from 1.35 to 4.35 kDa were angiogenic, while the size of our fragments were 200 kDa. HA fragments are generated at sites of inflammation and are also

increased with carcinogenesis, which may correlate with increased hyaluronidase activity (42-44). Although native HA has not been closely associated with induction of proinflammatory gene expression, it does itself have distinct signaling properties. Our results support the observations that HA and HA fragments transduce distinct signals.

Although this is the first study to demonstrate induction of COX-2 via the engagement of CD44, these two molecules display a parallel expression pattern in many physiological and pathological conditions. Expression of both CD44 and COX-2 can be induced by cytokines, growth factors, and tumor promoters (45-48), and COX-2 is a source of PG formation during inflammation, embryogenesis, and tumor growth, processes in which CD44 also plays major roles. Both CD44 and COX-2 are overexpressed in a variety of malignancies and have been linked to matrix metalloproteinase expression/activity, cell migration, invasion, and tumor metastasis (49, 50). Both COX-2 and CD44 (particularly variant CD44) have been implicated in the pathogenesis of colon cancer (51, 52). Dohadwala et al. (53, 54) investigated the impact of COX-2 expression in lung cancer invasiveness. They demonstrated that nonsmall cell lung cancer cells transduced with COX-2 cDNA showed enhanced invasive capacity and also over-expressed CD44.

In this study, ligation of CD44 by mAbs or its natural ligand HA induced VEGF secretion by EC. HA was a potent stimulus for VEGF production. This HA-driven VEGF secretion was completely abrogated by blocking CD44 antibodies that inhibit HA-mediated signaling. In addition, CD44-mediated COX-2 induction was significantly inhibited by a neutralizing VEGF antibody, indicating that the VEGF pathway is driving COX-2 activation. VEGF mediates its effects through two receptors, VEGFR-1 and -2. VEGFR-2 mediates much of the signaling in EC (15, 16), which leads to actin reorganization, membrane ruffling, and proliferation, resulting in vascular growth and angiogenesis. EC proliferation stimulated through CD44 (via mAbs and HA) was also completely inhibited by blocking CD44 mAbs that attenuate HA signaling. Importantly, this HA-stimulated EC proliferation was also blocked by a COX-2 (but not a COX-1)-specific inhibitor. The effects of high molecular mass HA on EC proliferation and, ultimately, vessel formation are complex and are believed to be mediated by multiple HA receptors. Savani et al. (9) have demonstrated that CD44 is the major determinant of EC adhesion to HA and EC proliferation, whereas another HA receptor, receptor for HA-mediated motility (RHAMM), regulates EC migration through the basement membrane substrate matrigel (9). Our finding that HA-CD44-mediated signaling in EC stimulates proliferation is consistent with this.

Previous studies have observed individual roles for CD44, COX, and VEGF in processes such as embryogenesis, angiogenesis, cellular proliferation, and wound healing and in pathophysiological conditions such as cancer and inflammation. However, this is the first study to demonstrate a mechanistic link between engagement of CD44, induction of COX-2 gene expression, and production of VEGF in EC signaling. This novel pathway and its products are potentially central to all of the above processes and may be a target used in the design of therapeutic strategies to treat tumor growth, diabetic retinopathy, and inflammatory conditions such as rheumatoid arthritis.

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Fig. 1

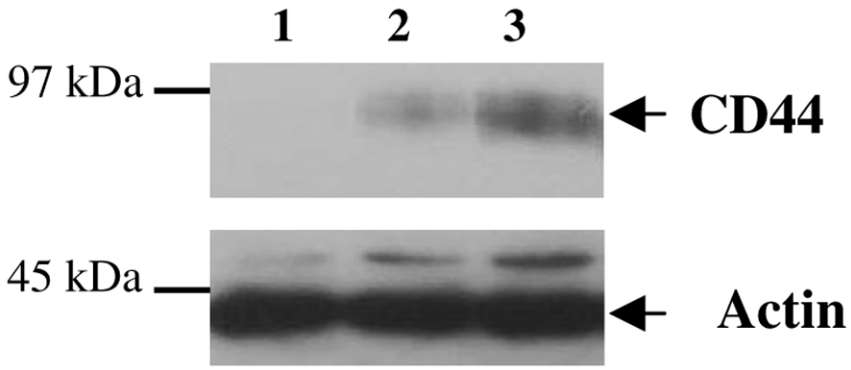

Figure 1. HUVEC express CD44. HUVEC (passage 2) were seeded onto 0.2% gelatin-coated T75 flasks at cell density of 1.5×10^5 per flask in M199 complete medium. They were then lysed 24 (lane 1), 48 (lane 2), and 72 (lane 3) h later and analyzed for CD44 and actin (loading control) expression by Western blot analysis. Anti-CD44 was Bric-238, and Anti-actin (polyclonal) was from Sigma.

