Delivery of the bacterial nitroreductase gene into endothelial cells prolongs the survival of tumour-bearing mice by bystander mechanisms

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Abstract. A current target of cancer gene therapy is tumour vasculature. We present a gene-directed enzyme prodrug therapy (GDEPT) approach to target tumours *in vivo* by modifying endothelial cells (ECs) with the *Escherichia coli* nitroreductase (*ntr*) gene. Firstly, we isolated two *ntr*-transfected clones of the human umbilical vein endothelial cell line (HUV-EC-C/*ntr*$^+$) that showed a differential sensitivity *in vitro* to the prodrug, dinitroaziridinylbenzamide (CB1954), with respect to untransfected HUV-EC-C cells (HUV-EC-C/*ntr*$^-$). Then, these cells were injected subcutaneously into nude mice, either in association with the murine melanoma cell line, B16-F10 ('co-injected' groups), or into tumour-bearing animals ('post-injected' groups). After intratumoural injection, we demonstrated, using PCR analysis, that human ECs resided in the site of the injection without spreading to other organs, such as the liver or lung. After the treatment of mice with CB1954, we observed a prolonged survival of animals carrying the HUV-EC-C/*ntr*$^+$ clones with respect to control animals injected with HUV-EC-C/*ntr*$^-$ cells. Significant differences in tumour growth were also observed and, after immuno-histological analysis, tumours carrying HUV-EC-C/*ntr*$^+$ clones showed large areas of tumour necrosis, probably due to tumour ischemia, as well as the presence of major histocompatibility complex class-II (MHC-II) positive cells. Collectively, our data indicate that targeting of the tumour vasculature by this GDEPT strategy may be an efficient approach for cancer treatment *in vivo*, depending on two possible bystander mechanisms based on tumour ischemia and immune cell activation.

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Abbreviations: BM, bone marrow; CMV, cytomegalovirus; ECs, endothelial cells; EPCs, endothelial progenitor cells; FCS, foetal calf serum; GCV, ganciclovir; GDEPT, gene-directed enzyme prodrug therapy; H-GAPDH-PG, human glyceraldehyde-3-phosphate dehydrogenase pseudogene; HSV/*tk*, herpes simplex virus-thymidine kinase gene; HUV-EC-C, human umbilical vein endothelial cell line; MHC-II, major histocompatibility complex class-II; MMPs, matrix metalloproteinases; *ntr*, *Escherichia coli* nitroreductase gene; s.c., subcutaneously; 3-D, three-dimensional; VEGF, vascular endothelial growth factor

Key words: antiangiogenic gene therapy, bystander effect, endothelial cells

Introduction

Tumour angiogenesis plays an important role in tumour development, vascular invasion and haematogenous metastasis. Hence, angiogenesis represents a major focus for novel therapeutic approaches to the treatment of cancer. During tumorigenesis, an angiogenic switch perturbs the local balance of proangiogenic and antiangiogenic factors (1), corresponding to molecules produced by tumour cells and the surrounding host tissue. Degradative enzymes, such as matrix metalloproteinases (MMPs), also contribute to tumour blood vessel development by destroying local tissue architecture and basement membranes to allow tumour invasion and metastasis (2).

Among the different strategies used in the development of antiangiogenic agents (3,4), gene therapy has been proved to allow the *in situ* expression of some of these angiogenic inhibitors (5,6). This provokes the reversion of the angiogenic switch preventing the growth of tumour vasculature. We and others recently demonstrated that the gene-directed enzyme prodrug therapy (GDEPT) approach, usually adopted to directly target cancer cells (7,8), can also be exploited for the selective delivery of cancer therapeutics to endothelial cells (ECs) (9,10). GDEPT only requires a fraction of the target cells to be genetically modified, providing that the resultant cytotoxic prodrug metabolites redistribute efficiently, by a phenomenon known as the bystander effect (11,12). This transfer of cytotoxicity to neighbouring non-targeted cancer cells is central to the success of the GDEPT-based strategy. It is well established that apoptosis, gap junction-mediated metabolic cooperation and the immune system are necessary components of the bystander effect observed with many GDEPT-based approaches (13,14). We recently demonstrated, in a three-dimensional (3-D) multicellular nodule model, that

the *ntr*-based GDEPT strategy is an efficient approach for cancer treatment, reliant on the spread of the apoptotis-mediated bystander effect from endothelial to tumour cells (9).

