ADAM28 Is Overexpressed in Human Breast Carcinomas: Implications for Carcinoma Cell Proliferation through Cleavage of Insulin-like Growth Factor Binding Protein-3

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Abstract
A disintegrin and metalloproteinases (ADAMs) are involved in various biological events including cell adhesion, cell fusion, membrane protein shedding, and proteolysis. In the present study, our reverse transcription-PCR analysis showed that among the 12 different ADAM species with a putative metalloproteinase motif, prototype membrane-anchored ADAM28m and secreted-type ADAM28s are selectively expressed in human breast carcinoma tissues. By real-time quantitative PCR, their expression levels were significantly higher in carcinomas than in nonneoplastic breast tissues. In situ hybridization, immunohistochemistry, and immunoblotting analyses indicated that ADAM28 is predominantly expressed in an active form by carcinoma cells within carcinoma tissues. A direct correlation was observed between mRNA expression levels and proliferative activity of the carcinoma cells. Treatment of ADAM28-expressing breast carcinoma cells (MDA-MB231) with insulin-like growth factor-1 (IGF-I) increased cell proliferation, cleavage of IGF binding protein (IGFBP)-3, as well as IGF-1 cell signaling; these processes were all significantly inhibited by treatment with ADAM inhibitor or anti-ADAM28 antibody. Down-regulation of ADAM28 expression in MDA-MB231 cells with small interfering RNA significantly reduced cell proliferation, IGFBP-3 cleavage, and growth of xenografts in mice. In addition, cleavage of IGFBP-3 in breast carcinoma tissues was correlated with ADAM28 expression levels and inhibited by treatment with ADAM inhibitor or anti-ADAM28 antibody. These results show that ADAM28 is overexpressed in an activated form in human breast carcinoma cells and suggest that ADAM28 is involved in cell proliferation through enhanced bioavailability of IGF-I released from the IGF-I/IGFBP-3 complex by selective IGFBP-3 cleavage in human breast carcinomas. (Cancer Res 2006; 66(20): 9913-20)

Introduction
A disintegrin and metalloproteinases (ADAMs) are a recently discovered gene family of proteins with sequence similarity to the reprotoxin family of snake venomases (1). Typical members of the ADAM family are type I transmembrane proteins with a unique domain structure composed of a signal sequence and the following domains: propeptide, metalloproteinase, disintegrin, cysteine-rich, epidermal growth factor-like, transmembrane, and cytoplasmic domains (2–5). Thirty-three members have been identified in mammalian species, Caenorhabditis elegans, Xenopus, and Drosophila. However, only one third of the members possess the zinc-binding consensus motif in their metalloproteinase domains (4, 5). These metalloproteinase-type ADAM proteins play a role in shedding of membrane proteins, cell adhesion, cell fusion, and cell signaling (2, 3, 6–9). ADAM28 is one of the metalloproteinase-type ADAMs and is expressed in two alternative forms [i.e., a prototype membrane-anchored form (ADAM28m) and a short secreted form (ADAM28s); ref. 10]. ADAM28 cleaves myelin basic protein and the ectodomain of CD23 (11, 12). In addition, we have recently reported that ADAM28, prepared by activation of the precursor with matrix metalloproteinase (MMP)-7, releases insulin-like growth factor (IGF)-I from the IGF-I/IGFBP binding protein (IGFBP)-3 complex by selective digestion of IGFBP-3 into the two major fragments (13).

A characteristic of malignant tumor cells is their ability to autonomously proliferate, invade surrounding tissues, and metastasize to distant organs. Accumulated lines of evidence have shown that MMPs play an important role in tumorigenesis and progression of various human cancers including breast carcinomas through degradation of the extracellular matrix such as basement membrane components (14, 15). Because the catalytic site within the metalloproteinase domain of ADAMs has high sequence homology to that of MMPs, it is expected that similar to MMPs, ADAMs may be involved in carcinogenesis as well as in invasion and metastases of human breast carcinomas, as they are expressed and activated within carcinoma tissues. The expression of ADAM9 (16) and ADAM12 (17) has been shown in human breast carcinomas, and ADAM10, 15, 17, and 20 are expressed in gastric carcinomas (18). Our previous study also showed that ADAM12 is overexpressed in an active form by glioblastoma cells in human astrocytic tumors and is involved in glioma cell proliferation (19). In addition, we have recently reported that ADAM28s and ADAM28m are overexpressed by carcinoma cells in human non–small-cell lung carcinomas (20). However, there have been no systematic studies on the expression of ADAM family members in human breast carcinomas and little is known about the role of ADAM28 in these carcinomas.

In the present study, we screened the expression of 12 different metalloproteinase-type ADAM species and found selective expression of ADAM28 in human invasive breast carcinoma tissues.

Note: Y. Mitsui and S. Mochizuki contributed equally to this work.

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3 See http://www.people.virginia.edu/%7Ejw7g/Table_of_the_ADAMs.html.
ADAM28 expression was compared with clinicopathologic variables and its tissue localization in carcinomas was determined by in situ hybridization and immunohistochemistry. We also examined the implications of ADAM28 in breast carcinoma cell proliferation, IGFBP-3 degradation, and IGF-I-induced cell signaling. The results provide the first evidence that ADAM28 is overexpressed in breast carcinoma cells and promotes carcinoma cell proliferation through degradation of IGFBP-3.

