CD44 Occupancy Prevents Macrophage Multinucleation

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Abstract. Cells of the mononuclear phagocyte lineage have the capability to adhere to and fuse with each other and to differentiate into osteoclasts and giant cells. To investigate the macrophage adhesion/fusion mechanism, we focused our attention on CD44, a surface glycoprotein known to play a role in hematopoietic cell–cell adhesion. We report that CD44 expression by macrophages is highly and transiently induced by fusogenic conditions both in vitro and in vivo. We show that CD44 ligands, hyaluronic acid, chondroitin sulfates, and osteopontin prevent macrophage multinucleation. In addition, we report that the recombinant extracellular domain of CD44 binds fusing macrophages and prevents multinucleation in vitro. These data suggest that CD44 may control the mononucleated status of macrophages in tissues by virtue of mediating cell–cell interaction.

Key words: CD44 • macrophage • adhesion • fusion • osteoclast • giant cell

Mononuclear phagocytes are cells which have the potential, in specific instances, to fuse and differentiate into either osteoclasts or multinucleated giant cells, in bone or in chronic inflammatory reactions, respectively. Multinucleation appears to endow mononuclear phagocytes with an added value, i.e., a powerful resorptive capability of extracellular components, i.e., bone or infectious agents (Vignery et al., 1989). Indeed, osteoclasts are responsible for the loss of bone that leads to osteoporosis.

Although osteoclasts and giant cells have long been recognized, the molecular mechanism by which their mononucleated precursors adhere and fuse with each other, a key step in their differentiation, remains poorly understood. Indeed, cell–cell fusion itself, whether it concerns that of sperm–oocyte or myoblast–myoblast, leading to fertilization and muscle development, respectively, has not been thoroughly investigated. It is thought that cell–cell adhesion leading to fusion involves a set of proteins similar to those used by viruses to fuse with host cells and inject their DNA or RNA (Hernandez et al., 1997). It has been hypothesized that viruses have stolen the fusion protein machinery from their target cells. It is now well accepted that virus–cell fusion requires both an attachment mechanism and a fusion peptide. One such example is HIV gp120 from the human immunodeficiency virus (HIV) which binds CD4 on T lymphocytes and macrophages (Dalgleish et al., 1984; Klatzmann et al., 1984), whereas the fusion molecule gp40, which arises from the same precursor molecule (gp160), is thought to trigger the actual fusion event. Although putative fusion molecules mediating sperm–oocyte and myoblast fusion have been reported (Blobel et al., 1992; Wakelam 1989), the actual protein machinery governing the attachment and fusion of these cells remains unknown.

