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FEMS Immunology and Medical Microbiology 30 (2001) 37–41

FEMS Immunology and Medical Microbiology

www.fems-microbiology.org

Improvement of *Clostridium* tumour targeting vectors evaluated in rat rhabdomyosarcomas

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Received 23 October 2000; accepted 27 October 2000

Abstract

Previous studies have demonstrated the feasibility of using apathogenic clostridia as a promising strategy for hypoxia-specific tumour targeting. The present study shows that the use of the vascular targeting compound combretastatin A-4 phosphate could significantly ($P < 0.001$) increase the number of *Clostridium* vegetative cells in rat rhabdomyosarcomas with sizes between 0.2 cm^2 and 3 cm^2. Furthermore, this study showed that administration of metronidazole for a 9-day period was sufficient to eliminate systemically administered *Clostridium* from the tumour. Moreover, previous *Clostridium* spore administration did not effect tumour colonisation, regardless of the immune response status of the host.

Keywords: Anti-cancer treatment; Gene therapy; Hypoxia; Vascular targeting; *Clostridium*

1. Introduction

The potential use of gene therapy as an oncologic treatment modality has been extensively addressed. Most viral and non-viral transfer systems developed so far are however hampered in their use due to the lack of tumour specificity and low transduction efficiencies [1]. An innovative approach for tumour-specific delivery of therapeutic proteins is to use bacterial vector systems [2–4]. Infections with anaerobic bacteria can spontaneously occur in rodent as well as in human tumours [5,6]. Tumours can also be infected by systemic injections of spores of anaerobic bacteria, as shown both in rodent tumours [7] and in cancer patients in clinical trials [8]. This is explained by the fact that rodent (e.g. [9]) and most human (e.g. [10]) solid tumours have size-related hypoxic/necrotic regions. Hypoxic conditions are normally absent in healthy tissues and necrosis is never detected [10].

Recombinant DNA techniques and specific transformation protocols are available to engineer *Clostridium* to express and secrete proteins that have a potential antitumoural effect [11,12]. Administration of such recombinant *Clostridium* to a tumour-bearing organism may thus likely result in a tumour-specific effect.

Using the rhabdomyosarcoma rat tumour model we have previously quantified the specific colonisation of tumours with volumes larger than 3 cm^3 following systemic administration of clostridial spores. Tumours with smaller size were inefficiently colonised. Spores did not germinate in normal tissues [2].

Experiments were now set up in order to improve the colonisation capacity of this bacterial-based vector system in much smaller tumours (< 3 cm^3). Such tumours inherently have less hypoxia and little or no necrosis. It was previously shown in several rodent tumour models that combretastatin A-4 phosphate (combreAp), injected at systemically non-toxic doses, induced a severe vascular

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0928-8244/01/$20.00
PII: S0928-8244(00)00231-5

shutdown within 3–6 h resulting in the development of extensive necrosis at 1–3 days (e.g. [13,14]). Based on this knowledge, administration of *Clostridium* spores was combined with combreAp administration and the effect on increase in bacterial colonisation was analysed.

We further investigated the efficiency by which *Clostridium* could be removed from a tumour-bearing organism following antibiotic treatment. This safety aspect is of much importance in case adverse effects should occur. Finally, the extent of the host immune response and the possible hindrance on colonisation capacity of the tumour with *Clostridium* after a first application was evaluated.

2. Materials and methods

2.1. Bacterial strain, tumour model and colonisation experiments

Clostridium sporogenes used in this study was isolated in our laboratory and identified following 16S rRNA sequence determination. This strain was cultivated in an anaerobic incubator (Forma Scientific, model 1024) with palladium as a catalyst on trypticase-soy-agar medium (TSA, BioMérieux) enriched with 5% defribinated horse blood for 10 days at 37°C. Spores were collected from the agar plates as described [2].

Male WAG/Rij rats with subcutaneously (s.c.) transplanted syngeneic rhabdomyosarcomas were used as in vivo model [2]. The test tumours were divided into groups of predetermined volume: <1 cm^3, 1–3 cm^3 and >3 cm^3, depending on the type of experiments. Before and during the follow-up period, the rats were given food and water ad libitum in a conventional housing facility. When tumours reached the predetermined volume, 10^8 spores were systemically administered to the rats and colonisation was analysed as described [2].

2.2. Vascular targeting compound

Freshly prepared solutions of combretastatin A-4 phosphate (CombreAp; Oxigene, Lund, Sweden) were given i.p. as a 0.5-ml saline solution in a concentration of 25 mg kg^{-1}, 4 h after *Clostridium* administration. This allowed the distribution of the bacteria throughout the vascular network and, specifically, in the tumours, prior to any possible tumour blood vessel damage. A number of tumour-bearing rats received a sham-injection of saline only.

