OVARIAN CANCER SUICIDE GENE THERAPY WITH GENETICALLY ENGINEERED, TRANSGENICALLY EXPRESSED, INTRACELLULAR scFv ANTIBODIES AGAINST ANTI-OXIDATIVE ENZYMES

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ABSTRACT

Ovarian cancer is the leading cause of death among all gynecological cancers. Some women choose bilateral oophorectomy as means of cancer prevention. In most patients, by the time this cancer is diagnosed, it has already metastasized. Treatment involves oophorectomy followed by radiation, chemo-, and immuno-therapies. However, oopherectomy results in infertility and fails to eliminate all cancer cells. Radiation and chemotherapy cause severe side effects and may lead to genetic mutations in DNA of the ova.

The ultimate goal of this project is development of a therapy which would target a therapeutic gene specific to ovarian cancer cells causing their apoptosis, but which would leave ova and other cells unharmed.

Herewith, we report the proof of concept for such a therapy in which genetically engineered single chain variable fragment (scFv) antibodies against HER2/neu, RON, and NK1R guided delivery of the therapeutic transgenes into the cancer cells of the ovaries. Under ovary specific promoters (OSP), the transgene expression generated intracellular scFv antibodies, which quenched cell anti-oxidative enzymes, thus raising levels of reactive oxygen species (ROS), inflicting oxidative stress, activating apoptotic signaling pathways, and causing cancer cell deaths.

Keywords

Ovarian cancer, anti-oxidative enzymes, signal transduction, genetically engineered, single chain variable fragment (scFv) antibodies,, HER2, RON, NK1R

INTRODUCTION

Ovarian cancer is the leading cause of deaths among all gynecological cancers. In the USA in 2007, 22430 women would be newly diagnosed with cancers of the ovaries, and 15280 of them would die according to the predictions of the
American Cancer Society (Jemal et al. 2007). While the exact causes of ovarian cancer have yet to be determined, BRCA1,2 gene mutations, which lead to dysfunctional gene products, put their carriers at high risk (Quinn et al. 2007, Silva et al. 2007). Therefore, some women carrying these mutations choose bilateral oophorectomy as a preventive measure.

The combinations of current diagnoses and therapies are less than adequate because the cancers’ origins, growth and metastases may remain non-symptomatic and hard to detect within abdominal cavity for a long time prior to diagnosis (Badgwell and Bast 2007). Upon diagnosis, cancers spread beyond the ovaries in more than 68% of patients. They reach stages III – advanced invasion of the neighboring tissues and/or IV – distant metastases in the WHO classification. Hence, ovarian cancer is often termed a “silent killer”. Currently, initial diagnosis often relies upon detection of the MUC16 gene expression product CA125 in blood (Suzuki et al. 2007). Computer assisted tomography (CAT) and magnetic resonance imaging (MRI) may help in diagnostic and therapeutic processes by showing images of tumor topography (Milam et al. 2007). However, the final diagnosis relies upon laparascopy and immunohistopathological examination of the biopsies. At that time, an evidence of HER2/neu oncogene being amplified and overexpressed is often demonstrated (Slamon et al. 1989, Shin et al. 2007, Tuma 2007). The level of its expression is associated with cancer malignancy (Berchuck et al. 2007, King et al. 1992). The ovarian or breast cancer cells may display approximately 1.5 x10⁶ HER2/neu receptors on their surfaces expressed from multiple copies of the gene, while healthy cells display only approximately 2 x10⁴ of these receptors. I have recently demonstrated that over-expression of preprotachykinin and RON genes led to the significant increase of NR1K and RON. These features of cancer cell surface biomolecules are the driving force for developing non-invasive means of diagnosis—in vivo immunocytochemistry—using scFv antibodies, which are genetically engineered for detection in gamma, single photon emission tomography (SPECT), positron emission tomography (PET), and magnetic resonance imaging (MRI) (Malecki 2006). Clinically, overexpression of HER2/neu leads to a large increase in stimulation of signal transduction pathways, accelerated cell cycles, and increased cell proliferation. As a consequence, HER2/neu positive cancers are the most invasive and have the worst prognosis.

