Abstract

Purpose: The propensity of tumor cells to escape immune elimination could limit, if not defeat, the long-term benefits of effective immunotherapeutic protocols. Immunologic targeting of tumor stroma could significantly reduce the ability of tumors to evade immune elimination. Murine studies have shown that inducing immunity against angiogenesis-associated products engenders potent antitumor immunity without significant pathology. It is, however, not known whether T cells corresponding to stromal products are present in humans. In this study, we describe a method to screen for human stromal products that have not triggered significant tolerance and could therefore serve as candidate antigens for cancer immunotherapy.

Experimental Design: To identify candidates for human stromal antigens, we used an *in vitro* screening method to determine whether dendritic cells transfected with mRNA encoding products, which are overexpressed in the tumor stroma, are capable of stimulating cytotoxic CD8+ (CTL) responses from human peripheral blood mononuclear cells.

Results: CTL responses could be consistently generated against fibroblast activation protein (FAP) but not against matrix metalloproteinase-9 (MMP-9) or MMP-14. To enhance the immunogenicity of the mRNA-translated FAP product, a lysosomal targeting signal derived from lysosome-associated membrane protein-1 (LAMP-1) was fused to the COOH terminus of FAP to redirect the translated product into the class II presentation pathway. Dendritic cells transfected with mRNA encoding the FAP-LAMP fusion product stimulated enhanced CD4+ and CD8+ T-cell responses.

Conclusion: This study identifies FAP, a protease preferentially expressed in tumor-associated fibroblasts, as a candidate human stromal antigen to target in the setting of cancer immunotherapy, and shows that differential expression of stromal products is not a sufficient criteria to indicate its immunogenicity in a vaccination setting.
receptor-2 expressed in the proliferating endothelial cells of the tumor vasculature can inhibit tumor growth in mice in the absence of significant pathology (8, 9). We have shown that vaccination of tumor-bearing mice with dendritic cells transfected with mRNA encoding several angiogenesis-associated products, including vascular endothelial growth factor receptor-2, Tie-2, or vascular endothelial growth factor, stimulated potent antitumor immunity in murine tumor models (10).

Stromal products are “self”-antigens and may have triggered various degrees of tolerance that would be reflected in the reduced frequency and avidity of cognate T cells. The degree of tolerance induced by self-antigens is inversely proportional to their potency as antigens in a vaccination setting (11). It is currently not known whether T cells with specificity to stromal products are also present in the periphery of human individuals which can be activated to exert antigen-specific effector functions. In this study, we describe a method to screen for human stromal products that have not triggered significant tolerance and could therefore serve as candidate antigens in the setting of cancer immunotherapy. We identify fibroblast activation protein (FAP), a product whose expression is largely confined to tumor-associated fibroblasts, as a candidate antigen to target in the setting of cancer immunotherapy. Our findings also show that differential expression of stromal products in the tumor milieu cannot be used as the sole or main criteria for their use in a vaccination setting and that functional measurements such as described in this study are required.

Materials and Methods

**Plasmid constructs and in vitro transcription of RNA: Cloning of matrix metalloproteinase-9, matrix metalloproteinase-14, and fibroblast activation protein plasmids.** The HindIII/NdeI fragment of pGEM4Z/A64 (12) was cloned into pSP73 (Promega, Madison, WI) digested with HindIII and NdeI to generate pSP73/A64. The pSP73-Sph plasmid was created by digesting pSP73 with SphI, filling in the ends with T4 DNA polymerase and religating. pSP73-Sph/A64/Not contains a NotI restriction site adjacent to the SphI site. Human MMP-9 was amplified by PCR (primers used are shown in Table 1) from a cDNA plasmid provided kindly by Dr. Stephen Weiss (University of Michigan, Ann Arbor, MI) and was cloned into the XhoI-BamHI site of pSP73-Sph/A64. The pSP73-Sph/A64 plasmid was created by digesting pSP73 with SphI, filling in the ends with T4 DNA polymerase and religating. pSP73-Sph/A64/Not contains a NotI restriction site adjacent to the SphI site. Human MMP-9 was amplified by PCR (primers used are shown in Table 1) from a cDNA plasmid provided kindly by Dr. Stephen Weiss (University of Michigan, Ann Arbor, MI) and was cloned into the XhoI-BamHI site of pSP73-Sph/A64. The cDNA encoding human FAP was amplified by reverse transcription-PCR from a breast cancer specimen. The full-length cDNA was cloned into the XhoI-BamHI sites of pSP73-Sph/A64 (primers used are shown in Table 1).

