Pilot study of anti-angiogenic vaccine using fixed whole endothelium in patients with progressive malignancy after failure of conventional therapy

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\textbf{A B S T R A C T}

Vaccines targeting tumour angiogenesis were recently shown to inhibit tumour growth in animal models. However, there is still a lack of information about the clinical utility of anti-angiogenic vaccination. Therefore, here, we aimed to test the clinical effects of a vaccine using glutaraldehyde-fixed human umbilical vein endothelial cells (HUVECs). Six patients with recurrent malignant brain tumours and three patients with metastatic colorectal cancer received intradermal injections of $5 \times 10^7$ HUVECs/dose (in total 230 vaccinations). ELISA and flow cytometry revealed immunoglobulin response against HUVECs’ membrane antigens. ELISPOT and chromium-release cytotoxicity assay revealed a specific cellular immune response against HUVECs, which were lysed in an effector:target ratio-dependent manner. Gadolinium-contrasted MRI showed partial or complete tumour responses in three malignant brain tumour patients. Except for a DTH-like skin reaction at the injection site, no adverse effect of vaccination could be observed. Our results suggest that the endothelial vaccine can overcome peripheral tolerance of self-angiogenic antigens in clinical settings, and therefore should be useful for adjuvant immunotherapy of cancer.

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\section{Introduction}

Angiogenesis, the growth of new blood vessels, is essential for tumour growth and metastasis.\textsuperscript{1–5} Whereas angiogenesis occurs minimally in normal adult tissues, it is intensive in developing tumours, enabling them to grow over the diameter of a few mm, the so called ‘angiogenic limit’. Angiogenesis-associated antigens over-expressed on tumour endothelium are specific molecular addresses targeted by anti-angiogenic therapy.\textsuperscript{6–10} Therapeutic damage of tumour endothelium activates the coagulation cascade, and consequently results in the obstruction of tumour vasculature, with hypoxia and shrinkage of tumours due to necrosis, whereas it does not affect blood supply in normal adult tissues.\textsuperscript{11} As a result, cancer...
patients coexist with small tumours that can neither grow nor metastasise, under the condition called ‘tumour dormancy’ or ‘cancer without disease’.  

Vaccines targeting tumour angiogenesis were recently shown by us and others to inhibit tumour growth in animal models. However, there is still a lack of information about the clinical utility of anti-angiogenic vaccination. Therefore, here, we aimed to test the clinical effects of a vaccine using glutaraldehyde-fixed human umbilical vein endothelial cells (HUVECs).

2. Patients and methods

2.1. Eligibility criteria

Patients with a recurrent malignant brain tumour or a metastatic colorectal cancer, previously treated in our hospital, were eligible. Eligibility criteria were as follows: A progressive disease resistant to conventional therapy, as assessed by contrasted computer tomography (CT) or magnetic resonance imaging (MRI), and classified according to the Response Evaluation Criteria in Solid Tumours; at least a 1 month period after finishing prior therapy; WHO performance status 0–3 (malignant brain tumours) or 0–2 (colorectal cancer); haemoglobin > 8 g/dl; bilirubin < 2.0 mg/dl; serum glutamic oxaloacetic transaminase <100 IU/L; serum glutamate pyruvate transaminase < 100 IU/L; creatinine < 2 mg/dl; blood urea nitrogen < 30 mg/dl; creatinine clearance > 90 ml/min; ejection fraction by echocardiography > 70% and prothrombin time > 70%. In addition, patients with a psychiatric disorder, extensive cachexia, multiple cancer types, active infection, autoimmune disease or myocardial ischaemia were excluded. The Institutional Review Board of the University of Tokyo approved this protocol. All patients provided written informed consent before enrolment on protocol, in accordance with the Declaration of Helsinki.

2.2. Study design

This single arm phase I study was carried out between August 2003 and August 2007 to assess the clinical utility of a vaccine using human umbilical vein endothelial cells (HUVECs). The enrolled patients were given intradermal injections of 5 × 10⁷ HUVECs/dose, first month weekly, and then every 2 weeks. Immune response was evaluated by examining patients’ peripheral blood mononuclear cells (PBMC) and sera, which were isolated monthly and frozen until being tested in immunological assays later at the same time. Tumour response was assessed monthly by contrasted computer tomography (CT) or magnetic resonance imaging (MRI) according to the Response Evaluation Criteria in Solid Tumours. Adverse effects were evaluated by monitoring the patients according to the Common Toxicity Criteria.

