Prostate Cancer Cell Proliferation *In vitro* Is Modulated by Antibodies against Glucose-Regulated Protein 78 Isolated from Patient Serum

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Abstract

Circulating autoantibodies against the glucose-regulated protein of 78 kDa (GRP78) are present at high levels in prostate cancer patients and are a biomarker of aggressive tumor behavior. We purified the anti-GRP78 IgGs and examined their effect on 1-LN, PC-3, DU145, and LnCap human prostate cancer cells. We also evaluated its effects on the breast cancer MDA-MB231 and melanoma DM413 cell lines. The anti-GRP78 antibody binds only to cells expressing GRP78 on the surface, to a site also recognized by its physiologic agonist, activated α_2-macroglobulin (α_2M*). This antibody is completely specific for a peptide, including the primary amino acid sequence CNVKSDKSC, which contains a tertiary structural motif mimicking an epitope in GRP78. Tertiary structural analysis suggested the linear GRP78 primary amino acid sequence LIGRTWNDPSVQQDIKFL (Leu[98]-Leu[115]) as the putative binding site, containing the tertiary structual arrangement described above, which was confirmed experimentally. The anti-GRP78 antibodies from prostate cancer patients recognize almost exclusively this epitope. We produced animal antibodies against both these peptides, and they are able to mimic the effects of the human antibody. Our experiments also suggest this epitope as highly immunogenic, thereby explaining the specificity of the immune response against this epitope in GRP78, observed in humans. Using 1-LN cells as a model, we show that anti-GRP78 IgG purified from the sera of these patients mimics the proproliferative effects induced by α_2M* via the common receptor, GRP78. Furthermore, increasing concentrations of human anti-GRP78 IgG show a dose-dependent protective effect on apoptosis induced by tumor necrosis factor α.

Introduction

Activated forms of α_2-macroglobulin (α_2M*) bind to two distinct cell surface receptors, the low-density lipoprotein receptor–related protein (LRP) with moderate affinity ($K_d \sim$ 2-5 nmol/L) and the glucose-regulated protein 78 kDa (GRP78) with high affinity ($K_d \sim$ 100 pmol/L). LRP is a scavenger receptor allowing rapid uptake and degradation of α_2M*, *in vivo* and *in vitro* (1, 2). LRP recognizes a wide variety of agonists that do not compete for binding with α_2M*. The receptor-associated protein binds to LRP, blocking the binding of all known ligands, but does not block intracellular signaling cascades induced by binding of α_2M* to cells. α_2M* binding to GRP78 is blocked neither by receptor-associated protein nor other ligands that bind to LRP (3–5). GRP78 is a chaperone protein induced by cellular stress and hypoxia (6), which is highly expressed in human prostate tumors (7). Although GRP78 functions as an endoplasmic reticulum chaperone (8), it also exists as a transmembrane protein capable of trafficking to tumor cell surfaces including highly metastatic prostate cancers (9–12). Binding of α_2M* to both these receptors occurs via a receptor binding domain (RBD), including the α_2M primary amino acid sequence KMVSGFIPLKPTVKMLERSNH (Lys[1361]-His[1381]; ref. 13).

The binding of α_2M* to GRP78 induces a number of signaling cascades. Initially, there is an inositol 1,4,5-trisphosphate-dependent increase in $[Ca^{2+}]_i$, and as a consequence activation of the Ras/mitogen-activated protein kinase–dependent signaling cascade (4, 5, 14, 15). The phosphatidylinositol 3-kinase pathway is also triggered, leading to activation of the downstream kinase Akt that regulates cell proliferation and apoptosis (10, 16). Silencing *GRP78* gene expression abolishes the rapid increases in IP_3 synthesis, the subsequent increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), and cellular proliferation induced by α_2M* in macrophages (10), thereby confirming its role as the α_2M* signaling receptor, originally reported by our laboratory in 1993 (17).

The circulating concentration of native α_2M may be as high as 5 μmol/L (18). However, in patients with prostate cancers, levels below 0.7 μmol/L have been reported (19–21). Prostate cancer cells may produce α_2M (22), which should increase the pool size of this protein. Prostate cancer cells, however, also produce the proteinase prostate cancer-specific antigen (PSA) and matrix metalloproteinases (23, 24), which convert α_2M to α_2M* (18). The lower levels of circulating α_2M are presumably a direct result of an increased clearance of the activated protein via the α_2M* receptor, LRP (25), because the half life of α_2M* is $\sim$ 2 minutes in both animals and humans (18). Clinically, α_2M levels of <0.7 μmol/L may indicate a possibility of bone metastases (21, 26), suggesting that in addition to increases in PSA levels, this disease requires new factors for expression of more aggressive cell phenotypes.

A recent study showed that an increase of anti-GRP78 antibodies in sera from cancer patients correlated positively with prostate cancer progression and shorter overall survival (23). For these reasons, we investigated the epitope specificity and function of anti-GRP78 antibodies produced by prostate cancer patients. The effect of anti-GRP78 IgGs was evaluated on 1-LN, PC-3, DU145, and LnCap prostate cancer cells. As controls, we used the breast cancer MDA-MB231 and melanoma DM413 tumor cell lines, both of which show up-regulated cytosolic expression of GRP78 (26, 27). Our data show that anti-GRP78 antibodies from prostate cancer patients bind to and stimulate proliferation of tumor cells expressing GRP78 on their surface, and protect them from apoptosis induced by tumor necrosis factor α (TNF-α) in a dose-dependent manner.

We also show that $\alpha_2 M^*$ blocks binding of the anti-GRP78 IgG to 1-LN cells. Finally, we identified a primary amino acid sequence in GRP78 that binds both to the antibody and $\alpha_2 M^*$.

Materials and Methods

Proteins and peptides. $\alpha_2 M$? isolated from human plasma was used to prepare the receptor recognized form $\alpha_2 M$-MeNH$_2$ ($\alpha_2 M^*$) as previously described (28). Recombinant GRP78 was obtained from Stressgen Bioreagents (Victoria, British Columbia, Canada), and keyhole lymphet hemocyanin (KHL) was purchased from Sigma (St. Louis, MO). The $\alpha_2 M$ peptides KMVSGFIPLKPTVKMLERSNH (Lys[1361]-His[1381]) and its mutant peptides K1370A and K1374A, LIGRTWNDPSVQQDIKFL (Leu[98]-Leu[115]) of GRP78, and CNVSDKSC were purchased from Genemed Synthesis, Inc. (San Francisco, CA, USA). The peptides CNVSDKSC or CLIGRTWNDP-SVQQDIKFLC (Leu[98]-Leu[115]) of GRP78 were conjugated to KLH via their terminal cysteine residues to KLH with the heterobifunctional cross-linker sulfosuccinimidyl 4-(N-maleidomethyl)-cyclohexane-1-carboxylate (sulfo-SMMC; Pierce, Rockford, IL). Briefly, 1 mL of a solution of KLH (2 mg/mL) was incubated in 0.1 mol/L sodium phosphate, 0.9 mol/L NaCl (pH 7.2) with 2 mg of sulfo-SMCC for 1 hour at room temperature. The activated carrier was purified by gel filtration using a column of Sephadex G-25 (15 × 2 cm) equilibrated in the same buffer. The first peak (2 mL) was incubated with 2 mg of the peptide for 2 hours at room temperature. Final concentrations of the conjugated KLH peptides were determined using the microbicinchonic protein assay (29). Protein A and Protein G-Sepharose were purchased from Sigma. Radioiodination was carried out by the method of Markwell (30). Incorporation of ^{125}I label was 2×10^7 cpm/nmol of protein. Radioactivity was measured in a Pharmacia-LKB Bioctechnology (Sweden) 1272 γ-radiation counter. RBF mutants K1370A and K1374A were expressed as previously described (13).