**Cloning of fibroblast activation protein/lysosome-associated membrane protein plasmid.** The human gp96 signal sequence was amplified by PCR from the plasmid pGEM4Z/H1ERT/lysosome-associated membrane protein (LAMP)/A64 (13). The LAMP-1 lysosomal sorting sequence was amplified by PCR from the same plasmid (primers used are shown in Table 1). The gp96 and LAMP signal sequences were isolated by digestion with HindIII and EcoRI, respectively. The fragments were ligated with pSP73-Sph/A64/Not cut with HindIII and EcoRI followed by digestion with SfiI. Human β-actin was amplified by reverse transcription-PCR from total human peripheral blood mononuclear cell (PBMC) RNA (primers used are shown in Table 1). The PCR product was digested with SfiI and ligated into the vector containing the gp96 signal sequence and LAMP sorting sequence. The full-length cDNA of FAP was subcloned into the SfiI-SfiI site by replacing β-actin cDNA.

**In vitro transcription.** Plasmids were linearized using the restriction endonuclease SpeI and in vitro transcribed using the mMESSAGEmMA-CHINE high yield capped RNA transcription kit (Ambion, Austin, TX) according to the manufacturer's manual. After digestion of the DNA template with DNase I, RNA was purified with an RNeasy kit (Qiagen, Valencia, CA).

**Dendritic cell generation from peripheral blood, RNA transfection, and maturation of dendritic cells.** All cellular material used in these experiments was obtained from human subjects following informed consent through protocols approved by the Duke University Institutional Review Board. Leukapheresis-derived PBMCs were incubated for 1 hour in AIM-V medium (Life Technologies, Grand Island, NY) at 37°C to allow plastic adhesion and then cultured for 6 days in AIM-V medium supplemented with 800 units/mL granulocyte macrophage colony-stimulating factor and 500 units/mL interleukin-4 (IL-4). In some experiments X-VIVO-15 medium (Cambrex Bio Science, Walkersville, MD) was used for the 6-day incubation without any significant difference. Electroporation was done as previously described (14). Immature dendritic cells were harvested on day 6, washed, and gently resuspended in Opti-MEM (Life Technologies) at 2.5 × 10^6/mL. The supernatant from dendritic cell culture was saved as conditioned medium for later use. Cells were electroporated in 2-mm cuvettes; 200 μL of dendritic cells (5 × 10^6 cells) at 300 V for 500 microseconds using an Electro Square Porator ECM 830 (BTX, San Diego, CA). The amount of in vitro–transcribed RNA used was 2.5 μg/10^6 dendritic cells. Cells were immediately transferred to 60-mm tissue culture Petri dishes containing a 1:1 combination of conditioned dendritic cell growth medium and fresh medium. Cells were matured overnight with IL-1β (5 ng/mL), tumor necrosis factor-α (5 ng/mL), IL-6 (150 ng/mL), and prostaglandin E2 (1 μg/mL). IL-4, tumor necrosis factor-α, IL-1β, and

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**Table 1. Primer sequences for PCR and reverse transcription-PCR**