2.3. Vaccine preparation and quality control

HUVECs were isolated from healthy donors as described, and cultured on 0.1% gelatin (w/v)-coated dishes in EC-SFM (Life Technologies, Grand Island, NY), according to the manufacturer’s recommendations. Specific properties of angiogenic endothelium were confirmed by measuring the expression of CD31, CD51, CD105, and binding of UEA (Ulex europaeus) lectin by flow cytometry (FACSCalibur, BD Bioscience, San Chose, CA; antibody concentration was 5 μg/ml). Negative presence of endotoxin in the cell cultures was confirmed by Toxicolour test (Seikagaku Co., Tokyo, Japan). HUVECs of up to ten passages were harvested, and fixed with 0.025% glutaraldehyde (v/v) as described, and stored at –80°C in single dose aliquots, containing 5 × 10⁷ cells/ml physiological saline for injection.

2.4. Enzyme-linked immunosorbent assay (ELISA)

96-well immunoplates (NUNC, Roskilde, Denmark) were coated with 10 μg/ml HUVECs’ membrane proteins overnight at 4°C, and blocked with 1% BSA (w/v), for 2 h at 37°C. Patients’ sera, diluted 2×, were used in duplicates as the primary antibody, and horseradish peroxidase conjugate of anti-IgG (Zymed, San Francisco, CA), diluted 2000×, as the secondary antibody. Colour-development by ABTS (ICN, Aurora, OH) was followed by determining optical density (O.D.) at 405 nm. For negative control, wells coated with K-562 (ATCC, Manassas, VA) were used. IgG reactivity was expressed as the ratio to the values measured in the pre-vaccination samples that were considered 100%.

2.5. Isolation of patients’ B cells and flow cytometry

Patients’ PBMC were cultured first in the supernatant of B95-8 (cotton-top tamarine lymphocyte cell line secreting Epstein–Barr virus, obtained from Japanese Collection of Research Bioresources, and cultured in RPMI - 10% FCS) supplemented with 5 μg/ml CpG-B-DNA (HyCult Biotechnology, Uden, The Netherlands), 10 ng/ml IL-6 (Strathmann Biotec, Zurich, Switzerland) and 2 μg/ml membrane proteins of HUVECs for 1 week, and then in RPMI-1640 supplemented with 10% FCS (v/v), 10 ng/ml IL-6, and 1% antibiotic/antimycotic (v/v); at final concentration of 100 U/ml penicillin G, 100 μg/ml streptomycin sulphate and 250 ng/ml amphotericin B; Life Technologies) for 3 weeks. Specific phenotype of selectively immortalised B cells was confirmed by analysing their expression of CD19 (B cell marker) and CD138 (plasmatic cells marker) by flow cytometry (FACSCalibur, BD Biosciences).

The supernatants of B cell media were added in the amounts of 200 μl to 2 × 10⁵ HUVECs for 30 min at 4°C. The cells were washed two times, incubated with FITC-labelled antibodies against human IgM or IgG (Zymed), diluted 40×, for another 30 min at 4°C. Finally, the cells were washed two times, and their fluorescence measured in the flow cytometer (FACSCalibur, BD Biosciences).

2.6. IFN-γ enzyme-linked immunospot (ELISPOT) assay

Human IFN-γ ELISPOT Kit (BD Biosciences) was used for the detection of patients’ PBMC secreting IFN-γ in the presence or absence of HUVEC membrane proteins, according to the manufacturer’s recommendations. The numbers and areas of colour-developed IFN-γ immunospots were determined by
2.7. Intracellular cytokine flow cytometry (CFC) assay

Re-stimulated patients’ PBMC were carefully removed from the immunoplates at the end of ELISPOT assays, cultured for another 6 h in a medium supplemented with 10 μg/ml Brefeldin A (Sigma, Saint Louis, MO), fixed with 3% paraformaldehyde (v/v), permeabilised with 0.5% Tween-20 (v/v), and stained with 5 μg/ml FITC-/PE-labelled monoclonal antibodies against IFN-γ and CD3 (BD Biosciences). % CD3(+)IFN-γ(+) cells were determined by flow cytometry (FACSCalibur, BD Biosciences), and expressed as the ratio to the values obtained in the pre-vaccination samples that were considered 100%.

2.8. Cytotoxicity T lymphocyte (CTL) assay

Patients’ PBMC were used as effectors against HUVECs or K562 at effectors:targets ratios 100:1, 30:1 and 5:1, in a 51Cr-release cytotoxicity assay as described,14 with some modifications as follows. Medium used for re-activation culture was RPMI-1640 - 10% FCS (v/v) - 100 U/ml recombinant human IL-2 (Sigma) - 1% antibiotic/antimycotic (v/v). % specific lysis was calculated using the formula (experimental release – spontaneous release) / (maximum release – spontaneous release) × 100. The results were expressed as the ratio to the values of the pre-vaccination samples that were considered 100%.

3. Results

3.1. Patient characteristics

Nine patients with a progressive malignancy, i.e. six patients with a recurrent malignant brain tumour and three patients with a metastatic colorectal cancer (median age: 53 years, range: 43–68 years), were enrolled in this study.

