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Targeting CD99 in association with doxorubicin: An effective combined treatment for Ewing's sarcoma

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ABSTRACT

CD99 is a 32 kDa surface glycoprotein that is involved in the migration of leukocytes, cell–cell adhesion and apoptosis of T cells and Ewing's sarcoma (ES) cells, two cell types with a high level of CD99 expression. Engagement of the molecule induces a rapid death signal that appears to be related to the level of expression of this antigen. The rapid apoptosis induced by agonistic anti-CD99 monoclonal antibodies is of clinical interest in ES, a tumour for which no new drugs have been described as clearly effective in the last 10 years. In this study, we show that an anti-CD99 monoclonal antibody can be used to advantage in association with doxorubicin. Striking effectiveness was observed against local tumours and metastases. No remarkably toxic effects of anti-CD99 monoclonal antibody were found in bone marrow against blood precursors. These results provide the necessary rationale and support for a novel modality of therapeutic intervention, which may have application in the care of patients with ES.

1. Introduction

Ewing's sarcoma (ES), the second most common malignant bone tumour, is an extremely aggressive neoplasm, occurring mainly in children and adolescents [1]. Combination chemotherapy with surgery or radiotherapy has become standard practice in the treatment of patients with ES and the use of these multi-modal treatments has increased the survival rate of ES patients with localised disease to 65–70% after 5 years [2,3]. However, the most recent improvements in the cure rate of these patients have been achieved by dose-intensification, therefore paying the price of severe toxicity and high rate of life-threatening late events, such as secondary malignancies [4]. This poses serious questions about quality of life, due to the young age of the patients and their long life expectancy. In addition, 25% of patients have metastases at the time of diagnosis, and for this high-risk group the survival rate at 5 years is still as low as 20% [5]. No new effective drugs have been described and proposed for sarcomas recently and, therefore, innovative treatment modalities are needed. In this respect, a new entry site for therapeutic intervention may derive from tailored therapies against CD99 [6], a cell surface transmembrane protein that is highly expressed in ES. Engagement of CD99 induces massive apoptosis of ES cells through

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caspaseindependent mechanisms and reduces their malignant potential [7–9]. Since the apoptotic functions of this molecule are of potential clinical interest, in this study we analysed the effects of a tailored therapy triggering CD99 in combination with doxorubicin (DXR), a leader drug in the treatment of sarcomas. Evidence is found for the pre-clinical effectiveness of these combined treatments against ES local tumours and distal (lung and bone) metastases in athymic mice and, by assessing the potential toxicity of anti-CD99-based therapies against normal bone marrow precursors, we offer the necessary rationale for the application of possible forthcoming clinical trials.

2. Materials and methods

2.1. Cell lines

The ES cell line SK-ES-1 was obtained from the American Type Culture Collection (Rockville, MD, United States of America (USA)). The ES cell line 6647 was a generous gift from T.J. Triche (Childrens Hospital, Los Angeles, CA, USA). Cells were routinely cultured in Iscove's modified Dulbecco's medium (IMDM) (Life Technologies Inc., Paisley, UK), supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 10% inactivated foetal bovine serum (FBS) (Biowhittaker Europe, Verviers, Belgium) and maintained at 37 °C in a humidified 5% CO_2 atmosphere.

2.2. Monoclonal antibodies and reagents

The anti-CD99 0662 monoclonal antibody (MAb) was produced in the Unité INSERM 343, Hopital de l'Archet, Nice, France, and clusterised during the Human Leukocyte Differentiation Agents International Workshop in 1989 and 1993 [10]. DXR was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. In vitro growth inhibitory effects of combined treatments with anti-CD99 0662 MAb and DXR

A total of 100,000 cells were seeded in 24-well plates in IMDM plus 10% FBS. After 24 h, cells were treated with varying concentrations of DXR (range 10 pg/ml to 10 ng/ml) without (control) or with anti-CD99 0662 MAb (1 μg/ml). As an additional control, an irrelevant antibody (MOPC-21, Sigma) (1 μg/ml) was also used. After 72 h of treatment, cell growth was evaluated on harvested cultures by trypan-blue vital cell count. For sequential treatments, two different experimental set-ups were used. In the first, cells were pre-treated with 1 μg/ml anti-CD99 0662 MAb for 12 h, a time corresponding to the in vitro doubling time of SK-ES-1 or 6647 cells, and then exposed to increasing doses of DXR for 48 h. In the second, cells were first exposed to DXR and then to 0662 MAb using the same schedule.