Patients. Blood samples for this study were taken from 13 patients from the Department of Urology, El Salvador Hospital, Santiago, Chile. Written informed consent was obtained from these subjects and approved by the Institutional Review Board of the University of Chile. The average age was 72.7 years old (range 55-86 years). The clinical stage was as follows: T_2 in one patient, T_3 in seven patients, and T_4 in five patients. All patients had serum PSA >10 ng/mL (mean 70.02 ng/mL), and were evaluated by histologic grading of transrectal biopsies for prostate cancer (31), before administration of any type of therapy. The Gleason score was 6 for one patient, 7 for four patients, and 8 to 10 for eight patients. All patients had bone metastasis confirmed by scintigraphy using 99m techtenium methylene diphosphonate. Control samples were obtained from age-matched blood donor volunteers.

Antibodies. Anti-GRP78 IgG from human serum was purified by affinity chromatography on Protein A-Sepharose (32), followed by immunoadsorption to GRP78 coupled to Sepharose 4B. Polyclonal antibodies against the GRP78 peptides CNVSDKSC and LIGRTWNDPSVQQDIKFL (Leu[98]-Leu[115]) were raised in rabbits. A polyclonal antibody against recombinant GRP78 was raised in sheep. The rabbit antibodies were purified by a combination of affinity chromatography on Protein A-Sepharose, and their corresponding peptides were covalently coupled to Sepharose 4B. The sheep antibody was purified by immunoaffinity on recombinant GRP78 immobilized on Sepharose 4B. The IRDye 800 DX conjugated affinity purified goat anti-human IgG was purchased from Rockland Immuno-chemicals, Inc. (Gilbertsville, PA). The rabbit anti-human $\alpha_2 M$ polyclonal IgG was purchased from Sigma. The mouse monoclonal anti-human $\alpha_2 M$ IgG (clone 1201) was purchased from Biodesign International (Saco, ME). Nonimmune human IgG was purchased from ICN Pharmaceuticals, Inc. (Aurora, IL). Horseradish peroxidase–conjugated IgG (HRP-IgG) to human IgG was purchased from Sigma.

Analysis of serum anti-GRP78 antibody and $\alpha_2 M$ levels. Antibodies against GRP78 in the serum of prostate cancer patients were assayed by an ELISA technique in 96-well culture plates coated with recombinant GRP78 (5 μg/mL) in 0.1 mol/L Na$_2$CO$_3$, 0.01% NaN$_3$ (pH 9.3). Incubation of these plates with serum samples and analyses of the data were done as previously described (33). The epitope specificity of the anti-GRP78 IgG in the serum of prostate cancer patients was also assayed by an ELISA technique. Briefly, 96-well culture plates were coated with the peptide CNVSDKSC conjugated to KLH (5 μg/mL) in 0.1 mol/L Na$_2$CO$_3$, 0.01% NaN$_3$ (pH 9.3). All assays were done in triplicate as previously described (33).

The concentration of $\alpha_2 M$ in the serum of prostate cancer patients was determined by an ELISA technique in 96-well culture plates coated with mouse anti-human $\alpha_2 M$ monoclonal antibody (mAb; clone 1201) as previously described (33). Calculation of specific $\alpha_2 M$ concentrations were done with calibration curves constructed with purified human $\alpha_2 M$ and a rabbit polyclonal anti-$\alpha_2 M$ IgG. Analyses and data processing were done as described above (33).

Analysis of serum PSA. Serum levels of PSA were determined by an ELISA technique using a UniCel Dxl 800, Access Immunoassay System, at the Duke University Medical Center, Core Clinical Laboratory, following instructions of the manufacturer.

Cell cultures. All tumor cell lines were grown in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/mL penicillin G, and 100 μg/mL streptomycin as described previously (34). The 1-LN cell line was a kind gift from Dr. Phillip Walther (Department of Urology, Duke University Medical Center). The PC-3, DU145, and LnCap prostate tumor and the breast cancer MDA-MB-231 cell lines were obtained from the American Type Culture Collection (Manassas, VA). The melanoma tumor cell line DM413 was a kind gift of Dr. Hilliard F. Seigler (Department of Immunology, Duke University Medical Center).

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ in 1-LN cells was measured by digital imaging microscopy using the fluorescent indicator Fura-2/AM (35).

On cell Westerns. Tumor cell monolayers were grown in 48-well culture plates until confluent and rinsed in HBSS. All binding assays were done at 4°C. Ice-cold 2% paraformaldehyde was added to each well and the plate was incubated at room temperature for 20 minutes. Cells were washed thrice with 400 μL PBS and incubated with a blocking solution containing 3% bovine serum albumin (BSA) and 5% nonimmune goat serum for 90 minutes at room temperature with gentle rocking. Blocking buffer was removed and cells were covered with 100 μL of a solution containing anti-GRP78 IgG (100 pmol/L) in blocking buffer. Cells were then incubated overnight at 4°C with gentle rocking. The next day, wells were rinsed thrice in PBS and incubated with 100 μL of a solution containing a 1:800 dilution of an IRDye 800 DX–conjugated affinity purified goat anti-human IgG in PBS. Plates were kept under low light conditions following addition of IR-conjugated antibodies. Following a 60-minute incubation at room temperature with gentle rocking, plates were again washed thrice with PBS. They were then dried and imaged using the LI-COR Odyssey System. Images were analyzed using Excel (Microsoft Corp, Redmond, WA) and Prism (GraphPad Software, San Diego, CA) software.