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>hMMP9 forward</td>
<td>5’-TATATACCTGAGGCCACCATGAGCCCTCTGCGCAAGC-3’</td>
</tr>
<tr>
<td>hMMP9 reverse</td>
<td>5’-TATATATCTAGACTAGTCTCCAGGCTAATCTGAC-3’</td>
</tr>
<tr>
<td>HMMP14 forward</td>
<td>5’-TATATATCTAGCACTGAGCACGACCCTGCGACGACG-3’</td>
</tr>
<tr>
<td>HMMP14 reverse</td>
<td>5’-TATATATCTAGCACTGAGCACGACCCTGCGACGACG-3’</td>
</tr>
<tr>
<td>hFAP forward</td>
<td>5’-TATATATCTAGCACTGAGCACGACCCTGCGACGACG-3’</td>
</tr>
<tr>
<td>hFAP reverse</td>
<td>5’-TATATATCTAGCACTGAGCACGACCCTGCGACGACG-3’</td>
</tr>
<tr>
<td>hgp96 forward</td>
<td>5’-ATTATATCTGAGGCGCCAGGCACTGCGACGACG-3’</td>
</tr>
<tr>
<td>hgp96 reverse</td>
<td>5’-ATTATATCTGAGGCGCCAGGCACTGCGACGACG-3’</td>
</tr>
<tr>
<td>hLAMP1 forward</td>
<td>5’-ATTATATCTGAGGCGCCAGGCACTGCGACGACG-3’</td>
</tr>
<tr>
<td>hLAMP1 reverse</td>
<td>5’-ATTATATCTGAGGCGCCAGGCACTGCGACGACG-3’</td>
</tr>
<tr>
<td>h-Actin forward</td>
<td>5’-ATTATATCTGAGGCGCCAGGCACTGCGACGACG-3’</td>
</tr>
<tr>
<td>h-Actin reverse</td>
<td>5’-ATTATATCTGAGGCGCCAGGCACTGCGACGACG-3’</td>
</tr>
</tbody>
</table>
IL-6 were purchased from R&D Systems (Minneapolis, MN); granulocyte macrophage colony-stimulating factor was from Immunex (Seattle, WA) under the trade name Leukine; and prostaglandin E2 from Pharmacia (Erlangen, Germany) under the trade name Miniprostin E2.

**Induction of CD4+ and CD8+ T-cell responses in vitro.** CD8+ T cells were isolated by positive selection using EasySep CD8 Selection Kit (StemCell Technologies, Inc., Vancouver, Canada). CD8+ T cells were cocultured with antigen-transfected, matured dendritic cells (ratio 10:1) in complete T-cell medium in the presence of IL-6 (1,000 units/mL), IL-12 (10 ng/mL), and anti-IL-10 antibody (2.5 μg/mL). On day 7, the T cells were restimulated with antigen-transfected dendritic cell (ratio, 10:1) in complete T-cell medium with IL-2 (20 units/mL), IL-7 (10 ng/mL), and anti-IL-10 antibody (2.5 μg/mL). T cells were harvested on day 12 and assessed for their ability to lyse autologous immature dendritic cells transfected with specific RNA in a standard 4-hour europium release assay (15). Europium release was measured by time-resolved fluorescence (Delta fluorometer; Wallac, Gaithersburg, MD). Specific cytotoxic activity was determined with the formula: % specific release = [(experimental release – spontaneous release) / (total release – spontaneous release)] × 100.

To measure the induction of CD4+ T cell responses, T cell–enriched PBMC were used as effector cells. PBMC were negatively selected using EasySep Human T Cell Enrichment Kit (StemCell Technologies) and cocultured with antigen-transfected dendritic cells (ratio, 10:1) with IL-7 (10 ng/mL) for 7 days and restimulated for 5 days in the presence of IL-2 (20 units/mL). CD4+ T cells (5 × 10^5) isolated by negative selection (EasySep CD8 Selection Kit, StemCell Technologies) and 5 × 10^5 RNA-transfected dendritic cells in 100 μL of complete medium were added to each well of flat-bottomed 96-well nitrocellulose plates (Multiscreen-IP; Millipore, Bedford, MA) precoated with 1 μg/mL IFN-γ capture antibody. Plates were incubated for 20 hours at 37°C; after washing, biotinylated IFN-γ detection antibody was added to each well. Wells were then incubated for an additional 2 hours at room temperature and then streptavidin-alkaline phosphatase and substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added. After washing, spots were counted using an automated Zeiss KS Elispot Comp reader (Carl Zeiss, Inc., Minneapolis, MN). Alternatively, the twice-stimulated T cells were used in a cytotoxicity assay as described above.

**Results**

The relative frequency of antigen-specific CD8+ T cells was determined by measuring the ability of autologous dendritic...
cells transfected with mRNA-encoding stromal products to stimulate in vitro CD8+ cytotoxic T cell (CTL) responses from the PBMC of human volunteers. Three products known to be overexpressed in the tumor stroma were evaluated in this study: MMP-9, MMP-14, and FAP. MMP-9 and MMP-14, as well as other MMPs, promote the invasion and metastatic behavior of tumors by remodeling of the extracellular matrix (5, 16, 17). MMP-9 and MMP-14 are expressed in virtually all human malignancies by stromal cell and/or tumor cells (16). FAP is a serine protease expressed by activated proliferating fibroblasts. It is broadly expressed in tumor-associated fibroblasts of most tumors examined (18–21). In nonmalignant adult tissue, FAP is transiently expressed in the proliferating fibroblasts of healing wounds. As a positive control in these studies, we measured the induction of CTL responses against survivin, an antiapoptotic protein expressed in many tumor cells and proliferating endothelial cells and shown to stimulate CTL responses in vitro from human PBMC (22–25).

