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MUC1-like tandem repeat proteins are broadly immunogenic in cancer patients

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Abstract

The identification of antigens mediating tumor rejection is an important goal of cancer immunology. The SEREX technology utilizes antibodies from cancer patients to identify candidate antigens from tumor-derived cDNA expression libraries. Using sera from a long-term surviving metastatic melanoma patient vaccinated with irradiated, autologous tumor cells engineered to secrete granulocyte-macrophage colony stimulating factor (GM-CSF), we identified an antigen reported to be a putative opioid growth factor receptor (OGFr). The human immune response to OGFr exhibits three features shared with other tumor antigens. First, the protein is an intracellular antigen found in both nucleus and cytoplasm. Second, part of the antibody response is directed at a putative protein product encoded by an alternative reading frame (ARF). Third, part of the antibody response is directed at a portion of the molecule that bears a striking resemblance to the extracellular domain of MUC1, both with respect to primary structure and size polymorphism. Antibody responses to OGFr and a synthetic peptide representing a putative alternative reading frame product (OGFr-ARF) were frequently found in cancer patients. 11/45 (24%) melanoma patients had antibodies to OGFr and 5/45 (11%) had antibodies to OGFr-ARF. Moreover, 5/24 (21%) lung cancer, 4/25 (16%) prostate cancer, and 5/6 breast or ovarian cancer patients had antibodies to OGFr, the alternative frame product, or both. These data add to the growing list of tumor antigens that appear to be translated in two frames, and suggest that OGFr and OGFr-ARF may be useful targets for vaccination.

Introduction

The identification of tumor rejection antigens is an important requirement for the development of effective immunotherapies. Ideally, these antigens should induce cancer-specific immune responses and be broadly applicable to diverse tumor types. Two major approaches to tumor antigen characterization have proven informative. One uses tumor-specific cytotoxic T lymphocytes to screen tumor-derived cDNA expression libraries

or peptide pools eluted from tumor cell-derived MHC molecules (1, 2). The second strategy (SEREX) uses endogenous antibody responses to screen tumor-derived cDNA expression libraries (3, 4, 5).

Of the many candidate tumor antigens identified to date using these technologies, no overriding structural theme to the proteins identified has yet emerged. Many gene products are transcription factors or other nuclear proteins. The biological functions of most of these antigens and their roles, if any, in carcinogenesis remain to be elucidated. Although many antigens detected by SEREX are intracellular, antibodies in cancer patients can also be directed against cell surface proteins. For example, antibodies to MUC1, a transmembrane protein expressed on breast and ovarian carcinomas, can frequently be found in patients with these diseases, and the presence of these antibodies is associated with improved survival (6, 7).

We previously reported the results of a phase I clinical trial in which patients with metastatic melanoma were vaccinated with lethally irradiated, autologous melanoma cells engineered to secrete GM-CSF (8). Although metastatic lesions resected before vaccination were minimally infiltrated with cells of the immune system in all patients, metastatic lesions resected after vaccination were densely infiltrated with T lymphocytes and plasma cells and showed extensive tumor destruction (at least 80%), fibrosis, and edema in 11 of the 16 patients examined. Vaccination stimulated the production of antibodies to numerous intracellular and cell surface antigens, as detected by immunoblotting and flow cytometry. We now report that a recently identified putative opioid growth factor receptor (OGFr) (9, 10) is one of the targets for antibody responses in these patients. Interestingly, the humoral reaction is directed at two different MUC1-like tandem repeat-containing proteins potentially encoded by the OGFr cDNA.

Results

Identification of OGFr and OGFr-ARF

Since vaccination with irradiated melanoma cells engineered to secrete GM-CSF stimulates the coordinated development of humoral and cellular anti-tumor immunity (8), we chose to identify candidate tumor rejection antigens using the SEREX approach (3). A melanoma cell line derived from long-term surviving patient K008 (no evidence of disease 7.5 years post-vaccination) was established from a metastasis densely infiltrated with T lymphocytes and plasma cells as a consequence of vaccination. A cDNA expression library was constructed in the Lambda Zap vector using K008 melanoma cell-derived messenger RNA, and a total of 1×10^6 independent plaques were screened with a 1:1000 dilution of patient serum obtained during immunization. One of the clones detected in this screen, designated K17.1, contained a partial cDNA sequence encoding a protein with 20 amino acid tandem repeats. We attempted to use K17.1 to identify the full-length cDNA by hybridization screening and 5' RACE in the K008 library, but these efforts were unsuccessful. We thus screened a U937 cDNA expression library using K17.1 as a probe and identified a full-length cDNA clone encoding a 578 amino acid protein with a predicted molecular weight of 63 kDa. While this work was in progress, an identical sequence encoding a putative opioid growth factor receptor (OGFr) was reported (10), and to maintain clarity in the literature, we have adopted this terminology.

Analysis of the sequence of OGFr revealed similarities between the carboxyl-terminal tandem repeats of this protein and the tandem repeats in the extracellular domain of MUC1 (20% identity, Figure 1), with striking positional conservation of 4 of the 5 prolines in each repeat (11, 12). Surprisingly, we noted that the tandem repeat region of phage clone K17.1 was fused with the *LacZ* promoter of Lambda Zap in a reading frame different from that reported for OGFr (we have designated this product OGFr-ARF, Figure 1). This suggested that

antibodies from patient K008 may have detected an alternatively translated sequence. The OGFr-ARF tandem repeat region has a higher overall identity to MUC1 (30%), but shows positional conservation of only 2 of the 5 prolines. All three structures, however, possess a proline at position 5.

