Tumor secreted grp170 chaperones full-length protein substrates and induces an adaptive anti-tumor immune response in vivo

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Abstract

Purpose—We employed a grp170-secreting tumor cell system to determine whether tumor cells engineered to secrete grp170 generate an anti-tumor specific immune response. Further, we examine the possibility that secreted grp170 can bind to and co-transport out of tumor cells full-length tumor antigens that may play a role in the anti-tumor immune response.

Materials and Methods—Wild type Colon-26 and Colon-26 engineered to secrete grp170 were subcutaneously inoculated into BALB/c mice. Tumor growth was monitored, and variations in immunoregulatory mechanisms were evaluated using immunohistochemistry, lymphocyte depletion, ELISpot assays, and Western blot analysis.

Results—Immunization of animals with grp170-secreting tumor cells results in rejection of the tumor by induction of antigen-specific, CD8-dependent immune responses. The secreted grp170 is able to deliver full-length tumor antigens to the tumor microenvironment, thus making them available for uptake by antigen presenting cells (APCs) to initiate tumor-specific immune responses.

Conclusions—These data parallel our studies showing that hsp110 or grp170 are able to chaperone full-length proteins, and when complexed with protein antigens and used as vaccines, these complexes elicit immune responses in vivo against the protein antigens. This cell-based approach has the potential to be utilized as a tumor-specific vaccine in tumors of various histological origins.

Keywords

vaccines; tumor immunotherapy; molecular chaperones; heat shock proteins; tumor antigens

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Introduction

Stress proteins are molecular chaperones that, either alone or in-concert with other chaperones and co-chaperones can inhibit the aggregation of proteins and refold and reactivate damaged proteins that form during cellular stress. Molecular chaperones also participate in numerous other cellular processes such as protein folding, transport, and trafficking of full-length proteins [1]. Grp170 is a major stress protein/molecular chaperone resident in the ER [2–4] and it is induced by conditions such as hypoxia, ischemia and interference in calcium homeostasis [5, 6]. In addition, grp170 is associated with the folding/ processing of secretory proteins such as thyroglobulin and immunoglobulin chains [2, 7] and may also be involved in protein/peptide import into the ER [8, 9].

While it has been reported that elevated levels of a particular type of stress protein, often referred to as heat shock proteins (hsp), can promote cancer by inhibiting programmed cell death and by promoting autonomous growth [48], several others have shown that hsps, including hsc70, grp94/gp96, Calreticulin, hsp110 and grp170 can serve as tumor rejection antigens [10–14, 41]. Further, it has been shown that some stress proteins can interact with various receptors on APCs leading to their uptake along with associated peptides that stimulate specific immune responses [15, 16, 36, 42], secretion of pro-inflammatory cytokines [17, 18], and maturation of DCs [15]. There is also an abundance of literature reporting a role for hsp70 in the activation of natural killer (NK) cells (reviewed in [47]). Thus, the adjuvant activity of some hsps/stress proteins appears to be multi-fold in that they induce both innate and adaptive immunity by stimulation of pro-inflammatory responses and priming of T cells [20]. These immunostimulatory advantages have made heat shock proteins (hsp) promising candidates in various immunotherapeutic approaches. For detailed reviews regarding the utility of hsps as immunotherapeutic agents, refer to Manjili et al. [44] and Wang et al. [45].

Murine tumors of several histological backgrounds exhibit decreased tumorigenicity and increased immunogenicity *in vivo* when transfected to secrete gp96 [19, 43]. Similarly, mice inoculated with tumors engineered to secrete hsp70 display strong CTL and NK cell responses, which impair tumor growth and increase survival [21]. In the studies presented here we use a grp170 secreting tumor cell system to determine if tumor cells engineered to secrete grp170 generate an anti-tumor specific immune response. We also explore the possibility that secreted grp170 can bind to and co-transport out of tumor cells a full-length tumor antigen that may play a role in the anti-tumor immune response.

Materials and Methods

Construction of sgrp170- pcDNA expression vector

Murine grp170 cDNA is amplified by PCR using Taq polymerase, a forward primer 5'ACCATCTCGCAAATAAATAGT-3', and a reverse primer 5'GTACAGTCTAGATTAATGGTGATGGTGATGATGTGAAGGCCGCTTCTGTCC3'. The PCR product which encodes for grp170 with deletion of KNDEL sequence (ER retention signal)[22, 23] and addition of a six histidine-tag at the C-terminus was then inserted into BamH I and Xba I restriction sites of a plasmid vector pcDNA3.1 (Invitrogen, Carlsbad, CA) which contains human cytomegalovirus immediate-early (CMV) promoter and Neomycin resistance gene.

Tumor cells transfection and selection

Colon-26 Murine colon adenocarcinoma cells originally derived from BALB/c mice treated with N-nitroso-N-methylurethane (ATCC, Rockville, MD) were transfected with sgrp170- pcDNA or mock-transfected with pcDNA3.1 using Lipofectamine reagent (Invitrogen,

Carlsbad, CA). Stable transfectants were selected using a limiting dilution culture with 1mg/ml of Geneticin (G418) (Sigma-Aldrich, St. Louis, MO) [24]. Cells were maintained in RPMI 1640, supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100U/ml penicillin, and 100g/ml streptomycin.

