G250: A Carbonic Anhydrase IX Monoclonal Antibody

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Current Oncology Reports 2005, **7:**109–115
Current Science Inc. ISSN 1523-3790

G250 or carbonic anhydrase IX (CA IX) is a membrane-associated carbonic anhydrase (CA) thought to play a role in the regulation of cell proliferation in response to hypoxic conditions and may be involved in oncogenesis and tumor progression. G250 refers to a monoclonal antibody (mAb) that was raised by immunization of mice with human renal cell carcinoma (RCC) homogenates. The RCC-associated transmembrane protein designated G250 has since proven to be identical to tumor-associated protein MN or CA IX. Previous studies using a mAb against CA IX have shown that CA IX is induced constitutively in certain tumor types, but is absent in most normal tissues with the exception of epithelial cells of the gastric mucosa. Furthermore, previous immunobiochemical studies of malignant and benign renal tissues revealed that CA IX was also highly expressed in RCC. Studies on tumor-bearing kidneys demonstrate selective uptake of mAb CA IX in antigen-positive cells versus antigen-negative cells. Furthermore, extraordinarily high uptake and the requirement of a low protein dose to obtain tumor saturation with respect to tumor targeting occur with mAb CA IX. These studies formed the basis of numerous clinical trials aimed at mAb-guided therapy in patients with metastatic RCC.

Introduction

Renal cell carcinoma (RCC) is estimated to account for over 35,000 new cases and over 12,000 deaths per year in the United States and over 100,000 deaths worldwide [1]. The incidence of RCC is steadily increasing at a rate of about 2.5% per year across all population groups [2]. Approximately one third of RCC patients present with metastatic disease, and one third of patients undergoing nephrectomy for clinically localized disease eventually progress to metastatic disease [3]. Metastatic RCC poses a therapeutic challenge because of its resistance to conventional modes of treatment such as chemotherapy and radiotherapy. During the past two decades, significant advances in the diagnosis, staging, and treatment of patients with RCC have brought improved survival of a select group of patients and an overall change in the natural history of the disease [2]. However, despite advances in biologic and immune-based therapies, response rates for patients with metastatic RCC remain at approximately 15% to 30% [1,4–7]. As a result, an urgent need remains for the development of novel therapies to manage patients with metastatic or high-risk, locally advanced RCC. RCC continues to be a hallmark disease for the testing of new immunologic approaches.

G250 or Carbonic Anhydrase IX

G250 refers to a monoclonal antibody (mAb) that was raised more than 10 years ago by immunization of mice with human RCC homogenates [8]. The RCC-associated transmembrane protein designated G250 has since been proven to be identical to tumor-associated protein MN or CA IX [9], a tumor-associated antigen (TAA) first identified in HeLa cells, and expressed in cervical cancer, using murine mAb 75 [10]. The *MN* gene appears to be chimeric in nature, arising from exon shuffling. Its sequence has been published [11], the original cDNA sequence corrected [10], and the sequences from tumors and normal tissue were shown to be identical [12]. Northern blot analysis and reverse transcription polymerase chain reaction (RT-PCR) studies showed that G250 transcripts were not detected in normal adult and fetal kidney specimens. In addition, hybridization of G250 cDNA with a cDNA library constructed from normal kidney did not identify any clones with G250 homology. These observations indicate that G250 transcription is not involved in renal organogenesis but is induced or upregulated upon malignant transformation of proximal tubular cells of the kidney. Targeted disruption of CA IX gene expression in a murine model results in gastric glandular hyperplasia with proliferation of mucus-secreting pit cells but otherwise normal development [13].