Antibody binding studies. The binding assays were done in confluent 1-LN cell monolayers cultured in 48-well tissue culture plates. Before use in binding assays, cells were washed thrice in HBSS. All binding assays were done at 4°C in RPMI 1640 (Life Technologies, Inc., Carlsbad, CA) containing 2% BSA. Cell viability was determined by trypan blue stain of cells detached with 20 mmol/L EDTA in RPMI 1640 from four wells and then by counting in a Neubauer chamber under a microscope. About 98% (1×10^5 per well) of the cells used in the binding experiments were viable. ^{125}I-labeled IgG was incubated with the cells at 4°C for 1 hour. Free ligand was separated from bound by removing the incubation mixture (300 μL) by aspiration and washing the cell monolayers rapidly with RPMI 1640 containing 20 mg/mL BSA. The cells were then lysed with 0.1 mol/L NaOH and 2% SDS (400 μL) and radioactivity was measured in a Pharmacia-LKB 1272-γ radiation counter. Bound IgG was calculated after subtraction of nonspecific binding measured in the presence of 50 μmol/L nonlabeled ligands. Estimates of dissociation constant (K_d) values and maximal binding of the ligands (B_{max}) were determined by fitting data directly to the Lagmuir isotherm using the statistical program SYSTAT for Windows: Statistics, Version 11 (Systat, Inc., Evanston, IL).

1-LN cell proliferation assays. 1-LN cells suspended in RPMI 1640 containing 5% FBS at a density of 1×10^5/mL were plated in 96-well culture plates (0.1 mL/well) containing increasing concentrations of human $\alpha_2 M^*$, human anti-GRP78 IgG, or nonimmune human IgG at a final volume of

0.2 mL/well. Cell proliferation was determined at 72 hours using a BrdUrd labeling and colorimetric immunoassay detection method (Roche Molecular Biochemicals, Indianapolis, IN). Results were expressed as absorbance at 372 nm (reference wavelength: 492 nm). Control cell proliferation was determined in the presence of nonimmune human IgG added at the same concentration as anti-GRP78 IgG.

DNA fragmentation. Tumor cells were suspended in RPMI 1640 containing 5% FBS at a density of 1×10^5 cells/mL and plated in 96-well culture plates (0.2 mL/well) containing increasing concentrations of TNF-α or a fixed concentration of TNF-α (100 ng/well) and increasing concentrations of human anti-GRP78 IgG. DNA fragmentation was determined at 72 hours by quantitation of cytosolic oligonucleotide-bound DNA using an ELISA kit (Roche Diagnostics Corporation, Indianapolis, IN), according to the instructions of the manufacturer. Briefly, the cytosolic fraction (13,000 × *g* supernatant) from cells in each well was used as an antigen source in a sandwich ELISA with a primary antihistone antibody coated to the microtiter plate and a secondary anti-DNA antibody coupled to peroxidase. From the absorbance values, the percentage of fragmentation compared with controls was calculated as described by Leist et al. (36).

SDS-PAGE and immunoblotting. Electrophoresis was done on polyacrylamide gels (1.2 mm thick, 14 × 10 cm) containing 0.1% SDS. A discontinuous Laemmli buffer system was used (37). Transfer to nitrocellulose membranes by the Western blot method was carried out as described by Towbin et al. (38). The dye-conjugated M_r markers (Bio-Rad, Richmond, CA) used were as follows: myosin (M_r 200,000), β-galactosidase (M_r 116,000), phosphorylase b (M_r 96,000), BSA (M_r 66,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 31,000), and soybean trypsin inhibitor (M_r 27,000).

Statistics. The levels of anti-GRP78 IgG and α_2M in sera from prostate cancer patients, as well as differences in tumor cell proliferations induced by anti-GRP78 antibodies, were evaluated by a Student's *t* test using the program SYSTAT for Windows: Statistics, Version 11 (Systat).

Results

Anti-GRP78 IgG, α_2M, and PSA levels in prostate cancer patients. Consistent with previous reports (23), the analysis of serum anti-GRP78 IgG in our subject group (Fig. 1*A*) shows titer increases that are statistically significant between prostate cancer patients and controls (means, 0.405 ± 0.381 and 0.062 ±

0.038 µmol/L, respectively; *P* < 0.0004). Because the peptide CNVSDKSC has been shown to contain a motif recognized by anti-GRP78 antibodies produced by prostate cancer patients (23), we developed an ELISA assay with this peptide conjugated to KLH and immobilized on 96-well culture plates. This assay (Fig. 1*B*) shows serum titers very similar to the ones determined with intact GRP78 (means 0.416 ± 0.337 and 0.052 ± 0.044 for prostate cancer patients and controls, respectively, *P* < 0.0004). Furthermore, this assay suggests that most of the anti-GRP78 IgG in the serum of prostate cancer patients is specific for a motif contained in the primary structure of the peptide CNVSDKSC. The analysis of total serum α_2M (Fig. 1*C*) showed titer decreases that are statistically significant between prostate cancer patients and controls (means 0.435 ± 0.285 and 0.990 ± 0.338 µmol/L, respectively; *P* < 0.005), and are consistent with observations reported by other investigators (19–21). Analyses of total serum PSA in our subject group (Fig. 1*D*) shows high levels of this protein (mean 70.02 ± 37.12 ng/mL) when compared with control subjects (mean <0.1 ng/mL).

1-LN cell surface reactivity with anti-GRP78 IgG analyzed by on cell Western analyses. Nonpermeabilized tumor cell monolayers grown on 48-well culture plates were used for these experiments (Fig. 2*A*). The cells were incubated first with 100 pmol/L purified anti-GRP78 IgG, followed by an incubation with a secondary IRDye 800 DX–conjugated secondary goat anti-human IgG, and visualized by IR fluorescence. GRP78 seems to be expressed only on the surface of 1-LN, DU145, and DM413 cells (Fig. 2*A, top, lanes 1, 3,* and *6,* respectively), whereas the prostate PC-3, and LnCap or the breast MDA-MB-231 cell lines does not show expression of this protein on their surface (Fig. 2*A, top, lanes 2, 4,* and *5,* respectively). As expected, the nonimmune human IgG did not show any reactivity with the cells (Fig. 2*A, bottom, lanes 1-6,* respectively).

The anti-GRP78 IgG is able to induce a increase in [Ca^{2+}]$_i$ with an oscillating pattern in 1-LN cells (Fig. 2*B*), thereby suggesting that engagement of GRP78 on the cell surface by this antibody
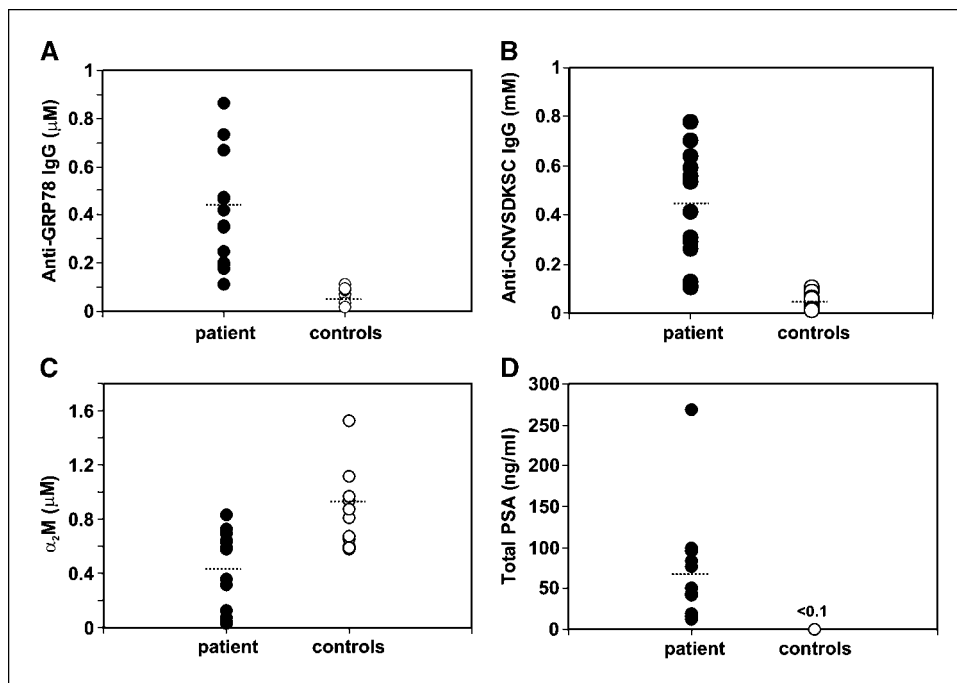