Materials and Methods

Clinical samples and histology. Fresh tissue samples were resected from patients with primary breast carcinoma (34 cases) who underwent surgery at Keio University Hospital, Tokyo, Japan. Control nonneoplastic breast tissues (16 cases) were obtained from sites remote from the carcinomas. All tumor tissues were obtained at primary resection, and none of the patients had been subjected to chemotherapy or radiation therapy before surgery. The samples were snap-frozen immediately after surgical removal and stored at −80°C. Human breast carcinomas were classified based on the standard criteria of WHO International Classification of Breast Tumors (21). The 34 cases of carcinomas included invasive ductal carcinomas (32 cases) and invasive lobular carcinomas (2 cases). Informed consent for experimental use of the surgical samples was obtained from each patient according to institutional ethical guidelines.

Reverse transcription-PCR and real-time quantitative PCR. Total RNA was isolated from frozen sections of breast carcinoma and nonneoplastic control breast tissues and evaluated with Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Reverse transcription-PCR (RT-PCR) for ADAM8, ADAM9 (ADAM9m and ADAM9s), ADAM10, ADAM12 (ADAM12m and ADAM12s), ADAM15, ADAM17, ADAM19, ADAM20, ADAM21, ADAM28m, ADAM28s, ADAM30, and housekeeping gene β-actin was carried out, and specific PCR amplification from the target mRNAs was confirmed by sequencing of PCR products as previously described (19, 20). For quantitative analysis of ADAM28s and ADAM28m expression, cDNA was used as template in a TaqMan real-time PCR assay (ABI Prism 7000 Sequence Detection System, Applied Biosystems, Foster City, CA), and sample data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) according to our previous methods (20).

In situ hybridization. To identify cells expressing ADAM28 mRNA, the carcinoma samples (five cases) were subjected to in situ hybridization according to our previous methods (20). BLASTN searches were done to confirm the specificity of the probe encoding ADAM28 nucleotides 952 to 1132. Paraffin sections of the tissues were hybridized with digoxigenin-labeled RNA antisense or sense probes, followed by immunohistochemistry using the peroxidase-labeled avidin-biotin-complex method. After the reaction, the sections were counterstained with hematoxylin.

Immunohistochemistry. Paraffin sections were subjected to immunohistochemistry for ADAM28 using mouse monoclonal antibody against ADAM28 (10 μg/mL; 297-2F3) and biotinylated secondary antibody to mouse immunoglobulin G (IgG) according to our previous methods (20). As control, sections were reacted by replacing the first antibody with nonimmune mouse IgG. For immunostaining of Ki-67, the sections were reacted with mouse monoclonal antibody against Ki-67 (MIB1; DAKO A/S, Glostrup, Denmark) following antigen retrieval (19, 20). The percentage of ADAM28-immunostained carcinoma cells to total carcinoma cells was measured by observing five different fields at ×200 magnification without knowledge of the clinical and real-time PCR data as previously described (22). MIB1-positive cell index was determined in a similar way (19, 20).

Immunoblotting and zymographical analyses. For immunoblotting of ADAM28, homogenate supernatants of breast carcinoma tissues (10 μg/ lane) were subjected to SDS-PAGE under reducing conditions and the resolved proteins on gels were transferred onto polyvinylidene difluoride membranes. The membranes were blotted with anti-ADAM28 antibody specific to the metalloproteinase domain of ADAM28 (5 μg/mL; 297-2F3; ref. 20), anti-ADAM28m antibody specific to the cytoplasmic domain of ADAM28m (1 μg/mL, CL1ADAM28; Cederlane Labs, Hornby, Ontario, Canada), or anti-β-actin antibody (0.2 μg/mL; Sigma-Aldrich, St. Louis, MO); immunoreactive protein bands were detected with enhanced chemiluminescence Western blotting reagents (Amersham Pharmacia Biotech, Piscataway, NJ; ref. 20). To study the effects of a synthetic ADAM inhibitor (KB-R7785; a kind gift from Dr. Koichi Yoshino, Carnabioscience, Kobe, Japan; refs. 8, 13) or anti-ADAM28 antibody (297-2F3; refs. 20) on IGF-FP-3 cleavage in breast carcinomas, carcinoma tissue fragments (−3 × 3 × 3 mm) were cultured in serum-free DMEM for 24 to 48 hours in the presence or absence of 1 μM/L KB-R7785 or 5 μg/mL anti-ADAM28 antibody, and the media (800 μL/lane) were subjected to immunoblotting with anti-IGF-FP-3 antibody specific to the COOH-terminal domain of IGF-FP-3 (0.2 μg/mL; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Inhibitor activity of KB-R7785 against ADAM species including ADAM28 was previously reported (8, 13). Inhibition of ADAM28 activity with anti-ADAM28 antibody (297-2F3) was shown in an assay of IGFBP-3 degradation by ADAM28s (data not shown).

