Colonisation of Clostridium in the body is restricted to hypoxic and necrotic areas of tumours

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CLINICAL MEDICINE/PATHOGENESIS

Colonisation of *Clostridium* in the body is restricted to hypoxic and necrotic areas of tumours

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The use of gene therapy is one of the most recent molecular strategies for the treatment of cancer. It is essential, however, to have an efficient transfer system by which the desired gene can be delivered to the correct environment. The experiments described in this report investigate apathogenic *Clostridium* as a possible vector to transfer a specific gene product into the extracellular microenvironment of the tumour which is hypoxic/necrotic in parts, using WAG/Rij rats with transplantable rhabdomyosarcomas as a model. Our data show that *Clostridium*, after systemic administration of at least 10^7 spores, specifically colonises the hypoxic/necrotic areas of our tumour model, the most efficient species being *C. acetobutylicum* (NI-4082) and *C. oncolyticum*. Although spores were also detected in normal tissues for up to 4 weeks, they did not germinate in these tissues. We conclude that it seems likely that these bacteria can be used as a selective transfer system into the extracellular environment of tumours which have hypoxic regions. This strategy would be more tumour-specific than various other strategies that are currently being investigated in anti-cancer gene therapy.

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Introduction

The use of metabolic suicide genes is a recently developed strategy in cancer treatment [1]. It is a promising, indirect approach to gene therapy, based on the delivery of specific non-mammalian genes to the target cell or its microenvironment. Successful gene therapy demands not only the identification of an appropriate therapeutic gene for treatment of the disease, but also a selective vector system by which the gene can be delivered to the desired site both efficiently and accurately. Viral vector mediated gene transfer has been the most extensively studied gene delivery system [2, 3]. Also non-viral mediated gene therapies (for example, targeted liposome vectors) are currently under development [4]. As an alternative non-viral gene delivery system, a novel tumour-specific transfer system using apathogenic anaerobic bacteria has been proposed [5, 6]. Several observations sustain the feasibility of the anaerobe-mediated targeting strategy: (i) Solid tumours are characterised by an aberrant capillary network. These capillaries exhibit many shunts, tortuosities and disruptions. As a consequence, a significant part of the tumour becomes hypoxic. Severe hypoxia is not found in non-pathological, normal tissues [7, 8]. (ii) Infiltration of anaerobic bacteria in human tumours is observed in the clinic [9, 10]. (iii) After intravenous administration certain obligate anaerobic bacteria proliferate in hypoxic and necrotic areas of solid tumours [11, 12]. This technique is safe since injections of 10^{10} spores of *Clostridium butyricum*, a strain that causes tumour lysis, have already been performed with healthy volunteers and cancer patients with little or no toxicity [13]. Although in many cases bacteria were found to be present in the tumours and oncolysis was observed, these trials failed to produce complete response and were discontinued. With the availability of genetic engineering, the possibility exists to improve the therapeutic effect of anaerobic bacteria. The specificity of a potential anaerobe vector system relies thus on the feature that solid tumours in contrast to healthy normal tissues have hypoxic/necrotic regions. In the body obligate anaerobes can, in theory, only proliferate in these hypoxic/necrotic tissues, which makes them thus potentially selective vectors in anti-cancer therapy. However, a certain degree of hypoxia has also been demonstrated in normal tissues [8]. Therefore, when using anaerobes as a vector system to specifically deliver therapeutic genes to a tumour, it is important to confirm experimentally the hypothesis that clostridia can grow solely in solid tumours and not in normal tissues. Furthermore, since different strains have different colonisation capacities, it was investigated among several apathogenic clostridia species which strains gave the best results.

