Interaction between the Adhesion Receptor, CD44, and the Oncogene Product, p185HER2, Promotes Human Ovarian Tumor Cell Activation*

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In this study we have examined the interaction between CD44s (the standard form) and the p185HER2 proto-oncogene in the ovarian carcinoma cell line. Surface biotinylation followed by wheat germ agglutinin column chromatography and anti-CD44-mediated immunoprecipitation indicate that both CD44s and p185HER2 are expressed on the cell surface and most importantly, that these two molecules are physically linked to each other via interchain disulfide bonds. We have also determined that hyaluronic acid stimulates CD44s-associated p185HER2 tyrosine kinase activity, leading to an increase in the ovarian carcinoma cell growth.

After transfection of the ovarian carcinoma cell line with the adenosine 5’EIA gene, which is known to repress p185HER2 expression, we observed that both surface CD44s expression and CD44s-mediated cell adhesion to hyaluronic acid are significantly reduced in the transfected cells compared with the control cells. These data suggest that down-regulation of p185HER2 blocks CD44s expression and subsequent adhesion function. Our findings also indicate that the CD44s-p185HER2 interaction is both functionally coupled and biosynthetically regulated. We believe that direct “cross-talk” between these two surface molecules (i.e. CD44s and p185HER2) may be one of the most important signaling events in human ovarian carcinoma development.

CD44 is a transmembrane glycoprotein that is widely distributed in different cell types and tissues (for reviews see Refs. 1 and 2). The extracellular domain of CD44 is extensively modified by N,O-glycosylation and glycosaminoglycan addition (2, 3) and is responsible for the binding of extracellular matrix materials such as hyaluronate and collagen (2–4) as well as fibronectin (5). The cytoplasmic domain of CD44 contains at least one ankyrin binding site (6). Post-translational modification of the CD44 cytoplasmic domain by either acylation (7), protein kinase C (8), or GTP binding (9) enhances the binding between CD44 and ankyrin. The transmembrane interaction between CD44 and the cytoskeleton appears to play an important role in the early signal transduction events leading to cell activation (10).

The gene encoding the CD44 protein (located on human chromosome 11) contains 20 exons, of which only 10 are expressed in the standard form (CD44s). The remaining 10 exons are expressed in different combinations in the extracellular domain of variant isoforms of the protein (CD44v), generated by alternative splicing of the mRNA (11, 12). Some of these CD44v isoforms are selectively expressed on certain tumor cell surfaces during metastasis (13–15). In addition, high levels of CD44s/CD44v expression have been correlated with the progression of various carcinomas (13–15). At the present time, information concerning possible factors involved in triggering CD44v formation and CD44s overexpression during tumorigenesis or metastasis is very limited.

Ovarian carcinoma is the most lethal tumor of the female genital tract and continues to be the major cause of mortality in female cancer patients. Ovarian cancer has a unique pattern of spreading, which is primarily by intraperitoneal seeding (16). Recently, it has been shown that ovarian cancer cells express CD44s predominantly, which causes very strong adhesion to peritoneal mesothelium (17, 18). It has also been reported that a significant reduction in tumor implants occurred in nude mice 5 weeks after intraperitoneal injection of ovarian cancer cells incubated with anti-CD44 antibody compared with injected cells pretreated with antibodies to other cell surface proteins (17, 18). These findings suggest that CD44 plays an important role in the implantation of ovarian cancer metastasis.

The HER2 oncogene (also called c-erbB-2 or neu) encodes a 185-kDa (p185HER2) membrane protein that contains a single transmembrane spanning region, two cystine-rich extracellular domains and a tyrosine kinase-associated cytoplasmic domain (19). This protein belongs to the epidermal growth factor receptor subgroup of the receptor-linked tyrosine kinase superfamily (20). Overexpression and amplification of HER2 oncogenes have been found to correlate with poor survival of many known cancers including ovarian cancer (21, 22). Hung and co-worker (23) have shown that the HER2 oncogene is also overexpressed in certain ovarian carcinoma cell lines, such as SKOV3.ip1, shown to display high tumorigenic and metastatic potential. Therefore, coexpression of both CD44s and p185HER2 appears to be closely associated with ovarian cancer progression.

