CM101 Treatment Overrides Tumor-induced Immunoprivilege Leading to Apoptosis

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ABSTRACT

CM101, a bacterial polysaccharide exotoxin produced by group B Streptococcus (GBS), also referred to as GBS toxin, has been shown to target pathological neovascularate and activate complement (C3), thereby inducing neovascularitis, infiltration of inflammatory cells, inhibition of tumor growth, and apoptosis in murine tumor models. Data from refractory cancer patients in a Phase I clinical trial with CM101 indicated a similar mechanism of tumor-targeted inflammation. To further our understanding of the mechanism of action of CM101 as an anti-tumor agent, we examined the role of the inflammatory response in inducing tumor apoptosis in a normal mouse and tumor-bearing mouse model. The i.v. infusion of CM101 into B16BL-6 melanoma tumor-bearing mice elevated p53 mRNA in circulating leukocytes as measured by reverse transcription-PCR (RT-PCR), and immunohistochecmistry demonstrated infiltration and sequestration of leukocytes. Whole tumor lysates from excised tumors exhibited an increase in binding to the murine p21\textsuperscript{N\textsubscript{a}t\textsubscript{C}p1}, derived p53 DNA binding sequence compared with control whole tumor lysates, in which minimal or no DNA binding was observed. CM101 infusion led to elevated levels of Fas protein within the tumors as well as a decrease in the expression of fas ligand (fasL). Furthermore, tumors were apoptotic as determined by terminal deoxynucleotidyl transferase-mediated nick end labeling and DNA fragmentation assays. Collectively, these data suggest that CM101 up-regulates p53 in tumor-infiltrating leukocytes, initiating a loss of tumor immunoprivilege and consequently rendering the tumor sensitive to Fas/fasL-mediated apoptosis. CM101 induced loss of tumor immunoprivilege through changes in the expression of leucocyte p53, tumor Fas and fasL coupled with neovascularitis and leukocyte infiltration, constitutes a plausible molecular pathway for tumor reduction observed in cancer patients.

INTRODUCTION

Infection of newborn infants with GBS\textsuperscript{1} is associated with a lung-specific inflammatory response, pulmonary hypertension, significant endothelial cell damage, and capillary thrombosis. The causative agent associated with these symptoms has been identified as a polysaccharide exotoxin, which, when injected into sheep, reproduces the lung pathophysiology observed in infected neonates (1–4). This observation led to the hypothesis that the binding of GBS toxin to embryonic receptors of the newborn lung neovascularate induced an inflammatory response that ultimately caused the respiratory distress syndrome known as “early onset disease” (1). It was further hypothesized that these receptors would be present in tumor neovascularate but not in mature vasculature, thereby rendering tumors susceptible to GBS toxin-induced inflammation. One active component of GBS toxin now referred to as CM101 has been further purified (5, 6) and shown to bind to human tumor neovascularate (7). The above-mentioned hypothesis has been substantiated in murine tumor models, in which CM101 has been demonstrated to inhibit tumor growth (7), promote long-term survival (8), and induce acute inflammation targeting the tumor neovascularate (9). Recently, we have demonstrated that CM101 in vivo binds to the tumor neovascularate within 5 min and activates complement C3 (10, 11). This results in the release of C3a, which effectively functions as a chemoattractant for leukocytes. C3-dependent infiltration of TNF-α-expressing macrophages of the tumor was shown to be coincident with up-regulation of TNFR II in mature endothelium, leading to apoptosis of this vasculature (11).

CM101 does not bind to leukocytes, tumor cells, or normal cells (with the exception of some primary endothelial cells in vitro) and has no apparent biological effect on any of these cell types.\textsuperscript{4} The p53 tumor suppressor gene has been identified as a transcriptional regulator of downstream effector genes associated with cellular proliferation and apoptosis (Ref. 12 and the references therein). In response to cellular stress including DNA damage and hypoxia, transcription of p53-dependent genes such as GADD45, p21\textsuperscript{N\textsubscript{a}t\textsubscript{C}p1}, KILLER/DR5, and Fas (18–20) is up-regulated. Expression of these gene products and others commits the cell to either cell cycle arrest, allowing for efficient DNA repair, or, alternatively, apoptosis, eliminating heavily damaged cells. Cellular proliferation in the absence of repair may lead to mutations that promote the growth of tumors. It is known that some human tumors harbor mutant or inactive p53 (21). In these tumors, p53 has lost the ability to bind to its DNA-binding consensus sequence and is therefore unable to activate transcription (22). Loss of p53-dependent transcription could negatively effect cellular homeostasis and could promote the proliferation of aberrant cells that might otherwise be eliminated. Although p53-independent mechanisms of apoptosis have been described previously (23, 24), studies investigating p53 as an important component of tumor suppression remain critical in the development of cancer therapies.

