Surgery and adjuvant dendritic cell-based tumour vaccination for patients with relapsed malignant glioma, a feasibility study

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Patients with relapsed malignant glioma have a poor prognosis. We developed a strategy of vaccination using autologous mature dendritic cells loaded with autologous tumour homogenate. In total, 12 patients with a median age of 36 years (range: 11–78) were treated. All had relapsing malignant glioma. After surgery, vaccines were given at weeks 1 and 3, and later every 4 weeks. A median of 5 (range: 2–7) vaccines was given. There were no serious adverse events except in one patient with gross residual tumour prior to vaccination, who repetitively developed vaccine-related peritumoral oedema. Minor toxicities were recorded in four out of 12 patients. In six patients with postoperative residual tumour, vaccination induced one stable disease during 8 weeks, and one partial response. Two of six patients with complete resection are in CCR for 3 years. Tumour vaccination for patients with relapsed malignant glioma is feasible and likely beneficial for patients with minimal residual tumour burden.

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In spite of modern oncological treatment, the prognosis of glioblastoma multiforme (GBM) remains dismal, with a median survival of less than 2 years (Young et al, 1981; Shrieve et al, 1999; Wolff et al, 2000). The prognosis at time of relapse is even worse (Finlay et al, 1995; Nieder et al, 2000; Brada et al, 2001; Tamber and Rutka, 2003; Rich et al, 2004). However, new treatment strategies are under development, one of them being immune therapy.

Brain tumours are considered to be located in a site of relative immune privilege (Walker et al, 2002). Malignant gliomas have immune suppressive characteristics locally (Black et al, 1992) and systemically (Elliott et al, 1990). In case of vaccination, immune responses are induced at sites remote from the tumour. Effector cells then recirculate to mediate their antitumour effects in the brain. The concept of tumour vaccination using dendritic cells (DC) has been demonstrated in animal models (De Vleeschouwer et al, in press). Phase I studies have demonstrated feasibility, safety and bioactivity of autologous peptide-pulsed DC vaccine for patients with malignant glioma (Yu et al, 2001). Early clinical experiences with immunotherapy using protein-pulsed DC suggest this to be a promising strategy for patients with recurrent malignant glioma (Wheeler et al, 2003; Yamanaka et al, 2003; De Vleeschouwer et al, 2004).

We summarise our experience in a group of patients with relapsed malignant glioma, who were treated with autologous DC loaded with proteins derived from autologous tumour homogenate.

MATERIALS AND METHODS

Patient population

All patients had a relapsing malignant glioma. Patients were considered candidates for adjuvant vaccination if a new operation with the intent to extensively debulk the tumour was deemed safe by the neurosurgeon. No further restrictions were applied to
recruit the patients. In this feasibility study, no patient dropped out after reoperation. Patient characteristics are described in Table 1. There were 12 patients (seven female and five male) with a median age of 32 years (range: 11–78 years). Eight patients were vaccinated at first relapse, while four patients had more than one malignant event prior to vaccination. All patients were reoperated upon and were off steroids and nonsteroidal anti-inflammatory drugs at the time of vaccination. Approval by the local ethical committee was obtained, and informed consent was provided before the start of the immunotherapy.

Assessment of extent of tumour resection before vaccination

Complete resection was defined as the absence of any residual tumour mass on early postoperative MRI or CT scan performed with and without contrast within 72 h after surgery. Any resection leaving a measurable residual tumoral mass <1 cm³ and <10% of the initial tumour volume was considered subtotal. All solid residual tumour of a measurable size ≥1 cm³ or removal of <90% of the tumour volume was classified as partial resection.