Immunoblotting analyses were also done using the human breast carcinoma cell lines MDA-MB231 and MCF-7. After synchronization and growth arrest, cells were treated with 1 μM/L KB-R7785, 5 μg/mL anti-ADAM28 antibody (297-2F3), or 100 μg/mL aprotinin (Sigma-Aldrich) 30 minutes before stimulation with 100 ng/mL IGF-I (Sigma-Aldrich) in the presence of each agent for 24 hours, and culture media were subjected to immunoblotting with anti-ADAM28 antibody or anti-IGF-FP-3 antibody as described above. Inhibition of IGFBP-3 degradation by the treatments was analyzed by densitometry using NIH Image 1.63. For analysis of IGF-I cell signaling, supernatants of the cell homogenates (10 μg/lane) were subjected to immunoblotting with anti–phospho-IGF-type I receptor (p-IGF-I) antibody (0.2 μg/mL; Cell Signaling Co., Beverly, MA), anti-IGF-IR antibody (0.2 μg/mL; Cell Signaling), anti–phospho-extracellular signal-regulated protein kinase (p-ERK)-1/2 antibody (0.2 μg/mL; Cell Signaling), or anti-ERK1/2 antibody (0.2 μg/mL; Cell Signaling). The effect of mitogen-activated protein kinase kinase (MEK) inhibitor on ERK1/2 phosphorylation in MDA-MB231 cells was examined by treatment of cells with 50 μM/L PD98059 (Calbiochem, San Diego, CA) 30 minutes before the IGF-I stimulation for 24 hours. Culture media of MDA-MB231 cells, treated with or without IGF-I under serum-free DMEM containing 0.2% lactalbumin hydrolysate, were subjected to gelatin and carboxymethylated transferrin zymography for detection of MMPs (23, 24).

Cell proliferation assay. The mitogenic effects of IGF-I on MDA-MB231 and MCF-7 cells were measured with 5-bromo-2-deoxy-uridine (BrdUrd) Labeling and Detection Kit III (Roche Molecular Biochemicals, Basel, Switzerland) according to the instructions of the manufacturer. After synchronization and growth arrest, cells were treated with 100 ng/mL IGF-I in DMEM containing 1% fetal bovine serum (FBS). After 6 hours, BrdUrd (10 μM/L) was added to the media and cultured for the next 42 hours. To determine the involvement of ADAM28 activity in the mitogenic effect of IGF-I, cells were incubated with 1 μM/L KB-R7785 or 5 μg/mL anti-ADAM28 antibody 30 minutes before the IGF-I treatment, and then reacted with BrdUrd for 42 hours in the presence of IGF-I. Similarly, the effects of MEK inhibition on cell proliferation were examined by treating the cells with 50 μM/L PD98059. For determination of MIB1-positive cell index, breast carcinoma cells cultured on Lab-Tek II Chamber Slides (Nalge Nunc, International, Rochester, NY) were treated with 100 ng/mL IGF-I after reaction with 1 μM/L KB-R7785 or 5 μg/mL anti-ADAM28 antibody. Cells were immunostained with antibody against Ki-67 as described above.

Transient transfection of ADAM28 small interfering RNA to cell lines. A 21-oligonucleotide small interfering RNA (siRNA) with the sequence of ADAM28 (GAGACTCTATCCACTGCTAA) and control nonsilencing oligonucleosome (AATTTCTGGAACGTTACGCT) were custom synthesized at Qiagen, Inc. (Valencia, CA). Transfection was done with the Nucleofector Kit (Amaxa, Inc., Gaithersburg, MD). siRNA (2 μg) was added to 1 × 10^6 cells suspended in 100 μL of solution T for MDA-MB231 cells and solution V for MCF-7 cells. The program A-23 (for MDA-MB231) or P-20 (for MCF-7) was selected for high density of transfection. The cells were then plated in 12-well plates and incubated in DMEM containing 5% FBS. After 48 hours, they were subjected to immunoblotting analysis or proliferation assay as described above.

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In vivo effects of ADAM28 siRNA on growth of xenografts in mice. MDA-MB231 cell suspension (2 \times 10^6/0.1 mL HBSS) was injected s.c. to male and female BALB/c-nu/nu mice (Charles River, Yokohama, Japan). Because there was no difference in tumor growth in male and female mice, male mice were used for further experiments with ADAM28 siRNA. To enhance the in vivo transfection efficiency of siRNA with less toxicity, cationic cardiolipin analogue (CCLA)-based liposome (NeoPharm, Inc., Waukegan, IL) was used as a transfection reagent (25). At 3 days after tumor cell injection, the mice were randomized into three groups. Two groups were treated with ADAM28 siRNA or nonsilencing siRNA in CCLA-based liposome. Based on data of a previous study (25) and our preliminary experiments, we determined optimal siRNA doses, charge ratios of siRNA to CCLA-based liposome, and injection schedule. Then, the siRNA solutions (100 μL) were injected into the s.c. tissues near the xenografts on days 3, 5, and 7 at 3.5 mg/kg/d of siRNA in CCLA-based liposome with a 1:2 charge ratio of siRNA to CCLA-based liposome. One group of mice were injected with an equal volume of 10% sucrose (v/v) as a vehicle control. The three dimensions, height (h), length (l), and width (w), of each tumor were measured at the indicated times, and volumes were calculated according to the following formula: volume = \( \frac{\pi h(l^2 + 3a^2)}{6} \), where \( a = \frac{w + l}{4} \) (ref. 26).

Statistical analysis. Results between the two independent groups were determined with the Mann-Whitney \( U \) test. Comparisons among more than three groups were determined by the Kruskal-Wallis test. Statistical analyses were carried out using StatView statistical software on a personal computer. \( P < 0.05 \) was considered significant.

