Characterization of Glycosylphosphatidylinositol-linked Molecule CD55/Decay-accelerating Factor as the Receptor for Antibody SC-1-induced Apoptosis


INTRODUCTION

The human monoclonal antibody SC-1 induces apoptosis of stomach carcinoma cells and is currently used in a clinical Phase II trial. The antibody binds to a target molecule that is preferentially expressed on diffuse- and intestinal-type stomach cancer cells and shows a very restricted expression on other normal and malignant tissues. In this paper, we show that the SC-1 receptor is a stomach carcinoma-associated isoform of CD55 [membrane-bound decay-accelerating factor (DAF)-B] with a relative molecular mass of approximately 82 kDa. The antigenic site of SC-1 is an N-linked carbohydrate residue. Cross-linking of the DAF receptor increases apoptotic activity. SC-1 binding induces tyrosine phosphorylation of three proteins of approximately 60, 75, and 110 kDa, whereas a serine residue of an approximately 35-kDa protein is dephosphorylated. Expression of caspase-3 (CPP32) and caspase-8 (FLICE) is elevated, and activation of these caspases occurs. These data show that a tumor-specific variant form DAF is involved in apoptosis and can be used for adjuvant therapeutical purposes on gastric carcinoma.

MATERIALS AND METHODS

Cell Culture

For all assays, the established stomach adenocarcinoma cell line 23132 (26) was used. Cells were grown to subconfluence in RPMI 1640 (PAA, Vienna, Austria), supplemented with 10% FCS and penicillin/streptomycin (both at 1%). For the assays described here, cells were detached with trypsin/EDTA and washed twice with PBS before use. The human hybridoma cell line SC-1 was grown in serum-free RPMI 1640 (PFHM-II; Life Technologies, Inc., Karlsruhe, Germany) using miniPerm Bioreactors (InVitro Systems & Services, Osterode, Germany).

Received 3/11/99; accepted 8/19/99.
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Purification of the SC-1 Antibody

The human monoclonal antibody was purified from mass cultures, using cation exchange chromatography followed by gel filtration as described elsewhere (27).

Purification of the SC-1 Receptor

For preparation of membrane proteins, harvested cells were resuspended in hypotonic buffer (20 mM HEPES, 3 mM KCl, 3 mM MgCl₂, incubated on ice (15 min), and sonicated (5 min), and the nuclei were pelleted by centrifugation (10,000 × g for 10 min). The membranes were pelleted by centrifugation (100,000 × g for 30 min) and resuspended in membrane lysis buffer (50 mM HEPES, pH 7.4, 0.1 mM EDTA, 1 mM NaCl, 10% glycerol and 1% Triton X-100). Complete protease inhibitor (Roche Molecular Biochemicals, Mannheim, Germany) was added to all solutions.

The purification of the antigens was performed by column chromatography using an Amersham Pharmacia Biotech (Freiburg, Germany) fast protein liquid chromatography unit. For size exclusion chromatography, an Amersham Pharmacia Biotech Superdex 200 column (XK16/60) was loaded with 5 mg of membrane protein preparation and run with Buffer A (100 mM Tris/Cl, pH 7.5, 2 mM EDTA, 40 mM NaCl, 1% Triton X-100). The eluate was fractionated and examined in Western blot analysis for reaction with antibody SC-1. Positive fractions were loaded on a MonoQ (5/5) column using Buffer A. The bound proteins were eluted with a linear gradient using Buffer B (100 mM Tris/Cl, pH 7.5, 1 mM NaCl, 2 mM EDTA, 40 mM NaCl, 1% Triton X-100), fractionized, and examined by Coomassie-stained SDS-PAGE and Western blot analysis. Positive bands were cut out from gel and sequenced.

Preparation of Cell Lysates after Induction with SC-1

Cell line 23132 was grown on 100-mm cell culture plates to subconfluency, and then the SC-1 antibody was added to a final volume of 30 μg/ml and incubated for the periods indicated. Then culture plates were washed once with PBS, and subsequently cells were directly lysed with SDS-buffer (50 mM Tris/Cl, pH 6.8, 10 mM DTT, 2% (w/v) SDS, 10% (v/v) glycerol). Cell debris was collected with a rubber policeman.