Increasing evidence suggests that the molecular machinery mediating virus–cell and cell–cell adhesion/fusion is more complicated than anticipated and involves numerous players. Although it had been thought that HIV needed only the T lymphocyte receptor CD4 to bind and infect cells, several chemokines have now been demonstrated to slow the growth of HIV in cultures. It has been determined that the chemokine family of G protein–coupled receptors, most notably CC-CKR4 and CC-CKR5, are involved in HIV infection (Alkhatib et al., 1996; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996). There are now at least 10 chemokine receptors identified as HIV coreceptors (Dimitrov, 1997). Furthermore, the interaction between the adhesion molecules leukocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1) has been described with respect to both virus–cell and cell–cell adhesion/fusion events. HIV-
induced syncytium formation is blocked by a monoclonal antibody directed against the β subunit of LFA-1 (Hildreth and Orentas, 1989), whereas cytokine induced multinucleate giant cell formation from peripheral blood monocytes (Möst et al., 1990; Kazazi et al., 1994) as well as osteoclast development in vitro (Kurachi et al., 1993) are both inhibited by antibodies directed against LFA-1 and ICAM-1. The expression level of these molecules, however, does not correlate with multinucleation (Kurachi et al., 1993). Members of the cadherin family of homophilic cell adhesion molecules have also been suggested to play a role in cell–cell multinucleation. Although N-cadherin appears necessary for myoblast fusion (Mege et al., 1992), inhibition of E-cadherin function prevents the fusion of osteoclast precursors in vitro (Mbalaviele et al., 1995). A set of proteins thought to enhance or induce cell fusion, initially termed FRP-1 and FRP-2 and now known to be CD98 and integrin α3, respectively (Ohgimoto et al., 1995; Higuchi et al., 1998), have been recently identified in a number of cell lines infected with several different viruses as well as on the surface of monocytes and macrophages. Monoclonal antibodies directed against these proteins stimulate polykaryocyte formation in CD4+ U937 cells transfected with the HIV gp160 gene (Ohta et al., 1994) and in HeLa and FL cells infected with Newcastle disease virus (Ito et al., 1992). In addition, anti-FRP antibodies inhibit giant cell formation in cultures of peripheral blood monocytes (Tabata et al., 1994). Most recently, overexpression of the purigenic P2Z/P2X ligand may participate in the adhesion/fusion event of macrophages. CD44 is an integral membrane glycoprotein which plays an important role in both cell–cell and cell–substrate adhesion (Belitsos et al., 1990; Stamenkovic et al., 1991; Sy et al., 1991). CD44 exists as different isoforms in a wide variety of cells and tissues due to posttranslational modifications and alternative splicing (Tolg et al., 1993; for review see Naor et al., 1997). Our interest in CD44 as a protein that might be important for macrophage multinucleation was prompted by the following criteria: first, CD44 is involved in cell migration, lymphopoiesis and lymphocyte homing, in which it mediates cell–cell and cell–substrate interactions. A substantial body of data has been published suggesting that certain molecules involved in cell–cell adhesion, such as CD4 and LFA-1, play an important role in the infectivity and cytopathicity of viruses. (Pantaleo et al., 1991). Second, CD44 recognizes and binds to extracellular matrix elements such as hyaluronate (Lesley et al., 1990; Miyake et al., 1990), collagen type I, fibronectin (Jalkanen and Jalkanen, 1992) and osteopontin (Weber et al., 1996). The interaction of CD44 with extracellular components, in addition to cell surface molecules, could provide an additional regulatory mechanism to control multinucleation in macrophages. Third, the 100-kD form of CD44, the most common so-called standard form expressed by hematopoietic cells, has been demonstrated to be involved not only in the attachment of poliovirus to HeLa cells (Shepley and Racaniello, 1994) but also in the infection of mononuclear phagocytes by HIV (Rivendell et al., 1995). CD44 does not, however, act as a viral receptor in either of these two instances. We therefore set out to investigate whether CD44 played a role in macrophage multinucleation. We report here that CD44 expression by macrophages is highly and transiently induced by fusogenic conditions, and that CD44 ligands prevent multinucleation. We also show that the recombinant soluble extracellular domain of CD44 binds macrophages and prevents multinucleation in vitro. This suggests that CD44 and its putative cell–surface ligand may participate in the adhesion/fusion event of macrophages.

Materials and Methods

Cells

Rat alveolar macrophages were obtained from 12-wk-old Fisher rats (Charles River, Kingston, NY) by tracheobronchial lavage and cultured in fusogenic conditions as previously described (Vignery et al., 1990). Osteoclast-like cells were elicited in vivo by implanting syngeneic bone particles intramuscularly for 10 d in 12-wk-old Sprague Dawley rats (Charles River) as previously described (Vignery et al., 1989; 1990). The National Research Council’s guide for the care and use of laboratory animals was followed. COS-7 cells were a gift of M. Solimena (Yale School of Medicine, New Haven, CT).

Chemicals

Hyaluronic acid from human umbilical cord was purchased from Calbiochem-Novabiochem (La Jolla, CA) and Pharmacia Biotech (Uppsala, Sweden). Chondroitin sulfate A from bovine trachea and chondroitin sulfate B were purchased from Calbiochem-Novabiochem. Osteopontin was a kind gift of W.T. Butler (Texas Medical Center Dental Branch, Houston, TX). Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Antibodies

Mouse anti-rat macrophage CD44 (MRC OX8), CD4 (W3/25), and MHCII (RT1B) which are of the IgG1 isotype, were obtained from Serotec (Raleigh, NC). Fluorescein isothiocyanate (FITC)-conjugated F(ab)2 goat anti-mouse IgG (H + L chains) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Indocarbocyanine (Cy3)-conjugated F(ab)2 goat anti-mouse IgG (H + L chains) and goat anti-mouse IgG horseradish peroxidase conjugate were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Goat anti-GST and rabbit anti-goat IgG conjugated to horseradish peroxidase were obtained from Pharmacia Biotech. Mouse anti-myec was purchased from Invitrogen (Carlsbad, CA), and sheep anti-mouse horseradish peroxidase conjugate was purchased from Amersham (Arlington Heights, IL).

