Biological Efficacy of a Dendritic Cell-Based Vaccine in a Patient with Metastatic Colorectal Cancer

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Abstract: Colorectal cancer is a serious health problem affecting de novo more than one million people every year in the developed world. Despite recent advances in the development of novel therapeutic agents, metastatic colorectal cancer remains mostly incurable and its survival rates ominous even when patients respond to the most advanced treatments. Here, we describe a case in which a patient with metastatic colorectal cancer and high risk of relapse remains disease-free while being treated solely with twelve doses of autologous dendritic cells vaccines pulsed with autologous tumor lysate. A sustained, specific immune response elicited by vaccination has also been documented. Prior to receiving this experimental treatment, the patient had undergone both tumor resections and chemotherapy treatments six times, invariably relapsing/progressing within a year from each resection. We believe that the use of autologous vaccines consisting in dendritic cells pulsed with tumor lysate should be further investigate in human clinical trials, particularly in patients with minimal tumor burden and high risk of relapse. We also believe that this type of immunotherapy is more likely to be successful when used as an early rather than merely compassionate treatment option, given the fact that the more toxicity the immune system has received from previous approaches, the less it will be able to respond to tumor vaccination.

Keywords: Cancer immunotherapy, dendritic cells, metastatic colon cancer, vaccine, tumor lysate, immune response.

INTRODUCTION

Colorectal cancer is a wide spread health problem which affects nowadays more than one million people per year in the developed world [1].

The prognosis of localized or locally-advanced colon cancer is highly dependent on the disease stage. In facts, five-year survival is about 80% for stage I or II localized tumors and about 60% for stage III, that is when loco-regional lymph nodes are involved [1]. All in all, surgery is a potentially curative treatment for localized or locally-advanced colon cancer. Moreover, the use of adjuvant chemotherapy has shown to reduce the relapse risk by 30-40% [2]. In particular, the combination of oxaliplatin, an alkylating agent, and fluoropyrimidines, that is antimetabolites, is considered the standard adjuvant treatment in these cases [2, 3]. Unlike what happens in metastatic colorectal cancer, the addition of biological therapies such as cetuximab, a monoclonal antibody targeting the epidermal growth factor receptor, or bevacizumab, a monoclonal antibody targeting the vascular endothelial growth factor, has not improved the results obtained with conventional chemotherapy [4]. In addition,

histopathological and molecular characteristics may substantially influence the relapse rates even among patients with the same disease stage [5]. Therefore, despite the apparent presentation homogeneity among localized or locally-advanced colon cancer patients, it is possible to identify subgroups with a particularly ominous prognosis [6], leading to the imperative necessity of finding new therapeutic approaches in order to possibly benefit patients with particularly poor prognosis.

Needless to say that when the focus is instead on patients with metastatic colorectal cancer, the presence or absence of negative prognostic factors loses most of its importance, and virtually all of these cases share in a very short life expectancy [7]. However, it has to be mentioned that among the 20% or more patients presenting at diagnosis with metastatic (stage IV) colorectal cancer, up to 25% will have an isolated liver metastasis that is potentially resectable. Interestingly, patients with such lesions who undergo curative resection may experience a 5-year survival now exceeding 50% [7].

Active Immunotherapy is one of the experimental strategies currently used as a complementary or alternative strategy to treat cancer. Specifically, cellular immunotherapy is based on the manipulation and injection of cells belonging to the immune system with therapeutic intentions. During the last two decades,