Figure 1. Serum levels of anti-GRP78 IgG, α_2M, and PSA titers in prostate cancer patients and normal controls. Serum samples (100 µL, 1:5,000 dil) from patients (*n* = 13) and normal controls (*n* = 13) in 0.2 mL PBS-Tween were added to 96-well culture plates and assayed as described in Materials and Methods. *A,* comparison of anti-GRP78 IgG titers of prostate cancer patients (●) and normal controls (○). *B,* comparison of α_2M titers between prostate cancer patients (●) and normal controls (○). *C,* total PSA titers of prostate cancer patients (●) and normal controls (○; values <0.1 ng/mL). *Horizontal lines,* mean titer values.
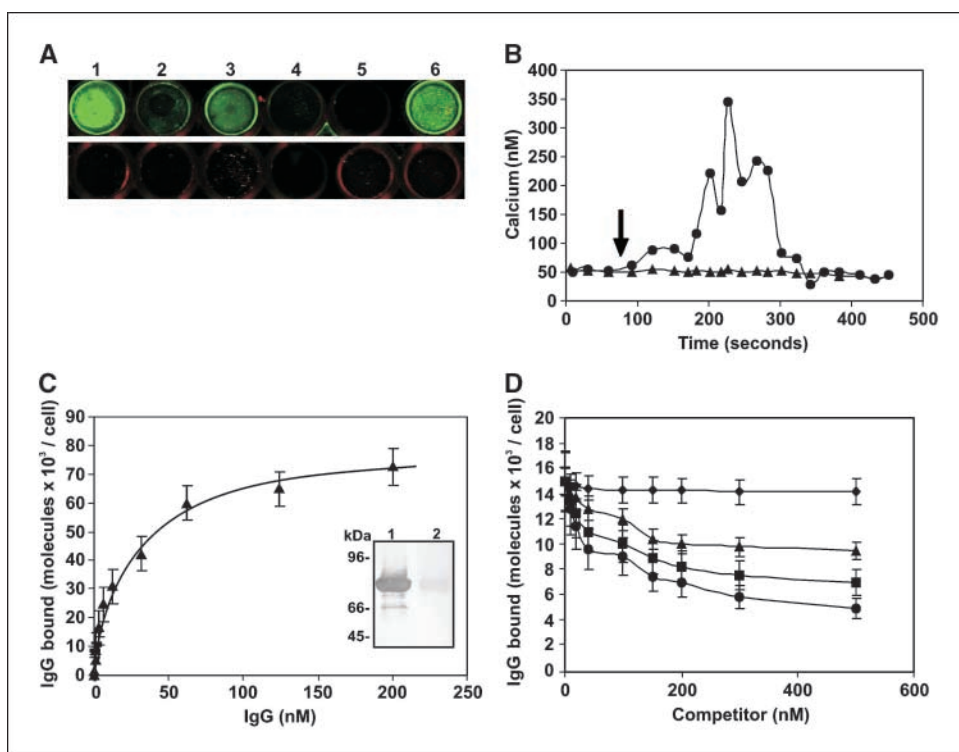
Figure 2. *A,* in cell Western immunocytochemical assay of GRP78 on tumor cell surfaces. Nonpermeabilized tumor cells were incubated with prostate cancer anti-GRP78 IgG or nonimmune human IgG. Bound antibody was detected with an IR-labeled secondary antibody as described in Materials and Methods. *Top,* IR images of 1-LN (*1*), PC-3 (*2*), DU-145 (*3*), LnCap (*4*), MDA-MB231 (*5*), and DM413 (*6*) cells, respectively, incubated with anti-GRP78 IgG. *Bottom,* IR images of 1-LN (*1*), PC-3 (*2*), DU145 (*3*), LnCap (*4*), MDA-MB-231 (*5*), and DM413 (*6*) cells, respectively, incubated with a nonimmune human IgG. *B,* changes in $[Ca^{2+}]_i$ in 1-LN cells exposed to prostate cancer patient anti-GRP78 IgG. The cells were preloaded with Fura-2/AM (4 μmol/L) and the changes in $[Ca^{2+}]_i$ were measured after addition of anti-GRP78 IgG (100 pmol/L). *Arrow,* time of addition of the ligand. (●), effect of anti-GRP78 on $[Ca^{2+}]_i$. (▲), effect of nonimmune human IgG on $[Ca^{2+}]_i$. *C,* binding of prostate cancer patient anti-GRP78 to 1-LN cells. Increasing concentrations of ^{125}I-labeled anti-GRP78 IgG were added to 1-LN cell monolayers cultured in 48-well plates (1×10^5 per well). IgG bound was calculated after subtraction of nonspecific binding measured in the presence of nonlabeled ligand (50 μmol/L) as described in Materials and Methods. *Inset,* blot binding assay of a SDS/10% PAGE (reducing conditions) of GRP78 (5 μg/lane). *Lane 1,* incubation with prostate cancer patient anti-GRP78 IgG (0.1 μg/mL); *lane 2,* incubation with prostate cancer patient IgG (0.1 μg/mL) in the presence of the peptide CNVSDKSC conjugated to KLH (0.25 μg/mL). *Points,* means from experiments done in triplicate; *bars,* SD. *D,* binding of prostate cancer patient anti-GRP78 IgG in the presence of α2M* or its RBD peptide Lys^{1361}-His^{1381}. 1-LN cells were incubated with ^{125}I-labeled anti-GRP78 IgG (100 pmol/L) in the presence of increasing concentrations of nonlabeled α2M* (●), RBD peptide (■), RBD mutant K1370A (▲), and RBD mutant K1374 (♦). Points, means from experiments done in triplicate; bars, SD.

initiates a signal transduction cascade. The ^{125}I-labeled anti-GRP78 binds to 1-LN cells (Fig. 2C) in a dose-dependent manner to a large number of sites (B_{max} of 86.27×10^3 binding sites per cell) with high affinity (K_d of 66 ± 6.56). The epitope specificity of the human anti-GRP78 antibody for the peptide CNVSDKSC was confirmed again using GRP78 immobilized on a nitrocellulose membrane. The Western blot results (Fig. 2C, inset, lane 1) show reactivity of GRP78 with this antibody, whereas incubation of the anti-GRP78 IgG with the octapeptide CNVSDKSC inhibits its binding to GRP78 (Fig. 2C, inset, lane 2), suggesting a specificity similar to that isolated by Mintz et al. (23).