Gel Electrophoresis and Blotting

SDS-PAGE under reducing conditions and Western blotting of proteins were performed using standard protocols as described elsewhere (13). Briefly, blotted nitrocellulose membranes were blocked with PBS containing 0.1% (v/v) Tween-20 and 2% (v/v) low-fat milk powder or 3% (w/v) BSA (for determination of phosphorylation), followed by a 1-h incubation with primary antibody. The antibodies were used at the following concentrations: SC-1/CD55-INDUCED APOPTOSIS 1:30,000 (Sigma, Munich, Germany). The secondary antibodies (peroxidase-coupled antiphosphotyrosine (clone PT-66), strepavidin-coupled antiphosphoserine (clone PSR-45), goat anticaspase-3 and -8 (SantaCruz, Heidelberg, Germany) and peroxidase-coupled extravidin (Sigma)) were detected with the SuperSignal chemiluminescence kit from Pierce (KMF, St. Augustin, Germany).

Protein Sequencing

A protein band having an apparent molecular mass of 82 kDa was isolated by one-dimensional PAGE and visualized by Coomassie staining. P82 band was in-gel digested with trypsin (Roche Molecular Biochemicals, unmodified, sequencing grade) as described (28). Unseparated pool of tryptic peptides was sequenced by nanoelectrospray tandem mass spectrometry as described (29). Sequencing was performed on a API III triple quadrupole mass spectrometer (PE Scieix, Concord Ontario, Canada). Peptide sequence tags (30) were assembled using tandem mass spectrometric data. Searching of comprehensive protein and expressed sequence tag databases was performed using PeptideSearch version 3.0 software, developed in-house.

Reverse Transcription-PCR

cDNA synthesis from total RNA from tumor cells 23132 was performed with 5 μg of total RNA using Life Technologies, Inc. (Eggenstein, Germany) Moloney murine leukemia virus reverse transcriptase according to the supplier’s manual. PCR products were carried out in a 25-μl volume with 1.75 mM MgCl₂, 0.4 pmol primer, 200 μM each dNTP, and 1 unit of Taq polymerase (MBI Fermentas, St. Leon-Rot, Germany). The PCR products for cloning the CD55 antisense vector were amplified using the following cycle profiles: CD55 (640-bp fragment spanning the sequence from bp 382 to 1022): 95°C for 2 min; followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s; and a final extension at 72°C for 4 min.

Cloning Procedures

Cloning for Sequencing. PCR products were purified from agarose gel using a JetSorb gel extraction kit (Genomed, Bad Oeynhausen, Germany). Cloning of PCR fragments was performed with pCR-Script Amp SK (+) cloning kit (Stratagene, Heidelberg, Germany).

Cloning of Antisense Vector pHook2-CD55anti. The CD55-PCR product was blunted with Pfu-polynucleotase and directly cloned into the Smal cut expression vector pHOOK-2 (Invitrogen, Leek, the Netherlands). One clone with antisense direction of the insert following the PcMV-promoter was selected for the antisense experiments.

DNA Sequencing

Eight positive clones were sequenced using the DyeDeoxy termination cycle sequencing kit (Applied Biosytems Inc., Weiterstadt, Germany) and analyzed with an automated DNA sequencer ABIPrism373. Both strands were sequenced using T3 and T7 primers. The sequences were analyzed using DNASIS for Windows software and the BLAST program from the National Center for Biotechnology Information.

Transfection

For transfection experiments, 2–5 × 10⁵ detached cells were washed in TBS and resuspended in 400 μl of TBS, 10 μg of plasmid DNA, isolated with Endotox-free Maxi-Prep kit (Qiagen, Hilden, Germany), were added, and the cells were pulsed with 240 V, 960 μF, using a Bio-Rad (Munich, Germany) electroporation unit. 5 × 10³ transfected cells were seeded on 60-mm cell culture and incubated for 24 h as indicated above. Apoptosis was induced by adding 50 μg/ml of purified SC-1 antibody to the growth medium. After 24 h, cells were trypsinized and used for preparing cytosins.