Immunolocalization

Tissues were prepared from both control and experimental rats implanted with bone particles (Vignery et al., 1989). The implants and the rat tissues were quick frozen and cut to 6-m-thick frozen sections using a Reichert-Jung cryostat (2800 Frigoulet; Leica, Deerfield, IL). The sections were first incubated overnight in PBS-milk (PBS supplemented with 5% nonfat dry milk [Carnation, Los Angeles, CA]), then for 2 h in PBS-milk containing anti-CD44, anti-CD4, anti-MHCII, or mouse IgG1. Sections were then incubated for 1 h in PBS-milk containing a 1:400 dilution of goat anti-mouse Cy3-F(ab)2. After three washes of 10 min each with PBS, the sections were imaged at 550 nm using the Cy3 excitation filter block on an Olympus microscope (Melville, NY) equipped with UV light. Cells were cultured on glass coverslips for the indicated time in MEM containing 5% human serum, fixed in formaldehyde for 1 h at 4°C, and then washed for 60 min in PBS-FCS (PBS + 10% FCS). The cells were incubated overnight in PBS-FCS supplemented or not with anti-CD44, anti-CD4, anti-MHCII, or mouse IgG1. After four washes of 15 min each in PBS-FCS (PBS with 10% FCS), the cells were incubated for an additional hour with...
Polymerase. The sequences of the primer pairs (used at a concentration of AmpliTaq DNA polymerase (Perkin Elmer), 1.25 mM MgCl₂, DNTP mix (New England Biolabs, Beverly, MA). The buffer condition pellets were resuspended in diethylpyro carbonate (DEPC)-treated water thawing the samples twice and then warming to 45°C. DNA was precipitated by the addition of 0.3 M sodium acetate and 3 vol of ethanol. The pellets were resuspended in diethylpyro carbonate (DEPC)-treated water and the concentration determined using optical density measurements taken in a Perkin Elmer UV/VIS spectrophotometer (Foster City, CA).

For Northern blot analysis, 8 µg of each RNA sample was electrophoretically separated on formaldehyde-agarose gels, blotted onto a nylon membrane (GeneScreen Plus; New England Nuclear, Boston, MA) and hybridized with a 32P-labeled PCR-generated DNA probe corresponding to the full-length CD44 cDNA. The signals on the autoradiogram were quantitated using a Linotype-Heil scanner (Eschborn, Germany) with a multi-analyst software for Macintosh (Bio-Rad Laboratories, Hercules, CA).

cDNA Synthesis and PCR

First-strand cDNA was synthesized as follows: 1 µg of total RNA was reverse transcribed using 200 U of MMLV reverse transcriptase (Boehringer Mannheim Biochemicals) in a 20-µl reaction primed with oligo dT primer (Invitrogen, San Diego, CA). The protocol followed was not published in the instruction manual from Clontech (Palo Alto, CA). The cDNA was aliquoted and stored at −70°C. For PCR reaction, 1 µl of cDNA was used as template in a 25-µl reaction mix containing 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer), 1.25 mM MgCl₂, and 0.1 mM DNTP mix (New England Biolabs, Beverly, MA). The buffer condition used for the PCR was a 1× dilution of the 10× mix provided with the polymerase. The sequences of the primers (pairs used at a concentration of 0.1 µg per reaction) were as follows: full-length CD44; primer forward (nucleotide [nt] 87–122): GATCCTTTGGTGCTAGCTGCAATCGAGCAAGG; reverse primer (nt 1187–1225): AAGTTAGTGGGACATGGACAAGG; primer forward (nt 86–122): AGATCCTTTGGCTCGAGCTGCACATGGACAAGG; reverse primer (nt 878–913): AGGTCTCCAGCTGCAATCGAGCAAGG; primer forward (nt 878–913): AGGTCTCCAGCTGCAATCGAGCAAGG; reverse primer (nt 878–913): AGGTCTCCAGCTGCAATCGAGCAAGG; reverse primer (nt 878–913): AGGTCTCCAGCTGCAATCGAGCAAGG; reverse primer (nt 878–913): AGGTCTCCAGCTGCAATCGAGCAAGG. The protocol followed was not published in the instruction manual from Clontech (Palo Alto, CA).