Materials and Methods

Bacterial strains, media and growth conditions

In this study we used *Clostridium beijerinckii* ATCC17778, *C. limosum* DSM1400, *C oncolyticum* DSM754, *C. acetobutylicum* ATCC824 and *C. acetobutylicum* NI-4082 [14], now reclassified as *C. saccharoperbutylacetonicum* [15]. To obtain sufficient spores for the administration to the rats, conditions for an efficient sporulation were examined, since a universal sporulation medium for clostridia is not available. Different culture media were compared including Trypticase-soy-agar medium (BioMérieux) enriched with 5% defribinated horse blood (Bio Trading Benelux), Reinforced Clostridial Medium (Difco) and Clostridial Basal Medium [16]. Incubation was carried out at 37°C in an anaerobic incubator (Forma Scientific) with 90% N_2 and 10% H_2 with palladium as catalyst. For the preparation of a spore suspension, *Clostridium* cells were collected from the culture plates using physiological water. Suspensions were centrifuged, pellets washed and resuspended in physiological water. The suspension was exposed to 73°C for 20 min to kill vegetative cells. Thereafter, serial dilutions were prepared to estimate the number of spores.

In vivo experiments

WAG/Rij rats with syngeneic rhabdomyosarcomas were used as the tumour model. The tumour was implanted subcutaneously in the flank of the rat. These fast-growing tumours do not produce early metastases and are known to be hypoxic [17]. When the tumours had a mean diameter of 2 cm, 10^5 to 10^9 spores suspended in 0.2 mL of saline were systemically administered to the rats, under a short general anesthesia with ether. At different time intervals after the injections, the animals were sacrificed. Tumours and pieces of normal tissue were removed and about 1 g tissue was used for examination. After mincing under sterile conditions, 0.05 mL cell suspension of the different dilutions was added to tubes containing 3.5 mL of thioglycolate medium to estimate the number of viable bacteria. To discriminate between the number of spores and vegetative cells in the samples, dilutions series were prepared from heated (at 73°C for 20 min) and non-heated samples. After 2 days of incubation at 37°C, reading of dilutions that showed *Clostridium* growth were done. The ethical committee of the University has approved these experiments.

To evaluate the eventual presence of *Clostridium* in urine, urine of the *Clostridium* treated and non-treated rats was collected in a tube. Therefore 0.05 mL from serial dilutions in saline was added to tubes containing 3.5 mL of thioglycolate medium or on blood agar plates to estimate the presence of bacteria. Each experiment has been repeated at least three times with a minimum of three to five animals per point and per experiment.

Histological examination

Tissues were fixed in Bouin's solution and embedded in paraffin. Sections (10 μm) were Gram-stained for bacterial visualisation and stained with hematoxylin-eosin for histological evaluation. For a number of tumours complementary staining was performed. In that case, the animals were intraperitoneally injected with 300 mg/kg 7-4′-2-nitroimidazole-1-yl-butyl-therophylline (NITP), a marker of hypoxic areas, 2 h before sacrifice [18, 19]. At 2−3 min before the sacrifice the animals were also injected intravenously with the fluorescent dye Hoechst 33342 (15 mg/kg) to estimate tumour perfusion [18]. After Hoechst injection the rats were killed and the tumour parts quickly removed, frozen and stored in liquid nitrogen. Perfusion and hypoxia were studied simultaneously in side-by-side frozen sections (5 μm) using a computerised digital image analysis system [19]. Briefly, the vasculature was visualised by staining with Collagen type IV polyclonal antibodies, followed by an incubation with a Tritc-labeled second antibody. After scanning the sections twice, using different filter-settings, the composite image with the vascular structures (red) and the composite image containing the perfused areas (Hoechst, blue) were combined. The overlapping structures showed the perfused vessels. Perfusion (Hoechst) and hypoxia (NITP) were visualised simultaneously by staining overnight with antibody against theophylline antibody, followed by two subsequent incubations with a Fitc-labeled antibody (green). Bacteria were Gram-stained. The necrotic areas were defined as regions with mainly debris and without cell structure.