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dendritic cell (DC)-based immunotherapy procedures have been steadfastly developed. Due to their efficiency at inducing robust antitumor immune responses [8, 9] and their relatively simple applicability, plenty of DC vaccine-based clinical trials have been carried out in oncology. DC are a leukocyte subpopulation whose main function consists in cross-presenting antigens to T lymphocytes. DC differentiate from hematopoietic precursors and are deployed in different tissues where antigens are processed for presentation. DC undergo maturation in the presence of pathogen-associated molecular patterns (PAMPs). Mature DC migrate from peripheral tissues to the secondary lymphoid organs where they encounter naive lymphocytes. The interaction of mature DC with these lymphocytes triggers the activation and the expansion of antigen-specific lymphocytes that are going to be the ultimate effector cells against the pathogen. In tumor immunotherapy, vaccination with mature DC loaded with tumor antigens has been very successful to mount potent anti-tumor immune responses in preclinical models [9]. The actual antitumor effect is typically mediated by cytotoxic T lymphocytes mediated. Therefore, the involvement of human leukocyte antigen (HLA) class I and II molecules, the generation of co-stimulatory signals through CD80 and CD86, as well as the production of interleukin 12 (IL-12) by DC is needed [9]. These events lead to the recruitment of more DC, monocytes and lymphocytes. DC maturation includes the acquisition of the ability to migrate to secondary lymphoid organs where they will activate T lymphocytes, leading to clonal expansion and differentiation to effector and memory cells [9]. Finally, DC can induce B cells proliferation, as well as immunoglobulin class switch and differentiation into antibody-secreting plasma cells [9].

Different approaches have been assessed to make DC a powerful immunotherapeutic tool. In particular, DC can be transformed by an exogenous DNA, pulsed with or more known antigens, or pulsed with autologous tumor lysate, which is assumed to contain the broadest possible pool of putative tumor-specific or -associated antigens [10, 11]. Indeed, this last option is based on the fact that each patient's immune system will be stimulated with his/her own, corresponding tumor material. In this respect, the strategy could be defined as twice customized, insofar as not only the DC, but also the tumor material must be patient-derived and not usable for other candidates to the same type of treatment. Compared to strategies focused only on one

or few tumor antigens, tumor lysate-pulsed DC might be less susceptible to fail in case of relapse sustained by a clone that has lost a single antigen against which an immune response had been previously elicited by DC vaccination. Moreover, tumor lysate proteins are internalized and processed into peptides which are cross-presented within class I and II HLA molecules. This fact allows the activation of either CD4$^+$ or CD8$^+$ T cells, which is important to possibly achieve an efficient and long-lasting antitumor immune response [10, 11].

All these features, together with the low toxicity profile, which is limited at most to local reactions, mild fever and transient malaise, and with the encouraging results published over the years, make DCs pulsed with autologous tumor lysate a very attractive tool to be used as one of the alternative therapeutic options against cancer. In particular, this strategy could be useful in patients with a minimal tumor burden after resection and standard chemotherapy procedures, especially in those with high risk of relapse.

In this paper, we report a successful such case, in which our experimental vaccination protocol based on DCs pulsed with autologous tumor lysate is keeping a patient relapsed, metastatic colorectal cancer disease free over the last 30 months in the absence of any other treatment.

PATIENT AND METHODS

Clinical Record

The patient, a Spanish surgeon, was diagnosed with metastatic colon cancer in February 2002. At the time of first resection, ultrasound revealed six liver metastases, some of which were successfully removed. He then underwent 6 cycles of an oxaliplatin and 5-fluorouracil chemotherapy regimen, achieving a documented complete response which lasted 12 months. In September 2003, new liver metastases were detected by positron emission tomography (PET). A second resection was performed to remove most of them, after which the patient completed other 6 cycles of the same chemotherapy regimen. Abdominal magnetic resonance imaging (MRI), hepatic ultrasound imaging and lung computer tomography (CT) confirmed the achievement of a second complete response, which lasted 15 months. In September 2005, a control MRI showed a small nodule in the inner side of the left thigh. After excluding myositis ossificans and confirming the original diagnosis, the patient underwent a new resection followed by local radiotherapy, which