The preferred therapy is radical surgery, which involves uni- or bi-lateral removal of the ovaries and hysterectomy. However, in advanced stages, metastases are often missed and become sources for relapses. Radiation and chemotherapy, while intentionally targeting cancer cells, indiscriminately affect healthy cells and cause severe side effects (Cannistra et al. 2007). Therefore, the majority of the ovarian cancer survivors become infertile, and the others risk carrying various mutations of genetic information in oocytes (Kesic 2007). Emerging therapies involve inhibitors of enzyme receptor HER2/neu (Kaye 2007, Palayekar and Herzog 2007), silencing RNA (Numnum et al. 2007), and Hsp70 (Chang et al. 2007). Immunotherapy trials, with antibodies targeting HER2/neu, are in progress; however, multiple rounds of monoclonal antibodies sooner or later elicit the patient’s response and worsen the outcome. These therapies are further complicated by mutations in the signaling pathways within ovarian cancer cells.

(Estep et al. 2007). In summary, current therapeutic approaches suffer from serious problems: multiple rounds of therapy with concomitant side effects, indiscriminate, non-specific delivery of therapy hurting healthy cells, toxic effects of dead cells, risk of developing mutations in oocytes, and women's infertility.

The ultimate goal of this project is development of a therapy, which would progressively eliminate only cancer cells, while leaving oocytes and all other healthy cells unharmed. Here, we tested the hypothesis, if therapeutics were delivered specifically to cancer cells only, then not only would healthy oocytes remain unharmed, but the patient's immune system (itself unaffected by therapy) would be capable of continued elimination of cancer cells. Furthermore, if only ovarian cancer cells were eliminated in progressive stages of apoptosis, then toxic effects of the therapy would be reduced. In addition, a targeted, single dose delivery of therapeutic transgenes would be far more advantageous than conventional multiple rounds of therapies. Finally, all these measures would protect oocytes, thus protecting a women's ability for healthy procreation.

Towards this goal, the constructs for antioxidative enzymes and for single chain variable fragment (scFv) antibodies against antioxidative enzymes were genetically engineered with the gene products being scFv antibodies of high specificity and affinity (Malecki et al. 2002). The targeted antioxidative enzymes were manganese superoxide dismutase (SODMn), copper-zinc superoxide dismutase (SODCuZn), catalase (CAT), glutathione reductse (GR), and glutathione peroxidase (GPX). The scFv antibodies were capable of quenching these antioxidative enzymes; thus disabling their ability to perform redox reactions and manage reactive oxygen species. Expressed under CMV and OSP intracellularly from the transgenes, scFv inflicted oxidative stress and caused activation of apoptosis signaling pathways. Using the same genetic engineering platform, the constructs were engineered for scFv antibodies targeting domains of HER2/neu, EGFR-vIII, NK1R, RON, and TfR on surfaces of cancer cells. They were expressed in myeloma cells and used to guide receptor-targeted delivery of the transgenes (Malecki 2006).

Briefly, I report success in scFv antibody-guided targeting of HER2/neu receptors on the ovarian cancer cells and effective delivery transgenes for the scFv antibodies against antioxidative enzymes, expressing them intracellularly, inducing apoptotic signaling pathways, and causing ovarian cancer cell deaths.

METHODS

Cell cultures. The cell line NIH OVCAR-3 HTB-161 was derived from the cells in ascites of a patient with malignant adenocarcinoma of the ovary. The cell line was grown in RPMI-1640 Medium (ATCC) supplemented with 0.01 mg/ml bovine insulin and donor bovine serum to a final concentration of 20%. The epithelial cells were positive for both estrogen and progesterone receptors. They formed colonies and spheroids when grown in soft agars and were tumorigenic in nude mice.