Luciferase aggregation assay

The assay was performed as described previously [25]. Briefly, 150 nM Luciferase (Sigma-Aldrich, St. Louis, MO) was incubated in 25mM Hepes (pH 7.9), 5mM magnesium acetate, 50 mM KCl and 5mM β-mercaptoethanol at 43C for 30 min with or without an equal molar of sgrp170 purified from culture supernatant of sgrp170transfected Colon-26 cells by nickel-nitrilotriacetic acid (Ni-NTA) chromatography (Qiagen, Valencia, CA). Protein aggregation was monitored by the increase of optical density at 320nm.

Tumor rejection assay

2×10^6 wild-type Colon-26, pcDNA3.1-transfected Colon-26 or sgrp170-pcDNA Colon-26 were inoculated subcutaneously into the left groin of six to eight week old female BALB/c mice. All mice used in these experiments were bred and cared for in accordance with the regulations of the Department of Laboratory Animal Resources at Roswell Park Cancer Institute. Tumor growth was monitored by measurements of the shortest diameter (A) and the longest diameter (B); tumor volume is then calculated using the formula $V= (A^2B)/2$.

Immunohistochemistry (IHC)

For IHC, 5μm sections of formalin-fixed, paraffin embedded tumor specimens taken on day 8 of growth were deparaffinized in xylene, rehydrated and blocked for endogenous peroxidase in 3% H2O2 in methanol. High temperature antigen retrieval was carried out by steam heating the slides in DakoCytomation Target Retrieval Solution in a Black and Decker vegetable steamer for 20 minutes, followed by a 20 min cool-down period. Sections were incubated at room temperature for 1 hr with either anti-CD3 (DAKO rabbit anti-human) or isotype control antibodies, washed in PBS and visualized with the Zymed SuperPicture HRP polymer system for detection of rabbit primary antibodies.

In vivo lymphocytes depletion

The GK1.5 (anti-CD4) and 2.43(anti-CD8) antibodies were kindly provided by Dr. Drew Pardoll (John Hopkins University, Baltimore, MD). CD4+ and/or CD8+ cells were depleted in vivo using i.p. injections of 200 g of GK1.5 and/or 200 g of 2.43 antibodies administered every day for 3 days before immunization and once per week thereafter. Depletion of each subpopulation was verified by FACScan analysis of splenocytes after the last antibody injection using isotype-matched antibodies as a control for specific staining.

ELISPOT assay

The T cell responses against the AH-1 peptide of gp70 were evaluated using ELISPOT assay as described previously [26]. Briefly, 96-well filtration plates were coated with anti-mouse IFN-γ antibody, and then blocked with RPMI-1640 containing 10% fetal bovine serum. Effector splenocytes from individual mice (3 per group) taken at 21 days post-inoculation were prepared separately by lysing red blood cells and then added into wells. A 17-mer extended version of the AH1 (SPSYVYHQF) peptide was added to each well, followed by washing and staining with another biotinylated IFN-γ antibody and alkaline phosphate avidin. Spots are developed by adding BCIP/NBT solution and counted using an inverted microscope.

Western blot

Secreted grp170 was purified from the spent media of transfected Colon-26 cell culture under native conditions using Ni-NTA metal affinity chromatography (Qiagen, Valencia, CA) performed according to the manufacturer's instructions. The resulting eluant fraction was designated (T). The spent media of mock-transfected Colon-26 cells treated in the same way served as a negative control (E). Proteins in eluants (T) and (E) were resolved on reducing 10% Tris-HCl SDS-polyacrylamide gel electrophoresis [27]. Proteins were transferred onto a Polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) which was then blocked extensively with 5% non-fat milk in tris-buffered saline with 0.5% Tween-20 (TBST), and then incubated with anti-gp70 from hybridoma 83A25 (a kind gift of Dr. Frank Malik, National Institute of Allergy and Infectious Diseases, Hamilton, Montana). Specific anti-gp96 and anti-hsp70 (Stressgen, Victoria, BC, Canada), anti-H-2K^d (BD Pharmingen, San Diego, CA), anti-proteasome 26S (Calbiochem, San Diego, CA) or anti-Large T Antigen (Lab Vision, Fremont, CA) were also used as primary antibodies. Specific antisera to Horseradish peroxidase– conjugated corresponding secondary antibodies were added and immunoreactivity was detected by chemiluminescence (ECL) (PerkinElmer Life Sciences, Boston, MA).

Statistical analysis

Unpaired Student t-tests were used to compare different groups of mice presented in Figures 2 and 3, and to compare different sets of splenocytes from individual mice in Figure 5. P values < 0.05 were considered statistically significant.