In this study we have addressed the question of whether...
there is any interaction between CD44s and p185HER2 in ovarian tumor cells using various human ovarian carcinoma cell lines including SKOV3.ipl (established from ascites of a nude mouse given an intraperitoneal injection of SKOV3 human ovarian carcinoma cell line) and derivatives (e.g. two stable transfectant cell lines designated as SKOV3.ipl.E1A (expressing E1A genes) and SKOV3.ipl.Efs (E1A frameshift mutants)) as model systems. Our results clearly indicate that these two surface molecules (i.e. CD44s and p185HER2) are physically linked, functionally coupled, and biosynthetically regulated together. We proposed that the interaction between CD44s and p185HER2 may be critically important for the onset of tumor genesis and spreading during ovarian tumor development.

MATERIALS AND METHODS

Cell Lines and Culture—The SKOV3.ipl cell line was established from ascites that developed in a nude mouse given an intraperitoneal injection of SKOV3 human ovarian carcinoma cell line (obtained from the American Type Culture Collection) as described previously (23). Cells were grown in Dulbecco’s modified Eagle’s medium, F12 medium supplement (Life Technologies, Inc.) supplemented with 10% fetal bovine serum.

DNA Transfection—Two stable transfectant cell lines, SKOV3.ipl.E1A transfectants (expressing E1A genes) and SKOV3.ipl.Efs were prepared as a control cell line (to make sure the changes in transformation phenotypes (if any) in ipy.E1A transfectants were not due to the selection process or to transfection of the plasmids and the pSV2-neo gene) (23).

Immunoreagents—Monoclonal rat anti-CD44 antibody (Clone 620, Isotype IgG2α, obtained from CMB-TECH, Inc., Miami, FL) used in this study recognizes a common determinant of the CD44 class of glycoproteins including CD44s and other variant isoforms (15) and is capable of precipitating all CD44 variants. Monoclonal mouse anti-p185HER2 antibody (c-neu-ab-3) and anti-phosphotyrosine antibody were purchased from Oncogene Science, Inc. (Manhasset, NY) and Zymed Laboratories Inc. (PY-plus™, clone P720, IgGα, South San Francisco, CA), respectively.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Southern Blot Analysis—Total RNA was extracted from 1 g of human ovarian cells (e.g. SKOV3.ipl) and processed for RT-PCR (using specific primer pairs (e.g. exon 5 and 15 primer pairs)) and Southern blot analyses as described previously (15).

Northern Blot Analysis—Twenty μg of total RNA isolated from SKOV3.ipl cells was separated on a 1.2% agarose-formaldehyde gel, transferred to nylon membrane, and hybridized to the CD44s cDNA probe at 42 °C for 16 h.

Cell Surface Labeling Procedures—Human ovarian carcinoma cells (SKOV3.ipl) and derivatives (e.g. SKOV3.ipl.E1A2 and SKOV3.ipl.Efs) suspended in PBS were surface-labeled using the following boutinylaion procedure. Briefly, cells (107 cells/ml) were solubilized with sulfo-uccimimidobiotin (Pierce) (0.1 mg/ml) in labeling buffer (150 μM NaCl, 0.1 M HEPES (pH 8.0) for 30 min at room temperature) followed by extensive dialysis against PBS buffer (0.1 M phosphate buffer (pH 7.5) and 150 mM NaCl). This biotinylated material was analyzed by SDS-PAGE under reducing conditions, transferred to the nitrocellulose filters, and incubated with ExtrAvidin peroxidase. After an addition of peroxidase substrate, the blots were developed using Renaissance chemiluminescence reagent according to the manufacturer’s instructions.

Immunoprecipitation and Immunobloting Techniques—After SDS-PAGE, the 280-kDa gel band that is precipitated by all CD44 variants. Monoclonal mouse anti-p185HER2 antibody (c-neu-ab-3) and anti-phosphotyrosine antibody were purchased from Oncogene Science, Inc. (Manhasset, NY) and Zymed Laboratories Inc. (PY-plus™, clone P720, IgGα, South San Francisco, CA), respectively.