The cell line TOV-112D CRL-11731 was derived from primary malignant adenocarcinoma of the ovary at grade 3, stage IIIC. It was cultured in 85% of a
A 1:1 mixture of MCDB 105 medium and Medium 199 and 15% donor bovine serum (ATCC). When cultured in soft agar, these cells formed colonies and spheroids. The cells tested positive for HER2/neu expression and the p53 mutation. The cells also induced tumors in nude mice.

**Genetically engineered single chain variable fragment (scFv) antibodies against antioxidative enzymes.** The constructs for the single chain variable fragment (scFv) antibodies were genetically engineered with the high specificity and affinity of the gene expressed scFv antibodies previously demonstrated (Malecki et al. 2002). The four targeted antioxidative enzymes were: manganese superoxide dismutase (SOD$_{Mn}$), copper-zinc superoxide dismutase (SOD$_{CuZn}$), catalase (CAT), and glutathione peroxidase (GPX). Coding sequences for the scFv antibodies targeting HER2/neu were selected from the surface displayed libraries and cloned into pM vectors designed with CMV and OSP immediate early promoter and SV40 poly(A) termination with hexahistidine and pentaglutamate and neomycin-resistance coding sequences (Malecki 1996). Constructs for these bi-functional antibodies were electroporated into human myelomas. Expressions of these constructs resulted in secretion of hetero-specific, poly-functional, mono-valent antibodies. For therapeutic endeavors, the constructs were modified to specifically target the transgene expression products to ER, mitochondria, peroxisomes, cytoplasm, or nuclei by introducing organelle specific targeting signal coding sequences as described earlier (Malecki et al. 2002). Chelating sites were saturated with the metal ions Gd and Eu. Purification from non-bound metal was performed on affinity columns. The antibodies were produced in modified roller bottles.

Affinities of these antibodies were determined on Biacore loaded with either the recombinant or purified enzymes: SOD, CAT, or GPX. Specificities were determined on Western blots from denaturing PAGE-SDS and in-native-gel immunolabels (Malecki 2006). Abilities of these scFv antibodies for quenching the antioxidative enzymes were determined in colorimetric reactions. The constructs coding the non-specific intracellular scFv antibodies against the main groups of blood served as the controls (Malecki 2006).

Effects of the transgene expression products exerted upon the ovarian cells were determined by evaluation of the cell membrane organization (LSCFM, FESEM), reactive oxygen species (ROS) levels (LSCFM), and reorganization of genome (pulse-electrophoresis, LSCFM, EFTEM).

**Genetically engineered, superparamagnetic, single chain variable fragment (scFv) antibodies against HER2/neu and TfR.** Details of plasmid constructs were described previously (Malecki et al. 2002). Coding sequences for scFv antibodies targeting HER2/neu were selected from the surface displayed libraries and cloned into pM vectors designed with CMV and OSP promoters and SV40 poly(A) termination. The vectors also contained coding sequences for hexahistidine and pentaglutamate, biotin or digoxigenin, and neomycin-resistance. Transgenes with the coding sequences for the antibodies against the main groups of blood inserted into excised sites of antioxidative scFv antibody sequences served as controls. These constructs were electroporated into human myelomas, which were then grown in the modified roller bottles. Expressions of these constructs resulted in secretion of hetero-specific, poly-functional, mono-valent antibodies.
The scFv antibodies were absorbed onto superparamagnetic, core-shell Fe Au nanoparticles and purified from non-bound antibodies with magnets.

**Rapid freezing and freeze-drying.** The details for cryoimmobilization and follow-up molecular imaging techniques were described earlier (Malecki et al. 2002). The cells injected into the chambers were rapidly frozen in nitrogen slurry down to -196°C. The frozen samples were further processed in two ways: freeze-drying for field emission scanning electron microscopy (FE-SEM) on LEO 1530 or Hitachi 3400 and freeze-substitution for energy filtering transmission electron microscopy (EFTEM) on Zeiss or Philips. For FESEM, the frozen cells were freeze-dried followed by fast atom beam sputter coating (freeze-drying and cryocoating, oil-free vacuum station unit built based upon NSF grant). For EFTEM, the cells were freeze-substituted at -90°C, -35°C, and 0°C and infiltrated with Lowicryl followed by polymerization with UV at -35°C and ultramicrotomy.