Results

Expression and characterization of secreted grp170 (sgrp170)

To design a secretory form of grp170, we used an approach that had been applied in other laboratories to gp96 [19, 20]. This involves removal of the KDEL or analogous C-terminal retention sequence that characterizes resident endoplasmic reticulum proteins. In this case, grp170 has KNDEL at its C-terminus [3]. For this purpose a sgrp-pcDNA3.1 expression vector was constructed by insertion of the cDNA of murine grp170 with the C-terminal ER retention signal (KNDEL) replaced by a six histidine tag into pcDNA3.1 plasmid (Invitrogen). sgrp-pcDNA3.1 was then used to transfect the Colon-26 cell line, an aggressive murine colon carcinoma that can be grown *in vivo* and *ex vivo*. Expression of a secreted form of grp170 (sgrp170) was detected in the spent culture media of transfected cells using an anti-grp170 polyclonal antibody. A stable clone with the highest level of expression of sgrp170 was isolated by limiting dilution culture in the presence of the selective antibiotic G-418 (Sigma) [24]. Empty plasmid (mock) transfected Colon-26 did not secrete grp170 as determined by Western blotting (Figure 1A). Intracellular levels of grp170 were similar between wild type and transfected Colon-26 as measured by Western blot indicating that sgrp170 did not accumulate in the cells (data not shown). The chaperoning function of sgrp170 was examined using a well-established *in vitro* aggregation assay [25] to verify that it possessed chaperoning function. Purified sgrp170 proved to be highly efficient in inhibiting the heat-induced aggregation of a substrate protein (Luciferase) at 43 C at a 1:1 substrate to chaperone molar ratio (Figure 1B). Thus, protein substrates associated with sgrp170 described in the studies to follow are likely to be products of molecular chaperoning.

Secreted grp170 results in tumor rejection accompanied by a specific anti-tumor immune response

All mice inoculated with wild-type Colon-26 developed rapidly growing tumors until they had to be sacrificed when tumor size reaches 2000 mm^3 starting at day 27 after tumor inoculation (Figure 2A). Mice inoculated with mock-transfected Colon-26 developed tumors in a pattern similar to wild-type Colon-26 (Figure 2B). However, all mice inoculated with sgrp170-transfected Colon-26 exhibited initial tumor growth followed by tumor regression and rejection (P = 0.000197, comparing wild-type to sgrp170-transfected Colon-26 tumor volumes at day 22). The tumor regression was first noticed on day 8 and continued until all tumors disappeared at day 16 (Figure 2C). The data presented in Figure 2 represents one of two independent experiments that were consistent with regression of the sgrp170-transfected, but not mock-transfected Colon-26 tumors.

To determine the underlying immunological mechanisms for tumor rejection, a T lymphocyte subset depletion study was performed. In vivo depletion of both CD4+ and CD8+ T-lymphocyte subpopulations in BALB/c mice with GK 1.5 and 2.43 antibodies respectively abrogated the rejection of sgrp170-transfected Colon-26 tumors (P = 0.00314, comparing tumor volumes at day 22 of sgrp170-transfected Colon-26 in immunocompetent mice and mice depleted of both CD4+ and CD8+ cells) (Figure 3A). Similarly, the depletion of CD8+ cells alone abrogated the rejection of sgrp170 transfected Colon-26 tumors (P = 0.005617, comparing tumor volumes of sgrp170transfected Colon-26 in immunocompetent mice and mice depleted of CD8+ cells at day 22) (Figure 3B). On the other hand, depletion of the CD4+ subpopulation had no effect on tumor rejection in BALB/c mice (Figure 3C). These results support the notion that the inhibitory effect on Colon-26 growth induced by sgrp170 is a result of an adaptive immune response. While tumors arising from mock-transfected colon-26 cells (M) show few CD3+ cells by immunohistochemical staining (Figure 4A), significant numbers of CD3+ cells were found in the tumors originating from sgrp170 transfected cells at day 8, which showed a dense aggregation of CD3+ cells and few viable tumor cells (Figure 4B). However, we have not shown that these infiltrating cells are specifically cytolytic. Nevertheless, we have previously shown that CD8+ T-cells are involved in the antitumor responses elicited by grp170-secreting tumor cells in vivo [46]. The present results are consistent with our earlier studies and support the conclusion that CTLs are responsible for tumor rejection in the Colon-26 model.

To pursue this further, we examined the extent of the cellular immune response against the H-2d restricted (AH1) epitope of the envelope protein gp70 of the murine leukemia virus (MuLV), which is a known tumor antigen in Colon-26 [28] using ELISPOT assay. Splenocytes isolated from mice bearing sgrp170-transfected tumors showed significantly higher frequency of AH1-specific cells compared to the mice bearing wild-type Colon-26 tumors (P = 0.03) or naïve BALB/c mice (P = 0.002) (Figure 5). Background stimulation with media alone was minimal and Con A stimulation showed competent T-lymphocytes in all mice tested (Data not shown). We conclude therefore that targeting grp170 to the extracellular environment leads to an enhanced cellular immune response against the tumor-specific antigen of Colon-26.