To our knowledge, this is the first report showing the efficacy of the GDEPT-based approach in prolonging tumour-bearing animal survival after delivery of the *ntr* gene to ECs and treatment with dinitroaziridinylbenzamide (CB1954), without inducing animal toxicity. Altogether, our results suggest that targeting of the tumour vasculature by the *ntr*-based GDEPT strategy would be an efficient approach for cancer treatment *in vivo*, depending on two possible bystander mechanisms based on both tumour ischemia and immune system activation.

Materials and methods

Cell cultures, plasmid, DNA transfection and cloning of HUV-EC-C/ntr⁺ cells. HUV-EC-C (CRL-1730) and B16-F10 melanoma (CRL-6475) cell lines were purchased from the American Tissue Culture Collection (Rockville, MD, USA). Cells were grown and maintained in DMEM medium supplemented with 10% heat-inactivated FCS, 0.3 mg/ml L-glutamine and 1% antibiotics (penicillin and streptomycin) at 37˚C in a 5% CO_2 humidified atmosphere. Plasmid DNA pxLNC-NR (kindly provided by D. Peter F. Searle, University of Birmingham, UK), carrying the *ntr* gene under control of the cytomegalovirus (CMV) promoter, was purified using the QIAfilter Plasmid Maxi kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Transfection of HUV-EC-C cells was performed using the TransFast reagent (Promega, Charbonnieres, France) following the manufacturer's instructions. Briefly, HUV-EC-C cells were seeded on six-well plates at a density of 3-5x10^5 cells per well and, 48 h later, transfected with 5 μg of plasmid pxLNC-NR.

Forty-eight hours after transfection, the HUV-EC-C cells were rinsed with PBS, trypsinized and harvested. The cells were then plated on petri dishes and cultured for 3-4 weeks in medium containing 750 μg/ml G418. Two stably transfected clonal cell lines (HUV-EC-C/*ntr*⁺ clones 1 and 2) were isolated and cultured in selective medium.

Evaluation of ntr/CB1954 system activity in vitro. In order to evaluate the efficacy of the *ntr*/CB1954 system, both HUV-EC-C/*ntr*⁺ clones and genetically unmodified HUV-EC-C/*ntr*⁻ cells were grown in the presence of 0.1 μM of CB1954 (Enact Pharma, Salisbury, Wiltshire, UK). Cells were seeded in triplicate at a density of 2-4x10^3 per well on 96-well plates and cell viability was determined by trypan blue exclusion after 3, 5 and 7 days of culture in the presence of CB1954. Controls consisted of either HUV-EC-C/*ntr*⁺ clones or HUV-EC-C/*ntr*⁻ cells cultured in the absence of the prodrug.

In vivo experiments. Nude mice weighing 20 g and at 6 weeks of age were purchased from Charles Rivers (L'Arbresle, France). The mice were maintained in a laminar airflow cabinet under specific pathogen-free conditions and used at 7 weeks of age. In the first *in vivo* study, the mice were separated into three groups ('co-injected' groups). Five animals of each group were injected s.c. in one flank with 7.5x10^5 B16-F10 cells mixed with 2.5x10^5 ECs (HUV-EC-C/*ntr*⁺ clone 1 or 2 or HUV-EC-C/*ntr*⁻ cells) in 0.1 ml of PBS. CB1954 treatment (600 mg/kg once daily, i.p., according to the manufacturer's procedure) was started three days later and continued for one week. In another set of experiments, the mice were injected s.c. with 7.5x10^5 B16-F10 cells on day 0. The animals were divided into four groups (twelve per group) and injected intratumourally once (at day 3) or three times (days 3, 5, and 7) with 7.5x10^5 HUV-EC-C/*ntr*⁻ cells (groups 1 and 2) or HUV-EC-C/*ntr*⁺ clone 2 (groups 3 and 4) ('post-injected' groups). Two days after the last injection of ECs, two mice from each group were sacrificed for PCR analysis (see below) and the other animals (ten per group) were treated with CB1954 (groups 1 and 3) as described above or with diluent (groups 2 and 4). Tumour size was defined every two days by measuring the maximum horizontal diameter and the maximum vertical diameter with a caliper. Tumour volume (V) was calculated according to the formula $V= (4/3)\ \pi R_1^2\ R_2$; R_1 = the smallest radius and R_2 = the largest radius, measured on the largest section of the tumour). At the end of the experiments, the mice were sacrificed and the tumours, livers and lungs were removed and subjected to histological analysis. All animal procedures were in accordance with our institutional guidelines as well as those of the National Institutes of Health.