Double Immunofluorescence Staining—Human ovarian carcinoma cells such as SKOV3.ipl were washed with PBS buffer and fixed by 2% paraformaldehyde. Fixed cells were then incubated with rat anti-CD44 antibody (10 μg/ml) and fluorescein-labeled goat anti-rat antibody (10 μg/ml) to detect surface CD44 expression. Subsequently, these fluorescein-labeled cells were rendered permeable by ethanol treatment and stained with monoclonal mouse anti-p185HER2 followed by rhodamine-conjugated goat anti-mouse IgG. To detect nonsppecific antibody binding, fluorescein-labeled cells were incubated with normal mouse IgG followed by rhodamine-conjugated goat anti-mouse IgG. No labeling was observed in such control samples.

RESULTS AND DISCUSSION

CD44 Expression in Human Ovarian Tumor Cells—The expression of CD44 is known to be closely correlated with the metastatic and proliferative behavior of a variety of tumor cells including human ovarian tumor cells (13–15, 17, 18). To examine the expression of CD44 transcripts at the mRNA level, total
CD44-p185HER2 Interactions Activates Tumor Cells

RNA from ovarian tumor cells (SKOV3.ipi) was isolated and analyzed by RT-PCR with exon 5 and 15 primer pairs (designed to amplify the open reading frame region of CD44 involved in the alternative splicing of several exons) (15). By employing ethidium bromide staining (Fig. 1A, lane 1) of agarose gels followed by Southern blot hybridization (Fig. 1A, lane 2), we have identified a major PCR product of 156 base pairs that comigrates with an amplifier of CD44s (the standard form) in human ovarian tumor cells (e.g., SKOV3.ipi). Northern blot analysis also reveals a single 2.8-kilobase CD44s transcript that is expressed in these cells (Fig. 1B). Nucleotide sequence data confirm that this transcript represents the CD44 standard form, CD44s (data not shown).

CD44 expression at the mRNA level does not always correlate with cellular protein expression. Therefore, it is important to further characterize CD44 protein expression in the human ovarian tumor cells. To examine CD44 expression on the surface of ovarian tumor cells, we have utilized surface biotinylation techniques and a specific monoclonal anti-CD44 antibody that recognizes the standard form of CD44 (CD44s) as well as other variant isomers (15). Our results indicate that a single surface-biotinylated polypeptide (molecular mass ~ 85 kDa) displaying immunological cross-reactivity with CD44s is preferentially expressed on the cell surface of human ovarian cells (Fig. 1C, lane 1). No CD44s-containing material is observed in control samples when normal rat IgG is used (Fig. 1C, lane 2). Therefore, we believe that the major CD44 isoform expressed on the surface of the human ovarian tumor cells (SKOV3.ipi) is CD44s. These results are consistent with previous findings indicating ovarian cancer cells express predominantly CD44s (17, 18).