sgrp170 chaperones the full-length tumor antigen, gp70, and other proteins into the tumor microenvironment

Since grp170 and other stress proteins/molecular chaperones have been long recognized to bind full-length protein substrates *in situ*, we hypothesized that the secreted grp170 chaperones full-length protein substrates. We also hypothesized that this includes full length tumor antigens and that these antigens are delivered into the tumor microenvironment, thus making them available for uptake by APCs. To test this hypothesis the histidine-tagged

secreted grp170 was purified from the spent media of transfected Colon-26 cell culture using nickel-metal affinity chromatography (T). Purification of the spent media of mock-transfected Colon-26 cells served as a negative control (E). Both eluants (T) and (E) were initially analyzed by SDS-PAGE and several protein bands were observed. Bands that were present in T, but not E, were excised, in gel digested with trypsin and the proteins identified by liquid chromatography coupled to tandem mass spectrometry. Our preliminary analysis (data not shown) identified hsp70, gp96, MHC Class I and the proteosome 26s subunit as being present in these bands. Importantly, gp70, the envelope protein of the MuLV was also identified. This protein is known to be present in Colon 26 cells and gives rise to the Colon 26-associated tumor-specific epitope AH1. Three tryptic peptides of gp70 were identified by LC-MS/MS: 266-267, VPIGPNPVLSDR; 242–256, LYVSGHDPGLIFGIR; 450–464, VTYH**SPSYVYHQF**KR. Peptide 450–464 contains the H-2d-restricted AH1 epitope (shown in bold) which we used in ELISPOT assays to quantify the immune response.

In order to validate the presence of this protein, SDS-PAGE gels were immunoblotted with anti-gp70 specific antibody. Gp70 was detected at the correct molecular size region on the gel in the purified sgrp170 fraction (T) but not in the control (E) (Figure 6A). To investigate whether the secreted grp170 binds its substrate (gp70) intracellularly or extracellularly the spent supernatants of sgrp170-transfected Colon-26 (Sup T) and mock-transfected Colon-26 cells (Sup E) were compared for the presence of gp70 (Figure 6B). Gp70 was detected only in the supernatant of sgrp170-transfected Colon-26 cells suggesting that sgrp170 binds its substrate inside the cell and co-transports it into the extracellular environment by association with sgrp170. The chaperones gp96 and hsp70, and MHC Class I and the proteosome 26s subunit were also found by immunoblotting in fraction T and not E, suggesting that these proteins are also found in association with sgrp170 (Figure 6A).

To verify this finding in an independent cellular system, monkey Cos-7 cells were stably transfected either with sgrp170-pcDNA3.1 or mock-transfected with pcDNA3.1. Spent cell culture media from both transfectants were run over nickel-metal affinity chromatography. Eluants were checked for the presence of the small and large T-antigens (both are well studied tumor antigen in Cos-7 cells) using Western blotting with an antibody specific to the small and large T-antigens (Figure 7). Both the large and small T-antigens were found associated with sgrp170. These results, similar to those obtained with gp70 of Colon-26 cells, further strengthen our conclusion that secreted grp170 chaperones full-length protein substrates, including tumor antigens, into the tumor microenvironment.

Discussion

Grp170 is a major molecular chaperone/stress protein resident in the endoplasmic reticulum (ER). It is distantly related in sequence to both the hsp110 and hsp70 families, but represents its own grp170 stress protein family [3, 4]. Studies have suggested that grp170 is an important element of the protein processing machinery of the ER [2, 7, 8] and the molecular chaperoning properties of grp170 have been recently defined [29].

Although most chaperones are intracellular proteins they can be modified to be secreted. Chaperones secreted into the tumor microenvironment can recruit professional APCs and promote antigen presentation and priming of T-lymphocytes. Impaired tumor growth, increased immunogenecity and specific CTL responses have been shown for several murine tumor cell lines transfected to secrete gp96 [19, 20] or hsp70 [21]. We demonstrated here that targeting grp170 to the extracellular environment has immunological consequences resulting in tumor rejection that is CD8+ dependent. The stable clone we selected (T) grows at the same rate as mock-transfected (E) Colon-26 *in vivo* in mice depleted of CD8+ lymphocytes (Figure 4B), as well as in SCID mice (data not shown). This indicates that the

selected clone is growth-competent and that the regression of the sgrp170 transfected tumor was due to a CD8+ mediated immune response.

We also showed that the envelope protein gp70 of the MuLV, a tumor-specific antigen is associated with grp170 secreted from Colon-26 cells. Indeed, the precursor of gp70, gp90, is known to be present in the ER and to be processed in the Golgi, and this provides a venue for sgrp170 to bind to gp70 and co-transport it into the extracellular environment [30]. Similarly, small and large T-antigens co-purified with grp170 secreted from Cos-7 cells; we speculate that grp170-associated tumor-specific antigens may serve as a source of full-length antigen for cross-priming in APCs. Because these are full length proteins harboring multiple MHC Class I epitopes there is the potential for priming across a variety of MHC haplotypes, a phenomenon observed when using tumor-derived heat shock protein vaccine preparations [40]. However, further studies are needed to determine whether the tumor-specific responses are triggered by the chaperone itself, its associated tumor antigens or peptide fragments of these antigens.