Results

mRNA expression of ADAM species in human breast carcinomas. The mRNA expression of ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, ADAM19, ADAM20, ADAM21, ADAM28s, ADAM28m and ADAM30 was screened by RT-PCR in eight matched carcinoma and control nonneoplastic breast tissues, in the latter of which normal histology was microscopically verified. As shown in Fig. 1A, ADAM8, ADAM20, and ADAM30 were expressed in neither the carcinoma nor the nonneoplastic tissues, and the expression of ADAM19 was detected in only 12.5% of the carcinoma samples. In contrast, ADAM21 was constitutively expressed in all the carcinoma and control breast tissues. ADAM10 and ADAM17 were expressed in all the carcinoma samples, but they were also expressed in 37.5% and 12.5% of the control tissues, respectively. The expression of ADAM9, ADAM12, ADAM15, ADAM28s, and ADAM28m seemed to be selective in the carcinoma tissues because none of the eight control breast tissues showed expression of these ADAM species (Fig. 1A). Although the expression rate of ADAM9, ADAM12, and ADAM15 was 87.5% in the carcinoma tissues, ADAM28s and ADAM28m were expressed in all the carcinoma samples examined. Thus, we focused on ADAM28s and ADAM28m and analyzed their expression levels in a larger number of carcinoma and control breast tissues by real-time quantitative PCR.

mRNA expression levels of ADAM28 species and their correlation with MIB1-positive cell index. RNA samples of the breast carcinomas \( (n = 34) \) and control nonneoplastic breast tissues, some of which showed partial fibrocystic changes \( (n = 16) \), were subjected to real-time quantitative PCR for ADAM28s and ADAM28m. As shown in Fig. 1B, the relative expression level of ADAM28s mRNA in the carcinomas (3.26 ± 8.80, mean ± SD) was significantly 20-fold higher than that in the control tissues (0.09 ± 0.17; \( P < 0.0001 \)). Similarly, the expression level of ADAM28m in carcinomas (2.22 ± 3.67) was significantly 20-fold higher than that in the control tissues (0.11 ± 0.31; \( P < 0.0001 \); Fig. 1B). Most breast carcinoma samples (~65%) showed high expression of ADAM28s and ADAM28m, but others showed an expression level similar to that of nonneoplastic breast tissues (Fig. 1B), suggesting that breast carcinomas can be classified to carcinomas with high expression of ADAM28 and carcinomas with low expression of ADAM28.

MIB1-positive cell index determined by immunohistochemistry was 27.9 ± 26.0% in the breast carcinomas, and no staining was observed in the nonneoplastic breast tissues (data not shown). When the index was plotted against mRNA expression levels of ADAM28s and ADAM28m in each case, there were direct correlations between the index and the expression levels \( (r = 0.759, P < 0.001; n = 34) \) or ADAM28m (Fig. 1C), suggesting that the ADAM28 species play a role in cell proliferation. On the other hand, no significant correlations were obtained between the mRNA expression levels and the clinicopathologic variables of breast carcinomas including age, menopausal status, tumor size, histologic type, lymph node metastasis, and tumor stage (data not shown).

*ADAM28 in Human Breast Carcinomas*

**Figure 1.** The mRNA expression of ADAM28s and ADAM28m in the human breast carcinomas and the correlation with carcinoma cell proliferation. A, RT-PCR analysis of 12 different ADAM species with a putative metalloproteinase motif in the invasive breast carcinoma and nonneoplastic breast tissues. Total RNA was extracted from the carcinoma tissues (lanes 1-8, T) and the nonneoplastic control breast tissues (lanes 1-5, N), and reverse transcribed into cDNA followed by a PCR reaction as described in Materials and Methods. C, positive control for RT-PCR (see ref. 20). B, real-time quantitative PCR analysis of the relative mRNA expression levels of ADAM28s and ADAM28m in the breast carcinomas and nonneoplastic breast tissues. Bars, indicate mean value. ***, \( P < 0.0001 \) n.d., not detected. C, correlation between MIB1-positive cell index and ADAM28 mRNA expression levels in the breast carcinomas. Note that MIB1-positive cell index directly correlates with ADAM28s/GAPDH \( (r = 0.759, P < 0.001; n = 34) \) or ADAM28m/GAPDH \( (r = 0.577, P < 0.01; n = 34) \).
Localization and protein expression of ADAM28 in breast carcinoma tissues. By in situ hybridization, the signal for ADAM28 was observed predominantly in the carcinoma cells with the antisense probe, although the sense probe gave only a background signal in the carcinoma tissues (Fig. 2A). Immunohistochemistry showed that ADAM28 is localized predominantly to cytoplasm of the carcinoma cells in ~65% of the carcinoma samples (22/34 cases; Fig. 2B). A few samples (2 of 22 positive cases) showed membrane staining as well as cytoplasmic staining (data not shown). The proportion of ADAM28-immunostained carcinoma cells to total carcinoma cells was 35.4 ± 38.7%, and a significant correlation was observed between the immunoreactivity and the mRNA expression levels (data not shown). Negligible or no staining was observed with nonimmune mouse IgG (Fig. 2B). By immunoblotting with anti-ADAM28 antibody (297-2F3), an immunoreactive band of 42 kDa was identified in the carcinoma tissues (Fig. 2C, lanes 1-4), but no such species was recognized in the control nonneoplastic breast tissues with normal histology (Fig. 2C, lanes 5-7). After a longer exposure of the films, carcinoma samples showed, besides the 42-kDa band, the 55/57-kDa bands, which were also immunoreactive with antibody specific to the cytoplasmic domain of ADAM28m (Fig. 2D). No immunoreactive bands were detected in the tissue samples when the immunoblotting was done with nonimmune IgG (data not shown).