led to a third complete response, as documented by tomography PET-CT scan, lasting 6 months. In April 2006, both MRI and PET showed a perianal nodule as well as another nodule along the sphincter, which were surgically removed and diagnosed as colorectal cancer metastases. The patient underwent 6 cycles of chemo-immunotherapy according to the capecitabine, irinotecan and bevacizumab regimen, achieving the fourth complete response, which lasted 10 months, that is until in September 2007, when both a PET-CT scan and a MRI showed a novel metastatic lesion just at the site of the last resection. Surgery was once again performed to remove the nodule, after which the patient completed 6 cycles of chemo-immunotherapy according to the capecitabine, oxaliplatin and bevacizumab regimen. This fifth complete response lasted 10 months. In January 2009, a small, new lesion was detected in the perianal region, but treatment was deferred as the nodule did not grow any further over few months. However, in May 2009 several new lesions appeared both in the same region and in the liver. Some of the most superficial nodes were surgically removed and the material used to produce the DC vaccine below. Meanwhile, as the tumor was documented to feature the wild type K-RAS, the patient underwent 12 cycles of by-weekly administrations of irinotecan and cetuximab, which were concluded in December 2009. The patient resumed his professional activity and both PET and CT scan documented a sixth complete response in February 2010. Owing to the typical short duration of all previous responses to standard treatments, we begun to vaccinate the patient with autologous tumor lysate-pulsed DC in May 2010, with the goal of possibly inducing a robust and long-lasting T-cell response against some of his putative tumor antigens.

Vaccine Preparation

Exploiting the first step along the therapeutic algorithm of metastatic colon cancer, that is complete microscopy resection with proper safety margins according to the standard oncologic digestive surgery, we obtained enough tumor material for our immunotherapeutic purposes. Afterwards, tumor viable parts were separated and processed in sterile and controlled conditions. Cellular and tissue processing was carried out in the Cellular Therapy Laboratory of the University of Navarra Hospital under good manufacturing procedures (GMP). First, the tumor sample was converted into a cellular suspension by mechanic disruption. The cell suspension was then subjected to cycles of freezing in liquid nitrogen and

thawing at 37 °C. After pelleting, the supernatant was filtered and total protein content was quantified. The tumor lysate was irradiated, aliquoted and frozen in liquid nitrogen until use. DC were generated from monocytes. After an aphaeresis of peripheral blood mononuclear cells, monocytes were immunomagnetically selected by $CD14^+$ beads using a CliniMacs^{TM} (Miltenyi Biotec, Bergish Gladbach, Germany). Monocytes were cultured in standard AIM-V medium (Gibco, Grand Island, NY) supplemented with a cocktail of cytokines, Including IL-4 at 1000 UI/ml (R&D Systems, Minneapolis, MN) and GM-CSF at 1000 UI/ml (Leukine^{TM}, Genzyme Corporation, Bayer Healthcare, Seattle, WA) to generate the DC, which were then pulsed with the tumor lysate and cultured with a second cytokine cocktail, including TNF-α at 50 ng/ml (Beromun^{TM}, Boerhinger Ingelheim, San Cugat del Valles, Spain), IFN-α at 1000 UI/ml (Intron A^{TM}, Schering Corporation, Kenilworth, NJ) and Poly I:C at 20 ng/ml (Amersham/GE Healthcare, Piscataway, NJ) to promote DC maturation. Tumor lysate-pulsed, mature DC were pelleted, aliquoted in vials according to the established protocols and stored in liquid nitrogen until use. The final, therapeutic vaccine dose consists in approximately $1x10^7$ autologous, tumor lysate-pulsed DC resuspended in 1ml of physiological serum administered intradermally. Once again, the cell product fabrication was performed controlling sterile conditions, counting, cellular viability, phenotype, microbiologic cultures and mycoplasma screening according to the standard operating procedures enforced in the GMP lab of the Cell Therapy Area of the University of Navarra Hospital. The final product was administered only after passing all the required quality controls

Vaccination Schedule

After the patient had signed a detailed informed consent, the first four doses were administered monthly. The following four vaccinations were administered bimonthly, after which we switched to quarterly boosts till consumption of all doses or relapse.