Phospholipase Assay

Detached and pelleted cells were resuspended in RPMI 1640 with supplements and incubated for 90 min at 37°C. After this recovery period, 20 milliliters/ml PI-PLC (Roche Molecular Biochemicals) were added, and cells were incubated for another 60 min. Finally, cells were washed and used for preparing cytosins.

Glycosidase Assay

Detached and washed cells were resuspended in RPMI 1640 containing 10% FCS and incubated for 1 h on ice and then counted, and cytosins were prepared. After air drying, cytosin preparations were acetone fixed (10 min), washed, and incubated with 20 micromilliliters/ml O-glycosidase or 5 milliliters/ml N-glycosidase (Roche Molecular Biochemicals) for 4 h at 37°C.

Immunohistochemical Staining

The following antibodies were used for immunohistochemical staining: purified SC-1, anti-CEA (DAKO, Hamburg, Germany), anti-EMA (Loom, Dossenheim, Germany), and anti-CD55 (Biozol, Eiching, Germany). Cytosin preparations were fixed with acetone and stained as described elsewhere (31).

Apoptosis Assay

Cytosin preparations (5000 cells/slide) were fixed in acetone, followed by washings with TBS. Then the cytosins underwent staining with the FragEl-Klenow DNA fragmentation kit (Calbiochem-Novabiochem, Bad Soden, Germany), according to the supplier’s manual and as described elsewhere (15, 27).

MTT Assay

The MTT proliferation assay for measuring the apoptotic activity of antibody SC-1 on stomach cancer cells was performed as described elsewhere (14).
Caspase-3 and -8 Assays

The activation of caspase-8 was measured with the ApoAlert caspase fluorescent assay kits (Clontech, Heidelberg, Germany). Briefly, 1 × 10^6 cells were incubated with 40 μg/ml SC-1 for 7 and 20 h. Then cells were collected and resuspended in cell lysis buffer, and caspase activity was measured according to supplier’s manual.

RESULTS

Purification of the CD55/SC-1 Receptor. In Western blot analysis of extracts from whole cell lysates of the stomach carcinoma cell line 23132, which were prepared under low salt conditions (100 mM NaCl), SC-1 bound a protein with a relative molecular mass of approximately 50 kDa (12, 13). By changing the stringency (1 mM NaCl) and using only membrane preparations, additional molecules of approximately 70 and 82 kDa were detected (Fig. 1a, Lane 1). We isolated these proteins from membrane fractions and prepurified them by sequential size exclusion and anion exchange chromatography (Fig. 1a, Lanes 2 and 3). The molecules were excised from Coomassie-stained SDS-PAGE and directly sequenced by nanoelectrospray tandem mass spectrometry after in-gel tryptic digest. The protein of approximately 50 kDa was identified as dihydriipoamide succinyltransferase (E2K, GenBank accession no. L37418), and the protein of approximately 70 kDa as the human lupus p70 (Ku) autoantigen protein (GenBank accession no. J04611; data not shown). These reactivities are most likely due to nonspecific binding of SC-1 caused by protein denaturation in Western blot analysis, which is a common observation for monoclonal antibodies. Because the SC-1 antibody only binds cell surface antigens in immunohistochemical stainings, cytoplasmic and nuclear antigens, such as E2K and p70 (Ku), can be excluded from specificity.

The approximately 82-kDa protein was identified as CD55 (human DAF; GenBank accession no. M31516; Fig. 1b, panels 1 and 2), co-migrating with another protein, which was identified as hnRNP R (GenBank accession no. O43390). The molecular mass of hnRNP R is 71 kDa, but it is known to migrate at 82 kDa. We further investigated CD55, which exists in humans in two genetically determined isoforms (secreted DAF-A and membrane-bound DAF-B) caused by differential splicing (18). Through reverse transcription-PCR analysis, we found that the cell line 23132 expresses only the GPI-anchored (DAF-B) isoform of CD55 (data not shown).