The normal function of CA IX is to catalyze the reversible conversion of carbon dioxide and water to carbonic

acid, playing a role in the intra- and extracellular pH regulation of cells [14]. It has been implicated as an intrinsic marker of hypoxia and as a prognostic marker for several types of human tumors [15]. CA IX overexpression may be mediated by hypoxia-inducible factor (HIF)-α and may contribute to an acidic tumor microenvironment to help cancer progression and metastasis [15,16]. HIF-1 is made up of α-subunits (HIF-1α or HIF-2 α) and HIF-1β. HIF-1β is constitutively expressed, whereas the intracellular concentration of HIF-α is controlled at the biosynthesis level and at the posttranslational level by hypoxia through the von Hippel-Lindau (VHL) tumor suppressor protein [17,18•]. Under normoxic conditions, HIF-1α and HIF-2α are hydroxylated and bound to VHL, which leads to ubiquitination and rapid degradation of the HIF-αs. Conversely, under hypoxic conditions, the unhydroxylated forms of HIF-1α and HIF-2α do not bind to VHL and thus are not subject to degradation by the ubiquitin-proteasome pathway. In addition to hypoxia, VHL tumor suppressor gene alteration (mutation, deletion, or hypermethylation) gives rise to defective ubiquitination of HIF subunits, which causes the intracellular accumulation of HIF-1α and HIF-2α even in the absence of hypoxia. Upregulation of HIF-α is a common feature in *VHL* gene alterations in clear cell RCC and is significantly associated with an upregulation of vascular endothelial growth factor (VEGF), which promotes angiogenesis [19]. Studies confirm that CA IX expression in normal tissue is extremely limited, mainly to the gastrointestinal tract, gallbladder, and pancreatic ducts [14]. However, it is expressed in numerous malignancies, including cervical, uterine, breast, lung, esophageal, gastric, biliary tract, colorectal, bladder, and skin cancers [14,20].

CA IX has also been shown to be a useful prognostic biomarker for RCC. A study at UCLA found that 94% of clear cell RCC tumor samples stained positively for CA IX [21••]. Survival tree analysis determined that a cut-off of 85% CA IX staining provided the most accurate prediction of survival. Low CA IX staining was an independent prognostic indicator of poor survival in patients with metastatic RCC. CA IX significantly sub-stratified patients with metastatic disease when analyzed by T stage, Fuhrman grade, nodal involvement, and performance status. For patients with localized, high-risk RCC, low CA IX staining also implied a worse prognosis, similar to that of metastatic patients. Furthermore, all complete responders (8%) to interleukin (IL)-2 immunotherapy included patients with high CA IX (>85%) staining of the primary tumor. A recent study from Harvard also demonstrated that high CA IX expression was associated with response to IL-2 and that response was twice as likely with high CA IX expression [22]. Based on these data, CA IX appears to be the most significant molecular marker described in kidney cancer to date. Significantly, studies have shown a direct correlation between CA IX expression and loss of functional VHL gene product, postulated to be the cause of near universal CA IX expression in clear cell RCC [19]. A recent study also demonstrated a close correlation between HIF-1α expression and CA IX expression in clear cell RCC, whereas no relationship between HIF-2α expression and CA IX was observed [23•]. Whether the expression of CA IX in RCC is a function of *VHL* gene mutation, tumor hypoxia, or a combination of the two remains unclear. However, CA IX reflects significant changes in tumor biology, which may be useful to predict clinical outcome and identify high-risk patients in need of adjuvant immunotherapy and CA IX–targeted therapies.

Monoclonal Antibodies for Cancer Therapy

For more than 50 years it has been known that tumors in laboratory animals undergo regression after treatment with serum derived from immunocompetent animals immunized with tumor antigens [24]. The pooled serum used in early studies consisted of antibodies directed against antigens expressed by a given tumor as well as other non–tumor-specific antibodies and proteins. These experiments demonstrated that antibodies generated against tumors could inhibit tumor cell growth and induce tumor cell death. However, difficulty with antibody production and target antigen selection made this form of therapy far from ideal [24]. With the introduction of hybridoma technology, described in 1975 by Kohler and Milstein [25], the production of large quantities of specific murine mAbs became possible. This development spurred the first human clinical trials using mAbs against solid tumors in the 1980s [24].