Dendritic Cells Phenotyping

In order to characterize both mature and immature DC, surface markers were detected by flow cytometry. Three-color immunostaining using combinations of FITC- (fluorescein isothiocyanate), PE- (Phycoerythrin), and perCP (Peridinin-chlorophyll-protein complex)-labeled monoclonal antibodies (mAbs) directed to

human CD14, CD19, CD3, CD33, CD209, HLA-DR, CD11c, CD40, CD80, CD86 and CD83 (BD-Pharmingen) was performed to characterize the immunophenotype of both mature and immature DC. About 5×10^5 cells were resuspended in phosphate buffered saline (PBS) and incubated for 15 minutes at room temperature with mAbs. Then, they were washed with 4 ml of PBS and resuspended in 500µl of paraformaldehyde. Corresponding isotype-matched mAbs were used as controls. Flow cytometry was performed using a FACSCalibur™ (Beckton Dickinson Immunocytometry Systems, San Jose, CA), whereas the data were analyzed using the Cell Quest Pro software (Beckton Dickinson Immunocytometry Systems, San Jose, CA). Results are expressed as percentage of positive cells and mean fluorescence intensity (MFI).

Immune Response Assessment

The possible induction of tumor-specific cellular immune responses through DC vaccination was assessed by four independent methods: interferon-γ (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) assay, T-cell proliferation assay, cytokine release enzyme-linked immunosorbent assay (ELISA) and cytotoxic T lymphocytes (CTL)-mediated killing by Chromium^{51} (Cr^{51}) release assay. For all these tests, main controls were represented by autologous mature DC alone, autologous peripheral blood lymphocytes alone and the combination of both, with the lymphocytes having being obtained prior to the first vaccination.

The number of IFN-γ producing cells was measured by ELISPOT (Mabtech, San Diego, CA) according to the kit's manufacturer's instructions. PBMCs obtained prior to each vaccination were plated in 96-well plates at the concentration of 2×10^5 cells per well with culture medium alone or with 1×10^5 mature, patient's tumor lysate-pulsed DC. Spots quantification was performed using an automated ELISPOT reader (CTL, Aalen, Germany). The response was considered positive when the number of spots was at least two fold that of control at least at two different time points.

For the T-cell proliferation assay, PBMCs obtained before each vaccination were plated in 96-well plates at 2×10^5 cells/well with 1×10^5 mature, patient's tumor lysate-pulsed DC over 5 days. The supernatants were then collected to measure IFN-γ production by ELISA (Pharmingen, San Diego, CA) according to the kit manufacturer's instructions. A response was

considered response whenever the increase in INF-γ production was at least two fold that of control at least at two different time points. Meanwhile, PBMCs were pulsed with 0.5 µCi/well of [3H]-thymidine. To determine such incorporation in proliferating cells, they were incubated for 18 hours and harvested. The radionuclide incorporation was measured by scintillation counting (Topcount Packard, Meriden, CT). The response was considered positive when radioactivity was at least two fold that of controls at least at two different time points. A nonparametric correlation was performed to assess whether the number of vaccinations had an effect on the potency of the immune response.

Finally, in order to determine the cytotoxicity mediated by CTLs, cryopreserved PBMCs were stimulated with mature, patient's tumor lysate-pulsed DC (effector/DC ratio = 10:1) over five days in the presence of interleukin 2 (IL-2) at 10 IU/ml (Novartis, Emeryville, CA). Then, activated and differentiated CTLs (effector cells) were cultured with 1×10^6 DC (target cells) pulsed with patient's tumor lysate, radiolabeled with 100µCi of Na$_2$^{51}CrO$_4$ and incubated with effector cells at four different ratios. In particular, the effector/target ratios were 100:1, 50:1, 25:1 and 12.5:1. The supernatants were harvested four hours later. Percentage of specific lysis was calculated as follows: (cpm experimental − cpm spontaneous)/(cpm maximum − cpm spontaneous) × 100, where spontaneous lysis corresponds to target cells incubated in the absence of effector cells, while maximum lysis is obtained by incubating target cells with 5% Triton X-100. A response was considered positive when $P < 0.05$ by Student's paired t test comparing post- and pre-vaccine DC lysis was achieved. Chromium release was assessed by scintillation counting (Topcount Packard, Meriden, CT).

All methods above were carried out according to the standard operating procedures which are routinely used in our lab at the Center for Applied Medical Research of the University of Navarra and have been previously published [12].