2.4. In vivo treatments with anti-CD99 0662 MAb alone or in combination with DXR

Female athymic 4–5-week-old Crl:CD-1nu/nuBR mice (Charles River Italia, Como, Italy) were used. Tumourigenicity and tumour growth were determined after subcutaneous (s.c.) injection of 5×10^6 cells. Two different treatment schedules were considered. In the first, treatments started 24 h after ES cell inoculation. The animals were randomised into controls and three treated groups. In the group treated with anti-CD99 MAb alone, each mouse received s.c. injections of 0662 MAb (50 μg/injection) in the proximity of the tumour for each day. The second group received DXR intraperitoneal (i.p.) (50 μg/injection) on the first and the second day after cell seeding. The third group received 8 s.c. injections of 0662 MAb (50 μg/injection) for 8 d and i.p. injection of DXR (50 μg/injection) on the first and on the second day after cell seeding. Control mice received s.c. injections of PBS or the irrelevant MOPC-21 MAb (50 μg/injection; 8 injections). Tumour growth was assessed once a week by measuring tumour volume, calculated as $\pi/6 \times [\sqrt{(ab)}]3$, where a and b are the two maximum diameters. In the second treatment schedule, the effectiveness of the treatments was also assessed against established tumours. Mice were randomised into control and treated groups when tumours started to be measurable (7 d after cell inoculation). Experimental procedures were as described previously, with the only difference regarding the dose of anti-CD99 0662 MAb that was used (250 μg/injection). To evaluate the ability of the 0662 MAb alone or in association with DXR to inhibit the metastatic ability of 6647 cells, 2×10^6 viable 6647 cells were injected intravenously (i.v.) into a tail lateral vein; 24 h later animals were divided into four groups of 10 animals each: the first group was treated with anti-CD99 0662 MAb (50 μg/injection i.p., 8 times); the second group received an higher dose of anti-CD99 0662 MAb (200 μg/injection i.p., 8 times); the third group corresponded to control group (PBS i.p., 8 injections). For combined treatments anti-CD99 0662 MAb (200 μg/injection i.p., 8 times) was administered in association with DXR (50 μg/injection i.p., day 1 and 2 after cell inoculation). The volume of bone metastases was assessed weekly, as described above for primary tumours. For ethical reasons, mice with local tumours/bone metastases were killed when they achieved a tumour volume of 5 ml. Mice that did not display evident bone metastases were killed 3 months after cell inoculation and necropsied. Spontaneous and experimental lung metastases were determined by counting the metastatic foci in the lungs with a stereomicroscope after staining with black India ink.

2.5. Toxicity assay on haematopoietic progenitors

A total of 2×10^4 CD34+ cells were pre-incubated for 30 min or 24 h with 10 μg/ml anti-CD99 0662 MAb and then cultured in selected media consisting of IMDM supplemented with 20% FBS, 10^{-4} mol/l 2-mercaptoethanol (Sigma), 0.2 mmol/l bovine haemin (Sigma), 0.8% BSA (Sigma), 2 U/ml recombinant human erythropoietin (EPO) (Dompè Biotec, Milan, Italy), 50 ng/ml stem cell factor (SCF) (Amgen, Thousand Oaks, CA, USA), 10 ng/ml granulocyte-macrophage CSF (Immunex, Seattle, WA, USA) and 10 ng/ml interleukin-3 (Immunex). Human colony-forming cells (CFU) were cultured in methylcellulose (final concentration 1.32%). CFU-granulocyte-macrophage (CFU-GM), burst-forming units erythroid (BFU-E) and multilineage colonies (together referred to as CFU-C), were scored after 14 d of incubation at 37 °C in a fully humidified 5% CO_2 atmosphere.