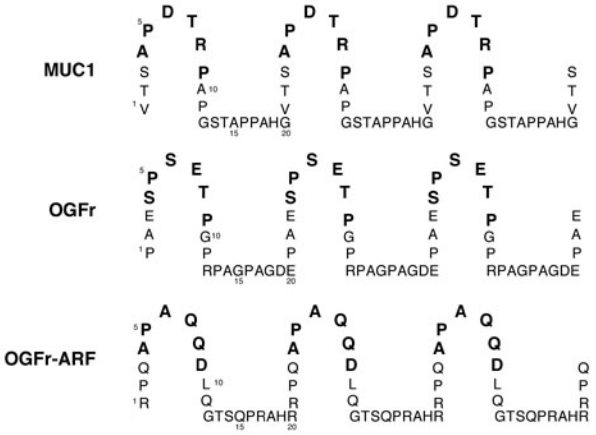

Figure 1. Sequence comparison of the tandem repeat regions of OGFr, MUC1, and the alternative reading frame of the OGFr tandem repeat region (OGFr-ARF). The sequences are derived from the central, most frequently occurring repeat. Minor variations in the sequence of both MUC1 and OGFr repeats occur in the flanking repeats. OGFr shares 20% identity with MUC1, and has positional conservation of 4 of 5 prolines with MUC1. OGFr-ARF shares 30% identity with MUC1. The projection showing the MUC1 amino acids involved in the poly-proline beta-turn "loop" secondary structure are from Fontenot *et al*. (13). The secondary structures of OGFr and OGFr-ARF remain to be determined experimentally.

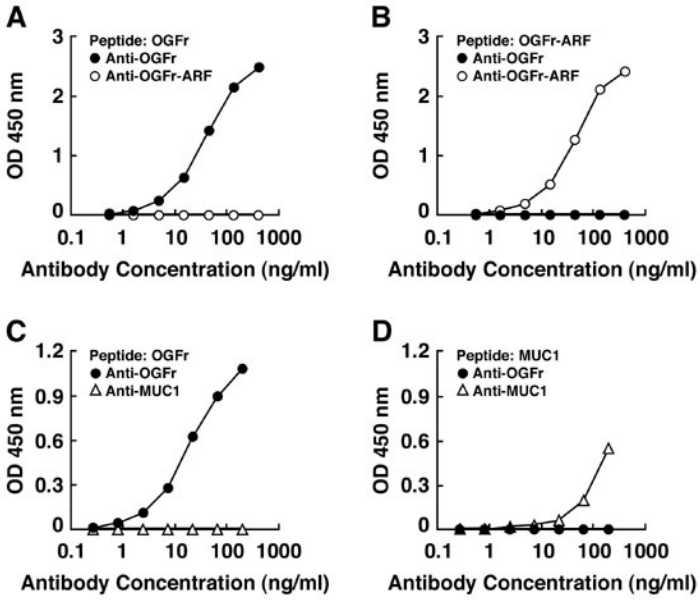

Figure 2. Specificity of peptide-selected antisera to tandem repeat motifs. Synthetic peptides (50 amino acids, 2.5 repeats) from the tandem repeat portion of OGFr (A, C), the alternative reading frame of the OGFr tandem repeat region (B) or MUC1 (D) were plated at 1 µg/well. Peptide-selected antisera to the tandem repeat region of OGFr (filled circles), the alternative reading frame of OGFr (open circles) or a monoclonal antibody to MUC1 tandem repeats, VU4H5 (open triangles) were titrated against the peptide at the indicated concentrations. The antibodies were detected with anti-rabbit or mouse (for VU4H5) antibodies conjugated to HRP.

The secondary structure of the tandem repeat domain of MUC1 has been solved by nuclear magnetic resonance spectroscopy, revealing an extended, rod-like structure with a "knob"-like motif, termed a poly-proline beta-turn helix (14). This secondary structure is effectively reproduced with synthetic peptides, especially those made to encompass two or more repeats (15). To characterize the protein encoded by the full-length cDNA and proteins containing the alternative frame translation, we generated rabbit antisera to synthetic peptides derived from their sequences. These antibodies demonstrated no shared antigenicity between the two peptides or the related tandem repeat motif from MUC1 (Figure 2).

OGFr cellular localization and polymorphism

To localize OGFr in the cell, we fractionated three cell lines into cytoplasmic and nuclear fractions and probed these using the antisera against the tandem repeat region. These experiments revealed that OGFr is in both the nucleus and the cytoplasm (Figure 3). Equal partitioning into these fractions is not a consequence of the cell cycle, as resting peripheral blood mononuclear cells showed a similar distribution in both fractions (data not shown). Interestingly, OGFr from these three lines demonstrated size heterogeneity, suggesting that this protein might possess varying numbers of repeats, analogous to MUC1 (12), or variations in glycosylation. To explore this hypothesis further, we prepared cytoplasmic extracts from peripheral blood mononuclear cells of five healthy donors, size-fractionated them on acrylamide gels, and probed the lysates with antisera to OGFr (Figure 4A). This analysis revealed considerable heterogeneity in the protein products, with 4 of 7 donors displaying homozygosity and 3 of 7 donors displaying heterozygosity in OGFr size. This heterogeneity was also reflected at the RNA level in the size of the transcripts (data not shown). The laddering effect of the proteins in the immunoblotting studies suggested that they might differ by defined size increments. Indeed, using K008 and U937 as reference points, we found that the varying sizes of OGFr fall on a line relating size to the number of repeats, suggesting that the proteins differ in the numbers of repeats and that the size of the proteins increases by increments corresponding to one repeat (Figure 4B).