Histological analysis and immunostaining. The tumours, livers and lungs were excised, fixed in Bouin and embedded in paraffin. Serial sections (6 μm thin) were stained with haematoxylin and eosin. The tumour sections were then treated for immunohistological analysis with M5/114 monoclonal antibody (rat IgG, ATCC TIB120), revealed by an alkaline phosphatase-conjugated goat anti-rat IgG (Southern Biotechnology Associates Inc.), for murine MHC-II detection. Counterstaining was performed using haematoxylin and eosin dye.

PCR analysis. Two mice from each post-injected group (see above) were sacrificed and DNA samples were extracted from the liver, lung and tumour tissue using DNAzol® Reagent (Invitrogen) according to the manufacturer's instruction. PCR of the pseudogene glyceraldehyde-3-phosphate dehydrogenase of human origin (H-GAPDH-PG) was performed using HotStarTaq Master Mix Kit (Qiagen). Twenty-one mer primers, matching with only the human DNA (forward, 5'-CTCTACTGGCACTGCGAAGGC-3'; and reverse, 5'-AG GAGACCACCTGGTGCTCAG-3'), amplified a 221-bp fragment. The cycling times and temperatures were as follows: 95˚C for 15 min followed by 35 cyc1es of 94˚C for 45 sec, 64˚C for 45 sec, 72˚C for 45 sec, and 72˚C for 10 min for final elongation.

Statistical analysis. Results were determined as mean ± SE. Survival data and other observed differences were compared using Fisher's Exact test and the unpaired Student's t-test respectively, to calculate the statistical significance, determined at the <0.05 level.

Results

The cytotoxic effect of the ntr/CB1954 system on ECs in vitro. We previously demonstrated, by using a 3-D multicellular

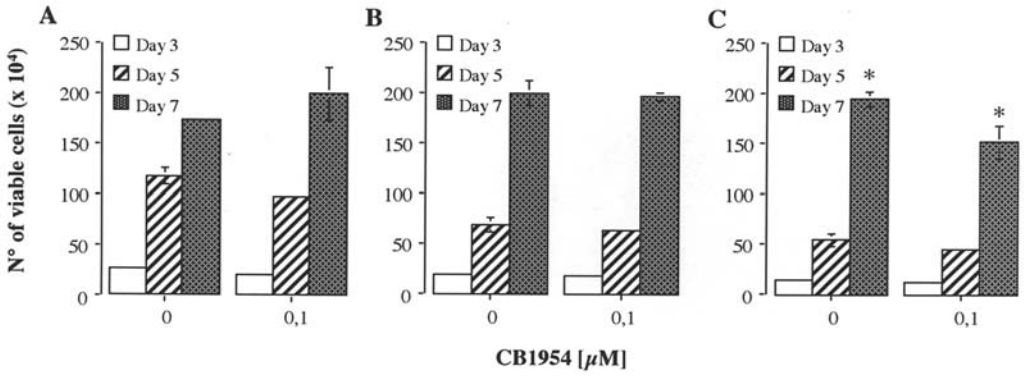

Figure 1. The evaluation of CB1954 activity on HUV-EC-C/*ntr*⁻ and HUV-EC-C/*ntr*⁺ clones *in vitro*. Untransfected HUV-EC-C/*ntr*⁻ cells (panel A) and *ntr*-transfected HUV-EC-C/*ntr*⁺ clone 1 (panel B) and clone 2 (panels C) were cultured in triplicate in the presence of 0.1 μM of CB1954. The control consisted of CB1954-untreated cells. CB1954 induced a time-dependent cytotoxic effect on both HUV-EC-C/*ntr*⁺ clones. Major and significant cytotoxic effects of CB1954 were observed on HUV-EC-C/*ntr*⁺ clone 2 after 7 days of treatment. *Statistical significance was determined at the <0.05 level.