A competition for binding to 1-LN cells of the ^{125}I-labeled anti-GRP78 IgG by either α2M* or its RBD (Lys^{1361}-His^{1381}) clearly shows the capacity of both these agonists to displace the antibody from binding to these cells (Fig. 2D), thereby suggesting binding of both to the same site on GRP78. The specificity of the RBD peptide for GRP78 was also evaluated. We have previously determined that mutations of the RBD affect binding of α2M* to GRP78 (13). The RBD mutant K1370A binds to GRP78 as well as the wild type, whereas binding of the mutant K1374A to GRP78 is blocked (13). Competition experiments with these mutants (Fig. 2D) show that K1370A competes with the anti-GRP78 IgG for binding on 1-LN cells, but K1374A has no effect, thereby confirming that Lys^{1374} is critical for binding of α2M* to GRP78.

Identification of the α2M binding site on 1-LN cell-surface GRP78. An extensive search for primary sequence similarities in the Protein Data Bank of the Swiss Institute of Bioinformatics between the octapeptide CNVSDKSC and GRP78 did not show any homologies. The similarity between this octapeptide and an epitope in GRP78 seems to be only conformational and may be evident only at the secondary or tertiary structural levels. We searched for such similarities and found a region in GRP78 where Asp^{105} is part of an α-helix, which is flanked by Ser^{107} and Val^{108} in the inverted position with respect to the octapeptide. This region is also flanked on the NH2-terminal side by the primary amino acid sequence LIGRT (Leu^{98}-Thr^{102}), which has been identified as a heparin-binding site in fibronectin and the 70 kDa family of heat-shock proteins (39). Because polysulfated drugs, such as suramin, inhibit binding of α2M* to cell surface receptors (40), the presence of this heparin-binding site served as additional evidence, suggesting this region as an α2M* binding site on GRP78. Therefore, we synthesized the GRP78 peptide LIGRTWNDPSVQQDIKFL (Leu^{98}-Leu^{115}), which includes both these regions, and assessed its capacity to interfere with the binding of the anti-GRP78 IgG to 1-LN cells.

The chemically synthesized α2M* RBD peptide (Lys^{1361}-His^{1381}) induces changes in $[Ca^{2+}]_i$ in 1-LN cells, in a similar fashion to those observed with cloned rat α2M* RBD (Lys^{1361}-His^{1381}; ref. 13).

Therefore, we used this peptide as an $\alpha_2 M^*$ substitute. Incubation of 1-LN with this peptide followed by anti-GRP78 IgG inhibits the oscillating $[Ca^{2+}]_i$ signaling response induced by the antibody (Fig. 3A). Anti-GRP78 IgG inhibits the $[Ca^{2+}]_i$ signaling induced by the peptide (Fig. 3B). A similar induction on $[Ca^{2+}]_i$ signaling response, followed by an inhibition to the response induced by anti-GRP78 IgG, is also observed with the mutant peptide K1370A (Fig. 3C). However, neither induction on $[Ca^{2+}]_i$ signaling response or inhibition of the response to anti-GRP78 IgG is observed with the mutant peptide K1374A (Fig. 3D).

Next, we evaluated the capacity of the GRP78 peptide (Leu[98]-Leu[115]) to affect $[Ca^{2+}]_i$ signaling induced by anti-GRP78 IgG. Preincubation of the antibody (100 nmol/L) with the GRP78 peptide Leu[98]-Leu[115] (100 nmol/L) for 1 hour, before addition to 1-LN cells, blocks almost completely the $[Ca^{2+}]_i$ signal induced by the anti-GRP78 IgG (Fig. 3E). Finally, incubation of 1-LN cells with heparin (1 µg/mL) before addition of the $\alpha_2 M^*$ RBD peptide (Lys[1361]-His[1381]) or anti-GRP78 IgG (both at 100 nmol/L) blocks almost completely the $[Ca^{2+}]_i$ signal induced by these agonists (Fig. 3F). Incubation of the RBD peptide with the GRP78 peptide (Leu[98]-Leu[115]) for 1 hour before addition to 1-LN cells also inhibited the $[Ca^{2+}]_i$ signal induced by RBD (data not shown). Taken together, these results suggest that the GRP78 primary amino acid sequence LIGRTWNDPSVQQDIKFL (Leu[98]-Leu[115]) is a common binding domain for both $\alpha_2 M^*$ and the anti-GRP78 IgG isolated from prostate cancer patients.

Specific recognition of the CNVSDKSC epitope by antibodies raised in animals. Rabbit polyclonal antibodies were raised against the peptides CNVSDKSC and LIGRTWNPSVQQDIKFL (Leu[98]-Leu[115]). A polyclonal antibody against recombinant GRP78 was raised in sheep. The three antibodies were initially purified by chromatography on Protein A-Sepharose (rabbit IgGs) or Protein G-Sepharose (sheep IgG), followed by specific peptide immunoaffinity chromatography (rabbit IgGs) or recombinant GRP78 immobilized on Sepharose 4B (sheep IgG). Each antibody (3 mg) was individually adsorbed on a resin (5 mL) containing the CNVSDKSC peptide immobilized, via an arm containing 6-aminohexanoic acid, to Sepharose 4B. Both rabbit polyclonal IgGs show 100% reactivity with the CNVSDKSC peptide (Fig. 4), whereas the polyclonal IgG raised in sheep against recombinant GRP78 shows two IgG populations, one that binds and another one that does not bind to this peptide (Fig. 4). As expected, both fractions reacted with GRP78 immobilized on nitrocellulose membranes (data not shown). Over 70% of the antirecombinant GRP78 IgG raised in sheep is specific for the peptide CNVSDKSC, suggesting that this region of GRP78 as highly immunogenic.