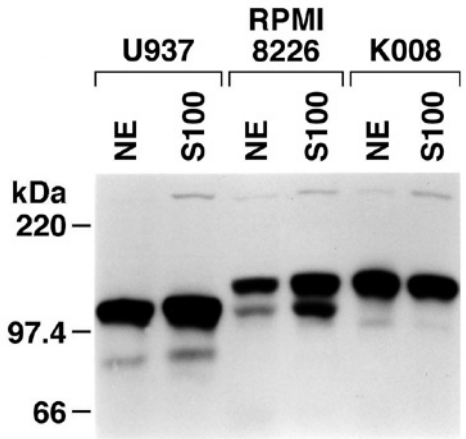

Figure 3. OGFr resides in both the nucleus and the cytoplasm of the cell. Nuclear (NE) and cytoplasmic (S100) extracts were prepared from the indicated cell lines as described in the materials and methods section. The proteins (35 µg) in each extract were separated on an 8% acrylamide gel, transferred to nitrocellulose and probed with antibodies to the OGFr tandem repeat region. Addition of the OGFr tandem repeat peptide to the blot completely blocks the detection of the protein (data not shown).

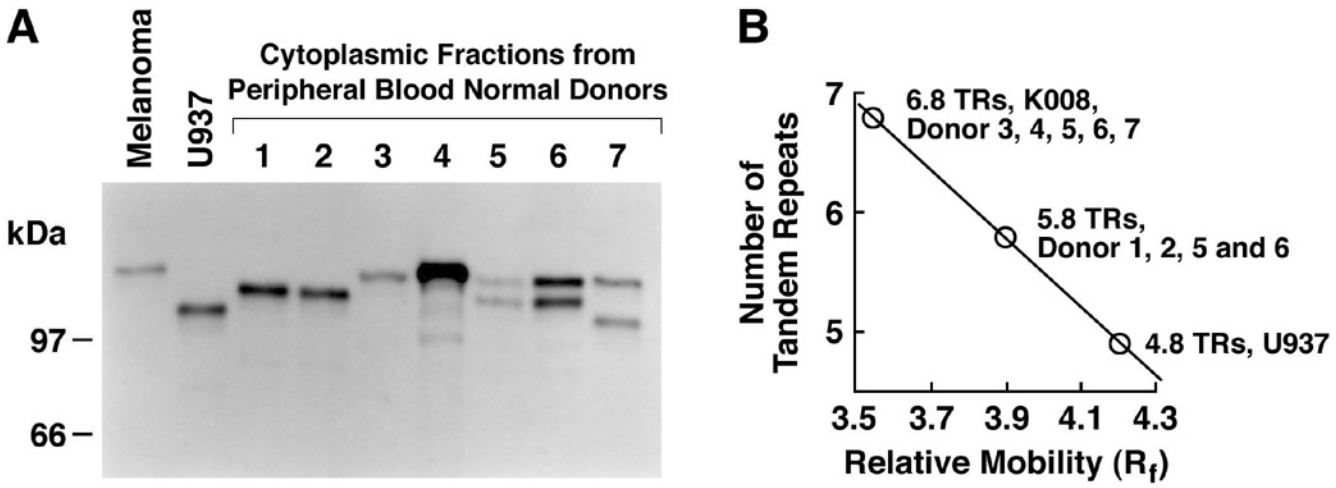

Figure 4. OGFr protein size polymorphism correlates with the number of 20 amino acid tandem repeats present. (A) Cytoplasmic fractions (20 µg/lane) from the cell lines K008, U937, or peripheral blood lymphocytes from seven normal donors were separated on an 8% acrylamide gel, transferred to nitrocellulose, and probed with an antibody to the OGFr tandem repeat region. (B) The R_f of the proteins correlates with the number of tandem repeats (TRs) contained in the protein using K008 (6.8 repeats) and U937 (4.8 repeats) as known points, and suggest that the size heterogeneity occurs in discrete 20 amino acid increments.

Identification of proteins reactive with anti-OGFr-ARF

Although the relationship between the full-length OGFr protein and a product containing the OGFr-ARF sequence remains to be clarified, the OGFr-ARF peptide is the target of antibody responses in both vaccinated and unvaccinated cancer patients (see below). To identify candidate proteins reflected by the OGFr-ARF peptide, we isolated nuclear and cytoplasmic extracts from melanoma cell line K008 and performed immunoblotting analysis with antibodies to OGFr-ARF (Figure 5). The sera identified two proteins in the extracts, a 54 kDa protein found in both nucleus and cytoplasm and a larger 174 kDa nuclear protein. Antibody specificity was established by the ability of the OGFr-ARF peptide, but not of a control (MUC1) peptide, to block detection. To test if these two species were restricted to melanoma cells, we examined other cell lines in similar studies (Figure 6). Extracts from myeloma (RPMI 8226) and myelomonocytic (U937) cells also showed multiple proteins detected by anti-OFGr-ARF, two of which were also present in K008 cells. U937 showed the most complex pattern, with 4 proteins detected (p174, 157, 129 and 54), some of which localized to both the nuclear and cytoplasmic fractions (p129 and 54). A similar pattern was seen in RPMI 8226, with three proteins detected specifically as determined by peptide blocking. Two proteins in RPMI 8226 had a similar relative mobility (R_f) to that of proteins found in U937 and K008 (p174 and p54). Two nuclear proteins (p147 and p157) were not common to the cell lines, but this might reflect a variation in the number of tandem repeats. The anti-OGFr-ARF antibodies could immunoprecipitate p174 and p54, and we have twice purified p54 on antibody columns and attempted to sequence the amino terminus, but in both cases the terminus was blocked. While further studies are necessary to characterize these proteins, their antigenic similarity to the OGFr-ARF peptide suggests that any or all of them may be responsible for eliciting the antibodies found in K008.