Tumour homogenate

Tumour tissue was immediately transported from the operation room into the laboratory and snap-frozen in liquid N₂ without additives. For further preparation, the tissue was thawed and put into NaCl 0.9% with 1% human serum albumin, and was homogenised mechanically. Afterwards, six snap freeze/thaw cycles were performed. The homogenate was filtered with a Falcon filter (70 μm). The amount of protein was measured using the method of the Bradford (1976). After irradiation (60 Gy), the homogenate was filtered with a Falcon filter (70 μm). The amount of protein was measured using the method of the Bradford (1976). After irradiation (60 Gy), the homogenate was filtered with a Falcon filter (70 μm). The amount of protein was measured using the method of the Bradford (1976). After irradiation (60 Gy), the homogenate was filtered with a Falcon filter (70 μm). The amount of protein was measured using the method of the Bradford (1976).

Preparation of autologous DC

In eight patients peripheral blood mononuclear cells (PBMC) were isolated from fresh blood samples. DC were differentiated out of the monocytes in the presence of 20 ng ml⁻¹ rIL-4 (Pepro Tech Inc., Rocky Hill, NJ, USA) and 1000 U ml⁻¹ rGM-CSF (Leuko-max®, Novartis) for 7 days as described (Sallusto and Lanzavecchia, 1994). In the other patients, PBMC were obtained from leukapheresis, and kept frozen until use. For each vaccination, part of the PBMC was thawed, and adherent cells were differentiated to immature DC as described (Thurner et al, 1999). Immature DC were loaded with 30–200 μg of tumour proteins per million DC, depending on the material available. For the loading procedure, 0.01% autologous plasma was used during the first 2 h, 0.04% for the next 4 h, and finally 0.28% for the last 20 h. At time of loading, rTNF-α (Stratham Biotec AG, Dengelberg, Germany), rIL-1β (Stratham Biotec AG) and PGE2 (Prostin®, Pharmacia) were added in a final concentration of 120, 120 and 20 μg ml⁻¹, respectively. After 24 h, mature loaded DC were resuspended in PBS with 0.5% human serum albumin (HSA) at a concentration of 2–6 × 10⁶ ml⁻¹. The syringes contained 1–2 million mature DC.

Vaccination

Vaccination was performed by intradermal (i.d.) injection of 2–4 million DC per lymph node region in the upper third of the arms at weeks 1, 3, and further with an interval of 4 weeks. The patients were kept in the hospital for 2 h after vaccination.

Skin tests

Delayed type hypersensitivity reaction (DTH) was tested after at least two vaccinations. For this, 100 μl tumour homogenate and 100 μl control PBS/HSA were injected i.d. After 24, 48, and 72 h, redness and induration were assessed by an independent observer. DTH reactions were judged as positive if the average perpendicular measurement of the reaction exceeded 5 mm.

Patient assessment

All patients were followed by clinical examination and MRI scanning. In eight patients, methionine-PET imaging was performed. Imaging studies were scheduled before each vaccine except the second vaccine. Afterwards, clinical examination and imaging studies were performed every 3–4 months.

RESULTS

Vaccines preparation and characterisation

The patients received 2–7 (median: 5) vaccines after surgery. The details of the vaccinations for each patient are described in Table 2. The median yield of DC from freshly isolated PBMC was 4.8 × 10⁶.
per injection (range: $0.8 – 13 \times 10^6$; $n = 37$), which was significantly (Mann–Whitney test: $P = 0.0007$) less than the median yield of DC from leukapheresis PBMC (median $10^6$, range: $3 – 18 \times 10^6$; $n = 22$). The quality of the DC was controlled by the expression of HLA-DR, CD80, CD86 and CD83 (Figure 1) (Thurner et al, 1999).

