REPORT

The 7A7 antibody: a new tool for the preclinical evaluation of anti-metastatic therapies against the epidermal growth factor receptor

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ABSTRACT

The epidermal growth factor receptor (EGFR) is considered one of the main targets for the treatment of epithelial tumors. Growing evidence shows the essential role of this receptor in disseminating metastasis. The Center of Molecular Immunology (CIM) has pioneered the development of this new therapeutic concept, generating different anti-EGFR agents that are currently under preclinical and clinical evaluation. One of these agents is the h-R3 monoclonal antibody (MAb h-R3), used to treat patients with metastatic tumors. The mechanisms by which the anti-EGFR MAbs exert their anti-metastatic effect is not yet clear, since studies have been made in athymic mice transplanted with human tumors, where the role of T cells cannot be characterized. We obtained here for the first time a MAb that can recognize the extracellular domain of the murine EGFR, named 7A7. It allowed us to confirm the potent anti-metastatic effect of anti-EGFR MAbs in immunocompetent mice. The EGFR signaling inhibition, mediated by anti-proliferative, anti-migratory and pro-apoptotic effects contributes to its anti-metastatic effect. Also, these results suggest the induction of cytotoxic responses through the Fc region of 7A7 could increase its anti-tumor effect. This was also the first report of a MAb that depends on $CD4^+$ and $CD8^+$ T cell functions to exert its anti-metastatic effects.

Introduction

In spite of the current advances in medical science, cancer is still a major health problem in the new millennium. The formation of metastatic niches is the main cause of cancer mortality. Metastasis is a sequential process, beginning with tumor cells that spread from the primary tumor, circulate in the blood and ultimately colonize distant organs. For this reason, the therapeutic agents available may not be as effective as needed in controlling metastasis. The epidermal growth factor receptor (EGFR) and its ligands are expressed in almost all cell types in the body, with the exception of hematopoietic cells, while they have been found to be highly expressed in most of the human epithelial tumors [1]. The autocrine and paracrine activation pathways involving these proteins have been shown to increase tumor cell growth, survival and metastatic potential in preclinical studies [2]. Potent and selective signaling antagonists through the EGFR have been identified from these observations, most of which are currently under clinical assessment [3]. They include the monoclonal antibodies (MAbs) that are specific for this receptor, which were evaluated in patients with metastatic tumors, with encouraging results [4]. One of them, the h-R3 MAb, was developed by the Center of Molecular Immunology and registered in Cuba at the Center for the State Control of Drug Quality (CECMED), to be used in patients with advanced head and neck tumors [5].

The mechanisms involved in the anti-metastatic activity of anti-EGFR agents have, however, been only partially dilucidated. The main drawback in these studies is that they were carried out in athymic mice transplanted with human tumors. In these models, it was possible to determine how the blocking of the EGFR by MAbs contributed to the anti-metastatic effect, but it was impossible to know if T cell-dependent immunological mechanisms were involved or not. This drawback was especially relevant in the light of recent reports showing that the anti-tumoral action of certain MAbs are mediated by cells from the adaptative immune system, called the "vaccine effect" [6, 7]. In fact, results from clinical trials using the h-R3 MAb indicate that the development of the clinical response to this MAb could take several months, suggesting that effectors of adaptive immunity could be involved [5, 8].

This significant shortcoming in the state of the art led us to develop new models to study the anti-metastatic ability of anti-EGFR MAbs and the mechanisms involved.

Results

In order to study the anti-metastatic effect of the anti-EGFR MAbs and their mechanisms in immunocompetent mice, we generated for the first time a MAb that specifically recognized the extracellular domain of the murine EGFR (DEC-EGFRm). This IgG subclass 1 MAb, named 7A7, was obtained by immunizing Balb/c mice with the recombinant DEC-EGFRm protein (rDEC-EGFRm) formulated in Freund adjuvants. Its specificity for recognition was verified by measuring its ability to immunoprecipitate the rDEC-EGFRm protein from culture supernatants of HEK

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293 cells expressing the recombinant protein. Additionally, the 7A7 MAb was able to recognize the complete receptor in Western blot and flux cytometry assays, detecting EGFR-positive tumor cells such as those of Lewis' lung carcinoma metastatic variant 3LL-D122. Moreover, the 7A7 recognized the native EGFR in immunohistochemistry assays using skin sections and EGFR-positive tumors from Balb/c mice [9].