RESULTS

At the time of this writing, the patient has received 12 vaccine doses, the next administration being imminent, and his ongoing complete response has already lasted 30 months since the last cycle of standard chemo-immunotherapy. Vaccine toxicity has been negligible at most. Given the fact that this sixth

Table 1:   Phenotypic Changes on the DC Upon the Addition of the Maturation Cocktail

	iDC		mDC	
	%	**MFI**	**%**	**MFI**
CD14+	3.73		4.33	
CD19+	0		0	
CD3+	0		0	
CD33+	98.91		99.4	
CD11c+	99.92		99.23	
HLA DR+	98.71	2037	99.09	3042
CD209+	99.79		99.36	
CD86+	57.63	233	93.38	325.78
CD40+	2.92	200	38.5	199
CD80+	18.97	107.28	97.3	338.68
CD83+	2.22	201	20.83	212.94

DC were matured with TNF-α, IFN-α and Poly I:C. Mature DC phenotype is characterized by the increase expression of CD40, CD80, CD83, CD86 and HLA-DR. iDC: immature dendritic cells; mDC: mature dendritic cells; MFI: mean fluorescence intensity; %: percentage of positive cells for each marker.

remission has outlasted any of the previous complete responses by at least a two-fold ratio and appears to be sustained only by vaccination, the follow-up of the patients systematically includes MRI, PET or CT scan every 2-3 months.

As for the quality of the patient's tumor lysate-pulsed DC, we were particularly interested in documenting their thorough maturation after the pulsing. In Table **1**, we show the substantial immunophenotype changes that the same autologous patient's DC underwent in their transition from the immature to the mature state. In particular, DC maturation is confirmed by the acquisition of surface markers like HLA-DR, CD40, CD80, CD83 and CD86, which are known to play crucial roles during antigen presentation.

In terms of DC vaccine-induced, tumor-specific cellular immune response, we were able to document it by three out of four of the methods employed. In particular, as the ELISpot results were negative, DC vaccination induced a specific IFN-γ production as documented by ELISA (data not shown), as well as some specific, direct tumor cytotoxicity at every effector/target ratio (data not shown). All in all, however, the T-cell proliferation assay provided the most clear-cut results (Figure **1**). Here, response data are expressed as stimulation index, that is the ratio of the response to DC pulsed with patient's tumor lysate (stimulus) over the mean response in the absence of stimulus. Indeed, the stimulation index was greater than two at every time point and kept increasing with

each dose of vaccine administered. As it can be seen in Figure **1**, the Spearman's Rho correlation coefficient is 0.829 with an associated $p \leq 0.05$ ($p = 0.042$), showing a strong lineal correlation between the number of vaccine doses and the stimulation index increase.
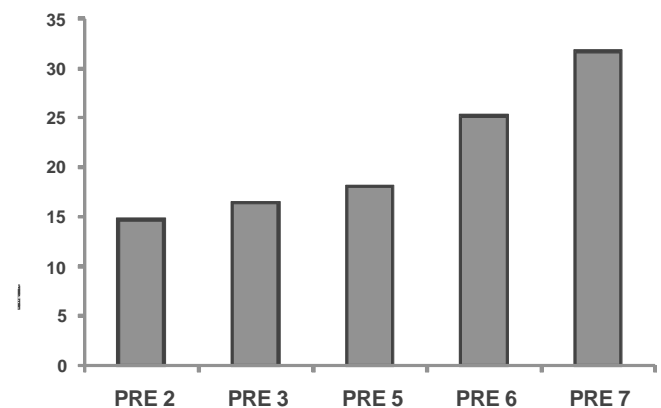

Figure 1: Vaccination of mature DC loaded with tumor antigens induces a tumor specific immune response. PBMCs were cocultured with DC pulsed with tumor lysate for 5 days. Afterwards, cells were pulsed with [3H]-thymidine in order to measure its incorporation. Data are reported as stimulation index. The stimulation index is defined as the ratio between the immune response detected against the dendritic cells pulsed with patient's tumor lysate versus the immune response detected in the absence of stimulus. Legend: PRE indicates that samples were obtained prior to the vaccination indicated by the following number (e.g. PRE 2 indicates that the sample was obtained before the second vaccine dose was administered).