Therapy-induced clinical effects

The details of the therapy-induced clinical effects are given in Table 3. The progression free survival (PFS) and overall survival (OS) at 36 months for the total group was 17% (median PFS = 3 months; OS = 10.5 months). In the six patients with residual tumour load after surgery and prior to vaccination, one stable disease (patient 1, Figure 2) and one partial response of a metastatic lesion (patient 6, Figure 3) were observed based on the volumetric analysis of the tumour. In the latter patient, the right temporal lesion, displaying a high grade malignant metabolic uptake ratio of 2.95 on methionine-PET, decreased in volume by 50% after the third vaccination (Figure 3). In two out of six patients (patients 2 and 3) who had complete resection of their tumour, continuous complete remission was observed at the moment of writing the manuscript, with a follow-up of 36 and 35 months respectively. Patient 3 had a transient contrast enhancement around the resection cavity after the fifth vaccine together with a transient increase of metabolic activity around the resection cavity, measured by methionine-PET (De Vleeschouwer et al, 2000).

Table 2

| Patient number | Surgery prior to vaccination | Source of PBMC   | Number of vaccinations | Amount of cells injected per vaccination ($\times 10^6$) | Skin test |
|----------------|-------------------------------|------------------|------------------------|--------------------------------------------------------|-----------|
| 1              | Partial resection             | Leukapheresis    | 5                      | 5/18/17/16/15                                          | Not done  |
| 2              | Total resection               | Leukapheresis    | 7                      | 4/3/4/16/10/15/14/13                                   | –V2, +V6  |
| 3              | Total resection               | Fresh blood      | 6                      | 3/1/2/9/1/2                                            | +V3      |
| 4              | Total resection               | Leukapheresis    | 4                      | 13/1/1/10/9                                           | –V3      |
| 5              | Total resection               | Fresh blood      | 5                      | 3/1/4/4/8/3/5                                          | Not done  |
| 6              | Total resection, second localisation | Fresh blood    | 3                      | 8/7/6                                                  | +V3      |
| 7              | Subtotal resection            | Fresh blood      | 5                      | 12/2/7/12/9                                           | –V3      |
| 8              | Partial resection             | Fresh blood      | 2                      | 13/6                                                   | Not done  |
| 9              | Subtotal resection            | Fresh blood      | 5                      | 9/3/2/2/5/0/8                                          | –V3      |
| 10             | Subtotal resection            | Fresh blood      | 5                      | 4/8.2/1.1/4.5/5/6                                      | –V3      |
| 11             | Total resection (no vital tumour) | Leukapheresis  | 6                      | 15/8/6/4/4/4/4                                        | Not done  |
| 12             | Total resection               | Fresh blood      | 6                      | 10/13/9/5/4/2/3                                        | +V3      |

$V_x$ = vaccine number.

**Figure 1** Quality control of dendritic cells. Representative example obtained by FACS analysis, of the expression of surface markers on loaded mature dendritic cells at the time of injection.
although the patient numbers were small, patient outcome was not depending on the procedure of making DC or on the amount of proteins to load DC or on the total amount of DC injected. Overall, in four out of 12 patients (patients 1, 2, 3, and 6), some evidence for tumour control due to immunotherapy was observed, and in three of them (patient 1, 3, and 6) an objective response was measured.

Immune response
In eight patients, a DTH skin test with crude autologous tumour homogenate was performed, of which only two tests remained negative at the time of third vaccination. In one patient, the skin test at the time of the second vaccination was negative, but became positive when the test was performed again at the time of the sixth vaccination. In patient 1, the peritumoral oedema related to each vaccination since the second vaccination was considered as immune-mediated. Therefore, no additional skin test was performed in parallel. Due to the small amount of tumour proteins available in the other patients, the skin test could not be carried out. No relation between positive skin test and response of the tumour or survival of the patient could be found in this small group of patients. There was also no trend in any direction that the amount of proteins used to load DC or the amount of DC injected had any effect on the induction of DTH reaction or on the clinical outcome.