2.6. Statistical analysis

Differences among means were analysed using Student's t-test. Fisher's exact test was used for frequency data. The analysis of drug combination effects was performed using the fractional product method. Kaplan–Meier and log-rank methods were used to draw and evaluate the significance of event-free survival curves.

3. Results

3.1. In vitro effects of combined treatments

In vitro studies were performed to identify the best drug–drug interaction schedules. In particular, the simultaneous combination of anti-CD99 0662 MAb with increasing concentrations of DXR as well as sequential treatments in which cells were exposed initially to anti-CD99 0662 MAb and then to DXR resulted in synergistic inhibition of 6647 or SK-ES-1 ES cell growth with respect to the therapeutic efficacy of DXR or anti-CD99 MAb used as single agents (Fig. 1A and B). Conversely, when cells were first exposed to DXR followed by anti-CD99 MAb, we observed an advantage with respect to DXR alone but not with the anti-CD99 MAb and the general effect of the two compounds was antagonist according to the fractional product method and the Chou–Talalay equation [11] (Fig. 1C). The in vitro inhibitory effects occurred at clinically relevant drug concentrations. The IC50 value of DXR, the dose of the drug that determines a 50% of growth inhibition, showed a 19–76-fold range decrease in 6647 and SK-ES

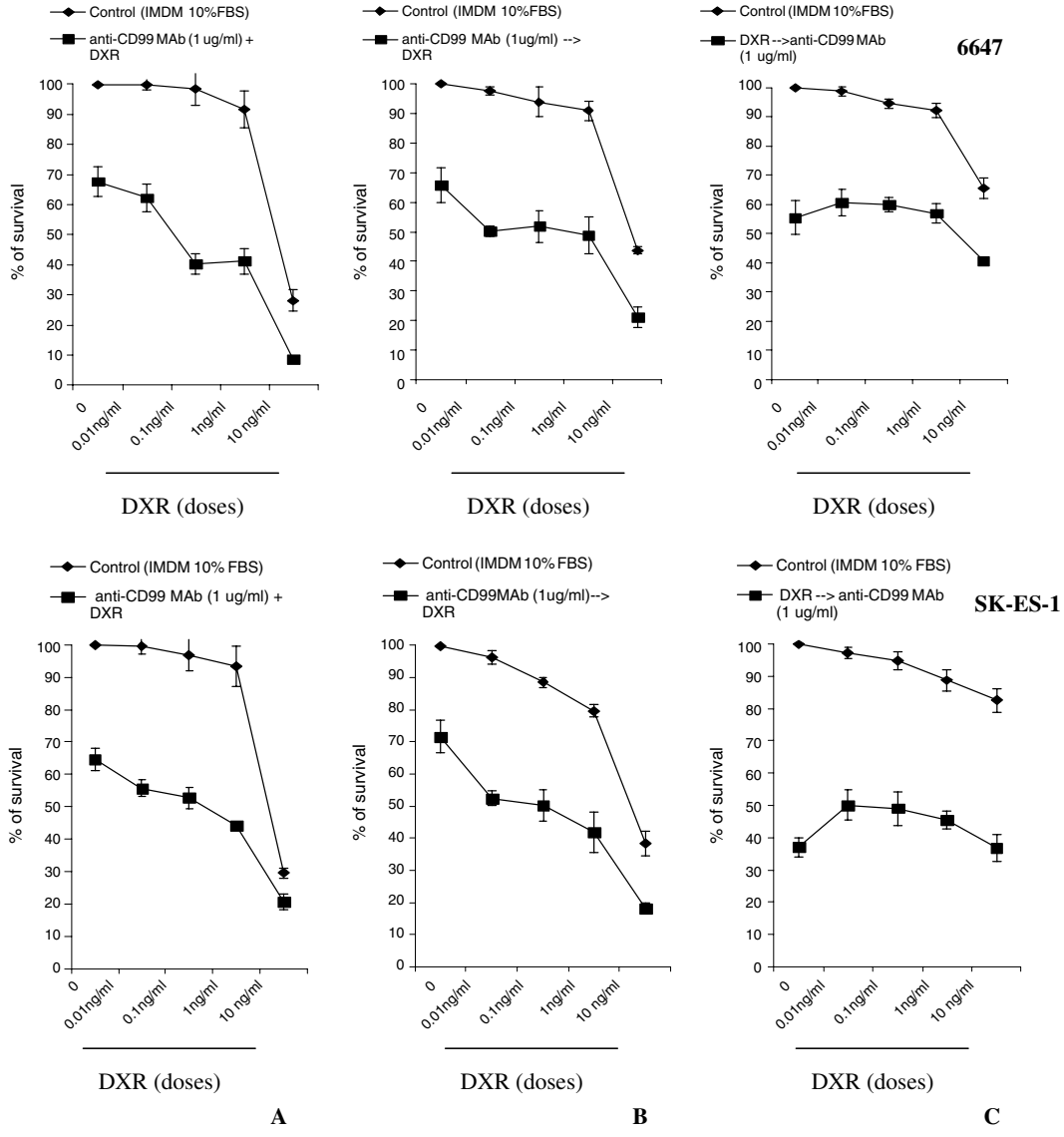

Fig. 1 – Inhibitory effects of doxorubicin (DXR) in combination with anti-CD99 monoclonal antibody (MAb) (1 μg/ml) after (A) simultaneous or (B,C) sequential treatments. Results represent the means ± SE of duplicate or triplicate experiments. For sequential treatments, two different experimental set-ups were used. In the first (B), cells were pre-treated with 1 μg/ml anti-CD99 0662 MAb for 12 h, a time corresponding to the in vitro doubling time of SK-ES-1 or 6647 cells, and then exposed to increasing doses of DXR for 48 h. In the second (C), cells were first exposed to DXR and then to 0662 MAb using the same schedule.