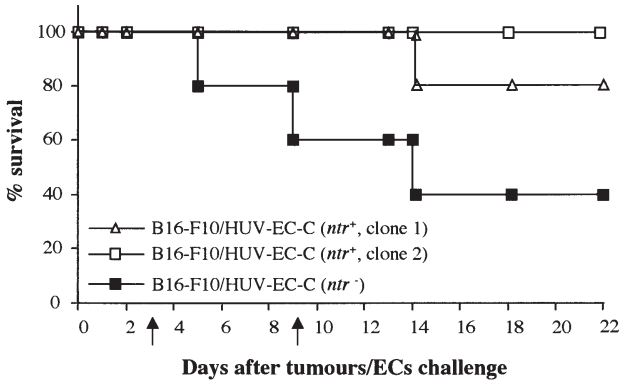

Figure 2. Evaluation of the *ntr*/CB1954 system *in vivo* after the co-injection of B16-F10 melanoma cells with HUV-EC-C/*ntr*⁺ or HUV-EC-C/*ntr*⁻ cells. Mice were injected s.c. with B16-F10 cells mixed with either HUV-EC-C/*ntr*⁺ clone 1 (△) or clone 2 (□). Controls consisted of B16-F10 cells co-injected with HUV-EC-C/*ntr*⁻ cells (■). CB1954 treatment was started three days after the cell challenge and continued for a week (arrows). A prolonged survival of mice bearing HUV-EC-C/*ntr*⁺ clones, as compared to control animals, was observed.

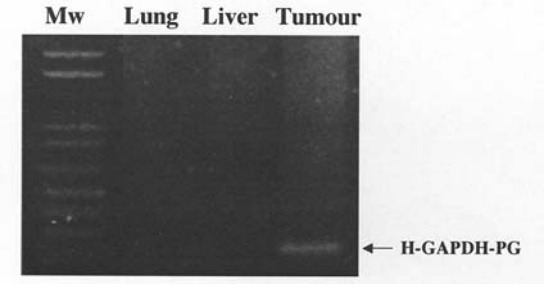

Figure 3. PCR analysis of tumour, lung and liver specimens. PCR analysis of the human pseudogene GADPH (H-GAPDH-PG) was performed on DNA samples purified from the lungs, livers and tumours of tumour-bearing mice injected three times with human ECs. The PCR product, a fragment of 221 bp in length (arrow), was detected only in tumour specimens, demonstrating that no human ECs spread from the site of the injection (tumour) to other organs (lung and liver). Mw, molecular weight marker.

nodule model, a cytotoxic bystander effect spreading from *ntr* bulk-transfected ECs to the unmodified B16-F10 murine melanoma cell line (9).

In the present study, we isolated two clones stably transfected with the *ntr* gene (HUV-EC-C/*ntr*⁺ clones 1 and 2) and we tested the *in vitro* effect of the prodrug, CB1954, on the viability of either HUV-EC-C/*ntr*⁺ clones or HUV-EC-C/*ntr*⁻ cells. Fig. 1 represents the effect of CB1954 treatment (0.1 μM) on these cells, 3, 5 and 7 days after the beginning of the treatment, with respect to CB1954-untreated cells. The viability of HUV-EC-C/*ntr*⁻ cells was not affected by the treatment with CB1954 (panel A), with respect to CB1954-treated HUV-EC-C/*ntr*⁺ clones that showed a reduced growth rate; not significant for clone 1 (panel B) but significant (p=0.037) for clone 2 (panel C).

Effects of the ntr/CB1954 system in vivo. In order to evaluate the *in vivo* effects of the *ntr*/CB1954 system, we injected into nude mice the HUV-EC-C/*ntr*⁺ clone 1 or clone 2 with the melanoma B16-F10 cell line.

Co-injections of HUV-EC-C/ntr⁺ clones in association with the B16-F10 cell line. In a first set of experiments, we co-injected each clone of ECs with B16-F10 cells into mice. Seventy-two hours later, the mice were treated with CB1954, which continued once daily for seven days, and their survival was analysed (Fig. 2). A prolonged survival of >22 days from the cell mixture challenge was observed for 100% of the mice injected with HUV-EC-C/*ntr*⁺ clone 2 and 80% of the mice injected with the HUV-EC-C/*ntr*⁺ clone 1, as compared to the control animals injected with HUV-EC-C/*ntr*⁻ cells, which showed a 40% survival. However, no significant tumour growth differences were observed (data not shown).