Analysis of a Complex Formed between CD44s and p185HER2—Both CD44s and the p185HER2 oncogene are often overexpressed (as much as 100-fold) in human tumor cells as a consequence of gene amplification and/or transcriptional regulation (15, 24, 25). In this study we addressed the question regarding whether there is an interaction between CD44s and p185HER2 in human ovarian tumor cells. Using the nonionic detergent Triton X-100 to extract surface-biotinylated cells followed by sequential wheat germ agglutinin-Sepharose column chromatography and anti-CD44-mediated immunoprecipitation, we found that only one surface-biotinylated 85-kDa CD44s band is revealed under reducing conditions by SDS-PAGE (Fig. 2, lane 1). However, under nonreducing conditions, we have detected a large polypeptide (~280 kDa) that is co-isolated with 85-kDa CD44s (Fig. 2, lane 2). To further characterize this large polypeptide, the 280-kDa protein was first eluted from the nonreducing SDS-PAGE gel and processed for biotinylation labeling. Under reducing conditions, we found that this biotinylated 280-kDa polypeptide is readily reduced into two proteins (Fig. 2, lane 3). One corresponds to p185HER2 (185 kDa) and the other is CD44s (85 kDa) as verified by anti-p185HER2 (Fig. 2, lane 4) and anti-CD44s immunoblot (Fig. 2, lane 5), respectively. Preliminary data indicate that the p185HER2 associated with the plasma membrane of SKOV3.ipi cells is not surface-biotinylated. However, if p185HER2 is denatured by SDS treatments, the sites for biotinylation of this molecule become available. This is the reason why we eluted the 280-kDa complex (Fig. 2, lane 6) and biotinylated again to detect both p185HER2 and CD44s (Fig. 2, lane 7). In addition, we carried out anti-p185HER2 or anti-CD44s-mediated precipitation followed by anti-CD44s immunoblot or anti-p185HER2 immunoblot, respectively, using reducing SDS-PAGE analyses. Our results indicate that the CD44s band is revealed in anti-p185HER2-immunoprecipitated materials (Fig. 2, lane 6). The p185HER2 band can also be detected in the anti-CD44s-immunoprecipitated materials (Fig. 2, lane 7). These findings clearly indicate that CD44s and p185HER2 are closely associated with each other in a complex involving interchain disulfide bonds in
Effects of HA on CD44s-associated Function—HA has been shown to play an important role in several important physiological functions such as maintaining cartilage integrity, balancing homeostasis of water and plasma proteins in the intercellular matrix, and promoting mitosis and cell migration (28). In addition, degradation products of HA containing 3–25 disaccharide units are known to promote angiogenesis, which involves cell proliferation, migration, and tube formation (29–31). CD44 is the major hyaluronan cell surface receptor (32) and cellular adhesion molecule in many different cell types (1, 2). Specific HA binding motifs have been identified and localized in the extracellular domain of a number of CD44 isoforms (33).

In this study we examined the ability of ovarian tumor cells to adhere to HA-coated plates. Our data indicate that ovarian tumor cells display a high level of CD44-specific cell adhesion to HA-coated plates (Table I). After treatment with specific rat anti-CD44 antibody, the cells were largely inhibited from displaying HA-mediated cell adhesion (Table I). These results confirm that CD44s is a hyaluronan receptor required for HA-mediated cell adhesion in these ovarian cells.

Adenovirus 5 E1A (E1A) products have been shown to be involved in regulating the expression of a number of important regulatory molecules (e.g. p185HER2 and nm23) in fibroblast model systems (34, 35). For example, the E1A products act as effective suppressors of invasion and metastasis in p185HER2-overexpressed 3T3 mouse fibroblasts (34). In addition, E1A is involved in elevating the expression of nm23 (a metastatic tumor suppressor gene) in ras-transfected rat embryo fibroblasts (35). These results suggest that E1A genes play an important role in suppressing certain cellular events leading to the metastatic cascade. Previously, it has been shown that introduction of the E1A gene into SKOV3.ipi ovarian cancer cells (e.g. SKOV3.ipi.E1A2) results in the suppression of p185HER2-induced metastatic properties, including tumor cell adhesion and invasion (23, 35).

In this study we determined the effects of E1A genes on regulating CD44s and p185HER2 interaction in ovarian tumor cells. Our results indicate that control cells (SKOV3.ipi.Efs) without E1A (Fig. 4, A–D, lane 1) express very high levels of p185HER2 (Fig. 4B, lane 1). However, in transfectants (e.g. SKOV3.ipi.E1A2) (Fig. 4A, A–D, lane 2) containing E1A (Fig. 4A, lane 2), p185HER2 oncogene expression is significantly suppressed (Fig. 4B, lane 2). These results are consistent with previous findings reported by Hung and co-workers (22, 23).

Most importantly, CD44s expression in those transfectants containing E1A is also concomitantly decreased (Fig. 4C, lane 2) as compared with that in the control cells (Fig. 4C, lane 1). Our recent results indicate that up-regulation of p185HER2 and CD44s promotes CD44s-mediated cell adhesion (36). The fact that CD44s-mediated cell adhesion to HA-coated plates dis-
In this study, we found that CD44s-associated to be closely associated with tumor progression and metastasis. This tightly coupled interaction between CD44s and p185