cells when cells were simultaneous treated with anti-CD99 MAb and DXR or when cells were pre-treated with anti-CD99 MAb (combined treatments: 5.3 ng/ml versus 278 pg/ml or 4.3 ng/ml versus 185 pg/ml in 6647 or SK-ES-1 cells, respectively; sequential treatments: 7.6 ng/ml versus 100 pg/ml or 6.4 ng/ml versus 225 pg/ml in 6647 or SK-ES-1 cells, respectively). The increased anti-tumour activity of DXR was not observed when the irrelevant MOPC-21 MAb, an isotype-matched MAb, was used (data not shown).

3.2. *Anti-tumourigenic and metastatic effects of combined treatments*

The inhibitory effects of anti-CD99 0662 MAb alone or in association with DXR was also investigated against xenograft growth in nude mice after s.c. injection of 5×10^6 6647 ES cells (Fig. 2). Animals were treated 24 h after cell injection or when tumours started to be measurable (7 d after cell inoculation). In the first group of experiments, we observed a highly significant inhibition of ES growth when mice were treated with anti-CD99 MAb alone and a complete remission when mice were simultaneously treated with anti-CD99 MAb and DXR. Indeed, at the end of the treatment period, the percentages of tumour-free mice were 10% in the control group, 0% in the MOPC-21 MAb group, 20% in the DXR group, 80% in the anti-CD99 0662 MAb group and 100% in the group treated with anti-CD99 0662 MAb and DXR ($P < 0.001$ with respect to controls; $P < 0.05$ with respect to DXR alone, Fisher's exact test). Moreover, while some other tumours developed in controls and single agent-treated mice after the end of treatments,