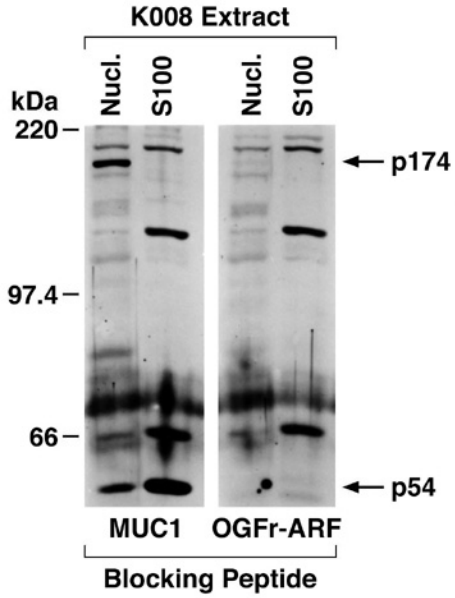

Figure 5. Antibodies to OGFr-ARF detect two proteins in the melanoma cell line K008. Nuclear and cytoplasmic (S100) extracts were size fractionated on an 8% acrylamide gel and probed with antibodies to OGFr-ARF. Two proteins were detected, a nuclear protein (p174) and a protein detected in both the nuclear and cytoplasmic fractions (p54). Antibody specificity was demonstrated by the ability of OGFr-ARF peptide, but not of a MUC1 peptide, to block detection.

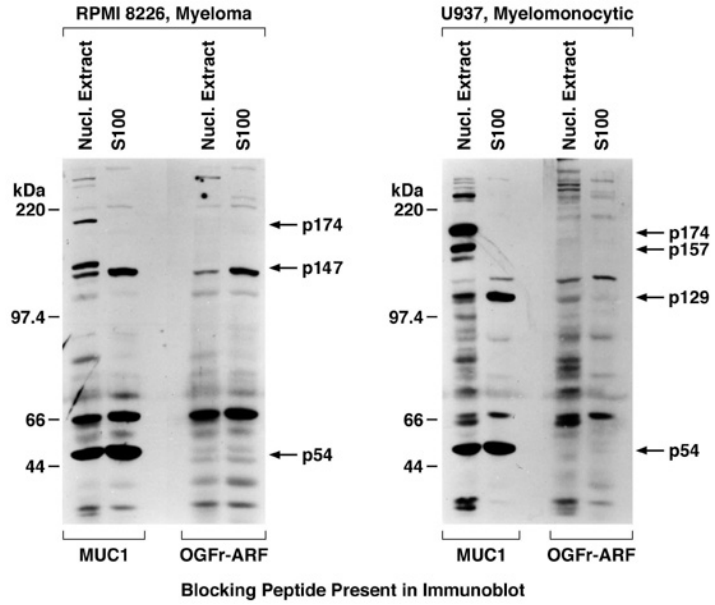

Figure 6. Antibodies to the OGFr-ARF peptide detected nuclear and cytoplasmic proteins in RPMI 8226 and U937. Peptide selected antisera to OGFr-ARF was used to probe nuclear and cytoplasmic (S100) extracts from myeloma and myelomonocytic cell lines. Arrows indicate proteins specifically detected by the antisera as judged by the ability of the immunizing peptide (OGFr-ARF), but not of an irrelevant tandem repeat peptide (MUC1), to block detection. The integrity of the extract was demonstrated by the presence of proteins in one of the two compartments, but not both. For example, while p54 was found in both nuclear and cytoplasmic fractions, p174 localized to the nuclear fraction only.

Expression profile of OGFr

One explanation as to how a single gene could give rise to two protein products related by a frame shift is by the generation of alternatively spliced transcripts (16). To explore the diversity of transcripts generated by the OGFr gene, we hybridized multi-tissue Northern blots with ^{32}P-labeled probes from the 5' portion of the cDNA (non-tandem repeat region, Figure 7A) or the tandem repeat region (Figure 7B). While both probes identified a widely expressed transcript of 2.4 kb, the 5' probe detected an additional 1.4 kb transcript in brain and heart, and the tandem repeat probe detected a 1.0 kb transcript in liver. Intriguingly, this liver transcript corresponded in size to clone K17.1 detected by the sera of patient K008, although Northern analysis of K008 mRNA revealed only the 2.4 kb transcript. 5' RACE reactions using liver mRNA failed to identify a specific product to date, and further work is necessary to elucidate the structure of this transcript.

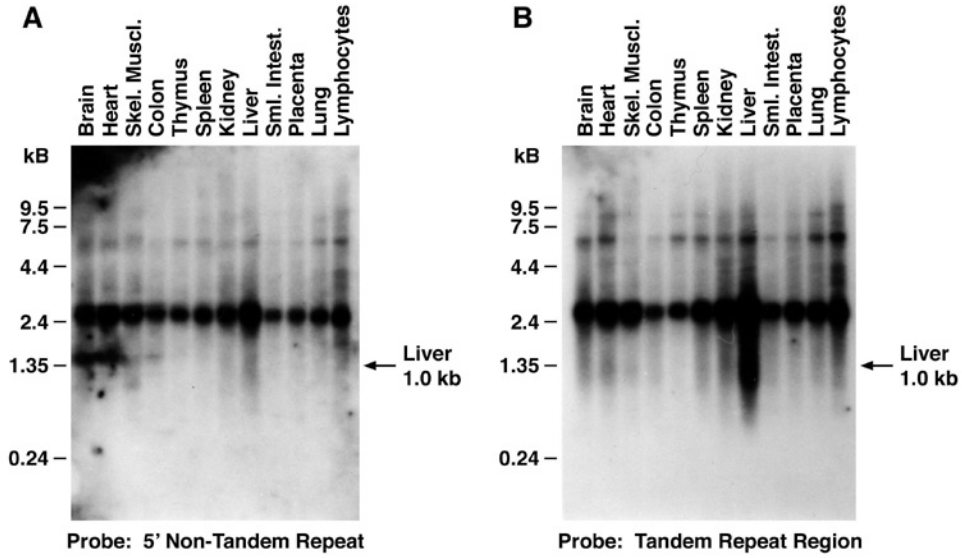

Figure 7. Multi-tissue Northern blots show a predominant 2.4 kb OGFr transcript, along with smaller tissue-specific transcripts. A DNA fragment derived from the 5' non-tandem repeat region (nt 486-1105) of the OGFr cDNA (A), or a restriction enzyme fragment (*Kpn*I-*Eco*RI, nt 1574-2127) (B) containing only the tandem repeat region of the cDNA was used to probe a multi-tissue RNA blot (Clonetech No. 7780-1). Besides the ubiquitously expressed 2.4 kb transcript, the 5' probe detects a smaller 1.4 kb transcript in brain and heart corresponding to a splicing product previously reported in fetal brain tissue that lacks the tandem repeat region (10). In contrast, the tandem repeat probe hybridizes to a 1.0 kb transcript in liver RNA, in addition to the predominant 2.4 kb species. The ubiquitous 6.5 kb transcript is likely a nascent transcript, as it corresponds in size to an unspliced genomic clone (see GenBank Accession No. AF112980). Hybridization of this blot with a beta-actin probe demonstrated the absence of RNA breakdown and that all 12 lanes were equally loaded (data not shown).