Transient Transfection with the CD55 Antisense Vector. To analyze whether inhibition of CD55/DAF expression influences SC-1-induced apoptosis, the cell line 23132 was transiently transfected with the CD55 antisense vector pHOOK-2-cd55anti and control vector pHOOK-2 by electrophoresis. Cytosins of transfected cells were immunohistochemically stained with SC-1, anti-CD55 (data not shown), and anti-CEA. Cells transfected with the control vector showed intensive staining with SC-1 and CEA (Fig. 2, a and c), but we observed hardly any staining with SC-1 in cells transfected with the CD55 antisense vector (Fig. 2b). The staining with anti-CEA antibody revealed that the expression pattern of CEA, also GPI-anchored, is not affected by the transfection with the antisense vector, but that expression of CD55 was specifically reduced due to expression of CD55 antisense RNA.

To analyze whether the expression of antisense CD55 RNA also inhibited SC-1-induced apoptosis, we incubated cells 1 day posttransfection with and without 30 μg/ml SC-1 for a 24-h period. Cytosins of cells transfected with the antisense and control vector were stained with the FragEl-Klenow DNA fragmentation kit for detection of DNA fragmentation induced by apoptosis. Whereas untreated cells transfected with either plasmid show hardly any spontaneous apoptosis, after incubation with SC-1, there is a clear decrease in apoptosis in cells transfected with the CD55 antisense vector when compared to cells transfected with the control vector (data not shown). A quantification showed that the incidence of spontaneous apoptosis in transfected 23132 cells was 6%, whereas 85% of cells transfected with the control vector showed apoptosis after incubation with SC-1. This apoptotic response was clearly reduced to 21% because of transfection with the CD55 antisense vector (Fig. 3).

Phospholipase Treatment. To confirm the reactivity of the SC-1 antibody with a GPI-anchored isoform of CD55/DAF, we investigated whether cleavage of the GPI anchor influences SC-1 binding in
immunohistochemistry and MTT activity tests. The GPI anchor was therefore cleaved by incubating stomach cancer cells for 1 h with 20 milliunits/ml phosphatidylinositol-specific PI-PLC. Cytospins of PI-PLC-treated and untreated cells were immunohistochemically stained with SC-1, anti-CD55, and anti-EMA as a control to exclude unspecific effects of PI-PLC treatment on non-GPI-anchored proteins. Compared to untreated cells (Fig. 4a), a clear loss of staining intensity can be observed on PI-PLC-treated cells stained with SC-1 (Fig. 4b). The cleavage of GPI anchor was confirmed by staining with anti-CD55, with a reduced staining after PI-PLC treatment (Fig. 4d), as compared to untreated cells (Fig. 4c). PI-PLC treatment did not have any effect on staining with anti-EMA (data not shown), which indicates that the PI-PLC treatment has no effect on non-GPI-anchored membrane proteins. In the MTT test, the treatment of cells with PI-PLC leads to a significant (P ≤ 0.05, Student’s t test) decrease in apoptotic cells (Fig. 5d).

**Glycosidase Treatment.** We investigated the effect of protein deglycosylation of the cell line 23132 on the binding of SC-1 by incubating cytospin preparations for 4 h with O- and N-glycosidases prior to immunohistochemical staining. Treatment of cells with N-glycosidase led to a dramatic decrease in SC-1 staining (Fig. 4f), whereas staining with anti-CD55, which binds to the unglycosylated SCR3 region, was not affected by protein deglycosylation (Fig. 4e). Incubation with phosphate buffer and digestion with O-glycosidase (data not shown) had no effect on SC-1 binding (data not shown). This shows that the specificity of SC-1 binding must be located in sugar residues and not in the primary protein sequence.

**MTT and Cross-linking of CD55/SC-1.** The apoptotic activity of SC-1 in vitro was measured in MTT assays, as shown here on two different stomach carcinoma cell lines (Fig. 5a). To investigate whether receptor complexing might be a part of a signaling pathway through CD55/DAF, we first incubated cells for 24 h with an increasing amount of SC-1, to evaluate the optimal apoptotic activity of SC-1 (Fig. 5b). Cross-linking was then performed with 40 μg/ml antibody with and without a rabbit antihuman IgM. After incubation for 48 h, we found a rate of death cells 47% higher than in control incubated with SC-1 (Fig. 5c).

