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14. ABSTRACT
As described in the 2008 progress report, the revised hypothesis is that agonist DR5 Ab induced by DNA vaccination will trigger tumor cell apoptosis without compromising T cell activity. The specific aims are to (1)Construct and test DR5 vaccines to induce anti-DR5 Ab, (2) Test the agonist activity of vaccine-induced anti-DR5 Ab, and (3) Amplify anti-tumor activity of DR5 vaccination with novel chemotherapeutics. Using three DNA constructs, phDR5 encoding full length human DR5, phDR5Δ encoding human DR5 with a premature termination signal in the death domain (aa.1-338) and phDR5ECTM encoding the extracellular and transmembrane domains of DR5 (aa. 1-223), we demonstrated the induction of agonist polyclonal antibodies that inhibited the growth of triple negative breast cancer (TNBC) in vitro and in vivo. Anti-tumor activity of DR5 agonist mAb was amplified when tumor cells were treated simultaneously with MS-275, an inhibitor of histone deacetylase (HDAC), indicating potential therapeutic advantage by combining HDAC inhibitor with DR5 vaccination. Because Her-2 positive and ER positive breast cancers are more resistant to DR5 agonists, the mechanism of resistance was tested. Activation of Akt survival pathway by TRAIL was shown in T47D and OVCA 432 cells. Strategies that block survival pathway may be exploited bo amplify DR5 vaccination efficacy.in Her-2 or ER positive b reast cancers.

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INTRODUCTION

As described in the 2008 progress report, the revised hypothesis is that agonist DR5 Ab induced by DNA vaccination will trigger tumor cell apoptosis without compromising T cell activity. The specific aims are to

(1) Construct and test DR5 vaccines to induce anti-DR5 Ab,
(2) Test the agonist activity of vaccine-induced anti-DR5 Ab,
(3) Amplify anti-tumor activity of DR5 vaccination with novel chemotherapeutics.

BODY

Specific Aims 1-2

(1) Construct and test DR5 vaccines to induce anti-DR5 Ab
(2) Test the agonist activity of vaccine-induced anti-DR5 Ab

A manuscript describing the results is being submitted for publication.

Three human DR5 vaccine constructs have been tested. pCEP4 hDR5 encoding full length human DR5 and pCEP4 hDR5Δ encoding human DR5 with a premature termination signal in the death domain (aa. 338) have been described (1). Coding sequences were obtained by restriction with BamHI and HindIII and subcloned into pVax-1, giving rise to pVax-hDR5 ((phDR5) and pVax-hDR5Δ (phDR5Δ). Since pVAX1 is suitable for clinical use, it will be possible to use the same vaccines in patients. The 3rd construct pVax-hDR5 ECD-TM (ECTM), encoding the extracellular domain and transmembrane regions of DR5 (aa. 1-223) was obtained by PCR amplification using the wild type sequence as template and primers: Upper 5'-AT ATC TAC AAG CTT GCG ACC ATG GAA CAA CGG GGA CAG A-3' and Lower 3'-GTA GAC GAG TCC ACC TCC GAC TCC TAG GTA GAT C-5' and cloned into BamHI and HindIII of pVax-1. Expression of the recombinant proteins was verified by flow cytometry.

<u>hDR5 immune sera suppress the proliferation of SUM159 TNBC cells</u>

Because triple negative breast cancer (TNBC) has been associated with elevated sensitivity to TRAIL induced apoptosis, the activity of DR5 immune sera was tested with TNBC cell line SUM159. DR5 expression on SUM159 was verified by mAb HS201 and hDR5 immune sera (Fig. 1A). Using a MTT-based assay, significant inhibition of tumor cell proliferation was demonstrated with phDR5 immune sera, but not control sera (Fig. 1B). Induction of apoptosis was further tested with a panel of breast cancer cell lines: TNBC SUM149, MDA MB231, triple positive BT474 and Her-2^{+}ER^{neg} SKBR3 cells (Fig. 1C). Significant apoptosis was induced in TNBC, with little annexin V staining in BT474 or SKBR3 cells, consistent with greater sensitivity of TNBC.