Antibody responses to OGFr and OGFr-ARF in patient K008

To evaluate whether OGFr was a target for antibody responses, we produced recombinant full-length OGFr in *E. coli* using the pET28b(+) vector. Recombinant OGFr produced by this method exhibited a single band on Coomassie stained gels (Figure 8). Antisera to the tandem repeat region detected the recombinant protein, and this binding was blocked by the addition of the immunizing peptide. Recombinant OGFr and the endogenous protein product from cells migrate significantly slower than the predicted molecular weight (approx. 63 kDa), and this anomalously slow R_f may reflect the extended conformation of the tandem repeat domain in the protein (17).

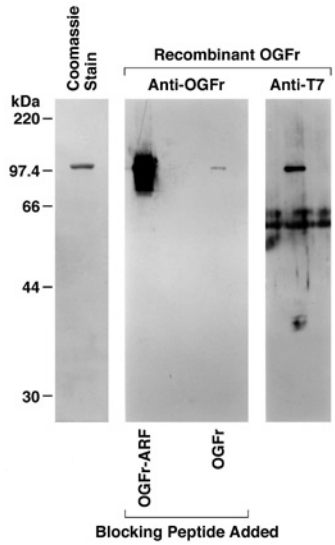

Figure 8. Analysis of the purified, recombinantly expressed OGFr. The full length OGFr protein was produced in *E. coli* and refolded as described in the materials and methods section. The final product is considered pure as it gave a single-band by Coomassie blue staining. The protein product is also detected by antisera to the tandem repeat region, a detection that is blocked by the addition of the OGFr tandem repeat peptide. The recombinant molecule is also detected by an antibody to the T7 epitope.

To determine whether patient K008 developed antibodies to OGFr and/or OGFr-ARF, we developed an ELISA assay using purified recombinant OGFr, the in-frame tandem repeat peptide derived from OGFr, the alternative frame tandem repeat peptide derived from OGFr-ARF, and a MUC1-derived peptide as a control (Table 1). Interestingly, sera from patient K008 specifically detected both the OGFr-ARF peptide and recombinant OGFr both in 1995 (pre-vaccination) and in 1999 (4 years after the completion of vaccination). Antibodies to the OGFr peptide, if present, were at much lower levels, and comparable to those found against MUC1. A detailed longitudinal analysis of antibody titers as a function of immunization remains to be performed.

Table 1. Antibodies to OGFr in melanoma patient K008[a].

Date	Tandem Repeat Derived Peptides			Recomb. OGFr
	OGFr	OGFr-ARF	MUC1	
November 1995[b]	0.16 ± 0.01	0.80 ± 0.00	0.13 ± 0.01	0.34 ± 0.00
September 1999[c]	0.06 ± 0.00	0.31 ± 0.03	0.05 ± 0.00	0.61 ± 0.02

[a]Values correspond to the OD_{450nm} (mean ± SD).
[b]Assay performed at a 1:800 dilution.
[c]Assay performed at a 1:1000 dilution.

Antibodies to OGFr and OGFr-ARF in cancer patients

To determine the frequency of antibodies to OGFr and OGFr-ARF in metastatic melanoma patients, we studied 44 other individuals receiving autologous, irradiated, melanoma cells engineered to secrete GM-CSF. Using a criterion of four standard deviations above the mean observed in a group of normal donors (Figure 9B), we found 11/45 (24%) responses to recombinant OGFr and 5/45 (11%) responses to OGFr-ARF (Figure 9A). Two responses to the OGFr "in frame" tandem repeat peptide were also found, but these were of significantly lower titer than those to the recombinant OGFr protein. Further testing of longitudinal serum samples from these patients is required to determine the influence of vaccination on these antibody responses.

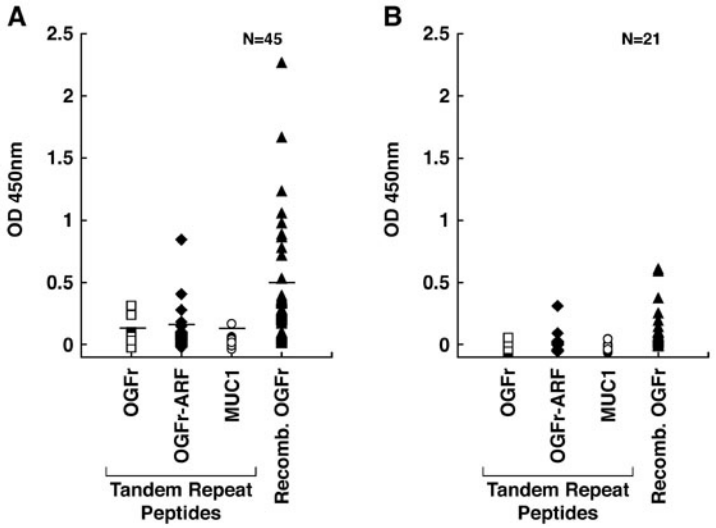

Figure 9. Antibody responses to recombinant OGFr and OGFr-ARF in melanoma patients. Synthetic peptides (0.5 µg/well) or recombinant OGFr (0.3 µg/well) were bound to ELISA plate wells and incubated with sera (diluted 1:1000) from 45 melanoma patients enrolled in the GM-CSF vaccine trial (A) or from 21 normal donors (B). Using a criterion of four standard deviations above the mean of the values observed in the normal donors (indicated by bars, panel A), the number of positive responses in the melanoma patients were as follows: OGFr 2/45, OGFr-ARF 5/45, MUC1 1/45 and recombinant OGFr 11/45.