Chaperones such as gp96 and hsp70 were also found associated with secreted grp170. The presence of chaperones in multiprotein complexes is well-documented [1, 7]. For example, grp170 has been previously shown to associate in situ with grp78 and gp96 [2]. It is nonetheless unclear how other chaperones could escape the ER despite possessing an ER-retention signal (gp96) or being cytosolic chaperones (hsp70). However, there are several reports on the secretion or cell surface expression of cytosolic and ER chaperones in immature thymocytes, rat exocrine pancreatic cells, a thyroid cell line and human prostate cell lines in a physiological state or following a forced overexpression [see reference 35 for review]. It should also be noted that these chaperones may also contribute to the immune response to colon-26 tumor cells observed in these studies. Also of possible interest and importance to the immune responses to Colon-26 is that parts of the intrinsic antigen processing and presentation machinery such as MHC class I molecules and the proteasome 26s subunit were also found associated with sgrp170.

It has been postulated that the immunogenicity of hsps stems from their ability to bind a diverse array of peptides in the cell including immunogenic peptides generated from proteasomal degradation of tumor antigens in a tumor cell. For the past several years attempts were made to identify peptides associated with chaperones using highly sensitive techniques such as mass spectrometry. However, most of these studies focus on a defined T-cell epitope sequence from tumor cells that are virally infected such as vesicular stomatitis virus (VSV) [31] and Hepatitis B virus (HBV) [34] or overexpress a certain tumor antigen (e.g. OVA and c-Akt) [32,33]. Recently, Binder et al. [37] provided evidence that that the specific immunogenicity of gp96 preparations isolated from β-galactosidase-expressing cells derives from gp96-chaperoned peptides rather than from full-length β-galactosidase itself. However, Kunisawa and Shastri [38] reported that hsp90 binds to large proteolytic intermediates or truncated proteins (10 ~30 kDa) containing an immunogenic OVA epitope, although there is some debate whether these peptide precursors are extended at both the N- and C- termini [39]. Our observations in the present study support the idea that hsp is involved in antigen presentation by directly carrying proteins/or peptide precursors, rather than precisely processed MHC-binding peptides.

From the studies described here, grp170 chaperones full-length proteins and, when engineered to be secreted from the cell, grp170 accompanies/carries immunologically relevant, full-length proteins into the extracellular compartment. The broader question of where and how cytoplasmic proteins such as gp70 encounter sgrp170 for co secretion is unclear. Although we conclude in our studies that grp170 chaperones full-length proteins, we cannot exclude the possibility that grp170 also chaperones small immunogenic peptides

in vivo, and this remains to be more fully examined. Furthermore, although we use the anti AH-1 response as an indication of the immune response to sgrp170-transfected Colon-26 cells, there may well be other as yet unidentified epitopes involved in the response.

Conclusion

Together, these results indicate that immunization of animals with grp170-secreting tumor cells results in rejection of the tumor by induction of antigen-specific, CD8-dependent immune responses. Furthermore, the secreted grp170 is able to deliver full-length tumor antigens to the tumor microenvironment, thus making them available for uptake by antigen presenting cells (APCs) to initiate a tumor-specific immune response. These data parallel our studies showing that hsp110 or grp170 are able to chaperone full-length proteins and when complexed with protein antigens and used as vaccines, the complexes elicit immune responses in vivo against the protein antigens [12, 13]. The cell-based approach described here has the potential of being exploited as a tumor-specific vaccine in tumors of various histological origins.

Acknowledgments

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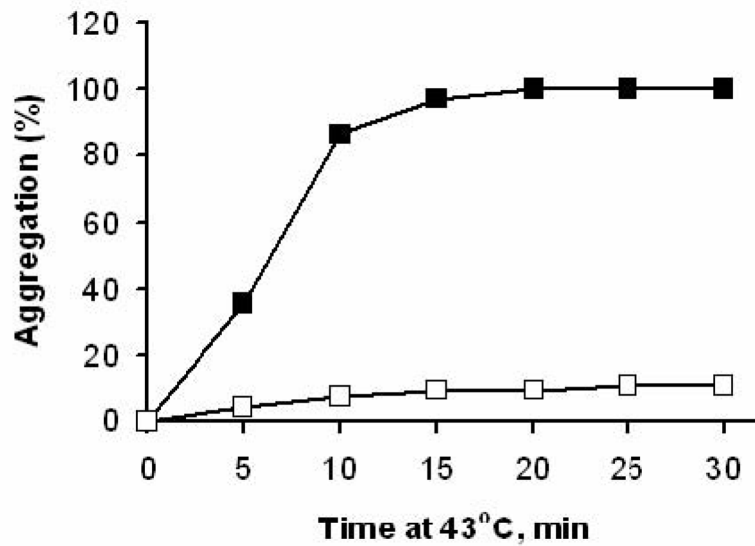
Figure 1A

Figure 1B

Figure 1.
Characterization of sgrp170. A, Western blot with polyclonal anti-grp170 on the spent culture media of wild type Colon-26 (lane 1), mock-transfected Colon-26 (lane 2) and sgrp170-transfected Colon-26 (lane 3).B, The effect of sgrp170 on the inhibition of protein aggregation *in vitro*. Luciferase alone (filled squares) or with equal molar ratio of sgrp170 (open squares) was incubated at 43 C for 30 min. The light scattering was measured by optical density change at 320nm every five minutes.