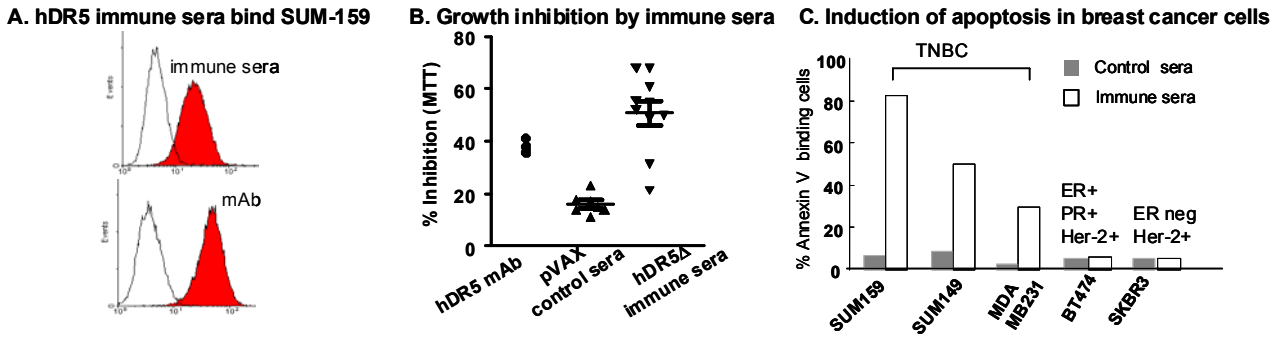

Fig. 1. Anti-tumor activity of hDR5Δ immune sera (A) SUM159 cells were incubated with immune sera (top panel, filled histogram) or control sera from pVax treated mice (open histogram). hDR5 mAb was the positive control (lower panel, filled histogram). (B) Inhibition of cell growth/survival by MTT assay. SUM159 cells were incubated with immune sera or DR5 agonist mAb631. Data represent % growth inhibition relative to media control. Greater inhibition represents higher agonist activity. n=10 per group. (D) Induction of apoptosis in breast cancer cells. Cells were stained with Annexin V and 7-AAD at 20h after treatment with immune or control sera.

hDR5 immune sera induces apoptosis through the extrinsic, death receptor pathway

To further test the mechanism of tumor cell death, the cleavage of caspase-3 and PARP was measured (Fig. 2). Within 5 hours of treatment with immune sera or mAb 631, caspase-3 cleavage was near completion (lane 3). Inhibition of caspase-8 with Z-IETD-FMK greatly reduced caspase-3 (lane 5) and downstream PARP cleavage (lane 6), consistent with the activation of extrinsic apoptosis pathway. With hDR5 immune sera (lanes 3 vs. 6) and DR5 agonist antibody (lanes 2 vs. 5) showing comparable level of signaling activity, the same extrinsic apoptosis mechanism is likely induced.

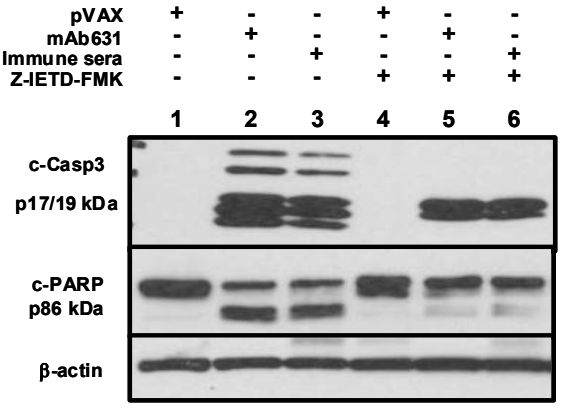

Fig. 2. Western blot analysis of SUM159 cells treated for 5 hours with 2% control (pVAX) or immune (pVAXhDR5) sera or 5 μg/mL mAb631 in the absence or presence of Caspase-8 inhibitor, Z-IETD-FMK as described. Whole cell lysates were analyzed for cleavage products of Caspase 3 and PARP and normalized to β-actin.

Inhibition of TNBC SUM159 growth by hDR5 immune sera

To test the effect of hDR5 immune sera on the growth of human TNBC cells in vivo, SUM159 cells were coated with either non-immune sera, immune sera or agonist mAb631 before they were injected s.c. into SCID mice. Immune sera from hDR5 vaccinated mice eliminated tumor growth in >85% (6/7) mice. Treatment with 5μg/mL of the control agonist mAb 631 delayed tumor onset without eliminating growth (Fig. 3). Therefore, hDR5 immune sera have potent tumor inhibitory activity and are significantly more effective ($p<0.0005$) at preventing tumor growth than agonist mAb suggesting that polyclonal immune sera maybe superior in controlling tumorigenesis.