Toxicity
No severe adverse events were noted, except for one patient. This patient suffered from peritumoral oedema that caused grade IV (NCI common toxicity criteria) neurological deficits and lethargy after vaccinations 2–5, but not after the first vaccination. Remarkably, the period of oedema appeared 30 h after V2, 4 days after vaccinations 2–5, but not after the first vaccination. In the latter patient, lumbar puncture at that time revealed 8.4 white blood cells per μl with 83% lymphocytes. The CSF was haemorrhagic, with proteins of 1511 mg l\(^{-1}\), glucose of 50 mg dl\(^{-1}\) and lactate of 2.92 mmol l\(^{-1}\). Bacterial cultures and viral PCR tests remained negative. In the other patients, no vaccine-related toxicity was observed.

Only patient 1 and patient 10 have been admitted into hospital due to vaccine-related symptoms. All the other patients received their treatment as outpatients.

Table 3
Clinical evolution and outcome

| Patient | Clinical data during vaccination | Radiological evolution | Therapy-induced clinical effect | Follow-up period after surgery (months) | Treatment after vaccination, current status |
|---------|---------------------------------|------------------------|---------------------------------|-----------------------------------------|---------------------------------------------|
| 1       | Peritumoral oedema with grade IV neurotoxicity, responding to steroids | Peritumoral oedema from V2; SD during 7–8 weeks, later PD | Yes | 7 | DOD at 05-2001 |
| 2       | Nil | — | Yes | 36 | CCR |
| 3       | Morning stiffness after V5 | Transient contrast enhancement after V5 Yes | No | 12 | Surgery, chemotherapy; DOD at 07-2002 |
| 4       | Nil | Relapse after 3 months | No | 23 | Temozolomide; Ommaya reservoir and repetitive punctures of cystic fluid; DOD at 11-2003 |
| 5       | At moment of V3; thrombocytes = 72 000 μl\(^{-1}\) | Relapse after 3 months | No | 17 | DOD at 11-2003 |
| 6       | Subdural hygroma after surgery; CCR of primary tumour; anaemia after V3: Hb = 9 g dl\(^{-1}\) | — | Yes | 7 | Abrupton of further vaccination; † at 09-2002 |
| 7       | Nil | PD after 2 months | No | 8 | DOD at 10-2002 |
| 8       | Nil | Immediate progression | No | 4 | DOD at 08-2002 |
| 9       | Night sweating after V4 | PD after 2 months | No | 7 | Chemotherapy; DOD at 12-2002 |
| 10      | Meningismus after V3 | PD after 4 months | No | 9 | Chemotherapy; DOD at 02-2003 |
| 11      | Nil | PD after 16 months | No | 14 | Chemotherapy; DOD at 11-2003 |
| 12      | Nil | Relapse after 3 months | No | 14 | Chemotherapy; DOD at 11-2003 |

CCR = continuous complete remission; DOD = death of disease; Hb = Haemoglobin; PD = progressive disease; SD = stable disease; Vx = vaccine number.
vaccines could be administered because of the rapid decline of the patient’s neurological status.

DISCUSSION

We summarised the observations on 12 patients with relapsed malignant glioma who were vaccinated with autologous DC loaded with autologous crude tumour homogenate after reoperation. In 25% of patients, we documented an objective clinical response. The report illustrates that, in spite of considerable logistical and technical difficulties, it is worthwhile to further develop the approach of protein-loaded DC as therapy against malignant glioma, even in the absence of known target antigens and for tumours in immunologically privileged sites such as the brain.

An increasing number of clinical trials evaluate DC-based vaccines in the therapy of cancer in adult (Jefford et al., 2001) and in pediatric patients (Geiger et al., 2001). Specific peptides were mostly used in DC vaccination strategies for melanoma, prostate cancer or breast/ovarian cancer. Also for malignant brain tumour, MHC class I-associated peptides have been eluted from cultured autologous glioma cells, and a mixture of peptides was used to load DC since no specific tumour antigenic targets are known (Yu et al., 2001). In other trials, tumour proteins instead of peptides have been used to load DC (Hsu et al., 1996; Schott et al., 2000; Geiger et al., 2001; Chang et al., 2002; Höltl et al., 2002; Wheeler et al., 2003; Yamanaka et al., 2003; De Vleeschouwer et al., 2004). The use of proteins from autologous tumours instead of peptides is now generally considered a valuable approach, certainly when tumour-specific epitopes are not known (Curiel and Curiel, 2002).