Injections of HUV-EC-C/ntr⁺ clones into tumour-bearing animals. In a second set of experiments, we injected HUV-EC-C/*ntr*⁺ clone 2, the more responsive clone to CB1954, or control HUV-EC-C/*ntr*⁻ cells once or three times into tumour-bearing mice. Some animals were sacrificed after EC challenge in order to evaluate the spreading of human ECs from the site of injection to other organs such as the liver and lungs. PCR analysis to determine the presence of the H-GAPDH-PG showed only the presence of human DNA in tumours, demonstrating that no human cell spread from the site of the injection to the liver and lungs (Fig. 3). The other animals were treated with CB1954. No significant differences in

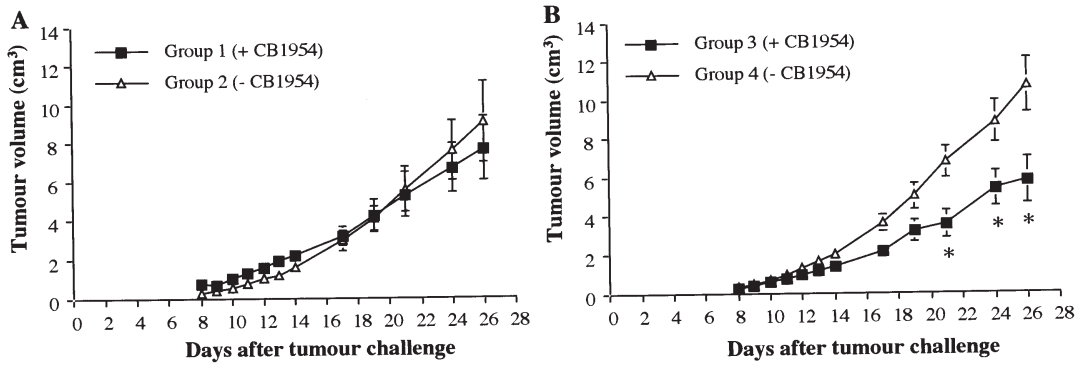

Figure 4. Evaluation of the *ntr*/CB1954 system *in vivo* after the injection of HUV-EC-C/*ntr*$^+$ or HUV-EC-C/*ntr*$^-$ cells into tumour-bearing mice. Three intratumoural injections of either HUV-EC-C/*ntr*$^+$ clone 2 or HUV-EC-C/*ntr*$^-$ cells were performed on nude mice. Then, the animals were treated with CB1954 as described in Materials and methods. No differences in tumour size were observed in mice injected with HUV-EC-C/*ntr*$^-$ cells, either treated (■) or untreated (△) with CB1954 (panel A). In contrast, mice injected with HUV-EC-C/*ntr*$^+$ clone 2 showed a significant reduction in tumour growth (panel B) in the CB1954-treated group with respect to the untreated animals. *Statistical significance was determined at the <0.05 level.