The five classes of antibodies are IgG, IgM, IgA, IgD, and IgE [26]. IgG is the most prevalent antibody in human serum. IgG antibodies have the ability to initiate the complement cascade and the capacity to bind to other cells of the immune system, such as macrophages, lymphocytes, and natural killer cells. All naturally occurring Igs are made up of at least two heavy and two light chains. The composite portion of the antibody that recognizes and binds the antigen is called the idiotype, whereas that area of the antigen involved in the antibody–antigen interaction is called the epitope. Antibodies manufactured from a particular clone of a hybridoma are identical and are termed monoclonal. The mAbs are generally more favored than polyclonal Abs, and they can be produced reliably in large quantities. With polyclonal techniques, the antibodies produced are of various classes, contain different idiotypes, and may show variable pharmacokinetics.

The potential applications of mAbs in the management of cancer can be divided into diagnosis, prognosis, and treatment. Specificity is the most important factor in decisions concerning whether a particular mAb can be successfully used, especially when clinical implementation is desired, but other factors can also influence success. Specificity can be viewed as tumor specificity, that is, no cross-reactivity with nonmalignant tissues, or tumor-type

specificity. To diminish any possible side effects, little or no cross-reactivity with normal tissues is most desirable. Tissues that cross-react may necessitate administration of large amounts of mAb for saturation, and unwanted side effects may occur when the mAb is conjugated to a radionuclide or toxin. However, overexpression of tissue differentiation antigens can provide a sufficient difference in mAb targeting levels, and normal tissue damage may be acceptable in selected cases.

Although it would be preferable that all tumors of a particular type express the antigen with as little heterogeneous expression as possible, few antigens with true tumor specificity have been detected [27•]. These antigens are expressed in various tumors but in only 30% to 40% of cases. Moreover, the expression within tumors is heterogeneous, which would hamper complete tumor destruction [27•]. Several other parameters besides antigen expression have been defined that may be equally important in mAb-guided tumor localization and therapy. The size of the tumor mass, the antigen density, the fate of antigen–antibody complex, the mAb dose, the presence of circulating antigen, the mAb format, the mAb circulating time, and the administration route have been recognized as important parameters that can differ from one tumor type to the other. Furthermore, many of these factors are interrelated. Parameters related to tumor physiology may also be important. The existence of leaky blood vessels, impaired blood flow patterns, neovascularization, high interstitial fluid pressures, and aberrant lymphoid vessels heavily influences the distribution of diffusion-driven therapeutics, such as mAbs [28].

Theoretically, tumor therapy using mAbs can be mediated via effector cell mechanisms, complement, or conjugation to toxins, drugs, or radionuclides [24]. Radionuclide conjugated mAbs have been studied extensively for the treatment of solid tumors. Killer isotopes (α or β emitters such as ^{131}I, ^{90}Y, ^{212}Bi, and ^{211}At) can be effective at distances of up to 50 or more cell diameters; therefore, the Ab does not have to bind to every tumor cell for a therapeutic effect. Radiolabeled mAbs also do not need to be internalized for cell killing. The advantage of radiolabeled conjugates is that they can be used at a low scout dose in situ for tumor detection, followed by their use for high-dose therapy. Dosimetry calculations for the use of a mAb in a particular patient can be performed, permitting patient-adjusted administrations.

Role of the Immune System in Renal Cell Carcinoma

Like melanoma, RCC has been considered an intrinsically immunogenic tumor. This designation is based on several observations. Spontaneous regression occurs in patients with metastatic RCC, and immunologic mechanisms are thought to have a dominant role in these events [1,4]. Tumor-specific antibodies have been isolated in sera obtained from patients with RCC [29]. Tumor-specific cytotoxic T cells (CTLs) have been identified. Several investigators have been able to isolate CTLs capable of specifically killing autologous RCC cells [30]. Durable responses have occurred with IL-2 and interferon (IFN)-2α treatment of patients with metastatic RCC [1,4].

Renal Cell Carcinoma Antigens Defined by Monoclonal Antibodies

Several investigators have defined mAbs that are reactive with RCC-associated antigens [8,31,32]. The vast majority of these mAbs recognize kidney differentiation antigens retained by subsets of RCC. Fine specificity studies have revealed strict kidney specificity for some mAbs, whereas other mAbs have shown cross-reactivity with many non-kidney tissues. Several mAbs recognizing RCC-associated antigens that are absent from normal kidney have been defined [8,32,33]. In general, only a proportion of the primary and metastatic RCCs tested were positive for the respective antigen, and cross-reactivity with other tumors and some normal tissues was observed. These mAbs are thought to identify different RCC-TAA because they display different cross-reactivity with normal tissues. In view of their restricted cross-reactivity with normal tissues and the expression of a given RCC-TAA in subsets of RCC, these mAbs are prime candidates for clinical investigation.