The significance of mutant p53 in tumors becomes important within the context of immune surveillance and tumor immunoprivilege. Fas (APO-1/CD95) is a member of the tumor necrosis family of type I membrane proteins capable of eliciting an apoptotic response on specific ligand interaction. The binding of fasL, or agonistic antibodies to the Fas receptor protein initiates a molecular signaling cascade resulting in apoptotic cell death (25, 26). Fas protein is constitutively expressed in many tissues such as the liver, thymus, heart, and ovary (27). In contrast, fasL protein has been shown to be primarily limited to activated cell lineages of the immune system such as T cells (28, 29), B lymphocytes (30), natural killer cells (31), monocytes/macrophages (32), and immunoprivileged tissues (27, 28, 33, 34). Recently, blockade of the Fas/fasL signal transduction pathway has been suggested to participate in the establishment of tumor immunoprivilege in a variety of nonlymphoid human tumors (35–38). In the tumors examined, fasL was markedly elevated throughout the tumor when compared with Fas protein. In this manner, the expression of tumor fasL would result in the induction of apoptosis of the Fas-presenting immune effector cells and contribute to the establishment of tumor immunoprivilege (reviewed in Refs. 33, 34, and 39). In a striking example, fasL-positive hepatocellular carcinoma cells increased lymphocyte cell death when Jurkat T cells were plated on hepatocellular...
carcinoma cryostat sections (40). Recently, human vascular endothelial cells have been shown both in vivo and in vitro to express fasL (41, 42) that is down-regulated after local administration of the inflammatory cytokine TNF-α. The down-regulation of fasL correlated with adherence and extravasation of leukocytes within the endothelial milieu, suggesting an important relationship between inflammatory signaling and activation of the endothelium. Our previous studies establishing the up-regulation of TNFR II on the tumor endothelium concomitant with leukocyte adhesion and infiltration (11) suggest that CM101 may be a potent mediator of a tumor-targeted inflammatory response.

It is evident that several molecular pathways are likely to be involved in the establishment of tumor immunoprivilege. Several hypotheses have been suggested that involve the inability of mutant p53, or alternatively, the absence of wild-type p53 to up-regulate Fas expression within the tumor (18–20). Although it is not clear how p53 would modulate such activity in vivo, the role of p53 in effective therapeutic strategies targeting tumor vascular dysfunction has become an area of great interest (21).

In this report, we present evidence that the CM101-induced inflammatory response elevated p53 mRNA in tumor-infiltrating leukocytes in vivo. Examination of whole tumor lysates demonstrated that CM101 treatment resulted in an increase in p53 sequence-specific DNA binding compared with control whole tumor lysates. This increase in DNA binding correlated with a concomitant increase of total p53 in whole tumor lysates. In addition, immunohistochemical data indicated that prior to CM101 treatment, tumors were immunoprivileged with high expression of fasL and little or no expression of Fas. However, treatment with CM101 down-regulated fasL and up-regulated Fas within the tumor cells. The data presented herein suggest that CM101 treatment reduces tumor immunoprivilege through down-regulation of tumor fasL, up-regulation of tumor Fas, and up-regulation of p53 mRNA in tumor-infiltrating leukocytes. Observations of tumor reduction in human cancer patients, coupled with both inflammatory cytokine and Fas/fasL data from pre- and post-CM101 treatment biopsies, suggests a similar mechanism of induced tumor apoptosis (43).

MATERIALS AND METHODS

Mice, Tumor Cells, and Treatment Protocol

C57BL/6 mice (body weight, 22–25 grams) were obtained from Taconic (Germantown, NY) and maintained at the Vanderbilt Animal Care facility according to established protocols. The B16BL-6 murine melanoma cell line used in this study was obtained from the Tumor Repository of the National Cancer Institute (Frederick, MD). B16BL-6 cells were expanded and briefly maintained in Eagles MEM (Life Technologies, Inc.) supplemented with 10% NaCl, 1% NP40, 1.5 mM MgCl2, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml aprotinin (44)) and maintained on ice for 30 min. The samples were then expressed through an 18-gauge needle and centrifuged at 12,000 rpm for 10 min, and the protein concentration was determined by using the standard Bio-Rad assay. The double-stranded oligonucleotide probe containing the p53 DNA-binding sequence in the murine p21Waf1/Cip1 gene promoter (45) was synthesized and end-labeled with [γ-32P]ATP by T4 kinase (Promega Corp.) DNA binding reactions contained 50 µg of whole tumor lysate in 1× binding buffer [20 mM Tris (pH 7.5), 50 mM KCl, 5 mM MgCl2, 1 mM DTT, 0.5 mM EDTA, and 30% glycerol] and 500