plays significant reduction in the E1A transfectants (Table I) suggests that down-regulation of these two molecules (e.g. CD44s and p185HER2 expression as described under “Materials and Methods.” A, immunoblot of E1A using anti-E1A in SKOV3.ipl.E1A2 (lane 1) and SKOV3.ipl.E1A2 (lane 2). B, immunoblot of CD44s using anti-CD44 in SKOV3.ipl.E1A2 (lane 1) and SKOV3.ipl.E1A2 (lane 2). C, immunoprecipitation of CD44 using anti-CD44 in surface-biotinylated SKOV3.ipl.E1A2 (lane 1) and SKOV3.ipl.E1A2 (lane 2). D, immunoblot of actin using anti-actin in SKOV3.ipl.E1A2 (lane 1) and SKOV3.ipl.E1A2 (lane 2).

other molecules (including epidermal growth factor, neuregulins, neu-activating factor, ascites sulfglycoprotein-2, a factor from bovine kidney (NEL-GF), and unknown factors in serum) have been implicated as potential agonists for the activation of p185HER2-linked tyrosine kinase activities (20). Therefore, we believe that the results of this study provide new evidence that the physiological ligand for CD44s, HA, may also play an important role in activating CD44s-associated p185HER2 kinase activity required for the onset of tumor cell growth and spreading during ovarian tumor development.

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Fig. 4. Characterization of E1A, p185HER2 and CD44 expression in human ovarian stable transfectants SKOV3.ipl.E1A2 and SKOV3.ipl.Efs. SKOV3.ipl.E1A and SKOV3.ipl.Efs transfectants were established according to the procedures described previously (23). These transfectants were then used for the analysis of CD44 and p185HER2 expression as described under “Materials and Methods.” A, immunoblot of E1A using anti-E1A in SKOV3.ipl.E1A2 (lane 1) and SKOV3.ipl.E1A2 (lane 2), B, immunoblot of p185HER2 using anti-p185HER2 in SKOV3.ipl.E1A2 (lane 1) and SKOV3.ipl.E1A2 (lane 2), C, immunoprecipitation of CD44 using anti-CD44 in surface-biotinylated SKOV3.ipl.E1A2 (lane 1) and SKOV3.ipl.E1A2 (lane 2). D, immunoblot of actin using anti-actin in SKOV3.ipl.E1A2 (lane 1) and SKOV3.ipl.E1A2 (lane 2).

Fig. 5. HA-stimulated tyrosine phosphorylation of p185HER2 (A) and ovarian cell growth in vitro (B). A, analysis of HA-stimulated tyrosine phosphorylation of p185HER2 (complexed with CD44). Surface-biotynlated SKOV3.ipl cells (5 × 10⁵ cells) (treated with HA (50 μg/ml) for 1 h, pretreated with anti-CD44 followed by HA treatment (50 μg/ml) for 1 h, or untreated) were processed for anti-CD44-mediated immunoprecipitation to obtain the CD44p185HER2 complex as described under “Materials and Methods.” Lane 1, anti-phosphotyrosine- mediated immunoblot of p185HER2 (upper panel) associated with surface-biotynlated CD44s (lower panel) in a CD44s-p185HER2 complex as shown in Fig. 2 in human ovarian tumor cells (SKOV3.ipl) without any HA treatment. Lane 2, anti-phosphotyrosine-mediated immunoblot of p185HER2 (upper panel) associated with surface-biotynlated CD44s (lower panel) in a CD44s-p185HER2 complex as shown in Fig. 2 in human ovarian tumor cells (SKOV3.ipl) pretreated with anti-CD44 antibody followed by HA treatment. B, measurements of in vitro cell growth. The in vitro cell growth of SKOV3.ipl cells (5 × 10⁵ cells/well) (treated with HA (50 μg/ml), pretreated with anti-CD44 followed by HA treatment (50 μg/ml), or untreated in Dulbecco’s modified Eagle’s medium, F12 medium for 24 h) were measured by the 3-(4,5-dimethyl thiazol-2-yl)-2,5-di-phenyl tetrazolium bromide assays as described under “Materials and Methods.” a, cell growth in the presence of HA treatment; b, cell growth without any HA treatment; c, cell growth with anti-CD44 antibody pretreatment followed by HA treatment.

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