Production of CD44e: The Recombinant Soluble Extracellular Domain of CD44

The recombinant extracellular domain of CD44 (CD44e) was expressed using two different systems. CD44e was expressed as a fusion protein using the GST fusion protein system (Pharmacia Biotech). PCR amplification of this region was performed with a sense (5'TTACAGTTGAGCAATCTCCGCAGCAAATCATGGAACAGG; nt 158–195), and an antisense primer (5'AGGTCCTCCGCTCGAGAAAGTTGGGTCACTCC; nt 878–913), using rat macrophage cDNA as a template (made from total RNA) and PWO as polymers (Boehringer Mannheim, Mannheim, Germany). The primers were designed to allow digestion of the resulting PCR fragment with EcoRl and XhoI by means of which it was ligated in frame into an EcoRl-XhoI cut pGEX-4T-1 vector. The resulting construct encoded a fusion protein of ~50 kDa and was used to transform the protease-deficient Escherichia coli strain BL-21. Soluble GST-CD44e was isolated from 1 liter of bacterial culture using the bulk GST purification module as described by the manufacturer. The eluted protein was extensively dialyzed against PBS and stored at −70°C until ready for use. As a control, a pGEX-calreticulin construct was obtained courtesy of A. Helenius (Yale University) and GST-calreticulin was isolated and stored as described above.

CD44e was expressed in mammalian cells with a Myc-His fusion tag using the mammalian expression vector pcDNA 3.1/Myc-His A, B, C (Invitrogen, Carlsbad, CA). PCR amplification of the extracellular region was performed with a sense primer (5'AGATCTTTGGTGGAATCTCCGCAGCAACGG; nt 86–122) and an antisense primer (5'AGGTCTCCGCTCGAGAAAGTTGGGTCACTCC; nt 878–913), and rat macrophage cDNA as a template and PWO polymerase. The PCR product was digested with EcoRI and ligated into the EcoRI site of pCDNA 3.1/Myc-His B. This construct was used to transfect COS-7 cells using lipofectamine (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. Transfected cells were cultured for 72 h in Opti-MEM I reduced serum medium (GIBCO BRL). The recombinant protein was purified from culture supernatant using Invitrogen's Xpress protein purification system. Recombinant GST–CD44e was quantified by running 5 µl on a 10% SDS-polyacrylamide gel and staining with Coo massie brilliant blue. The intensity of the stained band was tested against that of a serial dilution of bovine serum albumin (BSA) run on the same gel. The concentration of Myc-His–CD44e was then determined by immobilizing serial dilutions of both GST–CD44e and Myc–His–CD44e on nitrocellulose using a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories). The immobilized proteins were reacted with anti-CD44 mAb followed by horseradish peroxidase (HRP)-linked sheep anti-mouse IgG (ECL kit; Amersham). The enzyme reaction was performed according to the manufacturer's instructions and the blots exposed for 30 s on X-ray films. Recombinant Myc-His–CD44e concentration was determined by comparative scanning densitometry of the dots against the concentration of GST–CD44e.

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Deglycosylation of Recombinant Myc-His-CD44e

Approximately 250 µg of recombinant Myc-His-CD44e was deglycosylated in its native form by incubation with 10 U of N-glycosidase F for 48 h at 37°C in 0.1 M sodium phosphate, pH 7.4. Deglycosylation was analyzed by comparative Western blot analysis of both native and N-glycosidase F (Boehringer Mannheim, Indianapolis, IN)-treated Myc-His-CD44e using anti-myc antibody (Invitrogen).

Fusion Assay Using Recombinant Proteins

Freshly isolated rat alveolar macrophages were plated at 5 × 10⁶ cells/ml and cultured in fusogenic milieu supplemented with either GST–CD44e, GST–Cal, or GST at the indicated concentrations. The cells were examined daily until day 4 to determine the effects of fusion proteins on macrophage multinucleation.