Results

By comparing different culture media, it was observed that the best sporulation media were: Trypticase-soy-agar medium enriched with 5% defribinated horse blood for *C. acetobutylicum* NI-4082 (now reclassified as *C. saccharoperbutylacetonicum*), and *C. acetobutylicum* ATCC824; Reinforced Clostridial Medium for *C. oncolyticum*; and Clostridial Basal Medium for *C. beijerinckii* ATCC17778. Spores are less susceptible than vegetative cells to variable environmental conditions such as variation in O_2-concentration and they are much more resistant to heat. Spores survive fully after heating at 73°C for 20 min, while vegetative cells do not (Figure 1).

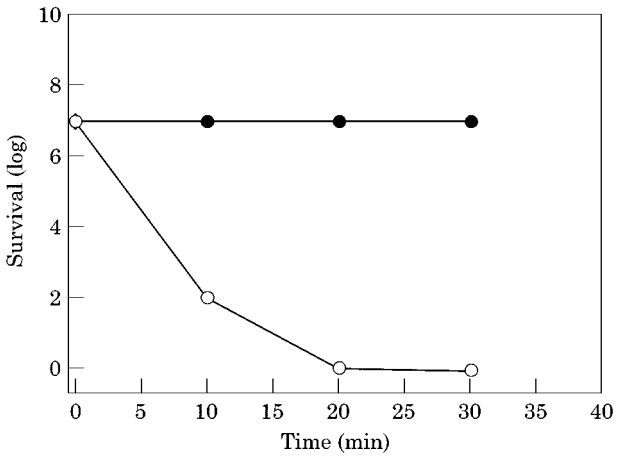

Figure 1. Effect of heating at 73°C on the survival of spores and vegetative cells of *Clostridium acetobutylicum* NI-4082: treatment at 73°C for 20 min kills the vegetative cells but not the spores. Key: ●, spores: ○, vegetative cells.

Bacterial colonisation in vivo

To obtain an efficient infiltration into the tumours, at least 10^7 spores have to be administered systemically. *Clostridium acetobutylicum* NI-4082 has superior infiltration properties compared with *C. beijerinckii*, *C. acetobutylicum* ATCC824 and *C. limosum*, but comparable infiltration properties to *C. oncolyticum*. When dilutions prepared from pieces of tissues sampled 4−5 days after the injection were incubated in thioglycolate medium to analyse the presence of clostridia, the number of colony forming units (cfu) found in tumours was up to 10^9 per gram of tissue. In normal tissues the cfu number ranged between 10^4 and 10^6 per gram of tissue. As indicated above, heat treatment can discriminate between the presence of spores and vegetative cells. After heating, the cfu count in tumours differed by an average factor of 100 (ratio vegetative cells/spores). In contrast the number of cfu in samples from normal tissues was not significantly changed after heating (Figure 2). Difference between the number of vegetative cells and spores in tumours and normal tissues got stronger when tissue analysis was performed at longer time periods after the *Clostridium* administration. These results demonstrate that in tumours *Clostridium* spores germinate into the vegetative, metabolically active cells, while in normal tissues *Clostridium* spores did not germinate. Consistent with

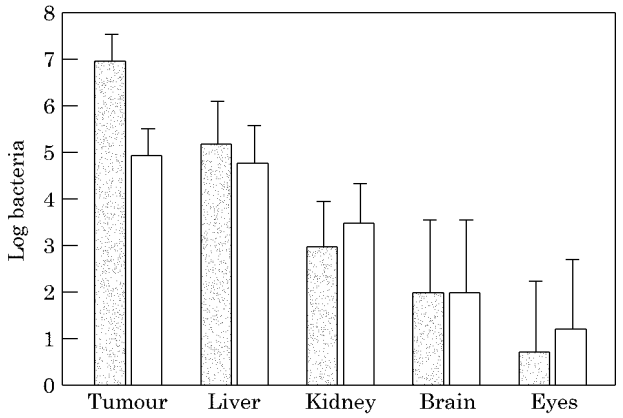

Figure 2. Differential between spores and vegetative cells of *Clostridium acetobutylicum* NI-4082 in tumours ($p < 0.05$) and in liver (NS), kidney (NS), brain (NS), and eyes (NS) (the confidence intervals are standard deviations). Key: ■, before heating; □, after heating.