Fig. 2

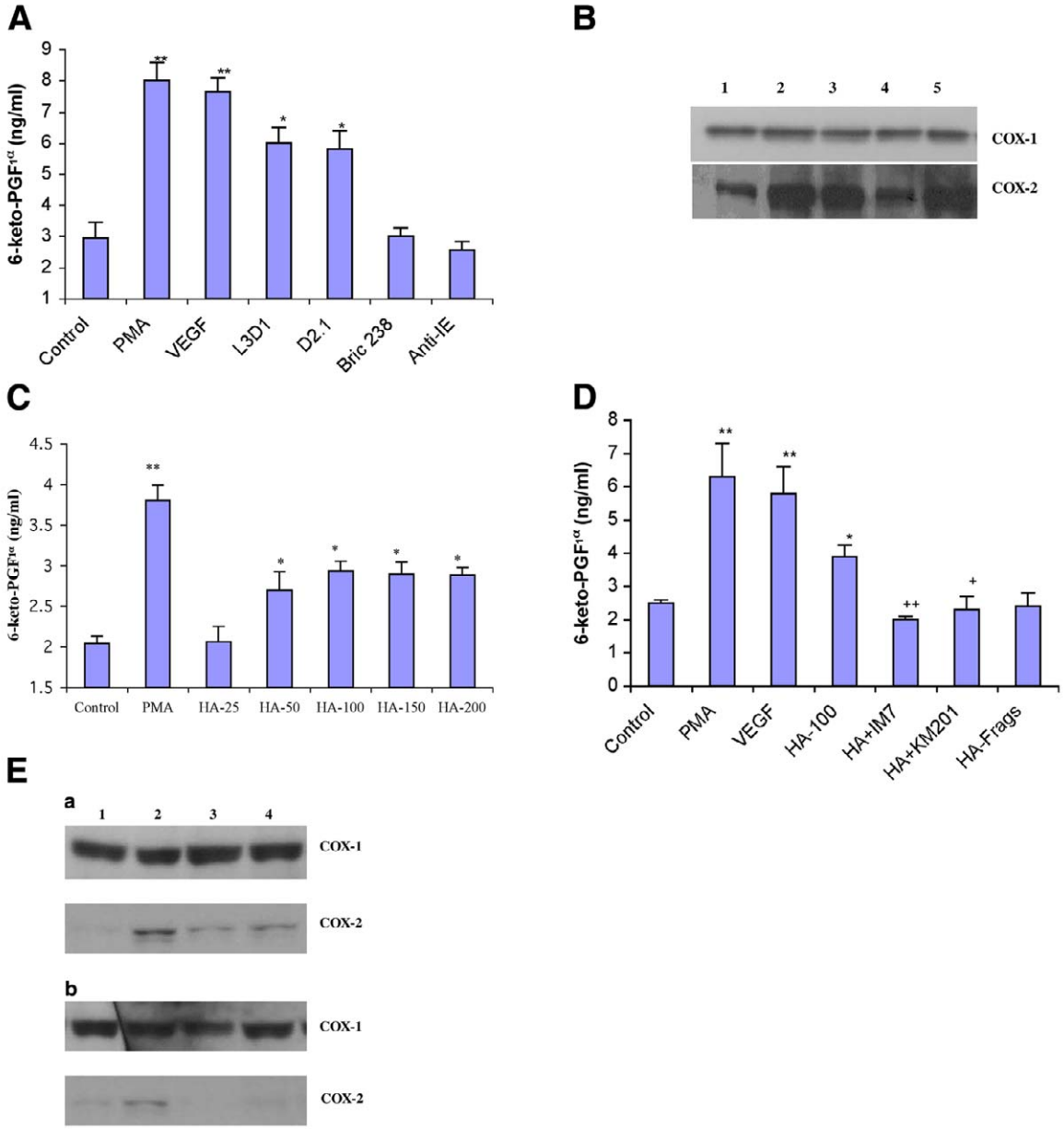

Figure 2. Anti-CD44 mAbs and high molecular weight HA induce COX-2. EC were plated onto 6- or 24-well plates and serum-starved overnight in M199/2.5% FBS. Aspirin (200 μM) was added for 45 min, washed, and then treated with mAbs, HA, HA fragments, or control agonist for a period of 3-4 h. Arachidonic acid was used as substrate to measure enzyme activity. COX protein expression was measured by Western blot analysis as described in Materials and Methods. Data are means ± SE from 5-6 independent experiments. ***A*)** 6-keto-$PGF_{1\alpha}$ generation after ligation of EC with stimulating CD44 antibodies (L3D1 and D2.1). PMA and VEGF were used as positive controls with the nonstimulating IgM anti-CD44 Bric 238 and an isotype Ig (Anti-IE) as negative controls. ***B*)** COX-1 and COX-2 protein induction by anti-CD44 mAbs as detected by Western blot analysis. Lane 1, control; lane 2, PMA; lane 3, D2.1; lane 4, Bric 238 (nonstimulating anti CD44); lane 5 L3D1. ***C*)** HA induces 6-keto-$PGF_{1\alpha}$ in a concentration-dependent manner. Cells were incubated with HA at concentrations ranging from 25 to 200 μg/ml. ***D*)** 6-keto-$PGF_{1\alpha}$ induction by HA is inhibited by blocking mAbs to CD44(IM7 or KM201). Blocking Abs were added 30 min before addition of HA. ***E*) *a*)** HA induces COX-2 but not COX-1 in EC. Lane 1, resting cells; lane 2, PMA (1 μM); lanes 3 and 4, cells incubated with high molecular weight HA (100 μg/ml) for 3 and 6 h, respectively. ***b*)** HA fragments induce neither COX-1 nor COX-2 in EC. Lane 1, resting cells; lane 2, PMA (1 μM); lanes 3 and 4, cells incubated with HA fragments (100 μg/ml) for 3 and 6 h, respectively. Data are means ± SE from 3-4 independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; HA vs. HA and mAbs $^{++}P < 0.01$; $^{+}P < 0.05$.