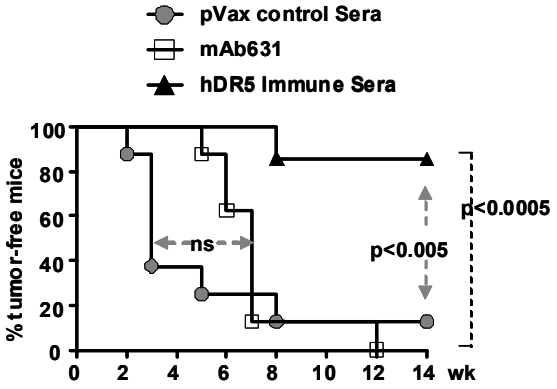

Fig. 3. Inhibition of tumor growth by hDR5 immune sera. SUM159 Cells were coated with hDR5 immune sera, pVax-1 control sera or mAb63, washed and then injected into SCID mice (3x106 cells). Animals were monitored weekly for tumor growth. The median time to palpable tumor in the control mice was 3 weeks (n=8) compared to 7 weeks in the mAb631 treated mice (n=8). This difference was not statistically significant (ns). The median time to palpable tumor was not reached in the hDR5 immune sera group (n=7) which was significantly different from the control ($p<0.005$) and the mAb631 treated ($p<0.0005$)

Specific Aim 3 Amplify anti-tumor activity of DR5 vaccination with novel chemotherapeutics.

Amplification of DR5 agonist activity with novel chemotherapeutic agent

To test the potential benefit of combining chemotherapeutic agents and DR5 agonist antibodies, we treated TNBC cell lines (SUM159, SUM149, MB231), HER2+ (SKBR3) and HER2+/ER+ (BT474) cell lines with agonist mAb 631 and histone deacetylase (HDAC) inhibitor MS-275 individually and in combination. TNBC cells were sensitive to hDR5 agonist, reducing growth >45%,

when compared with SKBR3 and BT-474 cells. TNBC cells are less sensitive to MS-275 alone when compared with SKBR3 cells. In combination, growth inhibition of TNBC was greater than each agent alone. HER2+ and HER2+/ER+ cells resisted DR5 agonists but were sensitive to HDAC inhibitor alone. There was no benefit in combining both treatment, indicating that HDAC inhibitor only sensitize TNBC to hDR5 agonist antibody.

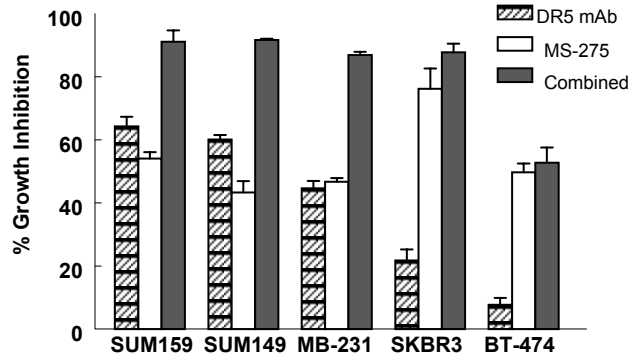

Fig. 4 HDAC Inhibitor MS-275 enhances growth inhibition induced by hDR5 agonist antibody in TNBC cell lines. Cells were treated with DR5 mAb631 at 2.5 μg/mL (SUM159 at 0.5 μg/mL) alone, 2.5 μM MS-275 (SKBR3 and BT-474 at 5 μM) alone or the combination and evaluated for growth over 72 hours. Data represent % Growth Inhibition relative to the diluent control after 72h by MTT assay. Data at Means+SEM, n=4.