Binding Assay Using Recombinant Fusion Proteins

Freshly isolated rat alveolar macrophages were plated at 5 × 10⁶ cells/ml in triplicate wells using 96-well dishes (5 × 10⁴ cells/well). The cells were cultured overnight in fusogenic milieu. At the indicated times, duplicate sets of cells were supplemented with GST–CD44e, GST–Cal, or GST at the indicated concentrations. Binding proceeded overnight at 4°C. Media was removed from one set of cells and replaced with fresh media lacking recombinant protein to allow for dissociation. Dissociation proceeded for 3 h at 4°C. Media from all wells was then removed and the cells were fixed for 30 min in 4% paraformaldehyde. Cells were rinsed three times with PBS and blocked for 1 h in 5% milk/PBS. After three washes of 5 min in PBS, the cells were incubated with goat anti-GST for 30 min at room temperature. After three washes of 5 min each in PBS, the cells were incubated with rabbit anti–goat IgG-HRP for 30 min. After three final washes of 5 min each in PBS, 100 µl of peroxidase substrate (Moss) was added to each well and the optical density (OD) was read at 650 nm using an ELISA plate reader. Specific binding was determined as the difference in average OD values between the two sets of cells.

Statistical Analysis

Data are expressed as mean ± SD of mean. Comparative analyses of the means were performed with appropriate controls using independent Student’s t test to determine the 99% confidence level (P < 0.01).

Results

High Expression of CD44 by Fusing Macrophages In Vivo and In Vitro

To investigate whether fusing macrophages expressed detectable levels of CD44, rat osteoclast-like cells were induced in vivo by implanting syngeneic bone particles intramuscularly as previously described (Vignery et al., 1989, 1990). Sections obtained from bone implants, as well as from long bones, brain, liver, spleen, lymph nodes, kidney, lung, skin, striated muscle, and pancreas were reacted with mAb anti-CD44, anti-CD4, anti-MHCII, or IgG1, followed by goat anti–mouse IgG conjugated to Cy3. Although each of these tissues hosts resident macrophages and many of them contain cells such as lymphocytes and epithelial cells known to express CD44, only osteoclast-like cells from the bone implants exhibited a strong fluorescent signal (Fig. 1A) that was not detected in the presence of anti-CD4, anti-MHCII, or IgG1 (Fig. 1B). The signal was restricted to both mono- and multinucleated cells that were closely apposed to the bone implants. In the larger cells attached to the implants, the signal appeared stronger distal to the bone, i.e., concentrated in the nonadherent domain of the plasma membrane. This is the domain that faces the incoming fusing macrophages. None of the surrounding cells, such as muscle cells, fibroblasts, or endothelial cells expressed a detectable level of fluorescence.

To investigate whether CD44 expression by macrophages was associated with cell–cell interaction leading to fusion, alveolar macrophages were isolated and cultured in fusogenic conditions, i.e., plated at confluency, as previously described (Vignery et al., 1990). Macrophages cultured in such conditions initiate fusion a few hours after

Figure 1. High expression of CD44 on fusing macrophages in vivo (A) and in vitro (C and D). Multinucleation was induced in vivo by implanting rats intramuscularly with syngeneic bone particles that were recovered 10 d later and processed for immunolocalization. Frozen sections from bone implants were incubated with either mAb anti-rat CD44 (A) or mouse IgG1 (B) followed by goat anti–mouse IgG F(ab’2) fragments conjugated to Cy3. Multinucleation was induced in vitro by plating alveolar macrophages on glass coverslips and culturing them under fusogenic conditions for either 1 (C) or 3 d (D). Cells were subjected to immunocytochemistry using anti-rat CD44 followed by goat anti–mouse IgG F(ab’2) fragments conjugated to Cy3. Note the differences in fluorescence intensity between fusing cells. Bars, 20 µm.
plating and reach 99% fusion within 3–4 d. Although both resident and freshly plated alveolar macrophages failed to express detectable level of CD44 expression by immuno-cytochemistry (data not shown), their fusion was associated with a strong fluorescent signal that was detected as soon as 24 h after plating (Fig. 1 C). By day three, the plasma membrane of the unfused mononucleated macrophages exhibited a strong signal whereas that of the multinucleated ones had become lesser (Fig. 1 D). Neither CD4 nor MHCII, which are both expressed by macrophages, were detectable by this technique suggesting that their level of expression remained low, i.e., similar to that of CD44 in nonfusing macrophages.