this finding, vegetative cells were detected in tumour specimens by microscopic investigation following Gram-staining, but they could not be detected in normal tissues (Figure 3). We also did not find any evidence of clostridia in urine at either 4 or 8 days following the administration of spores. Another important observation was that the number of viable cells decreased as a function of time in normal tissues but not in tumours (Figure 4). In the tumours *Clostridium* vegetative cells were still found 12 days after administration. Longer time periods could not be investigated due to the fact that tumours grew to the maximum size permitted. These results were qualitatively confirmed by combined histochemical

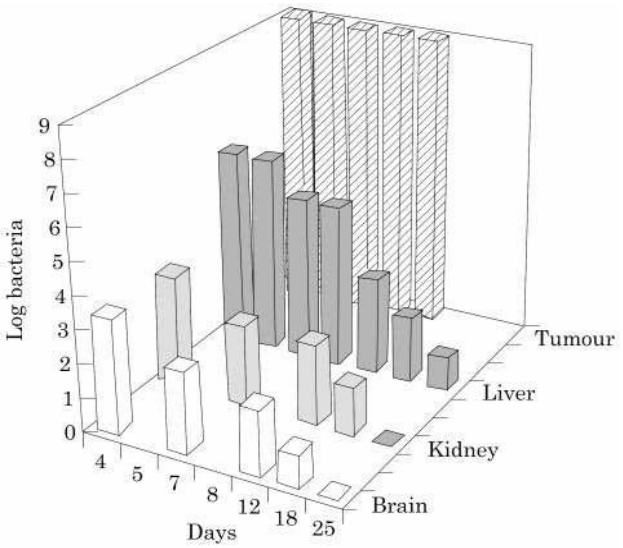

Figure 3. Presence of *Clostridium acetobutylicum* NI-4082 in tumours, liver, brain and kidney as a function of time after intravenous administration of 10^7 spores. Due to the fact that tumours grew to the maximum size permitted, longer intervals could not be investigated.

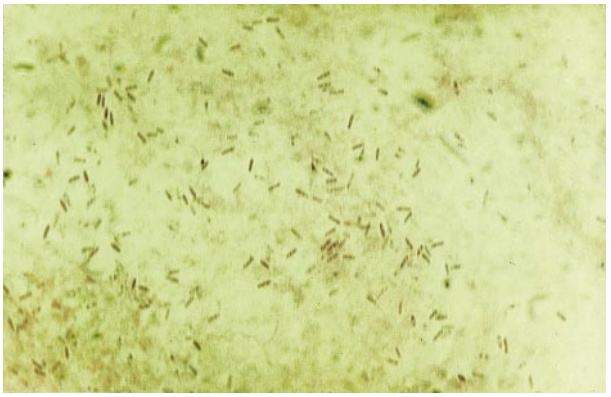

Figure 4. *Clostridium* cells visualised in the Rhabdomyosarcomas of the WAG/Rij rat after Gram-staining.

staining. Vegetative cells were found in areas stained by the hypoxic marker NITP or in acellular necrotic areas, but they were not detected in oxygenated areas, coloured by the perfusion marker Hoechst 33342.

Discussion

Infiltration studies of apathogenic Clostridium in tissues

The main aim of this work was to investigate whether after systemic injection of spores, *Clostridium* can specifically colonise tumours so that species of this genus could be used as a tumour-specific vector system. When we investigated pieces of brain, eye, liver, kidney, spleen and tumour at different time intervals after spore administration, bacteria were found in tumours and in normal tissues. However, in normal tissues *Clostridium* was present only as metabolically inactive spores and only for a period of time. In contrast, vegetative cells were predominantly present in the tumours. These vegetative cells were found in necrotic/hypoxic tumour areas which by definition have a poor blood supply. When clostridia were directly injected into the tumour, bacterial colonisation of the tumours always accounted for at least 10^8 cfu, with mainly vegetative growth. Spores could then also be detected in small amounts in the normal tissues, as observed following intravenous administration but again no vegetative cells were detected. Furthermore we have investigated which species might be most suitable because of superior colonisation capacity. From the obtained results it became clear that *C. acetobutylicum* NI-4082 and *C. oncolyticum* gave comparable results but superior to the other investigated species.