Fig. 3

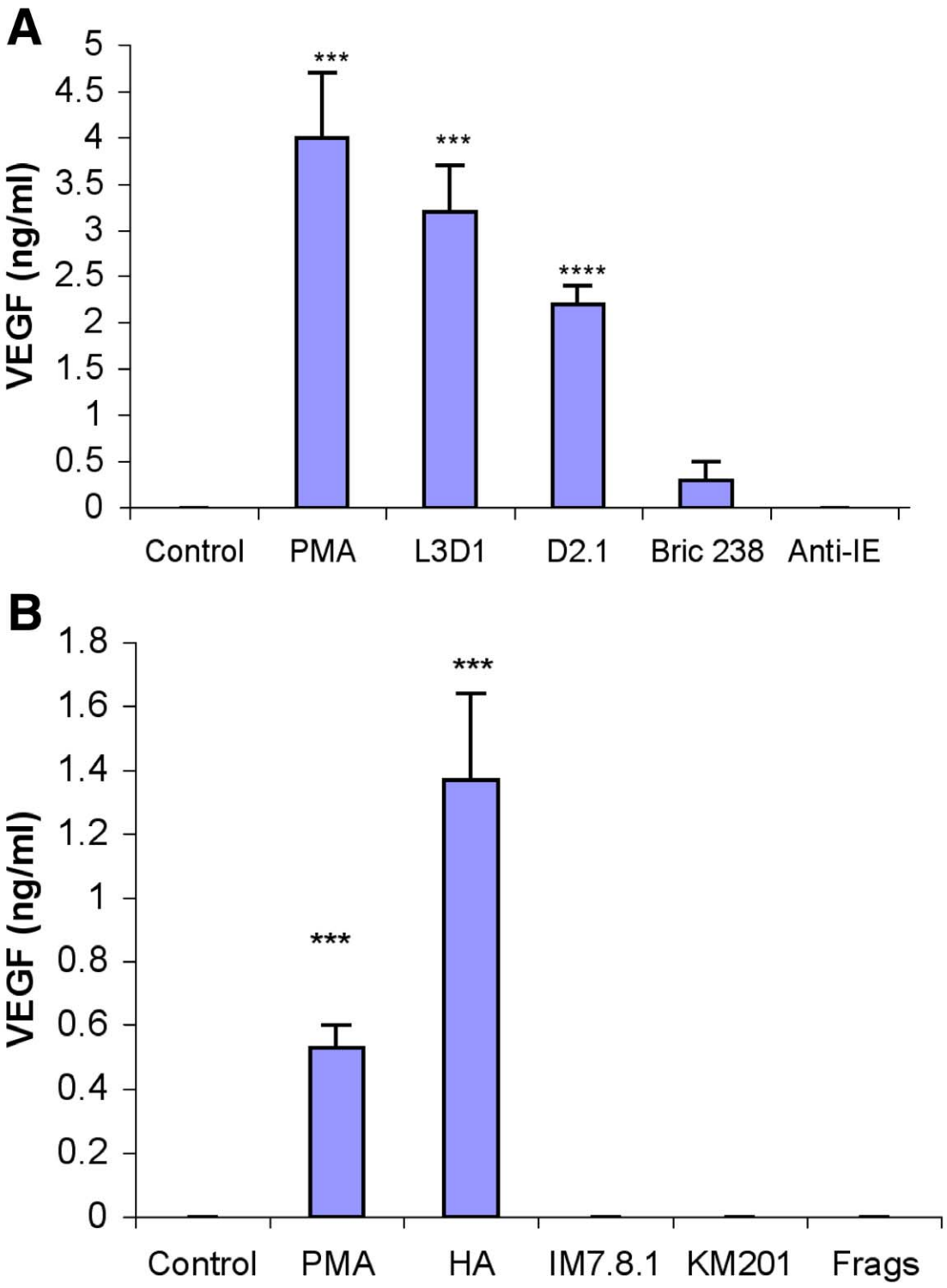

Figure 3. Effect of CD44 mAbs and HA on VEGF production. EC were plated onto 6- or 24-well plates and serum-starved overnight in M199/2.5% FBS. Aspirin (200 μM) was added for 45 min, washed, and then treated with mAbs, HA, HA fragments (Frags), or control agonist for a period of 3-4 h. At the end of the incubation period, supernatant samples were removed and assayed for VEGF by ELISA. ***A*)** Stimulation of VEGF production after ligation of EC with stimulating CD44 antibodies (L3D1 and D2.1). PMA was used as a positive control with the nonstimulating IgM anti-CD44, Bric 238, and an isotype Ig (Anti-IE) as negative controls. ***B*)** HA induces VEGF production that is inhibited by blocking mAbs. Blocking mAbs (IM7 or KM201) were added 30 min before addition of HA. Data are means ± SE from 3 independent experiments. ***$P < 0.005$; ****$P < 0.001$.