Activation of the Akt survival pathway counteracts TRAIL-induced apoptosis

To further investigate the mechanism of resistance to TRAIL or agonist induced apoptosis, we tested the hypothesis that activation of Akt survival pathway was induced in TRAIL resistant cells. Akt phosphorylation was increased in TRAIL resistant T47D cells after treatment with TRAIL relative to untreated cells, and Akt phosphorylation was blocked by the PI3-kinase inhibitor LY294002 (Fig. 5A). Importantly, inhibition of Akt phosphorylation caused PARP cleavage and apoptosis (Fig. 5A), which was correlated with TRAIL-induced growth inhibition (Fig. 5B). Similar results were obtained with TRAIL resistant ovarian cancer cell line OVCA432 (Fig. 5C and D). Therefore, while TRAIL induces apoptosis in sensitive cells, TRAIL can also activate the survival pathways. We will test if immune sera mediated antibody-dependent cell mediated cytotoxicity can overcome resistance to TRAIL or DR5 agonist.

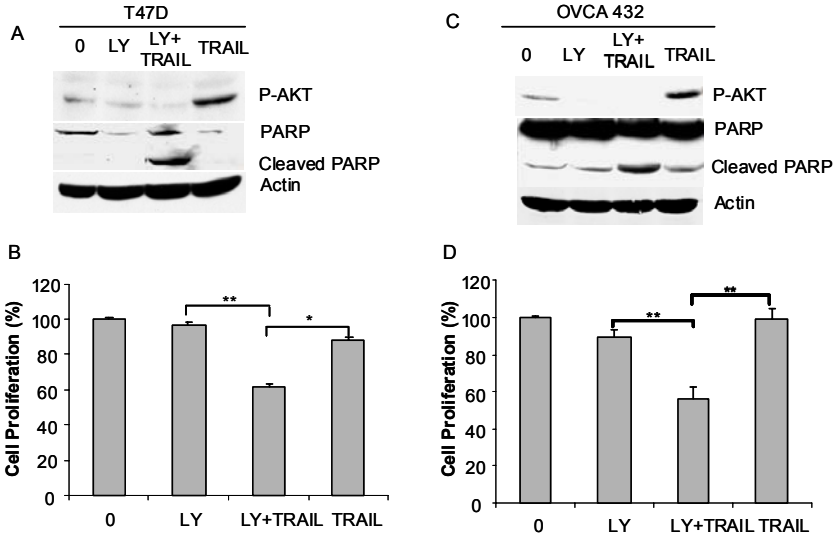

Fig. 5. Inhibition of AKT activity sensitizes resistant cancer cells to TRAIL. (A and C) Effect of the PI3K inhibitor LY294002 on TRAIL-induced apoptosis signaling. Breast cancer cell T47D (A) and ovarian cancer cell OVCA 432 (C) were left untreated or pretreated with 10 μM LY294002 (LY) for 30 min, and then incubated with or without 100 ng/ml TRAIL for 6h. Total protein was extracted. Cleaved PARP and total and phosphorylated Akt were determined by Western blotting. (B and D) Effect of LY294002 on TRAIL-induced growth inhibition. To measure cell proliferation, T47D (B) and OVCA 432 cells (D) were left untreated or pretreated with 10 μM LY294002 for 30min, and then incubated with or without 100 ng/ml TRAIL for 24h. Cell proliferation was determined by MTT assays. Cell proliferation data are expressed as percentage of untreated cells. *p<0.05, **p<0.005, by student's t test.

KEY RESEARCH ACCOMPLISHMENTS

1. Induction of agonist polyclonal antibodies by vaccination with human DR5 DNA to inhibit the growth of triple negative breast cancer in vivo.
2. Amplification of anti-tumor activity by combining DR5 agonist mAb with HDAC inhibitor.
3. Define a mechanism of escape from TRAIL or DR5 agonist activity by the activation of Akt survival pathway.

REPORTABLE OUTCOMES

"Vaccines targeting cellular death receptors" Preliminary patent application filed September, 2008

CONCLUSIONS

Agonist antibodies have been induced by hDR5 DNA vaccine to inhibit the growth of triple negative breast cancers. Anti-tumor activity can be further enhanced by combined treatment with HDAC inhibitor. One mechanism of tumor resistance to TRAIL or DR5 agonist is via activation of Akt survival pathway, indicating potential benefit of combining hDR5 vaccination with targeted therapy.

Reference List

1. Pai SI, Wu GS, Ozoren N, Wu L, Jen J, Sidransky D et al. Rare loss-of-function mutation of a death receptor gene in head and neck cancer. *Cancer Res* 1998;58:3513-8.