MAbs against the human EGFR have being evaluated in patients with advanced tumors of different origins, including lungs. This is one of the most deadly types of cancer worldwide. Only one out of ten patients diagnosed with lung cancer survives the following 5 years. The h-R3 MAb has been used in compassion studies for non-small lung cancer patients, improving their survival. We used 7A7 MAb dosages in the range of those employed for anti-EGFR in cancer patients (56 µg, equivalent to the 200 mg dose used in human clinical trials) to reproduce the clinical setting, and worked with an experimental metastasis model by intravenously administering the cancer cells for a successful lung metastasis in mice. The 7A7 MAb generated a significant anti-metastatic effect on the established 3LL-D122 lung metastasis (Figure 1) [10].

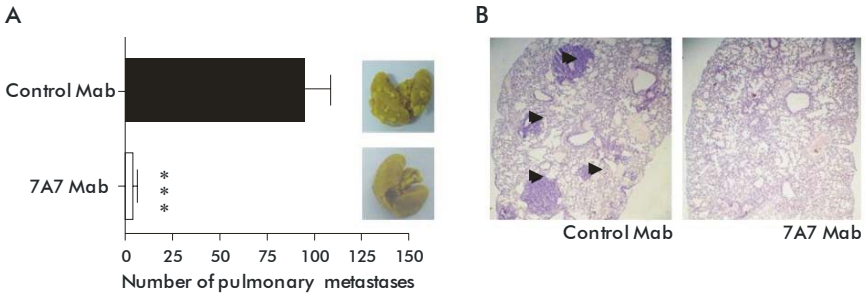

Figure 1. Anti-metastatic effect of the 7A7 MAb on 3LL-D122 tumor cells. (A) C57BL6 mice were inoculated on day 0 with 3LL-D122 cells by the intravenous route. The 7A7 or control MAbs were administered to mice by the same route, three times a week starting on day 6 after tumor inoculation. Mice were sacrificed on day 21 and the number of lung metastases was determined. Each bar represents the mean ± standard deviation for 10 mice per treatment (7A7 MAb *vs* control MAb: ***$p < 0.001$, Mann-Whitney U test). The graphic represents two independent experiments. A lung representation for each treatment is also shown. (B) Pulmonary lobes from mice treated either with the control or the 7A7 MAbs were stained with eosin and hematoxilin as previously described. The black arrowheads indicate metastatic nodes (40x). The pictures are representative slices of three separate staining experiments.

It has been demonstrated that the anti-EGFR antibodies block cell cycle progression and induce apoptosis in multiple tumor cell lines. The 7A7 MAb was able to inhibit intracellular signaling pathways involved in cellular proliferation and the anti-apoptotic stimuli such as those of the mitogen-activated protein kinases, the signal transducer and activator of transcription 3 and phosphatidylinositol 3-kinase pathways. It was also demonstrated here that this MAb induced an anti-proliferative effect in 3LL-D122 cells cultured in its presence, arresting cells in the G0-G1 transition. Furthermore, the treatment with 7A7 also induced apoptotic death in 3LL-D122 cells, among other programmed cell death mechanisms. The effects mentioned above were verified in 3LL-D122 lung metastasis, using a repeated treatment with 7A7 and provoking mitotic arrest, followed by nuclei condensation and the appearance of multiple apoptotic bodies, at levels similar to those reported for the *in vivo* treatment of murine carcinomas with taxol [10]. These results demonstrate that cell growth inhibition and apoptosis induction can contribute to the anti-metastatic effect of the 7A7 MAb on the 3LL-D122 tumor.

Many studies reveal an association between an increased EGFR expression and the metastatic capacity of certain tumors. The molecular mechanisms involved in this relationship are not completely dilucidated but there are reports that point to the MAPK pathway. Here we demonstrated that the 7A7 MAb completely decreased phosphorylation by the main MAPK kinases, also inhibiting the chemostatic migration of 3LL-D122 cells [10]. These results suggest an additional therapeutic effect of blocking the EGFR, which could be very relevant for the anti-metastatic activity of the 7A7 MAb.

The induction of complement-dependent cytotoxicity (CDC) by anti-EGFR MAbs has not yet been characterized in patients. *In vitro* experiments showed the ability of 7A7 MAb to mediate autologous CDC in 3LL-D122 cells [11]. The involvement of complement activation in the action of anti-EGFR MAbs should be verified *in vivo*, since the increased expression of certain complement decay factors have been reported as a CDC escape mechanism. In fact, our studies have revealed the resistance to complement activation as a tumor escape mechanism to the therapy with the 7A7 MAb [11].