β-actin. An equal aliquot of the reverse transcription reaction was subjected to PCR (11). The primer pairs were as follows: (a) p53 (GenBank accession number X01237), 5′-GGGACAGCCAATCTGTATGTGC-3′ (sense primer) and 5′-CTGTCTCCAGATCTCGGATAC-3′ (antisense primer); and (b) β-actin (GenBank accession number X03765), 5′-AGCAAGAGAGGCATCTTGCAC-3′ (sense primer) and 5′-CAGCTCATAGCTCCTTCCA-3′ (antisense primer). Primer pairs spanning intron/exon junctions were selected to ensure that the desired PCR product was derived from RNA. For each gene product, the optimum number of PCR cycles was determined for linear amplification. All PCR products were verified by hybridization with a probe internal to the PCR primers.

Electrophoretic Gel Mobility Shift Assay of Whole Tumor Lysates

Tumor sections were homogenized with a Teflon Potter-Elvehjem style grinder in ice-cold lysis buffer [20 mM Tris (pH 7.5), 20% glycerol, 100 mM NaCl, 1% NP40, 1.5 mM MgCl2, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml aprotinin (44)] and maintained on ice during the entire isolation period. Total cellular RNA was isolated from purified leukocytes with the RNeasy MiniKit (Qiagen, Valencia, CA) and quantitated, and cDNA was synthesized using the First Strand cDNA Synthesis Kit (Pharmacia Biotech). Total RNA (2–5 µg) was reverse transcribed at 42°C with antisense gene-specific primers for p53 and 3 R. J. Melder, R. K. Jain, and C. G. Hellenqvist, unpublished observations.

Fig. 1. Immunolocalization of CD45-positive cells in B16BL-6 melanoma tumors. Sections (7 µm) of tumors excised from CM101-treated animals were probed with either goat antimouse CD45 (A) or normal goat serum (B) followed by visualization with antigoat IgG HRP. In a representative tumor excised from a CM101-treated mouse, CD45-positive staining is observed within the tumor interstitium. Bar, 30 µm; magnification, ×400.
A

CM101

- 

- 

- 

+ 

+ 

P.C.

B

p53

actin

PBS

- 

- 

+ 

- 

P.C.

Fig. 2. A, RT-PCR analysis of p53 and β-actin mRNA isolated from total circulating leukocytes from non-tumor-bearing C57BL/6 mice (n = 3). Amplification of a p53-specific PCR product derived from exons 4–6 generated an expected product of 280 bp; β-actin amplification served as a control for RNA quality, generating an expected product of 500 bp. B, RT-PCR analysis of p53 and β-actin mRNA isolated from circulating leukocytes of B16BL-6 tumor-bearing mice treated with either PBS or 60 μg/kg CM101. Note the complete absence of p53 mRNA in the PBS-treated sample. The data are representative of two experiments. P.C., positive control.

Apoptotic Indices

Apoptosis was detected in tumor sections by labeling the 3' OH ends of DNA with biotin-14-dCTP (46). Briefly, paraffin-embedded material was deparaffinized, rehydrated, and then subjected to proteinase K [20 μg/ml in 10 mm Tris (pH 7.5), 10 mm NaCl, and 2 mm CaCl2] digestion for 20 min at room temperature. The labeling reaction was prepared on ice containing 1X TdT reaction buffer, 50 μM biotin-14-dCTP, and 0.22 unit/μl recombinant TdT (all from Life Technologies, Inc.). A sufficient volume of the labeling reaction was added to each slide and incubated at room temperature for 15 min. A reaction mixture without TdT was used as a negative control. Streptavidin-alkaline phosphatase (Life Technologies, Inc.) was diluted 1:1000 in 1× PBS (containing 0.1% NP40 and 3% BSA) and applied to each slide for 15 min at room temperature. Visualization of apoptotic nuclei was performed with 1-Step™ NBT/BCIP (Pierce) until sufficient color development occurred. High molecular weight DNA was isolated from mouse tumor tissue by lysis in a buffer containing 100 mM NaCl, 10 mM Tris (pH 8.0), 25 mM EDTA, 0.5% SDS, and 100 μg/ml proteinase K. The lysates were incubated for 4 h at 50°C, precipitated with ethanol, resuspended in 10 mM Tris (pH 8.0)-1 mM EDTA, and quantitated and analyzed on a 1.5% agarose gel.