Wild type Colon-26

Figure 2A

Mock-transfected Colon-26

Days after inoculation
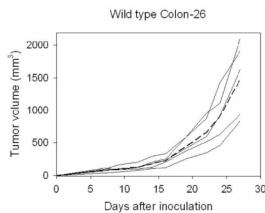
Figure 2B

sgrp170-transfected Colon-26
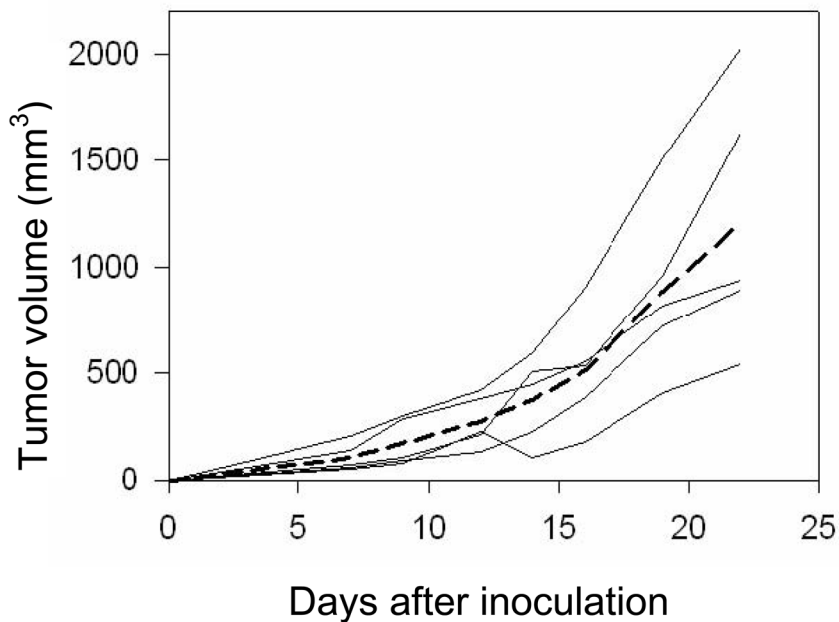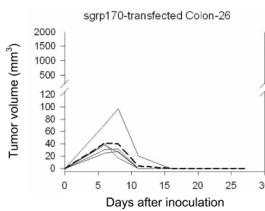
Figure 2C

Figure 2.
Transfection of Colon-26 with secreted grp170 results in tumor rejection in vivo. Balb/c mice were inoculated with wild type or transfected tumor cells as indicated (n=5 per group), and the tumor growth measured as described in Materials and Methods. Tumor growth curves for wild-type Colon-26 (Figure 2A), mock-transfected Colon-26 (Figure 2B) and sgrp170-transfected Colon-26 (Figure 2C) are shown. Growth curves for individual mice are represented by solid lines and the average growth curve is represented by a dotted line.
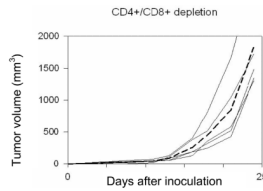
CD4+/CD8+ depletion

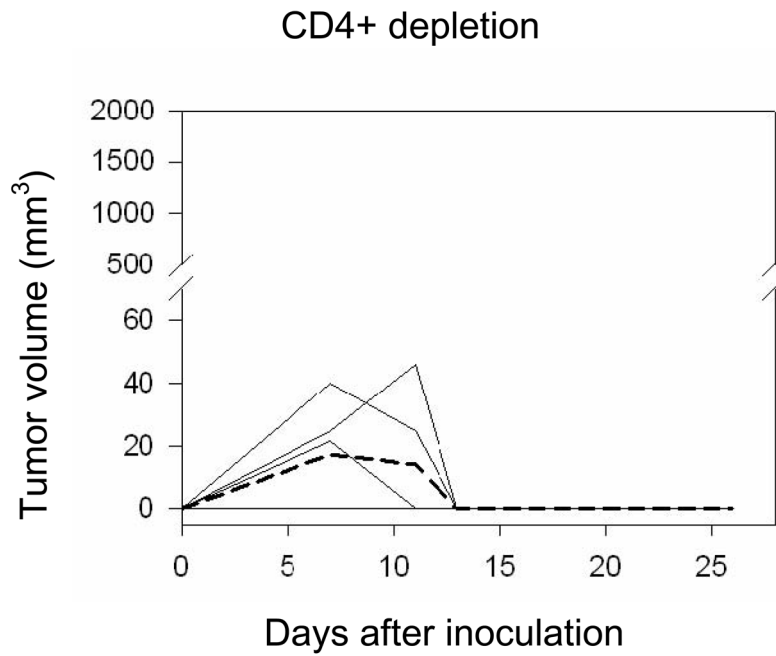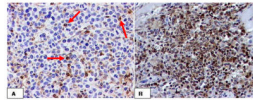Figure 3A