The presence of antibodies to recombinant OGFr and the OGFr-ARF peptide in patient K008 prior to immunization prompted us to determine if non-vaccinated patients also developed antibodies. We thus collected sera from individuals with metastatic non-small cell lung cancer or hormone refractory prostate cancer. Using criteria of three-fold elevations in mean OD readings compared to the response against control antigens to determine positive responses, we found that 5/24 (21%) lung cancer patients and 3/25 (12%) prostate cancer patients developed antibodies to OGFr-ARF (Figure 10A, B). Only one patient in the prostate cancer group had a three-fold elevation in the response to recombinant OGFr compared to that to the control antigen. A comparison of the data from the melanoma patients with the lung and prostate cancer patients reveals a striking absence of responses to recombinant OGFr in the lung and prostate groups, although these patients frequently developed responses to OGFr-ARF. Moreover, the responses to OGFr-ARF and recombinant OGFr did not strictly correlate. For example, in the prostate cancer group the highest responder to OGFr-ARF (OD at 450 nm of 1.6) had a negligible response to recombinant OGFr (OD at 450 nm of 0.06), while the highest responder to recombinant OGFr (OD at 450 nm of 0.36) showed only a negligible response to the OGFr-ARF peptide (OD at 450 nm of -0.03).

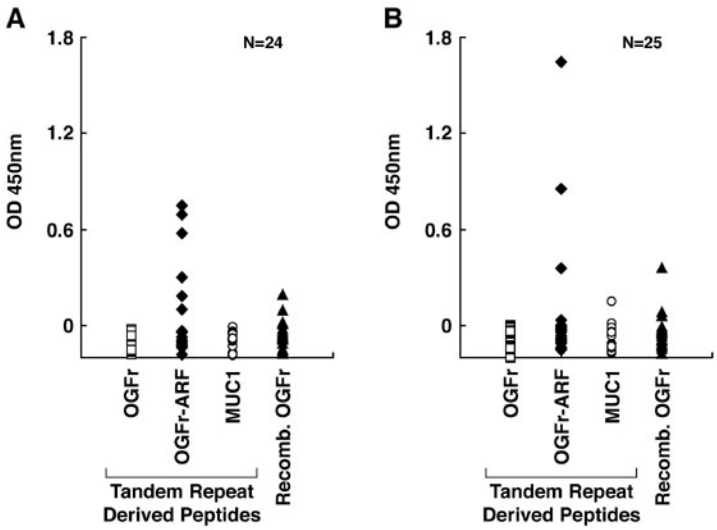

Figure 10. Antibody responses to recombinant OGFr and OGFr-ARF in metastatic non-small cell lung cancer (A) and hormone refractory prostate cancer (B) patients. ELISA plates were coated with the antigens indicated and incubated overnight at 4°C with serum diluted 1:800. Using a criterion of 3-fold elevation (OD at 450 nm) above that obtained with the control antigen (MUC1 peptide), there were 5/24 responses to OGFr-ARF in the lung cancer group. In the prostate cancer group, there were 3/25 responses to OGFr-ARF and 1/25 to recombinant OGFr.

Lastly, we tested a small sample of unvaccinated breast and ovarian cancer patients that developed antibodies to MUC1 (7, 18). We hypothesized that responses to MUC-1 in this patient population might be coordinately stimulated with those to OGFr tandem-repeat containing proteins. Indeed, we found that 5 of 6 patients evaluated showed positive responses to recombinant OGFr, in comparison to the three concurrently tested normal donors (Figure 11). One patient, BT-2, had antibodies to both the OGFr-ARF peptide and OGFr.

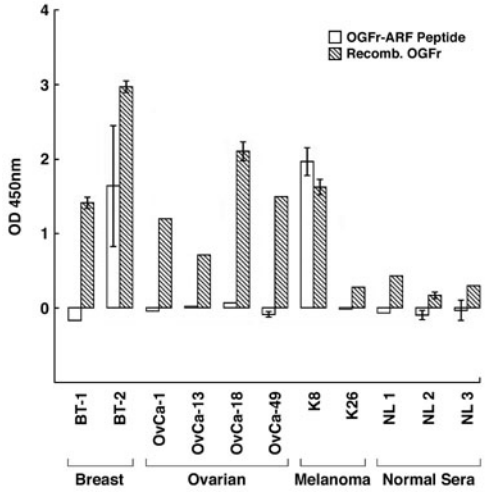

Figure 11. Antibody responses to OGFr and OGFr-ARF in breast and ovarian cancer patients with antibodies to MUC1. All serum samples were diluted (1:200) and tested as described above. A melanoma patient found to have antibodies to both OGFr-ARF and recombinant OGFr (K8), and one which had neither (K26), were included as controls. Also included are three normal donor serum samples. Using the criteria of four standard deviations above the mean of the normal donor response, 5/6 of the patient samples had an elevated antibody response to recombinant OGFr, and 1/6 (BT-2) to OGFr-ARF.