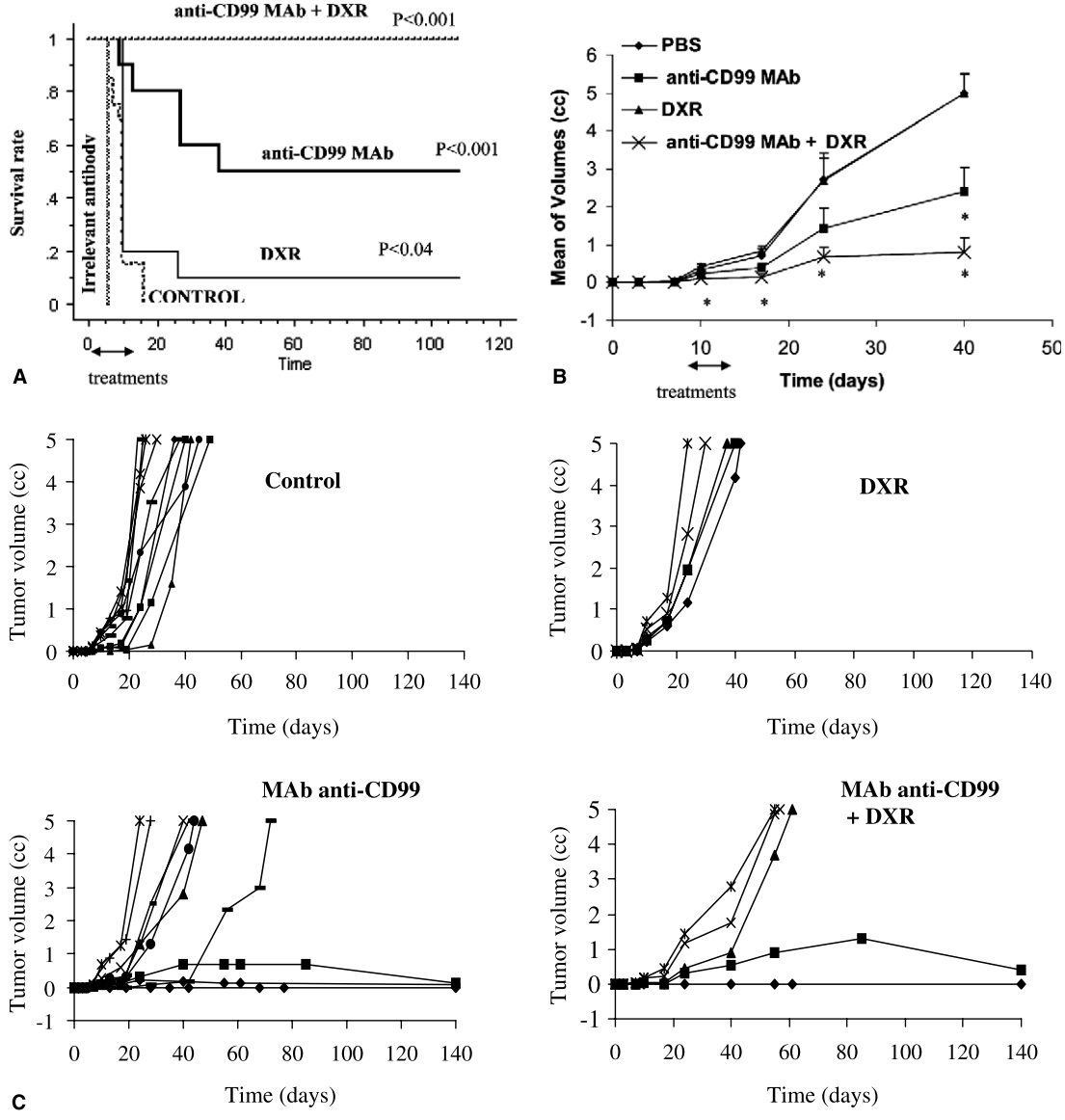

Fig. 2 – Effects of anti-CD99 0662 monoclonal antibody (MAb) alone or in combination with doxorubicin (DXR) on growth of 6647 Ewing's sarcoma (ES) cells injected s.c. into athymic mice. (A) Survival curves of mice treated with anti-CD99 MAb and/or DXR a day after cell inoculation. Kaplan–Meier curves and log-rank test; $n = 20$ for control and 10 for treated groups. (B,C) Mean tumour volume and growth curve of 6647 tumours in mice treated with anti-CD99 MAb and/or DXR when tumours started to be measurable (7 d after cell inoculation). In growth curve graphs each line corresponds to a single animal.