Effect of anti-GRP78 IgG on tumor cell proliferation. 1-LN, PC-3, DU145, LnCap, MDA-MB-231, and DM413 tumor cells in 96-well culture plates (1×10^4 per well) were incubated with anti-GRP78 IgG (100 pmol/L) for 72 hours in RPMI 1640 culture medium containing 5% FBS. Cell proliferation was determined using a BrdUrd labeling and colorimetric immunoassay detection method as described in Materials and Methods. Under these conditions, 1-LN, DU145, and DM413 tumor cells show statistically significant increases in proliferation induced by the anti-GRP78 IgG (Fig. 5A1, $P < 0.0001$; A3, $P < 0.004$; and A4, $P < 0001$, respectively).
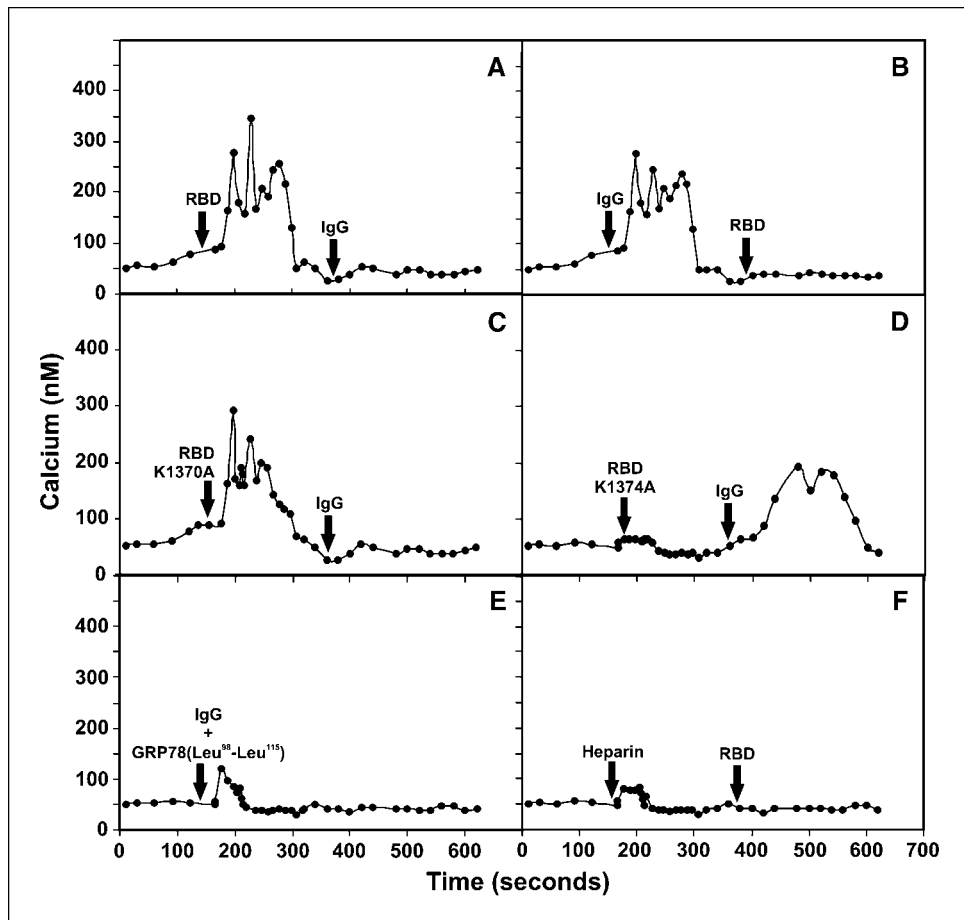

Figure 3. $[Ca^{2+}]_i$ response of 1-LN cells to the binding of anti-GRP78 IgG, $\alpha_2 M^*$ RBD peptide Lys[1361]-His[1381], and their antagonists. Cells were preloaded with 4 µmol/L Fura/AM and the changes in $[Ca^{2+}]_i$ were measured as described in Materials and Methods. *Arrows,* time of addition of the peptides (100 nmol/L) or anti-GRP78 IgG (100 pmol/L). *A,* incubation of the cells with $\alpha_2 M^*$ RBD peptide Lys[1361]-His[1381] for 180 seconds, followed by addition of anti-GRP78 IgG. *B,* incubation of the cells with anti-GRP78 IgG for 180 seconds, followed by addition of $\alpha_2 M^*$ RBD peptide Lys[1361]-His[1381]. *C,* incubation of the cells with RBD mutant K1370A for 180 seconds, followed by addition of anti-GRP78. *D,* incubation of the cells with RBD mutant K1374A for 180 seconds, followed by addition of anti-GRP78. *E,* incubation of the cells with an equimolar mixture of anti-GRP78 IgG and the GRP78 peptide Leu[98]-Leu[115]. *F,* incubation of the cells with heparin (1 µg/mL).
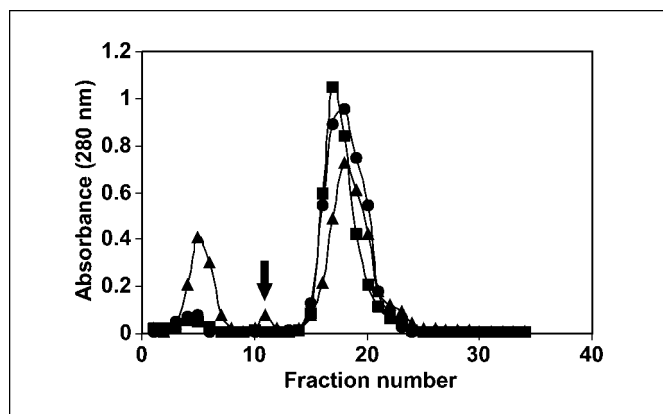
Figure 4. Immunoaffinity on CNVSDKSC-AHA-Sepharose. Rabbit polyclonal IgGs against the peptide CNVSDKSC (●), GRP78 peptide (Leu[98]-Leu[115]; ■), or sheep anti-recombinant GRP78 (▲) in PBS (3 mg) were individually filtered through a column (5 mL) containing the peptide CNVSDKSC immobilized on Sepharose 4B. The IgGs reacting with the peptide were eluted (*arrow*) with 20 mmol/L glycine-HCl (pH 3.0). Fractions of 1.0 mL were collected.

Increases on 1-LN cell proliferation were also induced by similar concentration (100 pmol/L) when compared with that of cells incubated with nonimmune IgG as controls. The rabbit antibodies against the CNVKSDKSC and GRP78 peptide (Leu[98]-Leu[115]) stimulate cell proliferation at a rate similar to that observed for the human IgG (Fig. 5*B2b*, $P < 0.0001$; and *B3b*, $P < 0.0001$, respectively). Similarly, the antirecombinant GRP78 IgG raised in sheep and purified by specific immunoaffinity to the CNVKSDKSC peptide induces cell proliferation at a rate similar to that of the human IgG (Fig. 5*B4b*, $P < 0.0001$). The anti-GRP78 IgG not reactive for this peptide did not induce any proliferation of 1-LN cells and shows an effect similar to that of nonimmune sheep IgG (Fig. 5*B5a* and *B5b*, respectively).