Involvement of ADAM28 in IGF-I-induced cell proliferation of human breast carcinoma cells. When the mRNA expression of ADAM28s and ADAM28m in human breast carcinoma cell lines, MDA-MB231 and MCF-7, was examined by RT-PCR, MDA-MB231 cells showed definite expression of ADAM28s and ADAM28m, but negligible expression of these ADAM28 species was observed in MCF-7 cells (Fig. 3A). By immunoblotting with anti-ADAM28 antibody (297-2F3), a 42-kDa immunoreactive band was detected in culture media of MDA-MB231 cells, but not MCF-7 cells (Fig. 3A). When the cell lines were treated with IGF-I, BrdUrd incorporation was significantly increased 2.5-fold and 1.4-fold with MDA-MB231 and MCF-7 cells, respectively (P < 0.0001; Fig. 3B). On the other hand, enhanced BrdUrd incorporation in IGF-I-treated MDA-MB231 cells, but not in MCF-7 cells, was significantly inhibited after treatment with KB-R7785 (P < 0.0001) or anti-ADAM28 antibody (P < 0.0001; Fig. 3B). KB-R7785 did not affect the proliferation of MDA-MB231 cells without IGF-I treatment (data not shown). Stimulatory effects of IGF-I on cell proliferation was confirmed by MIB1-positive cell index: 24.1 ± 3.1% versus 68.2 ± 6.0% for the control and IGF-I-stimulated MDA-MB231 cells and 29.4 ± 2.3% versus 48.7 ± 10.7% for the control and IGF-I-stimulated MCF-7 cells, respectively (Fig. 3C). MIB1-positive cell index in IGF-I-stimulated MDA-MB231 cells was significantly reduced after treatment with KB-R7785 (34.2 ± 7.4%; P < 0.001) or anti-ADAM28 antibody (38.0 ± 9.2%; P < 0.001; Fig. 3C). In contrast, no such inhibition was observed in MCF-7 cells (Fig. 3C). To further show a direct involvement of ADAM28 in the cell proliferation, we transfected the cells with ADAM28 siRNA. As shown in Fig. 3D, the mRNA and protein expression of ADAM28s and ADAM28m in IGF-I-stimulated MDA-MB231 cells was almost completely abolished with ADAM28 siRNA but not with nonsilencing siRNA. Importantly, transfection of siRNA to the cells significantly decreased IGF-I-mediated proliferation of MDA-MB231 cells (P < 0.05; Fig. 3D), whereas ADAM28 siRNA did not influence the proliferation of MCF-7 cells (Fig. 3D).

Growth inhibition of MDA-MB231 cell xenografts with ADAM28 siRNA. ADAM28 siRNA or nonsilencing siRNA in CCLA-based liposome was s.c. administered around the MDA-MB231 cell tumors on days 3, 5, and 7 after inoculation of tumor cells, and the average tumor volume was measured at 0, 3, 5, 7, 14, 21, and 31 days. As shown in Fig. 4A and B, the tumor volume on day 31 in the ADAM28 siRNA group (132 ± 21 mm³) was significantly reduced to <40% of the control groups treated with nonsilencing siRNA (356 ± 31 mm³) or 10% sucrose (347 ± 34 mm³; P < 0.01). No toxic effects of ADAM28 or nonsilencing siRNA in CCLA-based liposome and 10% sucrose were observed, and none of the mice died during the treatments. Immunoblotting analysis indicated that the 42-kDa immunoreactive band of ADAM28 in the xenografts was reduced on day 7 (at the point after the second treatment with ADAM28 siRNA), almost negligible on day 14 (7 days after the third treatment), and recovered on day 31 (Fig. 4C).

IGFBP-3 degradation and its inhibition by suppression of ADAM28 activity. Immunoblotting analysis of the conditioned

Figure 2. Tissue localization of ADAM28 mRNA and protein in the breast carcinomas and immunoblotting of ADAM28. A, in situ hybridization of ADAM28 in the breast carcinoma tissues. In situ hybridization was done as described in Materials and Methods. Note the positive and negative signals for ADAM28 in the carcinoma cells with the antisense and sense probes, respectively. Bar, 50 μm. B, immunohistochemistry of ADAM28 in the breast carcinoma tissues. Parafin sections of the carcinoma tissues were subjected to immunostaining with anti-ADAM28 antibody or nonimmune mouse IgG as described in Materials and Methods. Bar, 50 μm. C, immunoblotting with anti-ADAM28 antibody (297-2F3) in the breast carcinoma and nonneoplastic breast tissues. Note the positive 42-kDa band of ADAM28 in the breast carcinoma samples (lanes 1-4) but not in the nonneoplastic breast samples (lanes 5-7). β-Actin is used as a loading control. D, immunoblotting of the two carcinoma samples with anti-ADAM28 antibody (297-2F3) and anti-ADAM28 antibody specific to the cytoplasmic domain of ADAM28m (CL1ADAM28). A longer exposure of the membrane blotted with the 297-2F3 antibody gives 55/57-kDa bands besides the 42-kDa band, and 55/57-kDa bands are reactive with the CL1ADAM28 antibody.
media of IGF-I-stimulated MDA-MB231 and MCF-7 cells showed 45-kDa and 40-kDa bands of intact IGFBP-3 protein (27), but MDA-MB231 and MCF-7 cells showed different digestion fragments of 22 kDa and 26 kDa, respectively (Fig. 5A and B). Without IGF-I treatment, MDA-MB231 cells showed low-level production of intact IGFBP-3 and 22-kDa fragments (data not shown). Importantly, the fragment formation in IGF-I-stimulated MDA-MB231 cells was attenuated after treatment of the cells with KB-R7785, anti-ADAM28 antibody, or ADAM28 siRNA, but not aprotinin (Fig. 5A), although the 26-kDa fragments in MCF-7 cells were not influenced by these treatments (Fig. 5B). Densitometric analysis of the ratios of the 22-kDa fragments to the total amount of IGFBP-3 protein showed that treatment of MDA-MB231 cells with KB-R7785, anti-ADAM28 antibody, and ADAM28 siRNA gave 93%, 87%, and 88% inhibition, respectively, whereas aprotinin treatment resulted in only 31% inhibition. In the culture media of human breast carcinoma tissues, intact IGFBP-3 and 22-kDa fragments were also detected, and the intensity of the 22-kDa fragments was decreased when the carcinoma tissues were incubated with KB-R7785 or anti-ADAM28 antibody, but not with nonimmune mouse IgG (Fig. 5C). By densitometric analysis, treatment with KB-R7785 and anti-ADAM28 antibody resulted in 97% and 88% inhibition, but nonimmune IgG showed no inhibition. In addition, formation of the 22-kDa fragments in the breast carcinoma tissues seemed to correlate with the expression levels of ADAM28 (Fig. 5D). Zymographical analysis of the culture media of MDA-MB231 cells stimulated with IGF-I showed proteolytic bands corresponding to pro-MMP-1, pro-MMP-2, pro-MMP-3, pro-MMP-7, and pro-MMP-9, but not to their active forms (data not shown).