**CD44 Expression Is Induced by Fusogenic Conditions**

To investigate the kinetics of CD44 expression during induction of fusion, rat alveolar macrophages were plated in fusogenic conditions and CD44 expression was determined by ELISA as a function of time after plating. As shown in Fig. 2 A, CD44 expression increased to reach a peak at a time that varied between 24 and 48 h. CD44 declined thereafter. In some experiments, a dramatic increase was detected as early as 5–10 h after plating (data not shown). This confirmed our morphological observation that CD44 expression was less abundant in multinucleated than mononucleated fusing macrophages (Fig. 1 D). In contrast to CD44, the expression of CD4 and MHCII remained low, and was not altered by fusogenic conditions in macrophages (Fig. 2 A). When subjected to Northern blot analysis, CD44 transcripts were found to be abundant in freshly isolated macrophages, and increased by 36 and 13% in fusing alveolar and peritoneal cells, respectively (Fig. 2 B). This suggested that the regulation of CD44 mRNA expression in macrophages may be both transcriptional and posttranscriptional.

**CD44 Ligands Inhibit Macrophage Adhesion/Fusion**

To analyze whether CD44 ligands, hyaluronic acid (HA), chondroitin sulfate A (CSA) and osteopontin (OP), all of which are extracellular matrix components, altered multnucleation, alveolar macrophages were cultured in fusogenic conditions in the presence of increasing concentrations of HA, CSA, or OP. Although alveolar macrophages are all fused after 4 d of culture under fusogenic conditions, the addition of HA, CSA, or OP prevented multinucleation in a dose-dependent manner (Fig. 3 A). In the presence of 1 mg/ml of HA, and to a lesser degree 1 mg/ml CSA and 1 μM OP, macrophages failed to form giant cells. To investigate whether CD44 played a role in macrophage attachment to their substrate, cells were plated onto surfaces coated with PBS, HA, or CSA. Although neither PBS nor CSA altered the adherence of macrophages, the coating of the dish with HA strongly prevented macrophages from adhering (Fig. 3 B). Interestingly, this inhibition was reversed by the addition of 1 mg/ml of HA to the culture medium, and to a lesser extent by the same concentration of CSA (Fig. 3 B, center panel). This suggested that the interaction of CD44 with HA was not required for macrophage attachment to their substrate. Indeed, it suggested that the possible clustering of CD44 receptors to the adherent domain of macrophages driven by HA prevented macrophage attachment. If so, soluble HA added to the culture medium may have allowed for the redistribution of CD44 to the nonadherent domain of the plasma membrane and thereby secured the adherence of the macrophages. This could even suggest that given the high expression level of CD44 molecules, their clustering to one (adherent) domain of the plasma membrane prevented (mechanistically) adhesion molecules (non-CD44) from reaching and clustering there as well, thereby preventing macrophage attachment. Altogether, our data suggested
that macrophage attachment was not facilitated by HA-CD44 interaction, and that indeed other adhesion molecules might mediate their adhesion to a substrate.

**HA Reduces CD44 Increased Expression by Fusing Macrophages**

To initiate studies on the mechanism by which HA, and to a lesser extent CSA and OP inhibited macrophage multinucleation, fusing alveolar macrophages were cultured in the presence of CSA, HA, or OP, and CD44 expression was determined by ELISA. As shown in Fig. 2, the culturing of macrophages in fusogenic conditions was associated with a rapid and dramatic increase in CD44 expression. Fig. 4 shows that HA appeared to significantly (Fig. 2 A) and dose dependently (Fig. 2 B) prevent the increase in CD44 expression that accompanies the onset of fusion. This inhibition did not occur with either CSA or OP. BSA, used as a negative control, also failed to alter the binding of mAb anti-CD44 (Fig. 4 A). Because of the possibility that mAb anti-CD44 binding was prevented by HA itself which competed for the same site on CD44, fusing cells were cultured in the absence or presence of HA, and further treated or not with HA by adding HA to the mAb anti-CD44 incubation buffer. Fig. 4 C shows that mAb anti-CD44 binding was significantly reduced when mAb anti-CD44 incubation buffer was supplemented with HA. This suggested that HA and mAb anti-CD44 competed for the same epitope on CD44. But mAb anti-CD44 binding was further reduced when these cells were cultured in the presence of HA. This suggested that HA did reduce CD44 expression in fusing macrophages. Altogether, these data indicated that the interaction of CD44 with its extracellular ligands participated in the adhesion process leading to fusion of macrophages.