3.2. Properties of vaccine endothelium

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cords, cultured with angiogenic stimulators, fixed, and used as vaccines (Fig. 1a). The cells expressed CD31, CD51, CD105 and CD146, as well as bound UEA lectin, suggesting they were angiogenic endothelium (Fig. 1b).

3.3. Safety of vaccination

The patients received intradermal injections of 5 × 10^7 HUVECs/dose in the vaccination protocol (Fig. 1c), in a total amount of 230 vaccinations (Table 1; median number of vaccinations: 26 doses, range: 8–50 doses; median vaccination period: 12 months, range: 3–24 months). Except for a DTH-like skin reaction at the injection site, no adverse effect could be observed (data not shown).

3.4. Specific antibody response

Specific antibodies against HUVECs’ membrane antigens were detected in the sera of eight patients (Fig. 2a), and could also be detected in the culture media of patients’ B cells (Fig. 2b–d).

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**Fig. 1**– Study design. (a) Study protocol. Human umbilical vein endothelial cells (HUVECs) were isolated, cultured, harvested, fixed, and periodically injected in cancer patients. Vaccination effects, i.e. immune and tumour responses were evaluated monthly. (b) Confirmation of specific properties of HUVECs by flow cytometry. The cells expressed CD31, CD51, CD105 and CD146, as well as bound UEA lectin, suggesting they were angiogenic endothelium. (c) Vaccination protocol. The patients received vaccinations first month weekly, and then every 2 weeks.
These antibodies reacted neither with non-endothelial control cells K-562 by ELISA and flow cytometry, nor with human leucocyte antigens of platelets pooled from multiple donors by mixed passive haemagglutination as described \(^{18}\) (data not shown).

### 3.5. Specific cellular response

Increased secretion of IFN-\(\gamma\) as a response to the stimulation with HUVECs’ membrane antigens was detected in six patients (Fig. 3a–c). Patients’ cellular effectors specifically lysed HUVECs (Fig. 3d), but not K-562 (data not shown).

### 3.6. Clinical tumour response

Two partial and one complete tumour responses were observed on gadolinium contrasted MRI scans after 9 months of vaccination in three patients with recurrent malignant brain tumours (Fig. 4). In detail, in the patient #1 with pinealoblastoma, a recurrent disease accompanied by dissemination in the cerebrospinal fluid space that resulted in multiple new tumour lesions outside the post-operative radiation field (including one lesion being localised near the brain stem) was first detected 2 years after completed prior therapy. In the patient #2 with glioblastoma multiforme, and patient #3 with anaplastic oligodendroglioma, recurrences were first detected 9 and 20 months after completed prior therapy, respectively. Recurrent disease was treated first by chemotherapy with temozolomide, but without any effect, and therefore the patients were enrolled in the present study. After 9 months of subsequent endothelial vaccination, partial tumour responses lasting over 12 months were observed in the patients #1 and #2, and a complete tumour response lasting over 9 months was observed in the patient #3 (the protocol was still in progress at the time of manuscript preparation).

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Abbreviations: M, male; F, female; PBMrec, recurrent pinealoblastoma; GBMrec, recurrent glioblastoma multiforme; AODrec, recurrent anaplastic oligodendroglioma; CRCmet, metastatic colorectal cancer; sur, surgical therapy; cht, chemotherapy; xrt, radiation therapy; Period, vaccination period in months; Doses, number of vaccination doses; Ab, antibody response; Cell, cellular response; nd, not determined; Response, clinical tumour response; PR, partial response; CR, complete response; PD, progressive disease.

\(^{a}\) Still in progress.

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Fig. 2 – Specific antibody response. (a) Reactivity of patients’ serum IgG with HUVECs’ membrane antigens by enzyme-linked immunosorbent assay. Representative relative reactivities (Post/Pre) determined during best responses are shown. (b) Patients’ peripheral blood B cells. Representative growing colonies of patient #1 B cells, selectively immortalised and cultured, are shown. (c) CD19 and CD138 expression on patient #1 B cells measured by flow cytometry. (d) Reactivity of IgM and IgG secreted by patient #1 B cells with HUVECs measured by flow cytometry.
4. Discussion

In this study, we tested the clinical utility of vaccination using glutaraldehyde-fixed human umbilical vein endothelial cells (HUVECs) in patients with a progressive malignancy. HUVECs share specific angiogenic properties with tumour endothelium such as the overexpression of angiogenic antigens CD51\(^1\) and CD105\(^2\), and inhibit tumour growth in pre-clinical mouse models.\(^{14,21,22}\) Therefore, we hypothesised that HUVECs could also be effective in clinical settings, especially in patients with malignant brain tumours, which are known to be among the most vascularised tumours.\(^{23}\) In addition, we were interested in whether HUVECs could work in the patients with colorectal cancer, which on the one hand still remains a difficult target for cancer immunotherapy,\(^{24}\) but on the other hand was recently shown to respond to anti-angiogenic therapy.\(^{14,25}\)