Monoclonal Antibody CA IX: General Considerations

One mAb, G250 or CA IX, has been studied extensively because of its exceptional tissue distribution in combination with high antigen expression in RCC. On immunohistochemical analysis, this mAb was found to stain gastric mucosal cells and large bile canals, whereas all other tissues tested, including normal kidney tissue, had negative staining [8]. CA IX was present in greater than 80% of primary and metastatic RCCs, present in 95% to 100% of the clear cell RCCs, and absent or minimally expressed in normal tissues (Fig. 1) [8,21••]. Molecular definition of the *CA IX* gene has allowed investigations leading to the understanding that mutational events intimately related to the development of clear cell RCC and CA IX expression exist [34]. Various animal experiments have been performed as well as ex vivo experiments on tumor-bearing kidneys to show selective uptake of mAb CA IX in antigen-positive cells versus antigen-negative cells [35–39]. Extraordinarily high uptake and the requirement of a low protein dose to obtain tumor saturation were features that stood out with respect to tumor targeting. These studies formed the basis of numerous clinical trials aimed at mAb-guided therapy in patients with metastatic RCC.

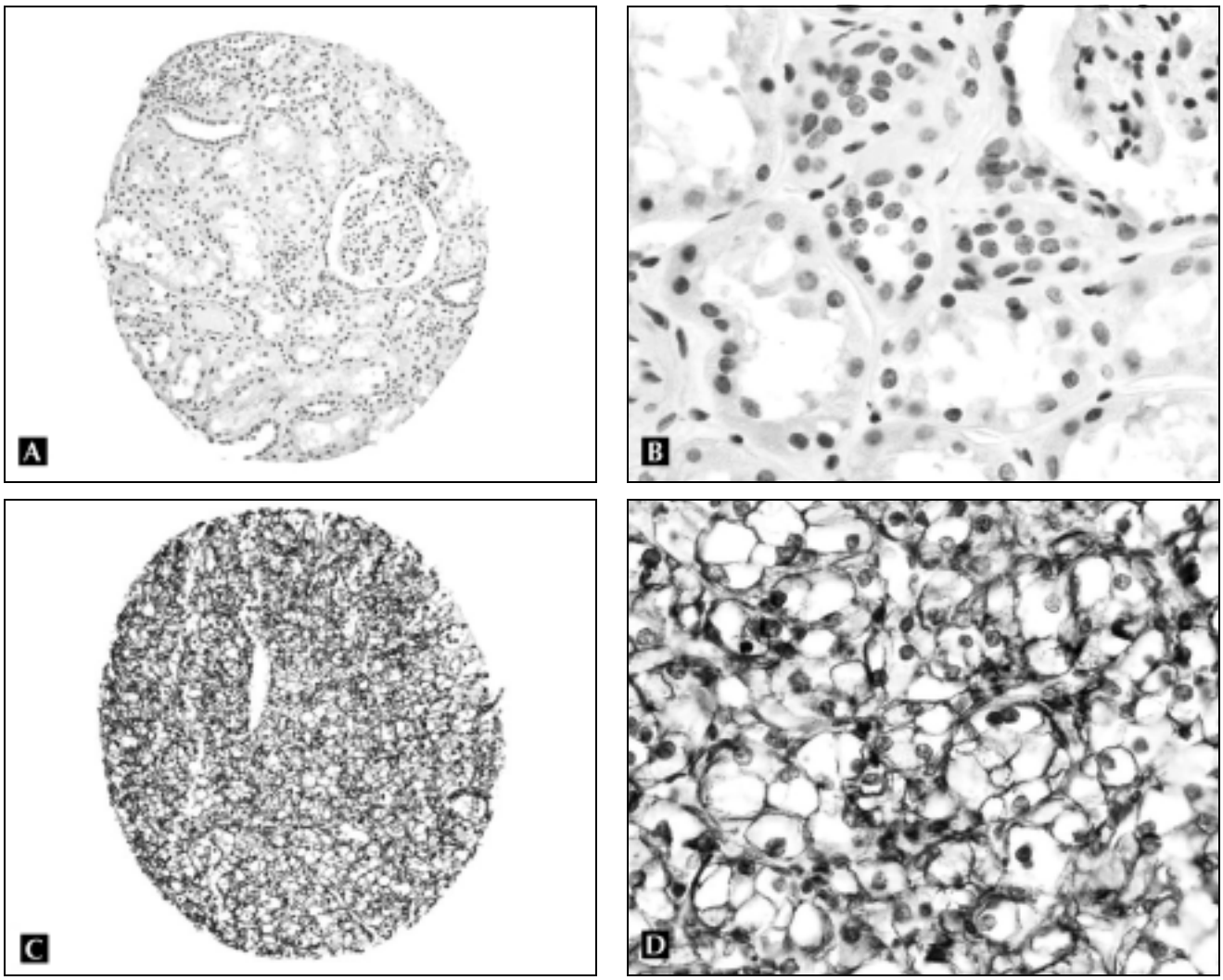

Figure 1. CA IX staining of normal kidney and clear cell renal cell carcinoma at x100 and x400 magnification. Normal kidney tissues were ubiquitously negative for CA IX (**A and B**), and clear cell renal cell carcinoma had intense membrane staining for CA IX (**C and D**).

Clinical studies with murine monoclonal antibody CA IX

Clinical trials with radiolabeled murine mAb CA IX (mG250) in patients with RCC have demonstrated selective and specific delivery of mAb to renal cancer sites [40,41]. Primary and metastatic RCC, including bone and soft tissue metastasis, were targeted and imaged. Oosterwijk *et al.* [40] performed a study of ^{131}I labeled to escalating mass amounts of mG250 to determine tumor uptake and mAb distribution. Tissue biopsies of approximately 30 imaged lesions from 23 patients receiving mG250 were all pathologically documented to represent site of renal cancer. There were no false-positive scans. Images taken immediately after injection did not show radioactivity in the tumor, but tumor uptake was evident 2 to 3 days after mAb administration. Antibody localization to tumor was selective and specific, with quantitative analysis of tissue samples demonstrating