Discussion

This work was undertaken as a first step to characterize the molecular targets of vaccination with irradiated, autologous melanoma cells engineered to secrete GM-CSF. Since whole tumor cells present a large pool of potential immunogenic moieties, the antigens eliciting immune responses in vaccinated patients may differ from or overlap with the antigens identified using sera and T cells from unvaccinated patients. Here we demonstrate that OGFr and OGFr-ARF are targets for antibody responses in some patients treated with GM-CSF secreting tumor cell vaccines. However, immunity to these antigens is not strictly dependent upon vaccination, as antibodies to OGFr and OGFr-ARF were present at significant frequencies in a variety of solid tumor patients. Moreover, OGFr (identical to the 7-60 gene) was recently identified as a target for immune-based remission in a chronic myelogenous leukemia patient receiving donor lymphocyte infusions (19). Together, these findings indicate that OGFr stimulates immune recognition in diverse tumor types.

Two emerging concepts from antigen discovery efforts are that many target molecules are nuclear in origin, and that non-primary open reading frames can elicit immune recognition (20, 21, 22, 23). We extend this work by illustrating that OGFr is partitioned equally between nucleus and cytoplasm, and that OGFr sequences can be translated in two different reading frames. Earlier investigations showed that tumor-specific cytotoxic T cells can respond to peptides derived from alternatively translated reading frames of NY-ESO-1/LAGE-1 (23, 24, 25), SART-1 (26), tyrosinase-related protein-1 (22), and intestinal carboxyl esterase (27). However, to the best of our knowledge, antibody responses to a tumor antigen translated in two different reading frames have not been previously observed.

Several mechanisms have been invoked to account for the generation of ARF products. These include translational initiation codon readthrough, frameshifting during translation, and the presence of internal ribosomal entry sites that guide the 40S complex to begin translation at sites different from the first AUG (28). An additional pathway involves out-of-frame splice variants, best exemplified by the tumor suppressor p16ink4a (16). A recent report by Shichijo and colleagues revealed that the squamous cell carcinoma tumor antigen SART-1 underwent a -1 frameshift during translation, resulting in a chimeric protein composed of amino acid sequences from two reading frames (26). Analogous to the SART-1 cDNA, we noted the presence of Shine-Delgarno sequences (AGGGGG) upstream of a region of putative frameshifting in OGFr (nt 1118-1123 and 1131-1136). While the role of these sequences in promoting frameshifting in eukaryotic mRNA translation is less clear than in prokaryotes (29), it is tempting to speculate that the larger molecular weight proteins detected in the OGFr-ARF immunoblots (Figures 5A and 5B) share with OGFr a common amino terminal domain, followed by a frameshift immediately upstream of the tandem repeat region. This hypothesis could be tested by generating antisera to the amino terminal domain of OGFr and then probing the immunoprecipitated proteins with sera against OGFr-ARF.

Several previous reports of antibody responses to tandem repeat containing proteins have suggested that these are of clinical importance. In this context, antibody responses to the extracellular tandem repeat domain of MUC1 are frequently observed, especially in breast and ovarian cancer patients, and have been shown to have prognostic significance (7). CDR34, a protein with 34 inexact six amino acid tandem repeats and which is expressed in the cerebellum, elicits antibody responses in some patients with paraneoplastic cerebellar degeneration (30). Allogeneic antibody screening of a melanoma cDNA expression library detected CT7, a protein with ten 35 amino acids repeats and some homology to MAGE 10 (31). Extending these reports, we found 14 patients with elevated antibody titers against OGFr-ARF. Such repetitive epitopes likely stimulate immunoglobulin production efficiently (32), analogous to the T cell-independent B cell responses directed to bacterial polysaccharides. Nonetheless, the in-frame OGFr-derived tandem repeat peptides were recognized much less often than the full-length recombinant protein, suggesting that additional epitopes in the molecule can also be immunogenic.

One prediction of SEREX is that the most useful tumor antigens will frequently stimulate high titer antibody responses in patients with diverse tumors. In this context, 24% of melanoma patients developed antibodies to recombinant OGFr and 11% to the OGFr-ARF peptide. Moreover, 21% of non-small cell lung carcinoma, 16% of prostate carcinoma, and 5/6 breast or ovarian cancer patients also generated antibodies to OGFr-ARF or recombinant OGFr. Additional studies are underway to characterize potential T cell responses to OGFr and OGFr-ARF.

The sequence homology between the tandem repeats of OGFr, an intracellular protein, and MUC1, a transmembrane molecule, highlights the issue of the possible functions of poly-proline beta-turn secondary structures. The murine MUC1 gene has been deleted in embryonic stem cells, but the homozygous deficient mice show only modest defects (33). Although MUC1 was initially thought to be restricted to surface epithelial cells, more recent work indicates that it is expressed in many tissues, including the immune system (34, 35, 36). Additional studies are required to determine the roles of OGFr, its putative ligands, and OGFr-ARF in health and carcinogenesis.

In conclusion, we have identified a new tumor antigen OGFr and a related translation product OGFr-ARF by performing SEREX analysis of a long-term responding patient vaccinated with GM-CSF secreting, autologous melanoma cells. While it remains unclear how the OGFr gene/cDNA gives rise to the ARF product, both appear to be targets of immune responses in at least five different types of solid tumors and chronic myelogenous leukemia. The biology of the human immune response to this molecule has revealed a complexity not evident by simple inspection of the gene sequence.

Abbreviations

ARF, alternative reading frame; OGFr, opioid growth factor receptor; SEREX, serological analysis of recombinant cDNA expression libraries

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Materials and methods

Tissues and cells

K008 is a cell line derived from a resected melanoma metastasis and has been described previously (8). The myelomonocytic and myeloma cell lines, U937 (ATCC CRL-1593.2) and RPMI 8226 (ATCC CCL-155) respectively, were obtained from the American Tissue Type Collection. Serum samples were obtained from patients with metastatic melanoma or metastatic non-small cell lung carcinoma upon enrollment into IRB approved vaccine trials (8). Serum samples (kindly provided by Dr. Phillip Kantoff) were also obtained from hormone refractory advanced prostate cancer patients at the Dana-Farber Cancer Institute. The breast and ovarian cancer serum samples were the kind gifts of Silvia von Mensdorff-Pouilly and have been previously described (7, 18). All sera were stored at -80°C until shortly before use. The anti-MUC1 monoclonal antibody VU4H5 was a gift of Jo Hilgers and was previously characterized (37, 38).