The vaccination protocol in this study was designed to provide strong antigenic stimulation to overcome the peripheral tolerance of angiogenesis. Therefore, the number of endothelial cells administered in one dose was \(5 \times 10^7\), i.e. five times more than commonly used in the vaccines based on tumour cells, to ensure that the amount of administered antigens is sufficient to induce a specific immune response. In addition, here, we decided to continue the vaccination protocol in a limited number of patients for long periods of time rather than to treat many patients with a limited number of doses; first, to reveal potential toxicity of the endothelial vaccine, and second, to ensure that the antigenic stimulation is sufficient for inducing a long lasting immune response. We found that except for a DTH-like skin reaction at the injection site, the endothelial vaccine caused no adverse effects to the recipients during a long term administration. In future studies, a minimum effective dose will be evaluated to improve the cost-to-performance ratio of the vaccination protocol.

One month after starting the vaccination protocol, specific antibodies and cellular effectors against HUVECs’ membrane antigens were detected in the patients with recurrent malignant brain tumours as well as metastatic colorectal cancer. Analysis of candidate target antigens has recently been ongoing so as to get better insight into the mechanisms controlling immune tolerance of angiogenesis, and to develop more effective protocols for anti-angiogenic endothelial vaccination.

In three patients with recurrent malignant brain tumours, marked tumour shrinkage could be observed over an
extended period of vaccination. Recently, it has been reported that in some patients with malignant brain tumours, immediate post-radiotherapy changes that mimic tumor progression on MRI, the so called ‘pseudo-progression’, can cause an overestimation of therapeutic results, and therefore the patients enrolled in a clinical study should be at least 3 months after completed radiation therapy. In the present study, recurrent disease was first observed in our patients more than 9 months after completed post-operative radiation therapy of the original tumour, some of the recurrent tumours being localised outside the radiation therapy field, and therefore suggested to be true progression. Given that neither recurrent tumour was treated by radiation therapy, and chemotherapy with temozolomide was ineffective, we concluded that the tumour responses observed in this study should be attributed to the effects of endothelial vaccination.

Human tumour cells share some properties with the angiogenic endothelium, such as expression of CD51 and CD105, and therefore it can be hypothesised that, theoretically, tumour cells might be potential co-targets of antiangiogenic endothelial vaccination. Although our preliminary data of in vitro immunological assays supported this hypothesis (data not shown), we suppose that under in vivo conditions, tumour endothelium should be a primary target. It is due to the fact that tumour endothelium is lining the intra-luminal surface of tumour vasculature, and consequently is first reached by immune effectors induced by endothelial vaccination. As mentioned in the Introduction section, therapeutic damage of tumour endothelium activates the coagulation cascade, and consequently results in the obstruction of tumour vasculature that makes the immune effectors unable to sufficiently reach tumour cells.

In contrast, with the three responding malignant brain tumour patients, neither tumour response nor other improvement of the clinical outcome could be observed in the other patients. The reason for the discrepancy between the immune response to vaccination and tumour response

Fig. 4 – Clinical tumour response. Partial tumour responses were observed in patient #1 with pinealoblastoma (a) and patient #2 with glioblastoma (b), whereas a complete tumour response was observed in patient #3 with anaplastic oligodendroglia (c). Representative gadolinium contrasted magnetic resonance imaging scans taken before vaccination (Pre), after 9 months of vaccination (Post-9 months), and after an extended vaccination period (Post-21 months and Post-18 months) are shown. Main target lesions are indicated by arrows.
in these patients is not yet known, but we can speculate that it might be caused by either strong immunosuppression in the tumour microenvironment, or by possible adaptation of some tumour cells to the consequences of anti-angiogenic therapy, as was recently described by others. Discrepancy between the immunological and anti-tumour effects was also reported by many other authors clinically investigating cancer vaccines, and therefore we suppose that there is a strong need for studies searching for factors that make cancer patients responsive or resistant to active immunotherapy.

In summary, the present pilot study showed the safety and potential clinical utility of the anti-angiogenic vaccine using fixed whole endothelium. Immune response, involving activation of both specific humoral and cellular immunity, was observed in eight of nine patients. Partial or complete tumour responses were observed in three of six malignant brain tumour patients, but not in three colorectal cancer patients.

To obtain further insight into the possibilities and limitations of this novel approach, another study employing different dose levels, adjuvants and combination with conventional therapy modalities against various tumour types is now ongoing.

Conflict of interest statement

None declared.

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