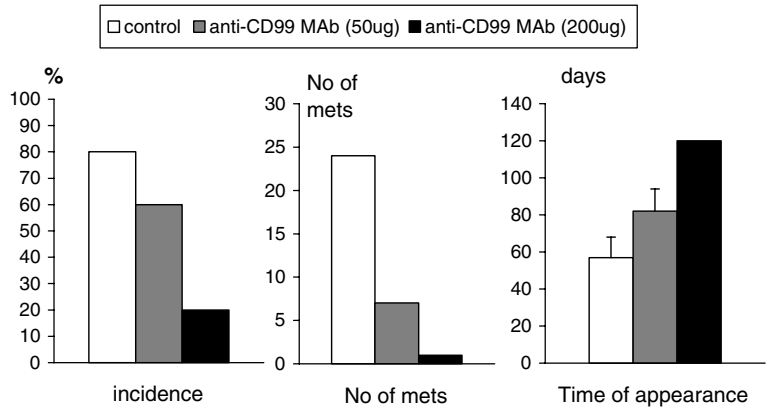

Fig. 3 – Dose-dependent effect of anti-CD99 monoclonal antibody (MAb) against bone metastases. Reduction in the incidence and number of metastases (median is indicated) as well as delayed time appearance was observed after systemic treatments with anti-CD99 MAb ($P < 0.05$).

Table 1 – Number of colonies obtained after treatment of CD34+ cells with anti-CD99 monoclonal antibody (MAb) (10 μg/ml)

Colonies	Control	Treatments	
		Anti-CD99 MAb (30 min)	Anti-CD99 MAb (24 h)
CFU-GM	148 ± 28	185 ± 2[a]	162 ± 40[a]
BFU-E	236 ± 77	314 ± 56[a]	239 ± 31[a]
CFU-C	394 ± 68	499 ± 54[a]	401 ± 61[a]

CFU-GM, CFU-granulocyte-macrophage; BFU-E, burst-forming units erythroid; CFU-C, colony forming units-cells.
a Not significant, Student's *t*-test.

the mice that received the combined treatments all remained tumour-free for at least 6 months. Survival curves of this first set of experiments clearly show the effectiveness of therapies triggering CD99 in association or not with DXR (Fig. 2A) ($P < 0.001$, Fisher's exact test). The efficacy of the combined therapy was also verified in a second set of experiments, in which treatments started when tumours begin to be measurable. A remarkable inhibition of the growth rate of 6647 ES tumours was observed. Significant differences in tumour size were observed among the different groups of animals until when control mice started to be sacrificed for achieving the tumour volume of 5 ml (Fig. 2B, $P < 0.05$, Student's *t*-test). In particular, while i.p. injections of DXR alone (50 μg/injection, for 2 days) induces a rather modest effect on local growth of ES xenografts, which never achieving statistical significance, mice treated with anti-CD99 MAb ± DXR showed remissions or stabilisation of tumour growth well after the end of the treatment period as indicated by the in vivo growth curve of 6647 tumours in individual mice (Fig. 2C). Systemic delivery of anti-CD99 0662 MAb alone or in combination with DXR also showed remarkable effects against metastatic potential of 6647 ES cells by reducing the number of metastases and delaying their appearance. A dose-dependent effectiveness was observed for anti-CD99 0662 MAb treatments. Fig. 3 shows the effects of 50 versus 200 μg/ml anti-CD99 MAb with respect to metastases. When the higher dose of anti-CD99 MAb was used in association with DXR (animals received anti-CD99 0662 MAb, 200 μg/injection i.p., 8 times, in association with DXR, 50 μg/injection i.p., day 1 and 2 after cell inoculation), neither bone nor lung metastases were observed.