Induction of CTL responses was evaluated in PBMC from six donors. T cells were stimulated with autologous dendritic cells transfected with mRNA encoding the tested antigen, and the generation of CTL was measured using standard cytotoxicity assays using mRNA-transfected dendritic cells as targets. Dendritic cells transfected with mRNA are a powerful antigen-presenting platform for stimulating human CTL responses in vitro and also provide broadly useful targets to measure antigen-specific cytotoxicity in view of the fact that autologous cells expressing the antigen in question are not readily available (26). For example, in this study, it would have been extremely laborious to generate autologous stromal cell lines expressing FAP, MMP-9, or MMP-14. In addition, dendritic cells transfected with irrelevant mRNA provide excellent specificity control in the cytotoxicity assay. Figure 1 shows the results from three donors in which all four antigens were evaluated in the same experiment. Consistent with previous reports, survivin-specific CTL responses could be generated from all four donors tested (22–25). Likewise, all three donors shown in Fig. 1 also stimulated a FAP-specific CTL response of comparable magnitude. In contrast, MMP-9- and MMP-14-specific CTL could be generated only from one of three donors shown in Fig. 1. As it is not uncommon in human in vitro CTL assays, PBMC from some donors tend to generate a high background lysis (Fig. 1: donor 3 MMP-9-CTL and MMP-14-CTL), perhaps reflecting elevated natural killer-like activity in the PBMC of this donor. A summary of all CTL assays done and numbers of donors tested is shown in Table 2. Clearly, FAP- and survivin-specific CTL could be consistently stimulated from the PBMC of human volunteers, whereas induction of MMP-9- or MMP-14-specific CTL was significantly less frequent.

Generation of robust and durable CD8+ CTL responses also requires the concomitant induction of CD4+ T helper responses (27). Vaccination with nucleic acid encoded antigens, such as mRNA-transfected dendritic cells used in this study, favors the generation of class I–restricted CD8+ T cell responses because the translated antigen will be channeled preferentially into the endogenous class I presentation pathway. To provide CD4+ T help, a lysosomal targeting signal derived from the LAMP-1 was fused to the carboxyl end of FAP to enhance access of the antigen to the class II presentation pathway (28). We have previously shown that dendritic cells transfected with mRNA-encoded LAMP-1 modified carcinoembryonic antigen or TERT antigens stimulated in vitro enhanced CD4+ T-cell responses as well as enhanced CD8+ CTL responses that were especially evident when IL-2 was omitted from the incubation reaction (13, 15). The potential value of vaccination with LAMP-modified antigens was underscored in a recent clinical trial whereby vaccination of patients with LAMP-modified but not with unmodified TERT antigen was capable of stimulating TERT-specific CD4+ T-cell responses (29).

T cells were stimulated with FAP or FAP-LAMP mRNA-transfected dendritic cells and the induction of FAP-specific CD4+ T-cell and CD8+ CTL responses were determined as described in Materials and Methods. As shown in Fig. 2, fusion of the LAMP-1-derived sequences to the COOH end of FAP enhanced the generation of FAP-specific IFNγ-secreting CD4+ T cells. Figure 3 shows that LAMP-modified FAP mRNA-transfected dendritic cells stimulated a more potent CTL response, especially evident when IL-2 was omitted from the culture. These observations are consistent with our previous observations (13, 15) and show that the endogenously expressed mRNA-translated LAMP-1 modified FAP product is

<table>
<thead>
<tr>
<th>Table 2. Human in vitro CTL assays</th>
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<tr>
<td>Donor</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>FAP</td>
</tr>
<tr>
<td>MMP-9</td>
</tr>
<tr>
<td>MMP-14</td>
</tr>
<tr>
<td>Survivin</td>
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NOTE: The numbers given reflect positive CTL results per total number of CTL assays done. CTL results are considered positive when (i) lysis of specific targets is higher than control targets in at least four of five E/T ratios and (ii) the difference between the lysis of specific and control targets is at least 2-fold and not <10%.