peak ratios of tumor to serum of 178:1, tumor to normal kidney of 285:1, and tumor to liver of 92:1 1 week after mAb administration [40]. Liver uptake decreased at higher protein dose levels, suggesting saturation of CA IX antigen sites by the mAb. Gamma camera images of ^{99m}Tc-labeled human serum albumin, administered immediately before surgery, and ^{131}I-mAb CA IX, were clearly disparate, demonstrating that the images were not a reflection of blood pool but of true selective antibody accumulation [40].

A phase I/II radioimmunotherapy trial with ^{131}I-mG250 in 33 patients with measurable metastatic clear cell RCC was initiated at Memorial Sloan-Kettering Cancer Center to determine its safety and therapeutic potential [41]. An escalating amount of ^{131}I (30, 45, 60, 75, and 90 mCi/m^2 of ^{131}I) labeled to 10 mg of mAb G250 was administered as a single infusion. Targeting to all known disease 2 cm or greater in diameter independent of location was excellent by the first imaging scan between 2 and 4 days after administration of ^{131}I-labeled G250. Targeting was comparable with the primary tumor (in

those who did not have a nephrectomy), as well as to bone, liver, lung, nodal, and subcutaneous metastases. Hepatic toxicity was invariable at dose levels of 45 mCi/m^2 of ^{131}I or greater, but the intensity was otherwise unrelated to the amount of radioactivity administered or the radiation dose absorbed to the liver. Furthermore, this toxicity was transient and not dose limiting. As is true in all radioimmunotherapy studies of radiolabeled antibodies, dose-limiting toxicity was hematopoietic, with the maximum tolerated dose (MTD) of activity determined to be 90 mCi/m^2 of ^{131}I. In the phase II arm of the trial, 15 patients were treated with 90 mCi/m^2 of ^{131}I-mG250 to determine efficacy. Seventeen of 33 evaluable patients had stable disease. No major responses were noted, but the overall survival of patients treated with ^{131}I-mAb G250 seemed to be increased compared with historical controls, and some minor responses were observed [41]. The development of human antimouse antibody (HAMA) in all patients within 4 weeks after therapy precluded retreatment. The excellent targeting and the lack of significant nonhematopoietic toxicity demonstrated the potential of radiolabeled mAb CA IX in the treatment of renal cancer. These characteristics, in combination with the necessity for multiple injections, led to the development of chimeric mAb CA IX (or cG250).