**Native electrophoresis.** An agarose gel was poured at 2% using a 10 mM Tris, 31 mM NaCl buffer of varying pH that did not contain any denaturing agents. The samples in their native state were loaded after mixing with glycerol to add density without denaturing the proteins. The gel was run in the same buffer that was used for pouring agarose at 60 mAmperes until the desired separation was reached. The gel was then stained for 30 minutes in Sypro Tangerine Gel Stain (Invitrogen) diluted in the running buffer prior to imaging using a Storm 840 (Amersham) or FluorImager (Molecular Dynamics).

**SDS-PAGE.** Electrophoresis was run on 12% polyacrylamide gel. Standard cell culture lysates were loaded on 0.75 mm thick combs with 2 mm lanes. The samples, after mixing with SDS and DTT containing sample buffers (Sigma) were loaded into the wells. The gels were run using Tris/Glycine/SDS/DTT running buffers. After each run, the gels were stained with colloidal silver or Sypro Tangerine for imaging using a FluorImager (Molecular Dynamics).

**Electrotransfer.** After electrophoresis, the samples were immediately transferred onto PVDF. The immunoblot was performed with the Mini Trans-Blot Cell (Bio-Rad) within CAPS: 10 mM 3-[Cyclohexylamino]-1-propanesulfonic acid (CAPS) and Tris/glycine transfer buffer (25 mM Tris base, 192 mM glycine, pH 8.3). Prior to the transfer the cooling units were stored with deionized water at -20°C. Immediately after electrophoresis the gel, membrane, filter papers and fiber pads were soaked in transfer buffer for 5-10 min. The pre-cooled transfer units were filled with cooled transfer buffer, and electrotransfer proceeded at 350 mA.

**Antibody guided receptor mediated transfection and magnetoporation.** AntiHer2/neu and antiTfR scFv antibodies carried the plasmid constructs for transgenic, intracellular expression of SOD$_{Mn}$, SOD$_{CuZn}$, CAT, or GPX. After docking into the ovarian cancer cell surface receptor sites, they were subjected to an electromagnetic field which forced the transgenic complexes to enter the cytoplasm without passing through lysosomal compartments. The nuclear localization signal enhanced their retention or delivery into the nuclei for transient expression.

**Laser scanning confocal microscopy.** Z-stacks of the immuno-labeled cells were acquired with an Odyssey laser scanning confocal system on an Olympus.
inverted microscope. The lines were generated by blue diode, helium and argon lasers at 337, 488, 543, and 588 nm. Images were acquired with Kernel averaging followed by deconvolution for the three-dimensional rotation or gallery display for analysis.

**Scanning electron microscopy and energy dispersive x-ray spectral imaging.** Analysis of the three-dimensional structure and distribution of antibodies were determined with a LEO 1530 Field Emission Scanning Electron Microscope (FESEM) equipped with a Hitachi 3400 Energy Dispersive X-Ray Spectral Imaging System (EDXSI). Complete elemental spectra were acquired for every pixel of the scans to create the elemental databases. From these, after selecting an element specific energy window, the map of this element atoms distribution was calculated with ZAF correction (NIST). As the antibodies were tagged with atoms of either Gd or Eu (exogenous elements incorporated into their structure), the location of antibodies could determined based upon the elemental maps as described in details earlier (Malecki 1996).

**Energy filtering transmission electron microscopy.** Supramolecular organization was determined with energy filtering transmission electron microscopy (EFTEM) using either a Zeiss with Omega as the in-column energy filter or Philips with a Gatan post-column energy filter. Ultrathin sections were maintained in the cryo-holder and protected with double-bladed cryo-traps. The instruments were operated at zero-loss or at a C-energy edge.

**RESULTS**

The success of this experimental therapy was contingent upon two main factors: specificity of transgene delivery and effectiveness of blocking of antioxidative enzymes.