The technical aspects of DC vaccination have recently been reviewed (Schuler et al., 2003). Intradermal administration of loaded mature DC seems to be preferable. Up to now, only empirical DC schedules are used. Our observational study pointed to some further practical issues according to feasibility. The size of the tumour sample and the yield of tumour proteins available to load DC were different for each patient. Based on laboratory data on antigen-presenting capacity and quality of DC (manuscript submitted), the range to load one million DC was kept between 30 and 200 μg of tumour proteins. Similarly, the number of DC per injection was different for each preparation and reflect an unavoidable heterogeneity commonly encountered in such studies (Geiger et al., 2001; Höltl et al., 2002). The fact that some or our patients had tumour control obtained after injection of lower numbers of DC is remarkable.

As this is a feasibility study, it is important to stress that the only selection of candidates for the adjuvant DC-based vaccination therapy was the surgical operability: all patients in whom an intended extensive tumour debulking was deemed feasible and safe by the neurosurgeon were eligible. The actual fraction of patients with a recurrent glioma, who possibly could benefit from this adjuvant therapy in this stage, can only be estimated and probably approaches 10%. Not a single included patient, however, dropped out after surgery: only in patient 2 (partial resection), we stopped after the second vaccination because of overwhelming tumour progression with rapid decline of her neurological status.

Immune monitoring was performed with skin tests, when enough tumour material was available. The DTH testing to antigen is one clinical monitoring tool to indicate cellular immunity, although it remains controversial whether or not DTH to autologous tumour can be a reliable correlate of clinical responses.
The group of patients was heterogeneous, because we wanted to assess general feasibility of tumour vaccination. Based on the safety (patient 1) and efficacy (patients 2 and 3) data obtained, (sub)total resection of the tumour should be the major inclusion criteria for upcoming DC vaccination strategies. The induction of serious and clinically important peritumoral oedema in our first patient shows a potential and unacceptable vaccination-related risk for patients with partially resected tumours. From an immunological point of view, tumour-induced immune suppressive mechanisms are limited when the tumour burden is lowered (Holladay et al., 1994). In addition, steroids can generally be weaned faster in case of (sub)total resection. Previous unpublished studies of our group showed that sufficient numbers of high quality DC cannot be obtained in glioma patients receiving steroids shortly prior to blood sampling. In fact, our observations illustrate in clinical practice that surgery-induced minimal residual disease is a prerequisite for a clinically relevant efficacy of DC vaccination (Smyth et al., 2001). Vaccination might induce better survivals in younger patients, in whom complete resection of malignant glioma can be reached more frequently. Moreover, the cytogenetic entity of malignant glioma at younger age differs from adult malignant glioma (Kraus et al., 2002) and might also be responsible for different immunological targeting, besides the differences in immune competence at younger age (Wheeler et al., 2003). To further implement these issues, the ongoing HGG-IMMUNO-2003 trial of our group includes patients below the age of 60 years with at least subtotal resection of the relapsed tumour. The amount of tumour proteins available should be enough to be able to provide at least five vaccines with at least $5 \times 10^6$ DC loaded with at least $50 \mu g$ tumour proteins per $10^6$ DC. The clinical effects will be evaluated by determining the PFS, OS and quality of life in a larger series, and in comparison to a matched historical control group. The immunological effects of DC vaccination in these patients will be further elucidated including Elispot immune monitoring.

After standard treatment for newly diagnosed malignant glioma, patients with early relapse and at least subtotally resectable tumours may particularly benefit from adjuvant immunotherapy. DC immunotherapy appears promising as an approach to successfully induce an antitumour immune response and long-lasting tumour control. This may prolong survival of patients with malignant brain tumours without compromising their quality of life.

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