Clinical studies with chimeric monoclonal antibody CA IX

A chimeric version of the antibody was constructed (cG250) by molecular techniques with the intention of diminishing the immunogenicity of mG250. The design of the first presurgical protein dose-escalation study was comparable with that for mG250 in order to determine the pharmacokinetics, toxicity, immunogenicity, and imaging characteristics of ^{131}I-labeled cG250 in patients with RCC [42]. The in vitro binding characteristics of cG250 were similar to those of mG250, demonstrating that chimerization of the antibody did not affect specificity, affinity, or avidity. In general, the in vivo behavior, including the half-life of cG250, was comparable with that of mG250. All 13 antigen-positive tumors showed excellent targeting of radioactivity, with uptake (measured at biopsy 1 week after infusion) as high as 0.52% ID/g (blood: 0.0028% ID/g). As was true for mG250, hepatic uptake was saturable at doses of 5 mg of cG250 and greater. Possibly more important, there was no evidence of host response as measured by enzyme-linked immunosorbent assay (ELISA) [42]. Therefore, multiple administrations became feasible.

In the subsequent phase I radioimmunotherapy trial, escalating doses of ^{131}I were labeled to 5 mg of cG250 [43]. To ensure that the metastases were G250 positive, patients received an imaging scout dose (6 mCi of ^{131}I labeled to 5 mg of cG250), and only patients showing targeting to tumor received the therapeutic infusion of ^{131}I-cG250 1 week later. Dose-limiting toxicity was hematopoietic (approximately 60 mCi of ^{131}I/m^2). Unlike the mG250 radioimmunotherapy trial, no hepatic toxicity was observed. This difference was most likely due to the saturation of the hepatic G250 antigen sites with the initial imaging dose of cG250 [41,44]. Another difference between the ^{131}I-mG250 and the ^{131}I-cG250 is the lower MTD of ^{131}I-cG250. The MTD with ^{131}I-mG250 was 90 mCi/m^2 of ^{131}I [41], whereas that of a single therapeutic dose of ^{131}I-labeled cG250 MTD was 60 mCi/m^2 of ^{131}I. This lower MTD can be explained by the longer circulation time of chimeric mAb cG250 compared with murine mAb mG250, with consequently higher bone marrow radiation absorbed dose. Targeting of ^{131}I-cG250 to disease was outstanding in patients with positive diagnostic scans.

Despite excellent targeting, uniform distribution of the radioactivity in tumors has rarely been observed. In a dual-labeling trial in which patients received two independent administrations of cG250 labeled with ^{131}I or ^{125}I followed by surgery, the importance of fluctuating factors on tumor uptake of mAb was addressed [45]. Ten patients with a clinical diagnosis of primary RCC were studied. Nine days before surgery, patients were administered with ^{125}I-cG250 (5 mg of cG250, 50 mCi of ^{125}I) followed by a second injection of ^{131}I-cG250 (5 mg of cG250, 3.5 mCi of ^{131}I) 4 days later. Tumors obtained at surgery were analyzed. Unexpectedly, cG250 distribution did not differ in the two administrations, demonstrating that intrinsic tumor factors have a prominent role in mAb targeting. Alternatively, it is possible that low uptake areas may consist of fast internalizing subpopulations in which internalized iodinated proteins are quickly degraded and the iodine lost, whereas avid mAb uptake occurs. Initially, this explanation was thought to be improbable because ^{131}I-cG250 retention in patients treated with therapeutic doses was in the order of weeks, suggesting low internalization rates. The use of residualizing radionuclides, such as yttrium or lutetium, may lead to a more noticeable homogeneous cG250 distribution and deliver more potent radiation more evenly. Preliminary studies in patients with RCC treated with cG250 labeled with a residualizing radionuclide (^{111}In) and ^{131}I have demonstrated increased uptake of the ^{111}In-labeled cG250 in comparison with ^{131}I-cG250 [46].