The targets for gene therapy were the antioxidative enzymes manganese superoxide dismutase (SOD$_{Mn}$), copper-zinc superoxide dismutase (SOD$_{CuZn}$), catalase (CAT), glutathione peroxidase (GPX). The genetically engineered scFv antibodies against SOD$_{Mn}$, SOD$_{CuZn}$, CAT and GPX had very high affinities and specificities towards their antigens as demonstrated in previous studies (Malecki et al. 2002). They were capable of blocking the functions of these enzymes as demonstrated in native-in-gel functional studies. In this endeavor, the genetically expressed scFv antibodies were redirected from the cell secretion pathways toward intracytoplasmic and intraorganellar sites as determined on Z-stacks from laser scanning confocal microscopy after three-dimensional reconstructions. The data were confirmed on organelle fractions with gradient centrifugation studies as demonstrated earlier (Malecki 1998). Likewise, the specificity of the targeting of genetically engineered scFv antibodies for HER2/neu and other EGFR family receptors, as well as, for TfR were all shown in preceding studies (Malecki 2006). Since they were guiding the delivery of transgenes to receptors, their high specificity and affinity were essential for specific delivery of the therapeutics.

In this study, the effects which these genetically engineered scFv antibodies had upon cancer cells were evaluated based upon the results of three basic, functional assays specific for detection of apoptosis and presented in this paper:
formation of apoptotic blebs (Figure 1), phosphatidylserine extracellular flip and chromatin structure collapse (Figure 2), and genome disintegration (Figure 3). Figure 1 is a representative result of the first group of assays. Rapid cryoimmobilization of cells secured retention of life-like cellular ultrastructure at nanometer resolution. This cellular ultrastructure was revealed with field emission scanning electron microscopy (FESEM) on the freeze-dried samples. Controls containing identical samples of ovarian cancer cells were transfected with constructs coding the non-specific intracellular scFv antibodies. A few hours after receptor-targeted delivery the transgenes for the scFv antibody against antioxidative enzymes, ovarian cancer cells formed abundant cell membrane blebs (Figure 1). These blebs are characteristic and early signs of apoptosis, while reflecting molecular compartmentalization of the cell. The identical ovarian cancer cells treated with non-specific antibody carrying transgenes were entirely healthy-looking and free from any effects upon the cells (not shown).

The panel in Figure 2 illustrates the representative results of the second group of assays which were detected with laser scanning confocal fluorescence microscopy (LSCFM). General view of the cells is shown with transmitted detector

Figure 1. Membrane blebs on the surface of the apoptotic ovarian cancer cell in the field emission scanning electron microscope. Horizontal field width = 2 microns.
Apoptotic alterations in cellular chemistry are manifested by two phenomena: translocation (flip) of phosphatidylserine (PS) and reorganization of chromatin. In both cases cells retain their viability and membranes remain intact. This phenomenon is demonstrated by propidium iodide exclusion (Figure 2, middle section). At the onset of apoptosis, PS relocates from the internal leaflet to the cell membrane exterior as demonstrated with annexin or with the genetically engineered scFv antibodies against PS in LSCFM (not shown). More specifically, chromatin suffers definite collapse (Figure 3, right section). The ovarian cancer cells transfected with the intracellular antibodies against the main groups of blood did not exhibit these effects (not shown).

Figure 3 illustrates the hallmark of apoptosis - chromatin collapse and condensation with disintegration of the genome. These phenomena were captured with the rapid cryo-immobilization technique. Therefore, they represent the life-like appearance of the occurring phenomena of apoptosis. Apoptotic disintegration of the genome was revealed on two ways: pulse gel electrophoresis of the genomic DNA (not shown here) and genome disintegration with EFTEM (Figure 3). The latter method offered a significant advantage over electrophoresis as it offered insight into structural changes which occur in the entire nucleus upon activation of the apoptotic signaling pathway. The image illustrates the compact cluster of genomic DNA characteristic of apoptotic degradation of the genome.