RESULTS

Infiltration of CD45-positive Cells into B16BL-6 Melanoma Tumors after CM101 Treatment. Consistent with our previously reported data in other tumor models (6), CD45-positive cells were found to infiltrate the B16BL-6 tumor after treatment with CM101 (Fig. 1A). However, in tumors isolated from mice treated with PBS, leukocytes were evident within the lumen of blood vessels, but no margination of the vessel wall or infiltration of the tumor was observed (data not shown).

Up-Regulation of Leukocyte p53 mRNA. In peripheral blood leukocytes isolated from non-tumor-bearing C57BL/6 mice, measurable levels of p53 mRNA were detected by RT-PCR in all animals tested (Fig. 3).
2A, Lanes 1–3). Similar analysis of peripheral blood leukocytes isolated 1 h after PBS treatment of a representative B16BL-6 tumor-bearing mouse demonstrated that PBS treatment did not stimulate an elevation in p53 mRNA (Fig. 2B, Lane 1). No measurable p53 mRNA could be detected in any PBS-treated tumor-bearing animal. However, within 1 h of i.v. infusion of CM101, a significant elevation of p53 mRNA could be measured in circulating leukocytes (Fig. 2B, Lanes 2 and 3). β-ACTIN mRNA was measured in each sample as a control for RT-PCR efficiency (Fig. 2A, and bottom panel).

CM101-induced p53 Sequence-specific DNA Binding in Whole Tumor Lysates. B16BL-6 melanoma cells in vitro express low levels of p53 by Western blot analysis (47) and RT-PCR (data not shown). However, these particular techniques do not indicate whether or not the identified p53 species is transcriptionally active. Whole cell lysates from B16BL-6 melanoma cells were tested for the ability to bind to the murine p21Waf1/Cip1-derived p53 DNA-binding consensus sequence. No DNA binding was observed when compared with an equal concentration of a control lýsate prepared from human C32 phorbol ester-induced cells (data not shown). The absence of sequence-specific p53 DNA binding suggests that the p53 expressed in these cells is a mutant phenotype and is therefore unable to participate in p53-dependent signaling pathways. However, low expression of wild-type p53 cannot be ruled out. This is consistent with the results of several studies exploring the role of p53 transcriptional activity in tumor growth suppression (22).

Whole tumor lysates were prepared from animals treated every Monday, Wednesday, and Friday with PBS or 60 μg/kg CM101 and subjected to the electrophoretic mobility shift analysis assay with the murine p21Waf1/Cip1-derived p53 DNA-binding sequence. In two independent experiments, whole tumor lysates from PBS-treated animals demonstrated a low background binding (Fig. 3A, Lane 1 and Fig. 3B, Lanes 2 and 3). However, whole tumor lysates from CM101-treated animals exhibited at least a 2-fold increase in p53-specific DNA binding (Fig. 3A, Lanes 2 and 3). p53 DNA binding in experiment 2 was more varied among the five tumor extracts analyzed (Fig. 3B, Lanes 5–9). The resulting shifts could be specifically competed with excess unlabeled, specific competitor DNA. No binding was detected in a liver lysate from a CM101-treated animal (Fig. 3B, Lane 4).

Increased p53 and Fas Proteins in Whole Tumor Lysates. To account for the variability of p53 DNA binding observed in experiment 2, Western blot analysis of total p53, Fas, and actin in whole tumor lysates was performed. No significant differences in the amount of tumor actin protein were observed among the tumor lysates examined (Fig. 4A, bottom panel). Animals treated with PBS demonstrated an even distribution of tumor p53 protein, although three of five tumor lysates from CM101-treated animals exhibited an increase in p53 protein (Fig. 4A, top panel). In the context of uniform actin detection, the differences in p53 protein levels are most likely the result of in vivo responses to CM101 and are not associated with protein quantitation or transfer to the polyvinylidene difluoride membrane. Whole tumor lysates that exhibited elevated total p53 protein levels correlated with those lysates that demonstrated an increase in p53 DNA-binding activity. Increased levels of Fas protein were also detected in the same tumor lysates of CM101-treated animals when compared with tumor lysates from PBS-treated animals (Fig. 4A, middle panel).