**The Extracellular Domain of CD44 Binds Fusing Macrophages and Prevents Multinucleation**

Since the fusion of macrophages requires close membrane–membrane attachment/adhesion, and since fusing macrophages express high levels of CD44 on their surface, we hypothesized that the extracellular domain of CD44 itself may bind a putative cell surface determinant on macrophages and by so doing interfere with adhesion/fusion. We therefore generated a recombinant soluble form of the extracellular domain of CD44 attached to GST, hence a GST–CD44e fusion protein. The addition of GST–CD44e to fusing macrophages strongly and dose dependently prevented multinucleation (Fig. 5 A). Neither GST–calreticulin (Fig. 5, GST-Cal) nor GST used as controls altered multinucleation. Of interest, GST–CD44e and GST–Cal bound macrophages (Fig. 5 B). Although calreticulin is known to bind many cell types in vitro, our data suggest that CD44, and possibly its cell surface ligand, participates in macrophage–macrophage interaction leading to fusion.
Figure 3. CD44 ligands inhibit macrophage adhesion/fusion. (A) Rat alveolar macrophages were isolated, plated at $5 \times 10^6$ cells/ml in quadruplicate wells, using 96-well dishes ($5 \times 10^4$ cells/well), and cultured in fusogenic conditions for the indicated times, in the absence or presence of hyaluronic acid (HA), chondroitin sulfate A (CSA), or osteopontin (OP) at the indicated concentrations. (B) Alveolar macrophages were cultured as in A, but in dishes coated with either HA (1 mg/ml) or CSA (1 mg/ml). Bars, 100 μm.
Fusing Macrophages Express the Standard (Hematopoietic) Form of CD44

To identify the CD44 isoform expressed by fusing macrophages, total RNA was isolated from fusing rat alveolar macrophages was subjected to reverse transcription (RT)-PCR. The use of primers located in the most 3' and 5' regions of rat CD44 generated a PCR product of 1,138 bp (data not shown). Using primers located at either end of the extra- and intracellular domains generated PCR products of 827 and 218 bp, respectively. This suggested that both the extra- and intracellular domains of CD44 from fusing macrophages belonged to the standard form. To ensure that these PCR products encoded CD44, the full-length RT-PCR 1,138 bp product was cloned into TA cloning vector and subjected to DNA sequence analysis. DNA sequencing confirmed that fusing rat alveolar macrophages express the standard form of CD44 (data not shown).

Discussion

We have presented evidence that surface expression of CD44 is highly and transiently induced by macrophages at the onset of cell–cell adhesion/fusion in vitro and in vivo. We have also shown that CD44 ligands, HA, CSA, and OP, and the recombinant extracellular domain of CD44 prevent multinucleation. We therefore propose that CD44, along with its putative cognate cell surface ligand, is possibly a potential member of a protein machinery that participates in macrophage–macrophage interaction leading to fusion.

Although multinucleated osteoclasts and giant cells have long been recognized, the mechanism by which their mononucleated precursors adhere to and fuse with each other remains unclear. Although a number of adhesion molecules and regulatory proteins have been suggested to participate in macrophage multinucleation, the nature of the assays reported in the literature used to identify these molecules did not allow one to investigate the direct role of these molecules in cell–cell adhesion and fusion. These assays used bone marrow cells and monocytes; thus, cells that are impure and have not yet acquired the status of the macrophage. These cells require culturing time to further differentiate and become fusion competent. In contrast, we have used a highly pure and fast assay whereby cells adhere to each other to initiate fusion within hours after plating, and reach 99% multinucleation within 3–4 d. This assay allows the investigation of molecules involved in...
early cell–cell interaction and possibly fusion (Vignery et al., 1989). Indeed, using this assay, we reported earlier the purification of a protein the expression of which is induced by fusogenic conditions in macrophages (Saginario et al., 1995). The subsequent cloning of the cDNA coding for that protein revealed that it belongs to the superfamily of IgG (Saginario et al., 1998). The question as to whether that protein associates with CD44 during cell–cell interaction remains open.