**Protein Phosphorylation.** We investigated the phosphorylation pattern after inducing cells with 40 μg/ml purified SC-1 antibody by Western blots of cytoplasmic and membrane extracts. We found an early tyrosine phosphorylation of proteins of 110 and 60 kDa 30–60 s after apoptosis induction (Fig. 6a). These proteins were found only in the cytoplasm (60 kDa) or in membrane extracts and cytoplasm (110 kDa). Furthermore, we found a slow tyrosine phosphorylation of a 75-kDa cytoplasmic protein, with the highest level after 10 min (Fig. 6b), as well as the complete disappearance of a serine phosphorylation of a 35-kDa protein, also 10 min after induction.

**Caspase Activity.** Western blot analysis showed that SC-1 triggers the up-regulation of caspases-3 and -8 of cell line 23132 (Fig. 7a). Also, activation of these caspases was observed. This activation occurs through proteolytic cleavage of the procaspases (22). This cleavage was shown for caspase-3 by identification of the p20 cleavage product, only minor activation of caspase-3 was observed with the ApoAlert caspase kit (data not shown). In contrast, we found a strong increase in caspase-8 activity 20 h after induction with SC-1, indicating the involvement of this caspase in the apoptotic process (Fig. 7b).
DISCUSSION

In this study, we characterized the receptor of the human monoclonal antibody SC-1. We isolated and sequenced the SC-1-specific molecule and showed that the antibody induces its apoptotic effect on stomach cancer cells by binding to a modified tumor cell-specific form of CD55/DAF, which is absent in normal tissue. We demonstrated that the SC-1 reactivity can be blocked by removing GPI-linked proteins from the surface of tumor cells and by transfection with CD55 antisense vectors. CD55/DAF is a highly glycosylated molecule and different isoforms show different glycosylation patterns (17). Treatment of tumor cells with glycosidases shows that the binding-site of SC-1 is a carbohydrate residue. The induction of apoptosis can be elevated by cross-linking of the SC-1 antibody, and binding of SC-1 induces higher expression and activation of caspase-3 and -8 and tyrosine phosphorylation of three proteins.

Induction of specific apoptosis is the most effective and safest way to remove tumor cells from the organism. Because tumor cells have lost control of cell proliferation by genetic alteration, most apoptosis mechanisms known from normal counterparts are ineffective. This becomes extremely evident in the approach to kill tumor cells through the Fas/APO-1 receptor ligand family, which plays a key role in normal immune development, homeostasis, modulation, and function but fails in the specific killing of malignant cells. Although this is a very promising line of investigation, neither antibodies against Fas/...
APO-1 nor the more restricted Fas ligand approach will cause a breakthrough in cancer therapy.

Because the easiest way to attack tumor cells is through the cell membrane, the obvious need is to search for other receptor/ligand/antibody systems through which apoptosis in tumor cells can be induced. Therefore, in an extensive study of the humoral immunity of stomach cancer patients, we screened for antibodies that influence cell growth. The human antibody SC-1 inhibits in vitro and in vivo growth of stomach cancer cells by inducing apoptosis through binding to CD55/DAF receptor.

There are two genetically determined isoforms of CD55, termed DAF-A and DAF-B, in existence, and in addition, there are different glycosylated isoforms of CD55 that are expressed by erythrocytes, lymphocytes, and sperm and in glandular fluid. Their molecular masses vary from 50 to 70 kDa, due to different glycosylation patterns, and they are different in size to the isoform defined by the SC-1 antibody on stomach cancer cells (82 kDa). CD55 is not a transmembrane molecule, but the DAF-B isoform is linked to the cell surface by a GPI anchor and contains no death domain (32). Interestingly, an up-regulation of CD55/DAF in the human gastric mucosa was found in Helicobacter pylori-infected individuals (33), and there is strong evidence that H. pylori is one cause for the development of gastric cancer and was correctly classified as carcinogen by the WHO (34).