Construction and immunoscreening of cDNA libraries

RNA was isolated from the melanoma cell line K008 using guanidine isothiocyanate and mRNA was selected with 2 rounds of oligodT cellulose. A cDNA library was constructed in the Lambda Zap vector using a commercial cDNA library kit (Stratagene). For screening, 1 x 10^6 plaques were screened with K008 sera that had been extensively pre-cleared on *E. coli* and lambda phage lysates and diluted 1:1000 in TBS, 0.1% Tween-20, and 2% non-fat dry milk (NFDM).

Production of peptide-selected antisera

All synthetic peptides derived from the tandem repeat regions of OGFr and MUC1 were 50-mers from a commercial source (SigmaGenosys, Houston TX). The sequences of the peptides were as follows: OGFr - (SPSETPGPRPAGPAGDEPAE) x 2.5, OGFr-ARF- (AHRRPQAPAQQDLQGTSQPR) x 2.5, MUC1 (PPAHGVTSAPDTRPAPGSTA) x 2.5. Peptides were conjugated to KLH and used to immunize New Zealand White rabbits (100 µg/injection) in incomplete Freund's adjuvant, except for the priming injection where complete Freund's adjuvant was used. Hyperimmune sera were diluted 1:2 in binding buffer (Pierce Gentle Binding Buffer, Pierce Chemicals) and passed over a sepharose column to which the immunizing peptide had been conjugated via cyanogen bromide activation (Amersham-Pharmacia Biotech). Peptide-specific antibodies were eluted from the column (Pierce Gentle Elution Buffer) and dialyzed against PBS. The antibodies were further purified on protein G, using the same binding and elution buffers as above.

Cell fractionation and immunoblotting analysis

Nuclear and cytoplasmic extracts were prepared exactly as described (39). The protein concentration was determined and the extracts were stored at -80°C in 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 20 mM Hepes, pH 7.9. For immunoblotting analysis, extracts (35 µg) were electrophoresed using the buffer system of Laemmli and transferred to nitrocellulose in 25 mM Tris, 192 mM glycine and 20% methanol transfer buffer. The membranes were blocked with PBS, 2% bovine serum albumin for one hour at room temperature and probed overnight at 4°C with the peptide-selected antisera (0.25 µg/ml). To show antibody specificity, the immunizing peptide was added to block antibody detection. The peptide (5 µg/ml) was added to the diluted primary antibody one hour prior to addition to the nitrocellulose membrane. Following incubation with the primary antibody, the membrane was washed extensively in PBS, 0.1% Tween-20 and incubated with anti-rabbit antibodies conjugated to horseradish peroxidase (Amersham Pharmacia Biotech). Finally, the blot was developed with chemiluminescence (NEN Life Science Products).

Expression of recombinant OGFr in *E. coli*

The cDNA from OGFr was subcloned into the expression vector pET28b(+) (Novagen) which incorporates a polyhistidine tag on the amino and carboxyl termini. Our OGFr cDNA lacks 177 nucleotides from the 5' end and initiates at methionine 60 of the published sequence (10). The plasmid was used to transform *E. coli* strain BLR(DE3), grown to an OD at 600 nm of 0.6, and induced for 4 hours with 1 mM IPTG. After the induction period, the bacteria were pelleted and resuspended in 25 mM sucrose, 1 mM EDTA, 50 mM Tris, pH 8.0 and protease inhibitors. The bacteria were frozen in liquid nitrogen and resuspended using a Dounce homogenizer. After treatment with lysozyme and DNAse/RNAse, the material was pelleted at 27,000 x *g*. The insoluble inclusion bodies were washed three times with 4 M urea, 2% Triton X-100, 5 mM DTT and 100 mM Tris, pH 7.0. Inclusion bodies were resolubilized in 6 M guanidine HCl, 5 mM DTT, 20 mM Tris, pH 8.0 and applied to a 25 ml Ni-NTA sepharose column (Qiagen). A linear gradient was used to remove the denaturant over 15 hours. Buffer A consisted of 6 M urea, 20% glycerol, 30 mM imidazole, 20 mM Tris, pH 7.9, and buffer B was identical to A except that it did not contain urea and the imidazole concentration was increased to 60 mM. Finally, the refolded protein was eluted with buffer B supplemented to 1 M imidazole.

Detection of patient antibodies to recombinant proteins and peptides

Purified recombinant OGFr or synthetic peptides (both at 0.5 µg/well) were allowed to bind to ELISA plate wells (Costar) overnight in a carbonate buffer, pH 9.5. The wells were washed and blocked for 2 hours with PBS, 2% NFDM. Serum samples were diluted in PBS, 2% NFDM added to the wells, and allowed to bind overnight at 4°C. The wells were washed, and the bound immunoglobulin was detected with anti-human IgG antisera conjugated to HRP (Amersham Pharmacia Biotech) for 2 hrs. The assay was developed with a one-step 3,3'-5,5'-tetramethylbenzidine (TMB) /peroxide reagent (DAKO).

Northern blot analysis

Human 12-lane multiple tissue Northern blots (Clontech, product number 7780-1) were hybridized with double stranded DNA probes. Probes were ^{32}P-labeled with a random primer labeling kit and had specific activities >1 x 10^9 cpm/µg DNA. Probes were derived from the 5' end of the OGFr cDNA (nucleotide 486 to 1105) by the polymerase chain reaction or derived from the tandem repeat region by restriction digest (*Kpn*I-*Eco*RI, nucleotide 1574 to 2127). The membranes were hybridized in Ultrahyb (Ambion) at 42°C overnight and washed in low and high stringency buffers (Ambion) equivalent to 2X SSPE and 0.1X SSPE, respectively.

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