DISCUSSION

I report successful development of a new gene therapy by means of intracellular expression of transgenes for the single chain variable fragment (scFv) antibodies against antioxidative enzymes (AOE). The four main anti-oxidative enzymes that were targets for the genetically engineered scFv antibodies were manganese superoxide dismutase (SODMn), copper-zinc superoxide dismutase (SODCuZn), catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPX). Their disabling leads to accumulation of reactive oxidative spe-
cies (ROS), thus oxidative stress beyond capabilities of managing it. Therefore, apoptotic signaling pathways become activated and start driving the ovarian cells into apoptosis.

Success of this experimental therapy approach can be attributed primarily to the high specificity and affinity of the genetically engineered scFv antibodies. The small size of the scFv antibodies allowed me to map individual domains on macromolecules. With this high precision, it also allowed me to target these antibodies to the active or neutral domains. Such a precise targeting efficiently allowed me to disable the functions of anti-oxidative enzymes.

Apoptosis of cancer cells induced by the increased levels of reactive oxygen species is a mechanism well described in radiation therapy. Exposure to the high levels of ionizing radiation leads to the rapid increase of free radicals beyond those which are manageable by AOE. Excessive levels of ROS cause induction of apoptotic signaling pathways. However, sustained therapeutic effects on the cancer cells via ROS require multiple sessions of exposures to radiation. Moreover, all the cells within the focal plane of the radiation beam are destined for destruction. This is also the case with the ova in proximity of the targeted ovarian cancer cells. Moreover, radiation causes mutations within DNA of the ova.

Figure 3. Condensation of collapsed chromatin in an apoptotic ovarian cancer cell. Cell membranes are retained, while some of them form surface blebs (upper left portion).
These problems are avoided in the protocol described here. This protocol will be
the subject of forthcoming studies in which molecules will be directly and spe-
cifically affected by the generated ROS to trigger apoptotic signaling pathways
activation.

Chemotherapy and immunotherapy require multiple rounds of applications.
These cause repetitive side effects, immunizations and hardships on the patient.
These problems would be greatly reduced in this experimental therapy. A single
session administration of a therapeutic is a clear advantage of the proposed ther-
apy. The antibody guided, ovarian cancer receptor targeted delivery of transgenes
may be sufficient for a long term, continuous expression of intracellular antibod-
ies. It has obvious advantages for the patient. Compared to immuno- and phar-
maco-therapeutics, one dose of the scFv antibody has a much lower potential
for immunizing the patient than applications of multiple rounds of monoclonal
antibodies or radio-immunotherapeutics.

Above all, the first and foremost advantage of this experimental therapy
is protection of oocytes. Retaining unharmed ova is equivalent to retention of
women's fertility, thus ability for procreation. Although, in classical therapies,
the chances for retaining fertility exist, the oocytes subjected to chemotherapy
and radiation therapies may suffer serious mutations with hard to foresee conse-
quences to the child.

Streamlining this experimental therapy faces at least three main challenges:
increased production, specificity of delivery in vivo, and innate response. Lab
scale production and affinity purification are very expensive already. Therefore,
the immediate attempts lead to reduction of the costs of production by express-
ing these scFv antibodies in plants. In silico simulations and in vitro works,
supplemented with tests on endothelial and a variety of other non-cancerous
cells, offer only limited numbers of molecules and molecular clusters which
may exhibit homology with those targeted by the scFv antibodies engineered
in this project. Therefore, their streamlining to the in vivo environment will be
the next step. Finally, innate response triggered by toll-like receptors and involv-
ing reticulo-endothelial cells may create problems for efficient delivery of the
transgene complexes to their destinations. These will require development of
means to bypass such responses by modifications of therapeutic complexes; e.g.,
by PEGylation. Expanding this therapy to treatment of other neoplasms will
be contingent primarily upon defining unique molecules displayed on their cell
surfaces.

To summarize, cancer suicide gene therapy opens the new routes for therapy
of ovarian cancer by modification of the women's own molecular mechanisms
and may lead to development of ovarian cancer vaccines.
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LITERATURE CITED