Figure 3. Involvement of ADAM28 in the IGF-I-induced cell proliferation in MDA-MB231 cells. A, determination of ADAM28 expression in MDA-MB231 cells. The expression of ADAM28 mRNA and protein was examined by RT-PCR and immunoblotting as described in Materials and Methods. Note that ADAM28 is expressed in MDA-MB231 cells but not in MCF-7 cells. β-Actin is used as a loading control. B, inhibition of the IGF-I-induced cell proliferation of MDA-MB231 cells by a synthetic ADAM inhibitor (KB-R7785) and anti-ADAM28 antibody. Breast carcinoma cells were treated with 1 μmol/L KB-R7785 or with 5 μg/mL anti-ADAM28 antibody, and then cultured in the presence or absence of 100 ng/mL IGF-I. BrdUrd incorporation was measured by a microplate reader as described in Materials and Methods. Columns, mean; bars, SD. C, culture in the absence of IGF-I. ***, P < 0.0001; **, P < 0.001. C, MIB1-positive cell index in MDA-MB231 and MCF-7 cells. Carcinoma cells were treated in a similar way as described in (B), and then MIB1-positive cell index (%) was determined by immunostaining with anti-Ki 67 antibody as described in Materials and Methods. D, inhibition of MDA-MB231 cell proliferation by transfection with ADAM28 siRNA. Breast carcinoma cells were transfected with nonsilencing siRNA (NS siRNA) or ADAM28-specific siRNA (ADAM28 siRNA), and then cell proliferation was assessed by BrdUrd incorporation. Effects of ADAM28 siRNA were assessed by RT-PCR and immunoblotting. Note that ADAM28 siRNA abolishes ADAM28 expression and decreases cell proliferation in MDA-MB231 cells. *, P < 0.05.
**Involvement of ADAM28 in IGF-I-triggered cell signaling in MDA-MB231 cells.** Because IGF-IR is tyrosine phosphorylated after binding with IGF-I (28), we examined phosphorylation of IGF-IR in IGF-I-treated MDA-MB231 and MCF-7 cells. As shown in Fig. 6A, a definite increase in the phosphorylation of IGF-IR was observed in MDA-MB231 cells after treatment with IGF-I (lanes 1 and 2), and their treatment with KB-R7785 or anti-ADAM28 antibody significantly inhibited the IGF-IR phosphorylation to basal level \( (P < 0.001; \text{lanes 3 and 4}) \). On the other hand, similar effects were not observed in MCF-7 cells (Fig. 6A, lanes 5–8). To study the IGF-IR-mediated post-receptor signal transduction, the phosphorylation of ERK1/2, one of the downstream molecules in this pathway (28, 29), was evaluated. Figure 6B shows that ERK1/2 is phosphorylated through IGF-IR activation in MDA-MB231 cells (lanes 1 and 2) and the phosphorylation is prevented by their incubation with KB-R7785 or anti-ADAM28 antibody \( (P < 0.001; \text{lanes 3 and 4}) \). However, these reactions of ERK1/2 phosphorylation were not observed in MCF-7 cells (Fig. 6B, lanes 5–8). In addition, the IGF-I-induced ERK1/2 phosphorylation and cell proliferation in MDA-MB231 cells were prevented by treatment with PD98059, a specific inhibitor of MEK1/2 (data not shown).

**Discussion**

In the present study, we provide the first evidence that among the 12 different metalloproteinase-type ADAMs, ADAM28 is selectively overexpressed in activated form by carcinoma cells in human invasive breast carcinomas with a direct correlation to carcinoma cell proliferation. In addition, our *in vitro* and *in vivo* experimental studies have shown that inhibition or down-regulation of ADAM28 activity attenuates cell proliferation and IGF-I-induced cell signaling, together with decreased IGFBP-3 degradation. Based on these findings, we propose the hypothesis that ADAM28 plays a key role in cell proliferation in human breast carcinomas through enhanced bioavailability of IGF-1 released from the IGF-I/IGFBP-3 complex by selective cleavage of IGFBP-3.