Tumour hypoxia and tumour necrosis

The system proposed here is quite different from the application of *Shigella* and *Salmonella* which grow in

hypoxic and in aerobic tissues [20, 21]. The proposed *Clostridium* transfer system uses hypoxia being a characteristic feature of the solid tumour. An extra advantage to targeting hypoxic cells is that they negatively influence treatment outcome, as they appear to be resistant to radiotherapy and chemotherapy [7, 24]. It has been shown that hypoxia induces DNA over-replication and enhances metastatic potential of murine tumour cells [22]. Good correlations were observed in two clinical studies in which the PO_2 distribution in cervix cancers and tumours of head and neck were measured by micro-electrodes and related to local tumour response after radiation therapy: the least well-oxygenated tumours showed the poorest response [25, 26]. Moreover, hypoxia selects for tumours that are clonal expansions of cells that have lost their apoptotic ability and have switched to a pro-angiogenic phenotype [22, 23]. It can be argued that only hypoxic tumours should be an indication for the application of the *Clostridium* vector system and it is known that not all solid tumours have a significant degree of hypoxia [8]. There is probably no strict parallelism between radiobiological hypoxia, which is a phenomenon at the cellular level, and the level of hypoxia needed to grow clostridia which probably also need a certain volume of necrosis. This point has to be further investigated. In the histological analysis, we have shown that *Clostridium* is present in hypoxic areas stained by NITP and in acellular-necrotic areas. This suggests that any treatment causing necrosis and/or hypoxia such as hyperthermia, radiotherapy or chemotherapy is likely to favour the tumour colonisation by *Clostridium*.

Immunological response of the rat to Clostridium

Would the immunological response of a host be a limitation for the applicability of such a vector? The clostridia are injected as spores and the proliferating clostrida are in poorly perfused areas that might act as an 'immunological sanctuary', like an abscess with pathological anaerobic bacteria. In the experiments we performed, we found that repeated injections of *Clostridium* spores did not provoke any change in body temperature of the rats, an indication that no severe immunological reactions occur (data not shown).

Potential advantages of the Clostridium transfer system for therapeutic applications

Successful gene therapy demands not only the identification of an appropriate therapeutic gene for treatment of the disease, but also an efficient delivery system by which the gene or its product can be delivered to the desired site both efficiently and accurately. The *Clostridium* transfer system has several advantages compared with the classical approach with viral vectors. As already discussed, this system is highly tumour specific because hypoxia is a feature of solid tumours, not of normal tissues. There is no need for transduction of the therapeutic gene into the genome of the tumour cell. Due to this, several restrictions of other gene transfer systems are avoided including the problem of the efficiency of transduction, the expression of a foreign gene in the tumour cell and of the genetic instability of recombinant tumour cells. Apathogenic anaerobic bacteria, classified as hazardous agent class 1, do not proliferate in healthy individuals because they need an oxygen-free environment. Moreover, injection of clostridia has been already performed in healthy volunteers and in cancer patients without major toxicity [27] and clinical trials were approved using antibodies to this organism to detect tumours [28].

In conclusion, our data show that *Clostridium*, after intravenous administration of at least 10^7 spores, specifically colonises the hypoxic/necrotic areas of our tumour model, the most efficient species being *Clostridium acetobutylicum* NI-4082 and *C. oncolyticum*. Although spores were also detected in normal tissues for up to 4 weeks, they did not germinate in these tissues. We conclude that it seems likely that these bacteria can be used as a selective gene transfer system into the extracellular environment of tumours which have hypoxic regions. This strategy would be more tumour-specific than various other strategies that are currently being investigated in anti-cancer gene therapy.

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