A phase I/II trial is ongoing in which patients are receiving two high doses of ^{131}I-cG250 separated by 3 months [47•]. Images obtained 2 weeks after cG250 administration demonstrated that ^{131}I-cG250 retention can be exceptional. In this ^{131}I-cG250 trial, toxicity is bone marrow related and seems to be more severe with the second administration, suggestive of bone marrow exhaustion by the first high dose. This trial is still open for recruitment, and, thus far, no major responses have been observed.

Administration of non-manipulated cold antibody may lead to recruitment of effector cells or complement activation, resulting in cell death. Although cG250 demonstrated little antibody-dependent cellular cytotoxicity in vitro, a phase II study was performed in which cG250 was

administered weekly by intravenous infusion to patients with advanced progressive RCC [48•]. Thirty-six patients received 12 infusions of 50 mg of cG250 per dose. The trial was powered to detect a true response rate of 15%. This primary objective was not reached, but a durable clinical benefit for at least 6 months or more was achieved in eight patients, which is considered clinically meaningful for this patient population. Six patients achieved stabilization of disease lasting more than 6 months. Beyond the official observation period, one patient achieved a complete response more than 6 months after the start of cG250 treatment, and another patient experienced a minor response. Both patients were free of disease progression for more than 1 year. Based on the good tolerability of this treatment with cG250 and the clinical benefit in 25% of this difficult treatment group, further investigation was warranted.

In vitro analyses of cG250 in combination with IL-2 showed strong enhancement and maintenance of cG250-mediated, antibody-dependent cellular cytotoxicity and K562 cytotoxicity when applied to peripheral blood mononuclear cells in culture for 7 days [49]. IFN-γ also enhanced the antibody-dependent cellular cytotoxicity of cG250 throughout the study period but was not as effective as the IL-2 treatment. In contrast, IFN α-2a and 2b increased cG250-mediated, antibody-dependent cellular cytotoxicity and K562 cytotoxicity for only 3 days of the study period. The potent and sustained immune effector activity observed with cG250 and cytokines in these in vitro studies suggested that combination immunotherapy with cG250 and cytokines, such as IL-2, might be advantageous in the treatment of RCC. Subsequent trials are focusing on the combination of cG250 with biologic response modifiers. In one trial, the effects of cG250 (20 mg of cG250 per dose) and low-dose IL-2 plus periodic IL-2 pulsing in advanced metastatic RCC are being studied [50]. In 30 evaluable patients with metastatic RCC, administration of 50 mg of cG250 over 12 weeks had an overall clinical benefit rate (objective response or stable disease of at least 6 months) of 25% and a median survival time of 15 months in patients with metastatic RCC. In another trial, the effects of cG250 combined with IFN-α are being studied [31]. The cG250/IFN-α trial has recently been initiated and is ongoing.

Conclusions

Strong indirect evidence suggests that the host immune system is intimately involved in the natural history of RCC. Immunologic studies are providing information on tumor-related differentiation antigens of the kidney and beginning to allow molecular subclassification of RCC subtypes. RCC was historically regarded as a single entity that expressed many possible histologic appearances. Today, RCC is more accurately recognized as a family of cancers resulting from distinct genetic abnormalities with unique morphologic features but a common derivation from the renal tubular epithelium. Lastly, mAbs, which have proven their value now that in vitro diagnostics are demonstrating a role as in vivo imaging and therapeutic agents in nonneoplastic and neoplastic disease. Although mAb G250 treatment may have a role in the management of metastatic RCC, particular subgroups of patients who are more prone to benefit from this treatment must be delineated. High-risk patients may benefit from adjuvant treatment with this nontoxic treatment modality. However, large cohort studies are needed to investigate this possibility.

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