Fig. 2. Stimulation of FAP-specific CD4+ T cell responses by chimeric FAP-LAMP mRNA-transfected dendritic cells. T cells were stimulated twice with mRNA-transfected dendritic cells as described in Materials and Methods. CD4+ T cells isolated by negative selection were incubated with mRNA-transfected dendritic cells as indicated, and the numbers of IFNγ-secreting cells was determined by ELISPOT.
be activated to exert effector functions. We have shown that responses in vitro. Restimulated T cells were incubated in the presence (top) or absence (bottom) of IL-2 and used in a cytotoxicity assay. Target cells were immature dendritic cells transfected with FAP mRNA (▲) or MMP-14 mRNA as control antigen (○).

Retargeted into the class II presentation pathway leading to enhanced induction of CD4+ T-cell responses which in the absence of an exogenous source of IL-2 provided “help” for the generation of CTL responses.

Discussion

In this study, we described a screening strategy to assess the relative suitability of tumor stromal products to serve as antigens in the setting of cancer vaccination. By measuring the ability of stromal products to stimulate CD8+ T-cell responses in vitro from donor PBMC, we could determine the relative abundance of antigen-specific T-cell precursors that can be activated to exert effector functions. We have shown that FAP, but not MMP-9 or MMP-14, precursors are present at high frequency in the PBMC (Fig. 1). Conceivably, despite the fact that self products such as MMP-9 or MMP-14 are overexpressed in the tumor environment, they have triggered a significant degree of tolerance as judged by the fact that compared with survivin or FAP it was more difficult to stimulate CTL responses against these antigens. These findings, therefore, suggest that FAP, a product whose expression is largely confined to tumor-associated fibroblasts, would be a more effective antigen to target in the setting of cancer immunotherapy. These observations also show that differential expression of antigenic candidates is not sufficient to guide their choice for use in a vaccination setting and that functional measurements such as described in this study are necessary. In addition, our study has shown that LAMP-modified FAP mRNA-transfected dendritic cells exhibit enhanced capacity to stimulate CD4+ and CD8+ T-cell responses in vitro. In light of the fact that LAMP-modified antigens were also capable of stimulating enhanced CD4+ T-cell response in human patients (29), LAMP-modified FAP would be a reasonable choice for further evaluation in murine tumor models and in a human clinical trials.

The screening method used in this study is semiquantitative and comparative in its nature; its primary value is to eliminate less suitable candidates, in this instance, MMP-9 or MMP-14. The use of mRNA as antigenic template and mRNA-transfected autologous dendritic cells as targets in the T-cell assay extends the applicability of this analysis to virtually every antigen. Nevertheless, in vitro human T-cell assays cannot evaluate the biological effect of vaccination against FAP or other (self) antigens on tumor growth and/or induction of autoimmune pathology. These variables can be assessed in rodent tumor models provided suitable homologous antigens exist. Indeed, preliminary observation from murine studies suggest that FAP, and in particular FAP-LAMP, vaccination is capable of stimulating potent antitumor immunity in the absence of significant toxicity. The not surprising variability among normal products in stimulating T-cell responses from human PBMC as shown in Fig. 1 suggests that screening of human antigenic candidates should precede murine studies assessing their biological effect (i.e., tumor growth and autoimmunity).

References

12. Li Y, Wang MN, Li H, et al. Active immunization against these antigens. These findings, therefore, suggest that FAP, a product whose expression is largely confined to tumor-associated fibroblasts, would be a more effective antigen to target in the setting of cancer immunotherapy. These observations also show that differential expression of antigenic candidates is not sufficient to guide their choice for use in a vaccination setting and that functional measurements such as described in this study are necessary. In addition, our study has shown that LAMP-modified FAP mRNA-transfected dendritic cells exhibit enhanced capacity to stimulate CD4+ and CD8+ T-cell responses in vitro. In light of the fact that LAMP-modified antigens were also capable of stimulating enhanced CD4+ T-cell response in human patients (29), LAMP-modified FAP would be a reasonable choice for further evaluation in murine tumor models and in a human clinical trials.

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1 Unpublished data.
Clinical Cancer Research

Induction of CD4\(^+\) and CD8\(^+\) T-Cell Responses to the Human Stromal Antigen, Fibroblast Activation Protein: Implication for Cancer Immunotherapy

Martin Fassnacht, Jaewoo Lee, Caterina Milazzo, et al.


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