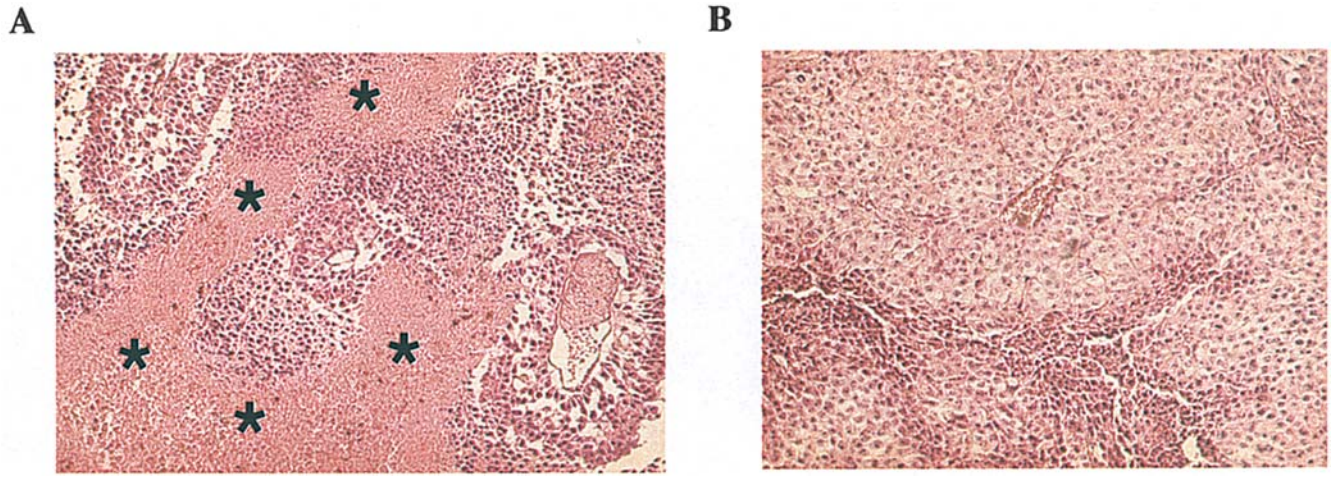

Figure 5. Histological analysis of tumour specimens. *Histological analysis of tumours showed large areas of necrosis in mice co-injected with B16-F10 and HUV-EC-C/*ntr*$^+$ cells, probably due to tumour ischemia, after treatment with CB1954 (panel A); asterisks correspond to areas of necrosis. These signs of cell death were not observed in CB1954-treated mice injected with HUV-EC-C/*ntr*$^-$ cells (panel B). Magnification, x100.

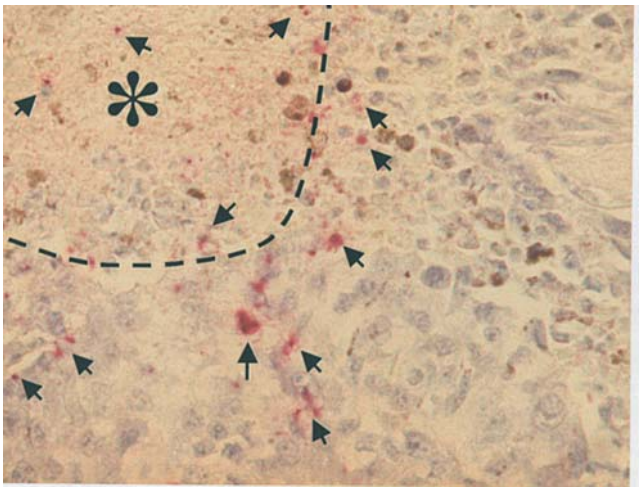

Figure 6. Evaluation of the presence of infiltrating inflammatory cells into tumour specimens. Immunohistological analysis demonstrated the presence of infiltrating inflammatory cells into tumour specimens containing HUV-EC-C/*ntr*$^+$ clone 2, as indicated by the presence of MHC-II positive cells (arrows). In these specimens, large areas of necrosis (* and dotted line) are also observed in tumour-bearing mice injected with HUV-EC-C/*ntr*$^+$ clone 2.

tumour growth or survival were observed when ECs were injected once (data not shown). The results obtained after treatment, of the mice injected three times with ECs, are shown in Fig. 4. The mice were sacrificed 26 days after tumour challenge. No differences in tumour size were observed in mice carrying HUV-EC-C/*ntr*$^-$ cells (panel A) either treated (group 1) or untreated (group 2) with CB1954, while a significant reduction in tumour volume was observed in CB1954-treated mice injected with HUV-EC-C/*ntr*$^+$ clone 2 (panel B, group 3) with respect to untreated animals (panel B, group 4).

Ex-vivo analyses. Surviving animals, either from co-injected or post-injected groups, were sacrificed because of excessive tumour burden and the tumour, liver and lung specimens were analysed histologically. The tumours from mice co-injected with B16-F10 cells and HUV-EC-C/*ntr*$^+$ clones, as well as specimens excised from tumor-bearing animals injected with HUV-EC-C/*ntr*$^+$ clone 2 (CB1954-treated mice of group 3), showed the presence of large areas of necrosis, probably due to tumour ischemia (Fig. 5, panel A). These signs of cell death were not observed in any of the

other animals (Fig. 5, panel B). Histological analysis of livers and lungs showed neither treatment toxicity nor the presence of tumour metastasis (data not shown).

Furthermore, we investigated the presence of infiltrating inflammatory cells involved in the first phases of tumour rejection in nude mice, such as macrophages, expressing the major histocompatibility complex class-II (MHC-II) molecules (15). It is well established that immunological mechanisms contribute significantly to the bystander effect following cell killing by GDEPT systems *in vivo* (16,17). Immunohistological analyses of tumour areas revealed the presence of infiltrating immune cells in the tumours excised from the mice in group 4 (CB1954-treated, carrying the HUV-EC-C/*ntr*$^+$ clone 2), as indicated by the presence of MHC-II positive cells (Fig. 6). No MHC-II positive cells were observed in tumours excised from animals in other groups (data not shown).