Our data suggest that the effect of the autoantibody is quantitatively modulated by the amount of GRP 78 expressed on the cell surface. For this reason, we evaluated its specificity as a receptor for α_2M^* or anti-GRP78 on 1-LN cells alone (Fig. 5*C*) plated in 96-well culture plates at a cell density of 1×10^4 per well in a 0.2 mL final volume. Anti-GRP78 IgG at two concentrations (50 and 100 pmol/L, respectively) do not modify the effect of α_2M^* (50 pmol/L) on 1-LN cell proliferation (Fig. 5*C1b,c*), as expected if both proteins are agonists of the same receptor. However, the peptide CNVSDKSC conjugated to KLH at two concentrations (50 and 500 pmol/L) inhibits the effect of α_2M^* (50 pmol/L) on cell proliferation (Fig. 5*C2b,c*). The same results were observed when α_2M^* (50 pmol/L) was incubated in the presence of the GRP78 peptide (Leu[98]-Leu[115]) at two concentrations (50 and 500 pmol/L, respectively; Fig. 5*C3b,c*). Once again, these results suggest that both α_2M^* and the anti-GRP78 IgG isolated from prostate cancer patients bind to a common site on GRP78 localized on the surface of these cells.

Because α_2M^* and the anti-GRP78 IgG share the same binding site and both induce proliferation in tumor cells, we assessed the capacity of this antibody to influence apoptosis induced by TNF-α on prostate, breast, and melanoma tumor cell lines (41–43). 1-LN, PC-3, DU145, LnCap, MDA-MB-231, and DM413 cells (1×10^4 per well) were incubated for 72 hours with increasing concentrations of TNF-α in RPMI 1640 containing 5% FBS (Fig. 6*A*). Apoptosis induced by TNF-α was evaluated on these cell lines by using a DNA
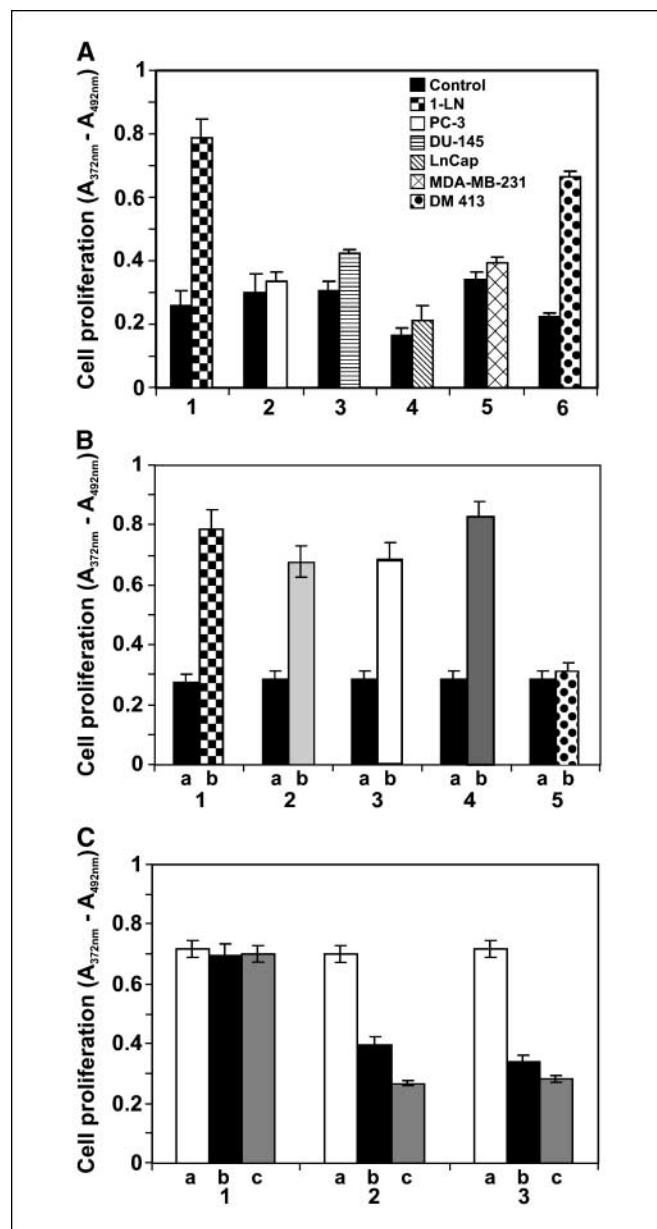

Figure 5. Effect of prostate cancer patient anti-GRP78 IgG on tumor cell proliferation. Cells in 96-well culture plates (1×10^4 per well) were incubated with anti-GRP78 IgG or control nonimmune human IgG at the same concentration (100 pmol/L) for 72 hours in RPMI 1640 culture medium containing 5% FBS. *A1,* 1-LN; *A2,* PC-3; *A3,* DU145; *A4,* LnCap; *A5,* MDA-MB-231; and *A6,* DM413 cells, respectively. *B,* comparison of the effect on 1-LN cell proliferation of the antibodies raised in animals with that of the human anti-GRP78 antibody and control nonimmune IgGs at a similar concentration (100 pmol/L). *B1a* (■), nonimmune human IgG. *B1b* (▨), human anti-GRP78 IgG. *B2a* (■), nonimmune rabbit IgG. *B2b* (■), rabbit IgG against the CNVKSDKSC peptide. *B3a* (■), nonimmune rabbit IgG. *B3b* (□), rabbit anti-GRP78 peptide (Leu[98]-Leu[115]) IgG. *B4a* (■), nonimmune sheep IgG. (B4b, ■) Sheep anti-recombinant GRP78 IgG purified by specific immunoaffinity to the CNVKSDKSC peptide. *B5a* (■), nonimmune sheep IgG. *B5b* (▨), sheep anti-GRP78 IgG not reactive with the CNVKSDKSC peptide. *C,* 1-LN cells were incubated with α_2M^* (50 pmol/L), in the absence or presence of antagonists, for 72 hours in RPMI 1640 culture medium containing 5% FBS. *C1a,* α_2M^*. *C1b,* α_2M^* in the presence of anti-GRP78 IgG (50 pmol/L). *C1c,* α_2M^* in the presence of anti-GRP78 IgG (100 pmol/L). *C2a,* α_2M^*. *C2b,* α_2M^* in the presence of the peptide CNVSDKSC conjugated to KLH (50 pmol/L). *C2c,* α_2M^* (50 pmol/L) in the presence of peptide CNVSDKSC conjugated to KLH (500 pmol/L). *C3a,* α_2M^*. *C3b,* α_2M^* in the presence of the GRP78 Leu[98]-Leu[115] peptide (50 pmol/L). *C3c,* α_2M^* in the presence of the GRP78 Leu[98]-Leu[115] peptide (500 pmol/L). Cell proliferation was measured as described in Materials and Methods. *Columns,* means from experiments done in triplicate; *bars,* SD.
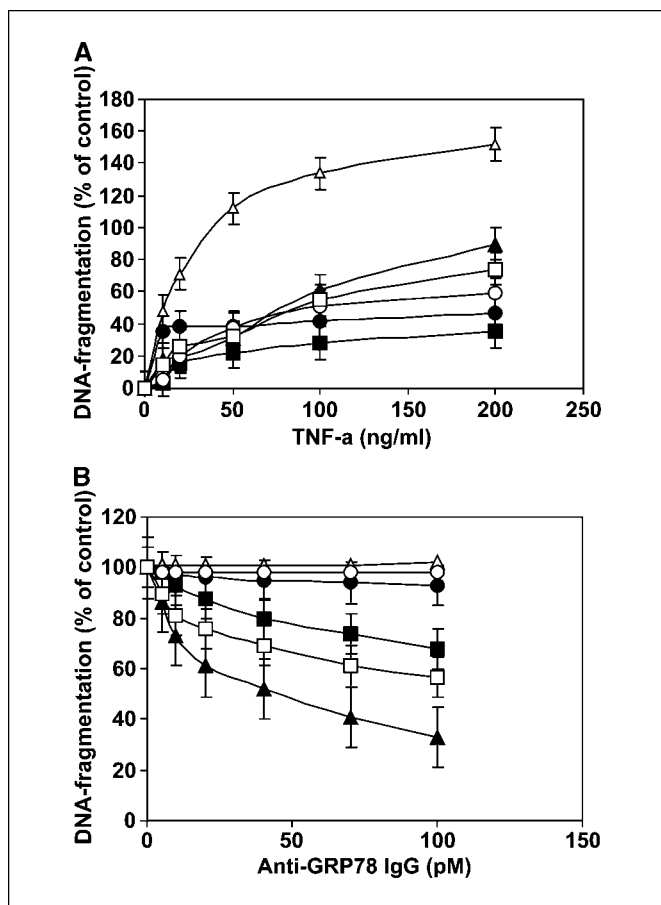
Figure 6. Protection from apoptosis by the prostate cancer patient anti-GRP78 IgG on tumor cells. *A,* tumor cells were incubated in RPMI 1640 containing 5% FBS and increasing concentrations of TNF-α. Apoptosis was evaluated by DNA fragmentation assays as described in Materials and Methods. DNA fragmentation of 1-LN (▲), PC-3 (●), DU145 (■), LnCap (△), MDA-MB231 (○), and DM413 (□) cells. *B,* cells were incubated with a single concentration of TNF-α (100 ng/mL) and increasing concentrations of anti-GRP78 IgG. DNA fragmentation assays of 1-LN (▲), PC-3 (●), DU145 (■), LnCap (△), MDA-MB-231 (○), and DM413 (□) cells.