Antibody-dependent cell cytotoxicity (ADCC) is one of the most relevant effector mechanisms for MAbs that are used to treat cancer patients. Various studies suggest that anti-EGFR MAbs could increase toxicity in tumor cells through ADCC activation in humans, without *in vivo* confirmation. These results confirm the potent ADCC induced by the 7A7 MAb on 3LL-D122 cells *in vitro*. The relevance of this mechanism in the anti-metastatic effect obtained was verified by depleting the population of natural killer (NK) cells during the administration of the 7A7 MAb. Under these conditions, the anti-metastatic effect remained unaltered (Figure 2A). A noteworthy result is that the anti-metastatic activity of the 7A7 MAb is able to revert the pro-metastatic effect obtained by depleting NK cells [10]. This result demonstrated that NK cells do not contribute to the anti-metastatic activity of the 7A7 MAb, although other immune cell populations cannot be excluded from such an effect. In fact, the depletion of $CD8^+$ and $CD4^+$ cells during the treatment with this MAb completely blocked its anti-metastatic effect over 3LL-D122 tumors (Figure 2 B and C) [10].

Further studies are, however, required to understand this phenomenon. A considerable amount of recent considerations support the assumption that anti-tumoral therapy that induce apoptosis could activate the acquired immunity. Apoptotic death has for long been considered as tolerogenic or non-immunogenic, by occurring in the absence of "danger signals". Nevertheless, it is currently established that it does not remain undetectable to the immune system. In certain situations, apoptosis is accompanied by "inflammatory signals" which activate potent immune responses. This is the case for the so called massive apoptosis, also named "secondary necrosis", in which apoptotic cells secrete significant amounts of endogenous danger signals activating dendritic cells (DCs). A plausible hy-

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pothesis is that of high levels of apoptosis induced by the 7A7 MAb at the metastatic site promoting the formation of an "inflammatory environment". Through this process, DCs which capture apoptotic bodies will become activated, presenting tumor peptides in the major histocompa-tibility complex class I molecules. This is a possible mechanism for generating tumor-specific CD8+ T cells by using 7A7 MAb.

These results confirmed that anti-EGFR MAbs are effective drugs for metastatic tumor treatment, offering new information on mechanisms controlling tumor dissemination. In this sense, the relevance of blocking the EGFR and inducing cytotoxic responses to reinforce the anti-metastatic effect of these agents was confirmed, and the first reports are given involving effector cells of the adaptative immune system in this effect. Particularly, these studies suggest that the anti-metastatic effect of the 7A7 MAb depends on the action of $CD4^+$ and $CD8^+$ T cells. It is crucial to validate these results in cancer patients treated with the h-R3 MAb.

Furthermore, these studies confirmed the use of 7A7 MAb as a tool for selecting EGFR-positive murine tumor models. This MAb could also be used to model therapeutic combinations of anti-EGFR MAbs with other anti-EGFR agents, conventional therapies or immunomodulatory agents, in immunocompetent mice. The possibility of carrying out these evaluations would allow us to extrapolate results from experiments in animals into the clinical setting.

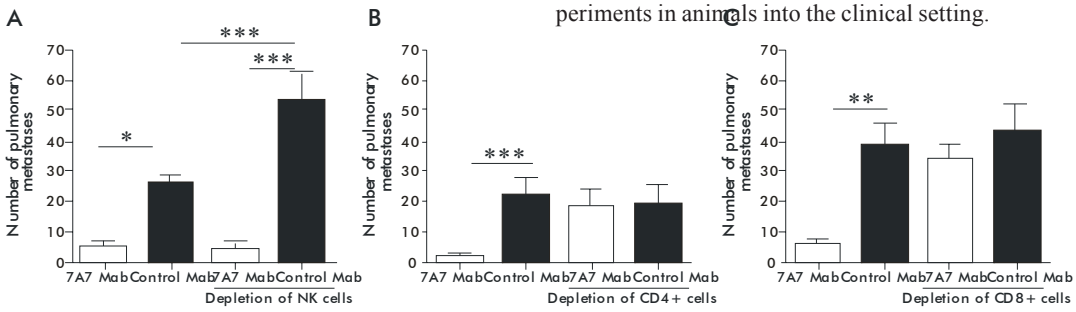

Figure 2. Effect of NK 1.1^+, $CD4^+$ and $CD8^+$ cells depletion on the anti-metastatic potential of the 7A7 MAb. C57/BL6 mice were intravenously inoculated with 3LL-D122 tumor cells. The 7A7 or control MAbs were administered to mice by the same route, three times a week starting on day 6 after tumor inoculation. Mice were simultaneously administered for four days with MAbs depleting the subsets of (A) NK 1.1^+, (B) $CD4^+$ or (C) $CD8^+$ cells. The animals were sacrificed on day 21 and the number of lung metastases was determined. Each bar represents the mean ± standard deviation for 10 mice per treatment (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Dunn's test). The results shown are representative of three separate experiments.