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DNA vaccination against membrane-bound Kit ligand: A new approach to inhibiting tumour growth and angiogenesis

Cristina Olgasi [a], Patrizia Dentelli [a], Arturo Rosso [a], Alessandra Iavello [a], Gabriele Togliatto [a], Valentina Toto [b], Marcella Liberatore [b], Giuseppina Barutello [c], Piero Musiani [b], Federica Cavallo [c,*], Maria Felice Brizzi [a,*]

[a] *Department of Medical Sciences, University of Torino, Italy*
[b] *Aging Research Centre, G. d'Annunzio University, Chieti, Italy*
[c] *Molecular Biotechnology Center, University of Torino, Italy*

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Abstract A functional c-Kit/Kit ligand (KitL) signalling network is required for tumour angiogenesis and growth, and therefore the c-Kit/KitL system might well be a suitable target for the cancer immunotherapy approach. We herein describe a strategy that targets membrane-bound KitL (mbKitL) via DNA vaccination. The vaccination procedure generated antibodies which are able to detect mbKitL on human tumour endothelial cells (TECs) and on the breast cancer cell line: TSA. DNA vaccination, interferes with tumour vessel formation and transplanted tumour growth *in vivo*. Histological analysis demonstrates that, while tumour cell proliferation and vessel stabilisation are impaired, vessel permeability is increased in mice that produce mbKitL-targeting antibodies. We also demonstrate that vessel stabilisation and tumour growth require Akt activation in endothelial cells but not in pericytes. Moreover, we found that regulatory T cells (Treg) and tumour infiltrating inflammatory cells, involved in tumour growth and angiogenesis, were reduced in number in the tumour microenvironment of mice that generate anti-mbKitL antibodies. These data provide evidence that mbKitL targeted vaccination is an effective means of inhibiting tumour angiogenesis and growth.

1. Introduction

Complex tumour-microenvironment interactions are known to drive tumour progression [1]. Several cell types involved in these processes express c-Kit, the receptor for the c-Kit ligand (KitL), and these include

* *Corresponding authors:* Address: Molecular Biotechnology Center, University of Torino, Via Nizza 52, 10126 Torino, Italy (F. Cavallo). Address: Department of Medical Sciences, University of Torino, Corso Dogliotti 14, Italy (M.F. Brizzi).
E-mail addresses: federica.cavallo@unito.it (F. Cavallo), mariafelice.brizzi@unito.it (M.F. Brizzi).

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endothelial cells (ECs) and pericytes [2,3]. KitL is expressed in two variants; a membrane bound (mbKitL) and a soluble (sKitL) form [4]. The soluble form contains an extracellular proteolytic cleavage site which permits the release of KitL from the cell surface [4]. Conversely, the spliced variant is not cleaved in humans and remains associated with the cell surface (mbKitL). mbKitL, unlike the soluble form which hastily activates the receptor and decays after prompt receptor internalisation, induces a more persistent signal [5,6]. c-Kit binding to the mbKitL expressed by bone marrow (BM)-derived stromal cells (SCs), supports stem cell survival *in vivo* [7]. Likewise, mbKitL, expressed by tumour ECs (TECs) [8], is crucial for survival signals in tumour vasculature [9,10]. A substantial body of data corroborates the involvement of paracrine/autocrine stimulation, via the c-Kit/KitL system, in cancerogenesis [11]. Recently it has been shown that c-Kit grades breast cancer cells including cells from Brca1-mutation-associated breast cancer [12]. These observations support the fact that the c-Kit signalling network is crucial not only to hematopoietic and vascular cells, but also to transformed mammary epithelial cells, and thus makes this system an ideal target for unconventional therapeutic strategies.

Targeted therapies that specifically inhibit the molecules involved in tumour progression have already been exploited in clinical settings [13,14]. However, tumours that are initially responsive to targeted therapies generally acquire resistance [15]. Immunotherapies targeting neoplastic lesion driving antigens have offered an alternative option [16,17]. However, the rate of tumour growth often exceeds immune system capabilities and the tumour microenvironment does not allow the local recruitment of immune cells and orchestrates a number of immunosuppressive activities, including the recruitment of a specific subpopulation of CD4+/CD25+ T lymphocytes called regulatory T cells (Treg) [18]. In addition, other genetic and epigenetic changes in tumour cells (TCs) and in the microenvironment commonly occur in advanced tumours following the initial genetic shift [16,17]. The adaptive immune response activated by vaccination against antigens expressed by a clinically evident tumour, is faced with genetic instability causing the selection of heterogeneous tumour clones and the reappearance of TCs that elude the immune system [16,17]. Therefore, targeting tumour antigens is unlikely to guarantee lasting tumour growth control. An alternative approach is the targeting of antigens expressed by SCs [19–21], crucial drivers of tumour drug resistance [22]. Therefore, if the target molecule expressed by TCs is also aberrantly expressed by SCs and contributes to tumour progression, immunotherapy approaches should be more effective.

The aim of this study is to investigate the impact of DNA mbKitL targeted vaccination on tumour angiogenesis and growth.

2. Materials and methods

2.1. Immunisation

The human mbKitL cDNA sequence (membrane domain of the KitL) was amplified by polymerase chain reaction (PCR) as a *HindIII/XhoI* fragment using the following primers; 5′ AGC TAA ACGGAT TCG CCA CAC C 3′ (sense) and 5′ ATA CTC GAG CTA CCA GTA TAA GGC 3′ (antisense). The resulting PCR product was verified by DNA sequencing and subcloned into the *HindIII/XhoI* restriction sites of a pVAX1 vector (Invitrogen, Carlsbad, CA, USA) to generate pVAX-mbKitL. 8–10-week-olds Balb/c mice (Charles River Laboratories, Calco, Italy) were immunised every 2 weeks for a total of three doses via intradermal (i.d.) injection of 25 μg of pVAX-mbKitL or the pVAX empty vector or left untreated as controls. The injection solutions were made up with 20 μl of 0.9% NaCl + 6 mg/ml polyglutammate. Immediately after the injection two 25-ms low voltage electric pulses were generated at the injection site. Pulse amplitude was set at 150 V and a 300 ms interval was used between the pulses. Two non-penetrating plates of 30 mm in linear length were connected to the electroporator (Cliniporator™, IGEA s.r.l. (Società a Responsabilità Limitata), Carpi, Italy). Mice were treated in conformity with European Guidelines and policies as approved by the University of Turin Ethics Committee.

2.2. Tumour challenge

To assess the effects of DNA vaccination on tumour growth untreated, pVAX and pVAX-mbKitL vaccinated mice were inoculated subcutaneous (s.c.) with 5×10^5 TSA cells. Tumour size was measured every 3–4 days and calculated using the formula; $V = 4/3\pi \times (d/2)^2 \times (D/2)$ (d = minor tumour axis; D = major tumour axis) and reported as tumour mass volume (mm^3, mean ± SEM of individual tumour volume/each mouse per cohort) obtained after 35 days. Mice were killed when the tumour exceeded 800 mm^3 [23]. Log-rank test/Kaplan–Meier survival plots for this study display the probability of survival as the percentage of mice whose tumours did not exceed 2000 mm^3 on the indicated days.

2.3. Histology, immunohistochemistry and immunofluorescence

Cryostatic sections of tumour samples were stained with haematoxylin/eosin while others were processed for immunohistochemistry or for immunofluorescence to evaluate; vessel and pericyte staining, microvessel and pericyte counts, in situ proliferating cells, inflammatory cell and Treg infiltration and vessel permeability.