Previous studies showed that ADAM9 or ADAM12 is predominantly expressed in the breast carcinomas by immunostaining and RT-PCR (16, 17) and that ADAM17 is overexpressed in breast carcinomas, although nonneoplastic breast tissues showed low-level expression (30). The present RT-PCR data confirmed the previous results on ADAM9 and ADAM12, but further indicated that ADAM15, ADAM28s, and ADAM28m are overexpressed in human breast carcinomas. Our real-time PCR and immunohistochemical analyses showed that ~65% of the breast carcinoma cases have strong ADAM28 expression at mRNA and protein levels. Importantly, *in situ* hybridization and immunohistochemistry indicated expression to be predominantly by the carcinoma cells,

![Figure 4](image_url)

**Figure 4.** Growth inhibition of MDA-MB231 cell xenografts implanted in mice by injection of ADAM28 siRNA. A, time-course changes in the tumor volume. Mice bearing tumors in the s.c. tissue received injections of ADAM28 siRNA in CCLA-based liposome \( (n = 9) \), nonsilencing siRNA in CCLA-based liposome \( (n = 9) \), or 10% sucrose \( (n = 6) \), and tumor volumes were calculated as described in Materials and Methods. **, \( P < 0.001 \). (B) macroscopic observation of representative mice bearing tumors (arrows) treated with nonsilencing siRNA or ADAM28 siRNA. C, immunoblotting analysis of ADAM28 and \( \beta \)-actin in the implanted tumors on days 3, 7, 14, and 31. Lanes 1, 3, 5, and 7, nonsilencing siRNA treatment; lanes 2, 4, 6, and 8, ADAM28 siRNA treatment.

![Figure 5](image_url)

**Figure 5.** Inhibition of IGFBP-3 cleavage by ADAM inhibitor, anti-ADAM28 antibody, or ADAM28 siRNA in breast carcinoma cell lines and breast carcinoma tissues. A and B, MDA-MB231 cells (A) and MCF-7 cells (B) were treated without (lane 1) or with 1 \( \mu \)mol/L KB-R7785 (lane 2), 5 \( \mu \)g/mL anti-ADAM28 antibody (lane 3), ADAM28 siRNA (lane 4), or 100 \( \mu \)g/mL aprotinin (lane 5) and then treated with IGF-I. Conditioned media were analyzed for IGFBP-3 proteolytic fragments. Note that formation of IGFBP-3 fragments \( (\text{closed arrowhead}) \) and MCF-7 cells \( (\text{open arrowhead}) \). C, breast carcinoma tissues were cultured in the absence (lane 1) or presence of 1 \( \mu \)mol/L KB-R7785 (lane 2), 5 \( \mu \)g/mL anti-ADAM28 antibody (lane 3), or 5 \( \mu \)g/mL nonimmune mouse IgG (lane 4), and then IGFBP-3 proteolysis was analyzed by immunoblotting. Note that formation of IGFBP-3 fragments \( (\text{closed arrowhead}) \) is inhibited by KB-R7785 and anti-ADAM28 antibody. D, immunoblotting of ADAM28 (top), IGFBP-3 (middle), and \( \beta \)-actin (bottom) in homogenate samples of the control nonneoplastic breast (lanes 1 and 2) and breast carcinoma tissues (lanes 3–5). Arrow and arrowhead, intact IGFBP-3 and 22-kDa IGFBP-3 fragments.
although our methods did not differentiate the isoforms of ADAM28s and ADAM28m. In a parallel study, we recently reported that human non–small-cell lung carcinomas selectively overexpress ADAM28s and ADAM28m (20). Our immunoblotting analyses with two different antibodies showed that ADAM28 is present in protein bands of 42 kDa and 55/57 kDa in breast and lung carcinoma tissues (data not shown for lung carcinomas). Based on the molecular weight and reactivity with the antibodies, the 42-kDa species is considered to be an active form of ADAM28s and/or ADAM28m shed from the cell membranes (20), and the 55–57-kDa species are propeptide-deleted ADAM28m. Altogether, these data suggest that ADAM28s and ADAM28m are selectively overexpressed in active form by carcinoma cells of these human solid tumors.

One of the interesting findings in the present study is that the mRNA expression of ADAM28s and ADAM28m directly correlates with MIB1-positive cell index of the breast carcinomas. Our recent study on human non–small-cell lung carcinomas (20) has shown a similar finding. Several growth factors such as IGFs (31–33), epidermal growth factor (34, 35), and transforming growth factor β (36) have been reported to modulate breast and lung carcinoma cell proliferation. Among them, however, IGF-I and IGF-II are considered to be the most potent mitogens for carcinoma cells (31, 37, 38). In the present study, we have provided in vitro and in vivo data that the IGF-I-induced proliferation of MDA-MB231 cells is significantly inhibited by treatment with KB-R7785, neutralizing anti-ADAM28 antibody, or transfection with ADAM28 siRNA, and that injection of ADAM28 siRNA to the MDA-MB231 cell xenografts significantly suppresses tumor growth. Thus, these data show that activity of ADAM28 is involved in IGF-I-induced proliferation of MDA-MB231 cells.