Fig. 4

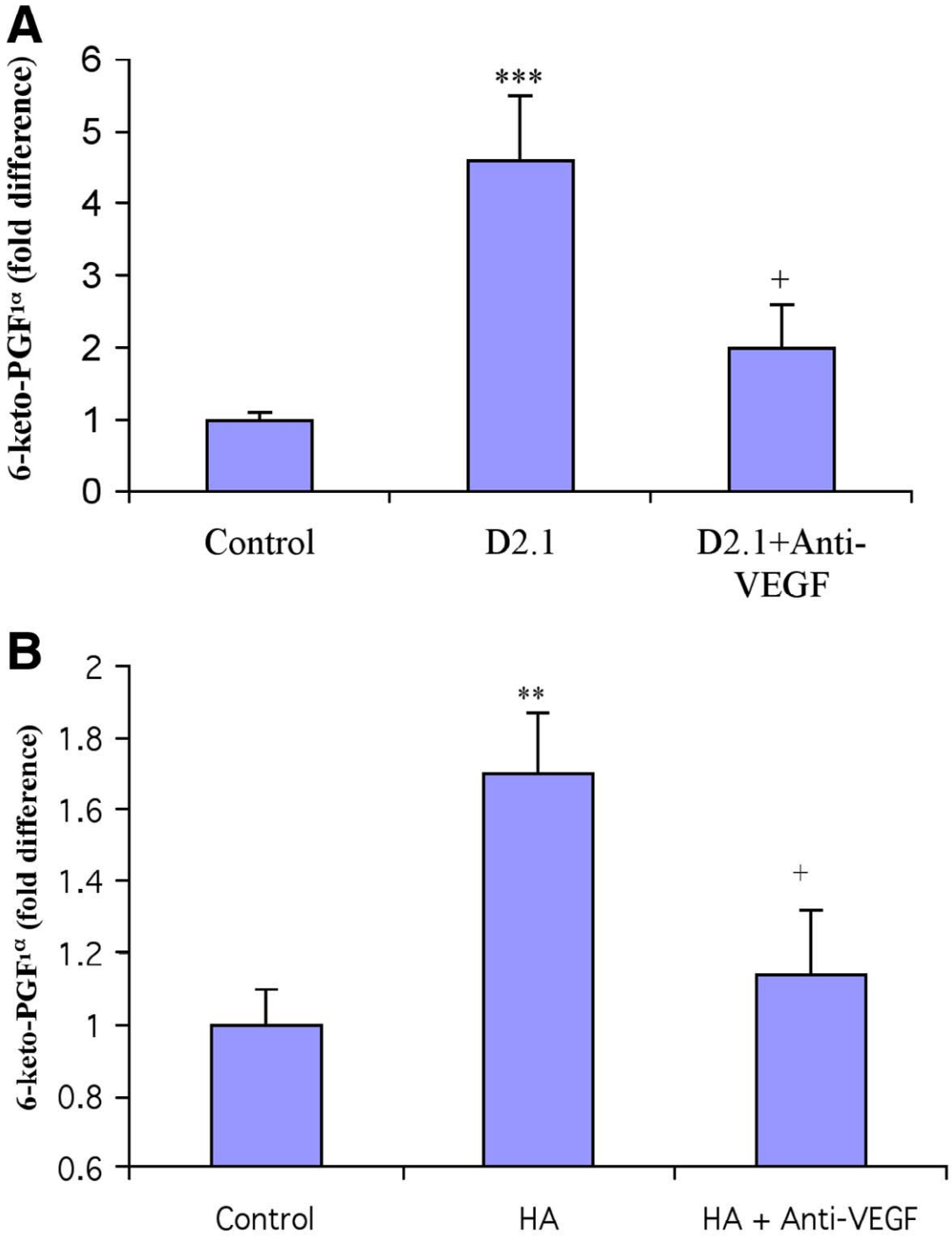

Figure 4. A neutralising antbody to VEGF inhibits Anti-CD44 mAb and high molecular weight HA induction of COX-2. EC were plated onto 6- or 24-well plates and serum-starved overnight in M199/2.5% FBS. Aspirin (200 μM) was added for 45 min, washed, and then treated with CD44mAb or HA for a period of 3-4 h. Neutralizing anti-VEGF was added 30 min before addition of anti-CD44 antibody or HA. Arachidonic acid was used as substrate to measure enzyme activity. ***A***) 6-keto-$PGF_{1\alpha}$ generation after ligation of EC with stimulating CD44 antibody (D2.1) in the presence and absence of neutralizing anti-VEGF. ***B***) 6-keto-$PGF_{1\alpha}$ generation after ligation of EC with HA in the presence and absence of neutralizing anti-VEGF mAb. Data are means ± SE from 3 independent experiments. ***$P < 0.005$, **$P < 0.01$, $^{+}P < 0.05$.