Indirect evidence for the participation of CD55 in apoptotic events has been provided by Jones and Morgan (35), who reported that reduced expression of CD55/DAF together with CD59 in human polymorphonuclear leukocytes correlates closely with the appearance of apoptotic morphology. On the other hand, Brodsky et al. (36) have shown that in paroxysmal nocturnal hemoglobinuria, which is a ge-

Fig. 5. MTT assay with human antibody SC-1 on stomach carcinoma cells. a, apoptotic effect of SC-1 (40 μg/ml) on stomach carcinoma cell lines 23132 and 4433, Control 1, cell survival without SC-1. Chromopure IgM, chromopure human IgM was added in equal amounts to exclude unspecific reaction of antibodies. Comparable amounts of apoptosis were obtained for both cell lines. b, titration of SC-1. Cells were incubated 24 h with indicated amounts of SC-1 antibody. c, effect of cross-linking of SC-1 with rabbit antihuman IgM antibodies, d, after incubation of cells with PI-PLC. Control, untreated cells; SC-1, cells treated with 40 μg/ml SC-1 antibody; SC-1-PI-PLC, cells treated with phospholipase, as described in “Material and Methods,” and then tested with SC-1 for apoptosis.

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Fig. 6. Phosphorylation pattern of cell line 23132 after induction of apoptosis. a, a rapid phosphorylation of tyrosine residues on proteins of approximately 110 and 60 kDa, and a dephosphorylation of a serine residue on a protein of approximately 35 kDa with 40 μg/ml SC-1. b, an increased phosphorylation of a tyrosine residue protein of approximately 75 kDa, with highest level after 10 min.

Fig. 7. Expression and activity pattern of caspases-3 and -8 after induction with SC-1. Cell line 23132 was induced with 40 μg/ml SC-1, and after the times indicated, samples were taken. a, Western blot analysis of caspase-3 and -8. After SC-1 induction, there is an increased expression of both caspases. Also, activation of caspase-3 by proteolytic cleavage is visible due to occurrence of the p20 cleavage product. b, activity of caspase-8. A 7-fold increase of the activity of caspase-8 was found in cell extracts tested with the ApoAlert system 7 h after induction of apoptosis.
selectively removed observed (15). In addition, about 70% of the patients are free of significant tumor regression as compared to the biopsies can be purified SC-1 prior to gastrectomy. The effect of antibody SC-1 on the cell into apoptotic bodies (22). The proteins are present as inactive phosphorylation of 97-, 66-, and 50-kDa proteins (21). A detailed analysis of these SC-1-associated molecules by protein sequencing is under way.

Cross-linking of the CD55/DAF receptor by the antibody SC-1 increases cell death of stomach cancer cells. Cross-linking of membrane molecules upon signals is essential for or increases the effect of external signals. The apoptosis signal of the Fas/CD95 antibodies on cell membranes, such as CD14 (38), are multispecific in function and are a commonly observed phenomenon, e.g., MUC1–6 in mamma carcinoma (41). These molecules are highly immunogenic (42), and it is therefore likely that an already strongly glycosylated membrane molecule, such as CD55/DAF, could be further glycosylated and modified by tumor-specific glycosylation machinery to also become immunogenic.

Furthermore, SC-1 increases levels of caspase-8 (FLICE) and caspase-3 (CPP-32) and leads to a strong activation of caspase-8, similar to other known apoptotic pathways. (24). Caspas are a group of intracellular proteases and are responsible for the disassembly of the cell into apoptotic bodies (22). The proteins are present as inactive pro-enzymes that are activated by proteolytic enzymes after apoptotic signals (44).

The human monoclonal antibody SC-1 is currently being used successfully in a clinical Phase II trial on patients with intestinal- and diffuse-type adenocarcinoma of the stomach. In this study, patients who were tested positively for SC-1 expression receive one dose of purified SC-1 prior to gastrectomy. The effect of antibody SC-1 on the primary tumor and lymphnodes is measured. In 90% of cases, a measurable induction of apoptosis can be observed, and in 50%, a significant tumor regression as compared to the biopsies can be observed (15). In addition, about 70% of the patients are free of lymphnode metastases. These data show that tumor cells can be selectively removed in vivo by a specific apoptotic signal.

ACKNOWLEDGMENTS

The authors thank E. Wozniak and N. Chudnovskaya for excellent technical assistance, E. Schmitt for preparing the artwork, and U. Hausmann for improving the manuscript.

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