Immunolocalization of Fas and fasL. The increase in Fas protein in whole tumor lysates suggested that a mechanism of action of CM101 might be the reduction of immunoprivilege established by the tumor. RT-PCR analysis of B16BL-6 tumor cells maintained in vitro indicated these cells express fasL (data not shown). Before CM101 treatment of tumor-bearing mice, expression of fasL is distributed throughout the tumor (Fig. 5A), and the pattern of cytoplasmic staining is localized exclusively to the tumor cells. In contrast, the expression of Fas protein was not detected (Fig. 5B). These results are consistent with the RT-PCR analysis. After CM101 treatment, a significant decrease in tumor cell fasL was observed (Fig. 5C). Some tumor cells were still positive for fasL; however, the number of cells expressing fasL was greatly diminished when compared with the number of cells expressing fasL in the PBS-treated tumor. In contrast, tumor cell Fas expression was significantly elevated in the CM101-treated tumor (Fig. 5D) and evenly distributed throughout the tumor. Normal rabbit IgG did not result in any positive staining (Fig. 5E).

Apoptosis Indications. Increased expression of Fas protein within the tumor cells after CM101 treatment suggested that the tumor might have become sensitized to apoptosis. TUNEL-positive nuclei were absent in the PBS-treated tumor examined (Fig. 6A). DNsase I digestion of a serial section of the same tumor before TUNEL labeling served as a positive control (Fig. 6C). In the CM101-treated tumor, a significant number of TUNEL-positive nuclei with a distinctive pattern of labeling were evident (Fig. 6B). Isolation of high molecular weight tumor DNA from PBS-treated animals indicated no DNA fragmentation (Fig. 7, Lane 1). However, tumor DNA isolated from a CM101-treated animal exhibited significant smearing of high molecular weight DNA and the generation of the classical DNA ladder (Fig. 7, Lane 2).

DISCUSSION

Our previous experiments in murine tumor models demonstrated that repeated i.v. infusion of CM101 resulted in a significant reduction in tumor volume of human tumor xenografts (7) and promoted the long-term survival of BALB/c mice bearing Madison lung tumors (8). In response to CM101, significant capillary
thrombosis, hemorrhage, and endothelial cell damage were observed within the tumor vasculature, with no evidence of toxicity to the vasculature of other organs. Furthermore, CM101 also induced an inflammatory response with increased numbers of leukocytes within both the tumor vasculature and the tumor itself (9). CM101 has no effect on normal physiological neovasculature such as that occurring during pregnancy (48), or wound healing (49). Recently, we have demonstrated that CM101 binds within 5 min to the endothelium of newly established tumor vasculature (11). CM101 activates complement C3, allowing activated leukocytes to adhere and extravasate into the tumor. This ability of CM101 to promote leukocyte adhesion on otherwise leukocyte-adhesion protected tumor vasculature (50) initiates the breakdown of tumor immunoprivilege (11). Therefore, these observations strongly suggest that CM101 selectively targets and inhibits pathological and not physiological angiogenesis through the engagement of the tumor endothelium. Up-regulation of TNFR II on the surface of endothelial cells in the murine B16-F10 melanoma is indicative of activated tumor-targeted inflammatory response. Indeed, a time- and dose-dependent systemic elevation of the inflammatory cytokines MIP-1α, TNF-α, IL-6, IL-8, and IL-10 has been demonstrated in human cancer patients in a successful Phase I study with CM101 (51), indicative of an activated tumor-targeted inflammatory response. Elevation of plasma levels of soluble-E-selectin provided further evidence of CM101-induced tumor apoptosis mediated through targeting of the vasculature. After CM101 treatment, expression of fasL was significantly reduced (C); however, CM101 significantly up-regulated Fas expression (D). No background staining was observed with normal rabbit IgG (E). Consistent with melanoma tumors, brown granular deposits of melanin were localized throughout the tumor (B and E). Bar, 30 μm; magnification, ×400.

Fig. 5. Immunohistochemical staining of B16BL-6 melanoma tumors by Fas and fasL. Sections (7 μm) of tumors excised from PBS- and CM101-treated animals were probed with either rabbit anti-fasL (A and C), rabbit anti-Fas (B and D), or normal rabbit IgG (E) followed by anti-rabbit IgG-biotin. Visualization of fasL was performed with avidin-HRP and DAB/H2O2, whereas Fas visualization was performed with avidin-alkaline phosphatase and Fast Red/naphthol. High expression of fasL in the PBS-treated tumor was localized to tumor cells and was evenly distributed (A), whereas no Fas expression was observed (B). After CM101 treatment, expression of fasL was significantly reduced (C); however, CM101 significantly up-regulated Fas expression (D). No background staining was observed with normal rabbit IgG (E). Consistent with melanoma tumors, brown granular deposits of melanin were localized throughout the tumor (B and E). Bar, 30 μm; magnification, ×400.