Fig. 5

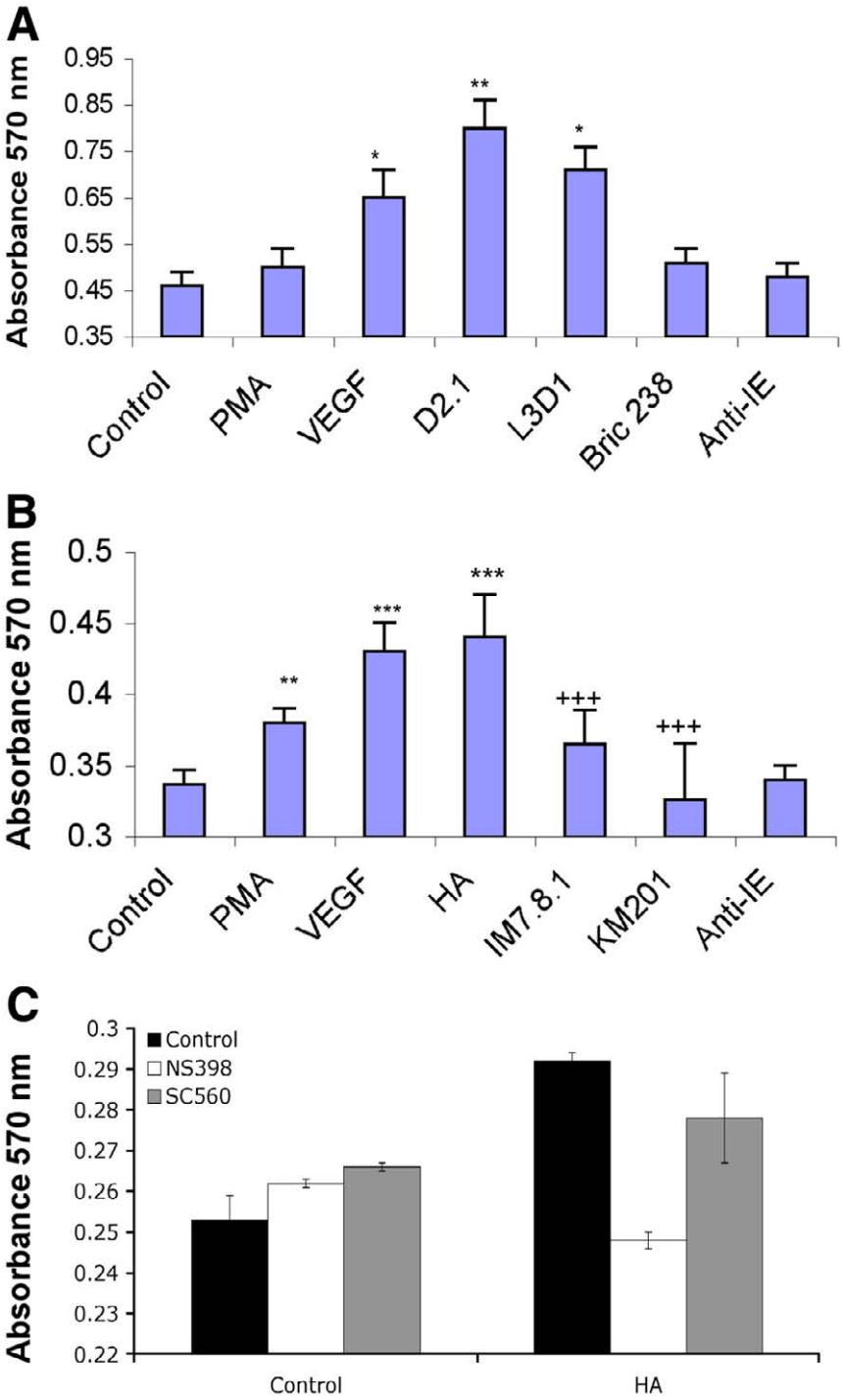

Figure 5. Effect of mAbs and HA on cell proliferation. EC were grown to 50-60% confluency in 96-well tissue culture plates, serum-starved (2.5% FBS) overnight, and then treated with aspirin for 45 min. MAbs, HA or HA fragments, in the presence or absence of blocking mAbs, were then added for 8-9 h. 3-[4, 5-dimethylthiazol-2-y]-2, 5-diphenyltetrazolium bromide (MTT) was then added for a further 1 h, cells were lysed in detergent, and absorbance was read at 570 nm. ***A*)** EC proliferation after ligation of cells with stimulating CD44 antibodies (L3D1 and D2.1). PMA and VEGF were used as positive controls with the nonstimulating IgM anti-CD44, Bric 238, and an isotype Ig (Anti-IE) as negative controls. ***B*)** HA induces EC proliferation that is inhibited by blocking mAbs. Blocking mAbs (IM7 or KM201) were added 30 min before addition of HA. ***C*)** HA-stimulated EC proliferation is attenuated by the COX-2-selective inhibitor NS398. COX-1 (SC 560, 1 μM) and COX-2 (NS398, 1 μM)-specific inhibitors were used, inhibitors were added 30 min before addition of HA. Data are means ± SE from 3-5 independent experiments. *P < 0.05; ***$P < 0.005$; ****$P < 0.001$; $^{+++}P < 0.005$; $^{++++}P < 0.001$.