fragmentation assay as described in Materials and Methods. The effect of increasing concentrations of the anti-GRP78 IgG at a single concentration of TNF-α (100 ng/mL) shows a dose-dependent protective effect on apoptosis, which is significant on 1-LN and DM 413 cells, and moderate on DU145 cells (Fig. 6*B*).

Discussion

Expression of GRP78 on the cell surface confers a protective cellular response against stress conditions present in solid tumors (44, 45). Because the immune response against GRP78 observed in prostate cancer is correlated with a bad prognosis of the disease (23), we assessed not only the titers of anti-GRP78 antibodies, but also the binding properties and physiology of these antibodies on 1-LN, PC-3, DU145, and LnCap prostate cancer cell lines.

The human prostate carcinoma cell line 1-LN, a subclone of the PC-3 cell line, are highly metastatic in athymic nude mice (46). 1-LN cells proliferate substantially when exposed to picomolar concentrations of α_2M^*, whereas the parent prostate cancer cell line PC-3 shows only a minimal proliferative response to α_2M^*

(10, 47). Expression of GRP78 is absent on the surface of PC-3 and LnCap, but it is moderately expressed in DU145 cells (10, 47). As shown in this study, the absence of GRP78 expression on PC-3 and LnCap cells not only accounts for the lack of proliferative stimulation by α_2M^* (48), but also the absence of stimulation of proliferation by anti-GRP78 IgG. DU145 cells, which show a moderate level of GRP78 expression (10, 47), also show a moderate response to anti-GRP antibodies in the present study. Our results suggest that the highly metastatic behavior of prostate 1-LN and DU145, and melanoma DM413 cells, may be explained in part by expression of GRP78 on their surface, which, at the same time, may protect them from apoptosis induced by TNF-α or from immune attacks. However, a note of caution must be expressed with respect to the situation in patients. Here, other effects of these autoantibodies, such as antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity, could play a role in the biology of tumors that would not have been detected in the current studies. The effects of autoantibodies directed against GRP78 ultimately must be addressed by well-designed *in vivo* models. Such models will need to mimic the low levels of circulating α_2M seen in prostate cancer patients (19–21) in addition to the high level of the autoantibodies.

Binding of α_2M^* to 1-LN cells is mediated almost entirely by GRP78, via a pertussis toxin-insensitive G protein, producing a rapid increase in the synthesis of IP_3, with a related increase in $[Ca^{2+}]_i$ (47), which leads to activation of protein kinase C (14), activation and phosphorylation of phospholipase Cγ1, and alkalinization of the cell cytoplasm (48). These last two effects, along with the regulation of the growth of smooth muscle cells, suggest that α_2M^*, in addition to serving as a sensor for proteolysis, also functions as a growth factor–like protein (48).

Our study confirms the presence of previously reported circulating anti-GRP78 antibodies in prostate cancer patients (23). We identified the GRP78 primary amino acid sequence LIGRTWNDPSVQQDIKFL (Leu[98]-Leu[115]) as a putative binding site for α_2M^*, and show that patient anti-GRP78 antibodies also bind to this structural segment. Furthermore, we also show that antibodies raised in rabbit or sheep against this GRP78 structural segment behave like the antibodies isolated from human serum. Interestingly, over 70% of the polyclonal antibodies elicited by recombinant GRP78 antibodies in sheep are specific for a motif included within the primary amino acid sequence Leu[98]-Leu[115], thereby suggesting this region as highly immunogenic.

In a previous study, we reported that 1-LN cells incubated with a mAb against the COOH-terminus of GRP78 blocked effectively the increase in the $[Ca^{2+}]_i$ response of 1-LN cells, and inhibited cell proliferation to some degree (9). In this study, we show that antibodies against a segment of GRP78 primary structure containing the amino acids Leu[98]-Leu[115] induce cell proliferation. The action of these antibodies may be restricted only to exposed GRP78 extracellular domains. For this reason, the topological localization of GRP78 on cell membranes is critical when designing antibodies for therapeutic purposes. We also must consider that drugs may not reach deeply beyond the perivascular region due to physical barriers by a stroma and to elevated interstitial pressure, which reduces fluid convection (49). Our results suggest a significant decrease in circulating α_2M in our study group of patients with prostate cancer. A decrease in circulating α_2M in patients with prostate cancer to concentrations below 0.7 μmol/L also has been reported by other investigators (19–21). This decrease in the concentration of circulating α_2M is most likely

the result of activation of large amounts of this protein by the tumor, a situation which, although undesirable in itself, may be further aggravated by increasing concentrations of circulating anti-GRP78 IgG. Therefore, we may hypothesize that this antibody, together with an aberrant expression of GRP78, facilitates the appearance of a more aggressive prostate cancer cell phenotype.

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