CD8+ depletion
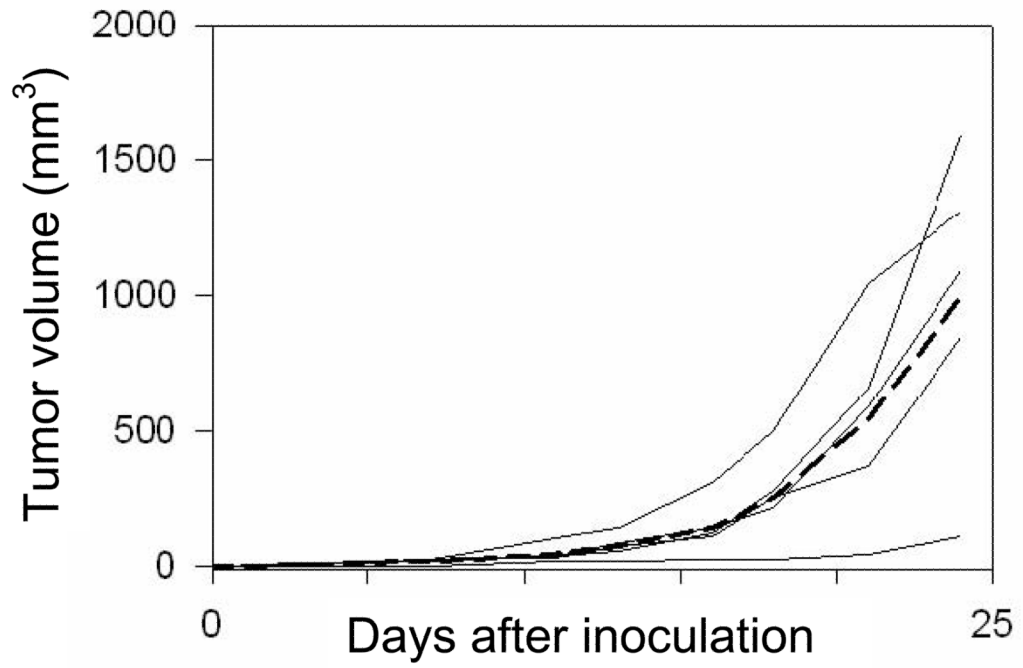
Figure 3B

CD4+ depletion

Figure 3C

Figure 3.
The effect on the growth of sgrp170-transfected Colon-26 tumors by in vivo depletion of both CD8+ and CD4+ T-lymphocytes (Figure 3A), depletion of the CD8+ subpopulation alone (Figure 3B) or depletion of the CD4+ subpopulation alone (Figure 3C). Tumor growth curves for individual mice are represented by solid lines (n=5 per group), and the average growth curve is represented by dotted line.