3.3. *Toxic effects of anti-CD99 MAb on blood cell progenitors*

Since CD34+ cells of bone marrow were found to express CD99 [12], we investigated whether anti-CD99 targeted therapies had any toxic effects on blood cell progenitors, possibly by inducing their apoptotic death. CD34+ cells were pre-treated with 10 μg/ml anti-CD99 MAb, a dose that induces massive in vitro apoptosis of ES cells (8), and then incubated with specific growth mediums to check the formation of CFU-granulocyte-macrophage (CFU-GM), burst-forming units erythroid (BFU-E) and multi-lineage colonies (CFU-Mix) (together referred to as CFU-C) colonies. No significant variations were observed in the growth of these colonies (Table 1), therefore suggesting that the expression of CD99 in blood cell precursors is too low to induce the delivery of an apoptotic signal when the molecule is engaged by agonist antibodies.

4. Discussion

CD99 is a transmembrane glycoprotein with no homology with other known proteins except the Xga protein [13] that has recently been implicated in cell adhesion, apoptosis, differentiation of T cells and thymocytes [14,15], the migration of monocytes [16] and the intercellular adhesion between lymphocytes and endothelial cells [17]. In pathological conditions, a role for CD99 has been indicated in ES cells, with the delivery of cell–cell adhesion and apoptotic signals [7–9]. Although the molecular mechanisms responsible for CD99-mediated apoptosis are still poorly defined [9], the capability

of this antigen to induce massive and rapid apoptosis of ES cells after engagement by agonistic MAbs has an obvious therapeutic interest. In this study, we explored the pre-clinical effectiveness of combined treatments with anti-CD99 0662 MAb and DXR. Simultaneous administration of the drugs or the use of anti-CD99 MAb before DXR induced synergistic in vitro growth-inhibitory effects. In athymic mice, the combination of the two agents was remarkably effective against local growth and metastases. Accordingly, a significant increase in the survival of mice was observed. Systemic delivery of anti-CD99 0662 MAb significantly reduced the number of lung and bone metastases and increased the time of their appearance. Combined treatments completely abrogate the metastatic ability of 6647 cells. Thus, the anti-CD99 0662 MAb could be a useful adjuvant to cytotoxic chemotherapy for the treatment of ES patients. Because CD99 is highly expressed on the cell surface of T cells, thymocytes and many haematopoietic cell types, including CD34-positive cells [12] and its engagement is able to induce apoptosis of double positive T lymphocytes [13], we explored possible toxic effects of anti-CD99 MAb against blood cell precursors. In vitro analysis of growth of the different blood cell populations after exposure of CD34+ cells to a dose of anti-CD99 MAb that is effective against ES cells excluded an important toxic effect at bone marrow level, further supporting the use of anti-CD99 MAb in therapy for ES. However, since the expression of CD99 has been reported in a variety of other human tissues, such as testis, prostate and gastric mucosa, we cannot completely exclude hypothetical toxicity of anti-CD99 MAb in human ES patients. In addition, in the clinical scenario the anti-CD99 MAb will be used in combination with chemotherapeutic agents that have deleterious effects on the haematogenic progenitors. Therefore, this treatment modality merits further in vivo evaluation for what concerns toxicity in an appropriate model, prior to human studies.

In conclusion, we provide evidence for the pre-clinical effectiveness of combined treatments with anti-CD99 MAb and DXR against ES local tumours and distal metastases in athymic mice and, by assessing the potential toxicity of anti-CD99-based therapies against normal bone marrow precursors, we offer the necessary rationale for the application of a tailored therapy with high clinical potentialities that could be used to advantage in association with conventional agents.

Conflict of interest statement

None declared.

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