Although CD44 was long known to mediate cell–cell interaction/aggregation, its possible involvement in macrophage–macrophage adhesion leading to fusion had not yet been investigated. From our data, it appears that fusion-competent macrophages express, in an inducible and transient manner, a very high level of surface CD44. This suggests that constitutive CD44 may secure the mononucleated status of macrophages in tissues. It also suggests that macrophage adhesion/fusion requires an overexpres-

Figure 5. Recombinant extracellular CD44 fusion protein (GST-CD44e) inhibits macrophage fusion. (A) Rat alveolar macrophages were isolated, plated at 5 × 10⁶ cells/ml in quadruplicate wells, using 96-well dishes (5 × 10⁴ cells/well), and cultured in fusogenic conditions for 3 d, in the presence of increasing concentrations of GST–CD44e, GST–Cal, and GST. (B) Cells were cultured as in A for 17 h and then incubated for 3 h at 4°C with GST–CD44e. Binding was determined by ELISA using anti-GST antibody coupled to horseradish peroxidase as described in Materials and Methods. Specific binding was determined by subtracting nonspecific binding from total binding for each concentration of GST–CD44e. Bar, 100 μM.
prevent the clustering of CD44 on the adherent domain of macrophages do not fuse with peritoneal macrophages (our unpublished observation). The role of glycosylation of CD44 in macrophage–macrophage interaction remains an important subject to investigate.

It is interesting to note that the expression of macrophage surface molecules known to participate in cell–cell interaction such as CD4 and MHCII was not altered by fusogenic conditions. Although their lack of induced expression does not exclude their participation in adhesion/fusion, this could be further suggestive of a possible role for CD44 in macrophage–macrophage interaction leading to fusion.

The question as to how HA prevents macrophage multinucleation is interesting. One possibility is that HA activates macrophages (Nobel et al., 1993) and we have previously reported that activation of macrophages prevents fusion (Vignery et al., 1990). Another possibility is that HA competes with the putative cell surface ligand for CD44, thereby blocking CD44 from interacting with its ligand. Indeed, we observed that partially deglycosylated COS cell produced CD44e-recovered binding of macrophages and prevented their multinucleation (data not shown). This suggested that sugar moieties added to CD44 by cells other than alveolar macrophages may not be functionally relevant. Macrophages appear to process CD44 posttranslationally in a tissue-specific manner (Camp et al., 1991), and although circumstantial, alveolar macrophages do not fuse with peritoneal macrophages (our unpublished observation). The role of glycosylation of CD44 in macrophage–macrophage interaction remains an important subject to investigate.

The fact that macrophages do not attach to HA-coated surfaces suggests that CD44 may not be required for macrophages to adhere onto a substrate. Indeed, it suggests that the possible clustering of CD44 molecules to the adherent domain of the plasma membrane of macrophages may mechanistically prevent adhesion molecules from accessing that domain, thereby preventing macrophages from adhering. This possibility is further suggested by the fact that the addition of HA, and to a lesser extent CSA, to the culture medium reverses this inhibitory effect of HA-coated surfaces on macrophage attachment. Indeed, this may be the case when macrophages adhere to, and fuse onto bone that contains OP. There again, the presence of HA and CSA in the extracellular microenvironment may prevent the clustering of CD44 on the adherent domain of the macrophages.

Although cell–cell adhesion leading to fusion appears to involve a complicated protein machinery, the present findings open possibilities to investigate the role of CD44 and its putative cell surface ligand in the interaction of not only macrophages but also potentially those of sperm cells with oocytes and myoblasts with myoblasts, the other developmental cell–cell adhesion/fusion events. Indeed, the mammalian oocyte–cumulus complex contains an extracellular matrix rich in HA (Cherr et al., 1990), and HA has been suggested to interact with the sperm surface, at the time of initiation of acrosome reaction (Vandevooort et al., 1997). Our observation that CD44 is highly expressed by fusing macrophages and that both CD44 ligands and soluble CD44 prevent multinucleation, opens avenues to study the role of CD44 in osteoclast and giant cell differentiation.

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