B16-F10 melanoma cells in culture express low but detectable levels of p53 protein and little or no Fas protein (47). RT-PCR analysis of cultured B16BL-6 melanoma cells revealed a positive signal for both p53 and fasL mRNA; however, no Fas mRNA could be detected (data not shown). Additionally, p53 protein in these cells is unable to bind DNA as determined by a specific DNA binding assay. p53 binds to DNA in a sequence-specific manner in response to a variety of extracellular signals initiating the transcription of genes associated with cellular proliferation and/or apoptosis (13–20). In the DNA binding studies described here, we used oligonucleotides representing the consensus p53-binding site in the murine p21Waf1/Cip-1 gene promoter known to be induced by p53 (14). Detection of a p53 sequence-specific DNA complex would serve as an indicator of active p53 within the tumor. After CM101 administration, whole melanoma tumor lysates exhibited
an increase in p53-specific DNA binding activity when compared with whole tumor lysates from PBS-treated animals (Fig. 3). Those extracts that demonstrated an increase in DNA binding activity also had an increase in the amount of total p53 protein. Because the tumor-infiltrating leukocytes demonstrated an up-regulation of p53 mRNA, they could constitute the source for increased p53 protein activity within the whole tumor lysates.

Within the context of the experiments described in this report, it is not entirely clear how the up-regulation of p53 mRNA in the infiltrating leukocytes contributes to CM101-induced apoptosis of the tumor cells. It is possible that the suppression of a host wild-type p53 allele in the tumor cells is overridden by the sequence of events associated with the tumor-targeted inflammatory response induced by CM101. Therefore, immunosuppression induced on the host by the tumor may be overcome through the activation of a suppressed wild-type p53 allele in the melanoma tumor cell that is not seen in vitro.

Studies by others of human colon and esophageal tumors have demonstrated that immune evasion through alteration of Fas/fasL expression is a possible mechanism of tumor growth and survival (35–37). Human esophageal carcinoma tumors displayed evidence of apoptotic CD45-positive tumor-infiltrating lymphocytes in regions of the tumors with elevated fasL expression. In contrast, fasL-negative regions of the tumors were positive for infiltrating CD45-positive cells, with no evidence of apoptosis. It has been suggested (40, 43, 53) that fasL-mediated depletion of tumor-infiltrating immune cells might contribute to tumor immunoprivilege. The data presented herein support and expand on these studies. Immunolocalization studies clearly show that after CM101 treatment, Fas protein is distributed throughout the tumor, which was previously negative for Fas protein expression. The elevated fasL protein seen in the tumors before CM101 treatment is down-regulated after CM101 treatment as the tumor becomes apoptotic, presumably via Fas-fasL interactions. The targeted inflammatory response induced by CM101 overrides the immunoprivilege and may be a plausible explanation for its antitumor effect.

Increased p53 activity has been correlated with Fas expression (18–20). Our data also show that leukocytes targeting the tumor vasculature after administration of CM101 have elevated p53 mRNA. These leukocytes may have the potential to deliver known or otherwise unknown p53-dependent effectors associated with apoptosis to the Fas-positive tumors cells.

Indeed, the development of therapeutic strategies reintroducing wild-type p53 into p53-deficient tumors is an area of great interest. Recently, adenoviral delivery of p53 into human colon cancer cells has been reported to inhibit tumor-induced angiogenesis (54). However, CM101 is unique in that it stimulates the immune system and targets and delivers leukocytes with elevated p53 mRNA to the tumor via the tumor neovasculature. This specific antipathoangiogenic mechanism of action leads to an apoptotic response in the tumor cells and in the endothelium (11).

The p53 DNA binding experiments with whole tumor lysates and the immunohistochemistry of tumor sections after CM101 treatment show that p53 activity is elevated within the tumors, fasL expression is reduced, and apoptotic tumor cells are present. The mechanism of up-regulation of transcription of p53 in the leukocytes of tumor-bearing mice and the issue of whether the same mechanism would apply to the onset of apoptosis in human tumors as observed previously (43) remain to be elucidated. Attempts to address this issue are in progress in p53 knockout mice.

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