Figure 4.
Immunohistochemical localization of CD3$^+$ immune cells in Colon-26 tumors. On the eighth day of growth, mock-transfected Colon-26 tumor contains a number of CD3 positive immune cells (red arrows) in close proximity to viable tumor cells (blue nuclei) (Figure 4A). In contrast, after eight days, the site of inoculation with the sgrp170 transfected tumor cells consists of a dense aggregation of CD3 positive immune cells and few viable tumor cells. (20x original magnification) (Figure 4B).
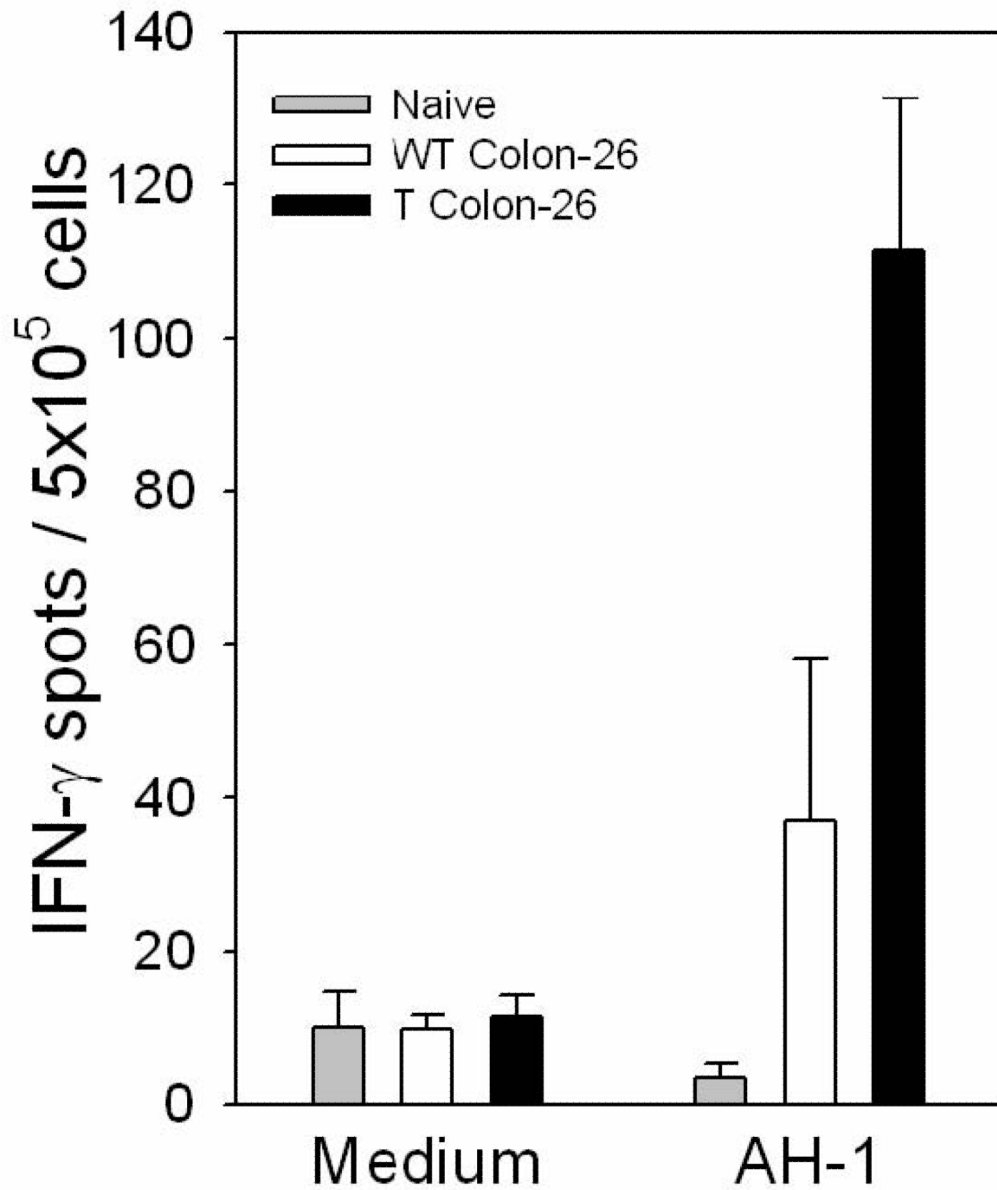
Figure 5.
Splenocytes from mice bearing sgrp170 transfected tumors have a significantly higher frequency of AH-1 reactive cells. Splenocytes isolated from naïve BALB/c mice (grey bars), mice bearing wild-type Colon-26 (white bars) or sgrp170-transfected Colon-26 tumors (black bars) were assayed for the frequency of IFN-γ-producing cells by ELISPOT in response to medium alone or the AH-1 epitope of gp70 of MuLV. The error bars indicate the standard error of the measurements from individual mice (n=3 per group).
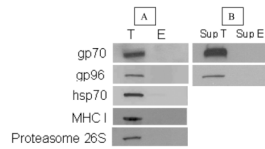
Figure 6.
Western blots of the eluants from nickel-chromatography of cell culture supernatants of Colon-26 cells transfected with sgrp170 (T) or mock-transfected Colon 26 (E). Western blot of the T and E fractions using specific antibodies to gp70, gp96, MHC Class 1, proteasome 26S and hsp70 (Figure 6A). Western blot of the unfractionated culture supernatants of sgrp170-transfected Colon-26 cells (Sup T) and mock-transfected Colon-26 (Sup E) using antibodies specific for gp70 and gp96 (Figure 6A).
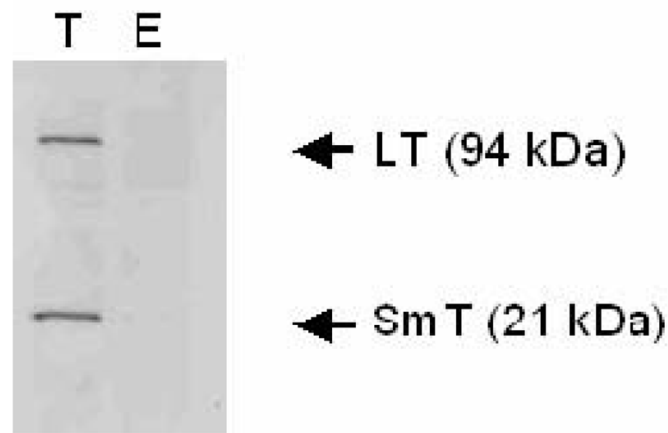
Figure 7.
Western blots of the eluants from nickel-chromatography of cell culture supernatants of sgrp170-transfected Cos-7 cells (T) and mock-transfected Cos-7 cells (E) using an antibody specific to both small and large T-antigens.