The cellular action of IGFs is strictly regulated by IGFBPs because the affinities of IGFs to IGFBPs are higher than those to IGF receptors (27). Therefore, proteolysis of IGFBPs directly controls the bioavailability of IGFs to the IGF receptors, and thereby indirectly modulates cell proliferation (27). Although the IGFBP family is composed of six proteins with high affinity to IGFs, the major IGF transport function is attributed to IGFBP-3, which is the most abundant circulating IGFBP species synthesized by liver and locally produced by cancer tissues (39). Proteolytic cleavage has been shown for IGFBP-3 and has gained wide acceptance as the predominant mechanism for IGF release from the IGF/IGFBP-3 complex (40). MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, and ADAM12 are capable of digesting IGFBP-3 (41–45). We have also reported that ADAM28 can release IGF-1 from the IGF-I/IGFBP-3 complex through selective cleavage of IGFBP-3 (13). MDA-MB231 cells have a potential to express IGFBP-3-degrading proteases, which include MMP-1, MMP-2, MMP-3, MMP-7, MMP-9 (46–48), and ADAM12 (49), as well as ADAM28. However, the COOH-terminal fragments of IGFBP-3 generated by the action of MMP-1, MMP-2, MMP-3, or MMP-7 (41, 42) are much smaller than the 22-kDa fragments observed in our present and previous studies (13), whereas MMP-9 degrades IGFBP-3 into the 30-kDa and 19-kDa fragments (43), which are also different from the ADAM28-digested fragments. In addition, MMPs are produced in inactive pro-MMPs under monolayer cultures used in the present study, and our zymographical analyses actually showed no active forms of MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9. ADAM12 digests IGFBP-3 into several small fragments of 10 to 20 kDa (45), which differ from those seen in the present study. In addition, down-regulation of ADAM12 by siRNA did not prevent the 22-kDa fragment formation in MDA-MB231 cells.4 In contrast, fragment formation was inhibited by treatment with anti-ADAM28 antibody and ADAM28 siRNA as well as KB-R7785. All these data strongly suggest that in MDA-MB231 cells, ADAM28 is involved in the degradation of IGFBP-3. Importantly, IGFBP-3 digestion in breast carcinoma tissues was also inhibited by treatment of the tissues with anti-ADAM28 antibody and KB-R7785, suggesting the possibility that a similar mechanism is present in the human breast carcinoma tissues.

The physiologic effects of IGFs are mainly mediated through IGF-IR (29). Our data that IGF-I-induced phosphorylation of IGF-IR and ERK1/2 in MDA-MB231 cells is prevented by treatment with KB-R7785 or anti-ADAM28 antibody suggest the possibility that proteolytic cleavage of IGFBP-3 by ADAM28 causes IGF-I-induced signaling in the cells. Because IGF-I-induced MDA-MB231 cell proliferation was also suppressed by treatment with a specific inhibitor of MEK (50), signaling of the IGF-I-induced cell proliferation may predominantly involve the ERK pathway in MDA-MB231 cells. Further work is necessary to show that in

Figure 6. Inhibition of IGF-I-triggered cell signaling by ADAM inhibitor or anti-ADAM28 antibody. A, serum-starved breast carcinoma cells were treated with 1 μmol/L KB-R7785 or 5 μg/mL anti-ADAM28 antibody in the presence or absence of IGF-I. Whole-cell lysates separated by SDS-PAGE were immunoblotted with anti-phospho-IGF-IR antibody (top) and membranes were reblotted with anti-IGF-IR antibody as a control for protein loading (bottom). Columns, mean densitometric values of phospho-IGF-IR/IGF-IR ratios from three independent experiments. **, P < 0.001. B, breast carcinoma cells were treated in a similar way as in (A), and ERK1/2 activation was evaluated by immunoblotting with an anti–phospho-ERK1/2 antibody (top) and anti-ERK1/2 antibody (bottom). Columns, mean densitometric values of phospho-ERK1/2/ERK1/2 ratios from three independent experiments. **, P < 0.001.

4 S. Mochizuki, Y. Okada, unpublished data.
human breast carcinomas, ADAM28 overexpressed by carcinoma cells promotes cell proliferation through the ERK pathway of IGF-IR signaling that is triggered with IGF-I released from the IGF-1/IGFBP-3 complex by the action of ADAM28.

Acknowledgments

References


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Correction: ADAM28 Is Overexpressed in Human Breast Carcinomas: Implications for Carcinoma Cell Proliferation through Cleavage of Insulin-like Growth Factor Binding Protein-3

In this article (Cancer Res 2006:66:9913–20), which appeared in the October 15, 2006, issue of Cancer Research (1), there were errors in Figs. 3C, 3D, 4C, and 5C, which the authors have corrected as follows:

Figure 3C. The authors repeated the experiment and obtained confirmatory results. By utilizing the data, they prepared a corrected version of Fig. 3C.

Figure 3D. The authors found that the loading control β-actin of Fig. 3A was erroneously duplicated for Fig. 3D. Figure 3D has now been corrected by using the appropriate β-actin controls.

Figure 4C. The authors could not find the original figure for Fig. 4C, so they repeated the experiments and obtained confirmatory data to those in the original Fig. 4C; the corrected Fig. 4C below consists of these new data.

Figure 5C. The authors were able to find the original figure for Fig. 5C and realized that lane 4 was inadvertently duplicated from the original figure. The corrected Fig. 5C uses the appropriate image for the control from the original data.

The corrected figures appear below. These errors were caused in large part by inadequate oversight of data management. These errors, which are now corrected, do not change either the results or the conclusions of the article. The authors regret these errors.
Reference

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ADAM28 Is Overexpressed in Human Breast Carcinomas: Implications for Carcinoma Cell Proliferation through Cleavage of Insulin-like Growth Factor Binding Protein-3

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