DISCUSSION

The relationship between the immune system and tumor development is well documented [13]. In general,

tumor growth is often caused by an imbalance between the mechanisms responsible of generating anti-tumor immune responses and those promoting the tumor immune escape. As it was previously described [10, 11], DC pulsed with autologous tumor lysate can serve as good tool to immunize hosts against tumors by eliciting strong and long-lasting immune responses. We believe that vaccination protocols based in DC pulsed with autologous tumor lysate should be able to cover the tumor antigen spectrum more widely than those in which DC are either pulsed with one or few known antigens, or transformed with an exogenous DNA or mRNA. Our data (Table **1**) show that the tumor lysate-pulsed DC that were administered to the patient were well matured and, in principle, featured optimal immunophenotypical conditions to function as excellent antigen-presenting cells. The results of the immune response assessment (Figure **1**) show that specific tumor T-cells proliferation increases with the number of doses. In particular, the correlation between vaccine doses and stimulation index increase (Table **2**) formally proves the biological efficacy of vaccine, that is its ability to elicit a tumor-specific immune response.

The only crucial question that this case report cannot formally answer is whether 30 months of disease-free survival in a patient experiencing his fifth relapse of metastatic colorectal cancer can have been caused and sustained solely by twelve biweekly cycles of chemo-immunotherapy according to the irinotecan and cetuximab regimen. The data from the literature seem to exclude it [14]. However, until large clinical trials are carried out to test our hypothesis, this remains a single case in which a high relapse risk patient is being maintained disease free now over two and a half years only by active immunotherapy. It is remarkable that, having received over and over again state of the art treatment without ever refusing resections at any reoccurrence of the disease, the patient never experienced a prolonged remission until our experimental vaccination strategy was started to prevent relapse. In fact, after the first five lines of combined modality treatments, the patient had been off therapy for 12, 15, 6, 10 and 10 months, respectively. Were we to assume that vaccination with autologous, tumor lysate-pulsed DC is in fact meaningless, then we would imply that the addition of cetuximab to irinotecan monochemotherapy has been able to keep the patient alive, disease-free and off therapy for at least 30 months. Needless to say that, precisely because this is highly unlikely and we cannot overemphasize the need for prudence, the patients keeps being closely watched through a strict radiological follow-up while receiving his quarterly vaccine boosts.

Our patient's disease history confirms that, after surgical resections, complementary treatment is needed. In principle, however, chemo-immunotherapy in which the immunotherapeutic component is passive, being represented by a monoclonal antibody, does not seem to assure long-term success. It may instead postpone tumor reoccurrence but, owing to its side effects and its costs, it cannot be used indefinitely. On the contrary, active immunotherapy like tumor lysate-pulsed DC vaccination, without being costless of course, is at least virtually deprived of any side effect. Moreover, it justifies more aggressive surgical de-bulking because the larger the amount of original tumor that is received, the greater is the number of vaccine doses that can be produced.

In the scientific literature, little is reported that may somehow resemble the results experienced by our patient [15]. However, what we have seen so far justifies the attempt to launch a formal clinical trial in order to assure that similar data may be reproduced in other cases of relapsing metastatic colorectal cancer. We are now actively working in this direction, hoping to expand on this extremely favorable, though limited experience.

Table 2: Tumor Specific Immune Response Increases with the Number of Vaccine Doses

Correlation				
			Stimulation index	**Number of vaccines**
Spearman's Rho	Stimulation index	Correlation coefficient	1.000	.829*
		Sig. (bilateral)	.	.042
		N	6	6
	Number of vaccines	Correlation coefficient	.829	1.000
		Sig. (bilateral)	.042	.
		N	6	8

*.Correlation is significant at level 0.05 (bilateral).
Nonparametric correlation value is provided by Spearman's Rho correlation coefficient and associated to a *p* value. Sig: significance (*p* value < 0.05).

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