Discussion

Angiogenesis is a fundamental process by which new blood vessels are formed from pre-existing ones. In physiological conditions, angiogenesis is a highly regulated phenomenon that plays an essential role in several physiological processes, such as embryonic development, the menstrual cycle, pregnancy and wound healing. Under these conditions, angiogenesis is highly regulated. However, several disorders, including tumour growth and metastasis, are characterized or caused by excessive angiogenesis (1).

Hence, inhibition of angiogenesis has been shown to be an efficient strategy in cancer therapy in humans (18). However, the administration of recombinant antiangiogenic factors may be limited by their short half-life. This limitation may be resolved by *in vivo* delivery and expression of antiangiogenic genes (6). Gene therapy presents many advantages over the transfer of recombinant proteins, including a decreased cost and long-term expression of the transgene.

The *ntr*/CB1954 system is based on the delivery of a suicide gene, the bacterial *ntr*, which codes for an enzyme able to convert a non-toxic prodrug, CB1954, into a potent cytolytic agent. This system, as well as other GDEPT combinations, is presently used to prove the efficacy of gene therapy by direct gene transfer into tumour cells (7,8) and it is characterized by the presence of a phenomenon known as the bystander effect, which allows the destruction of *ntr*$^-$ cells by diffusion of toxic metabolites from *ntr*$^+$ cells.

Based on previous observations that the bystander killing effect of CB1954 was much more efficient in multilayer cocultures of *ntr*-positive and *ntr*-negative tumour cells than in monolayer cultures (12), we previously analysed the spreading of the bystander effect from *ntr*-transfected ECs (HUV-EC-C) to the untransfected melanoma cell line (B16-F10) by using a 3-D multicellular nodule model (9). Nodules, which are *in vitro* systems allowing the coculture of cancer cells with other cell types, have the property of reconstituting some of the *in vivo* cellular architecture and interactions by providing an *in vitro* approximation of solid tumours *in vivo*. By analysing the ultrastructure of nodules, we observed the formation of capillary-like structures due to the presence of closely-associated cells, probably ECs, forming a sort of vascular lumen, as well as the presence of electron dense intercellular junctional complexes, probably corresponding to desmosomes and gap junctions. Afterwards, we observed an apoptotic-mediated bystander cell killing effect from endothelial to neighbouring melanoma cells.

In the present study, the evidence provided by our results gives support to the use of the *ntr*/CB1954 system to target tumour angiogenesis *in vivo*. Our observations are in agreement with previous data demonstrating that diffuse multifocal haemorrhages, occurring after intratumoural retroviral-mediated herpes simplex virus thymidine kinase (HSV/*tk*) gene transfer into malignant brain tumours treated with ganciclovir (GCV), as well as immune system activation, are necessary components of the bystander effect observed with many GDEPT-based approaches (19,20).

At present, the main concern is how to adapt this approach for clinical use. One of the main limitations of gene therapy is gene transfer *in vivo*. The currently used different methods, either viral or non-viral, show a limited ability to efficiently deliver transgenes to target cells. However, recent reports have shown that endothelial progenitor cells (EPCs), mobilized from bone marrow (BM) in response to cytokines and pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), may contribute to normal and pathological vascular organogenesis (21-25). Hence, the utilization of stem cells as vehicles to target the expression of a transgene seems to represent an interesting alternative strategy. EPCs home to sites of neovascularization and differentiate into ECs *in situ*. BM-derived EPCs have also been considered as therapeutic agents that supply the potent origin of neovascularization under pathological conditions. Indeed, BM progenitors genetically modified to express the HSV/*tk* gene home to sites of tumour neovascularization in mice and can be selectively eliminated after GCV treatment (10). This induces a substantial inhibition of both angiogenesis and tumour growth without systemic toxicity. Furthermore, some data demonstrated that adult organ-derived ECs, such as umbilical ECs or CD34$^+$ mono-nuclear cells collected from cord blood, also possess an *in vivo* tropism for tumour development with the potential to form a neovascular network in tumours (26,27).

In conclusion, angiogenic tumour vessels are promising targets for the activity and selective delivery of cancer therapeutics *in vivo*. The use of the *ntr*/CB1954 system to target tumour angiogenesis may be an efficient approach for cancer treatment, depending on the spreading of the bystander effect from endothelial to tumour cells, based on two possible mechanisms, tumour ischemia and immune cell activation. Thus, the transplantation of genetically modified EPCs with the *ntr* gene could represent a vehicle for the transport of gene therapy to tumours *in vivo* and, therefore, enhance the efficacy of GDEPT-based antitumour therapy by inducing little or no drug resistance.

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