2.3. Metronidazole treatment and subsequent tumour colonisation

To test the effect of antibiotic therapy, metronidazole (Flagyl®) dissolved in saline was injected intraperitoneally (i.p.) at 200 mg kg^{-1} twice a day for up to 9 days, starting at day 5 following spore administration. Control animals received the same volume of saline. Six hour after the last injection, bacterial colonisation was determined [2]. Each experiment was repeated three times, each time with a minimum of 3–5 animals per point.

The possibility of *Clostridium* to colonise tumours in animals that had been treated with metronidazole to eradicate previously administered clostridia was investigated. Non-tumour bearing rats were therefore injected systemically with 10^8 spores. To control the distribution of bacteria in the body, liver and spleen were removed from randomly selected rats at day 5 following injection and analysed for the presence of *Clostridium* spores (see [2] for technique). All other animals were after spore injection treated with metronidazole according to the optimal schedule and time (see Section 3). Five weeks after the antibiotic treatment, tumours were implanted s.c. in the flank of the treated animals. When the tumours reached a volume of at least 4 cm^3, clostridial spores were systemically injected and five days later, colonisation was evaluated. A control group of tumour-bearing WAG/Rij rats, which did not receive pretreatment with clostridial spores and metronidazole, were injected only once with a suspension of 10^8 spores. Tumour specimen were microscopically inspected at the time of removal. Quantification of *Clostridium*-specific antibodies was assessed using an ELISA-assay (Eurogenetics, Tessenderlo, Belgium). WAG/Rij rats with and without tumour, not previously injected with *Clostridium* were used as a negative control. During the course of the experiments body temperature and weight were followed.

2.4. ELISA assay

Blood samples were taken from each rat and the amount of *Clostridium*-specific antibodies in serum was estimated using an enzyme immunoassay. Therefore, clostridial cell lysates were generated by sonication, and 150 μl of 100-fold dilutions of the protein extracts were used to coat the solid phase in a 96-well microtitre plate. Following overnight incubation at 4°C, plates were washed three times with phosphate-buffered saline (PBS). For signal detection, 125 μl of the isolated rat antisera used as primary antibodies were diluted 1/2000 in PBS containing 7.5% BSA, incubated for 45 min at 37°C. 125 μl of horseradish peroxidase (HRP)-conjugated rabbit anti-rat immunoglobulins (1/1000) were taken as secondary antibodies (Dakopatts). It was subsequently added to the appropriate wells and incubated for 45 min at 37°C. After each antibody incubation step, blots were washed five times with PBS+0.1% Tween-80. For detection, 100 μl substrate was added to each well. The reaction was stopped after 20 min by adding 50 μl of 2N H_2SO_4 and the colour change in the plates was read at 450 nm. A standard curve was prepared by serially diluting a pool of maximal responders in PBS containing 7.5% BSA, and ranged from

1 to 1000 arbitrary units (AU) ml^{-1}. Samples and controls were added in duplicate to the appropriate wells.

3. Results

3.1. Improvement of clostridial tumour colonisation

In a first series of experiments, rats bearing tumours with volumes larger than 3 cm^2 were injected systemically with 10^8 *Clostridium* spores. The tumours were resected 4–5 days after the injection and dilutions of the tumour homogenates were prepared in saline. The analysis of bacterial growth in these larger tumours revealed the presence of at least 10^7 colony forming units (cfu) per g of tumour tissue (Fig. 1, left panels).

In a second series, 10^8 *Clostridium* spores were injected in rats bearing tumours measuring between 0.2 and 3 cm^2. In contrast to the larger tumours, the number of vegetative cells in these tumours was significantly lower ($P < 0.0001$) (Fig. 1, right panels) and varied between 0 and 10^3 cfu per g tissue, with exceptionally a tumour containing 10^6 cfu per g tissue. When these small tumours were classified in volumes between 1 and 3 cm^2 and volumes less than 1 cm^2, the differences in colonisation were even more striking (Fig. 2, left panels). In the very small tumours (< 1 cm^2), on average about $10^{0.5}$ cfu of *Clostridium* per g tissue were detected.

In parallel with these investigations, rats bearing rhabdomyosarcomas of closely matched sizes were treated with a single i.p. injection of combreAp at 4 h after administration of the *Clostridium* spores. This resulted in a strongly improved colonisation (Fig. 2, right panels). Particularly, tumours smaller than 1 cm^2 contained in most instances 10^6–10^7 cfu per g tissue. The bacterial colonisation of combreAp-treated tumours of 1–3 cm^2 or < 1 cm^2 was significantly higher than the colonisation of sham-treated tumours of a similar size (with $P < 0.001$ and $P < 0.0001$ respectively).

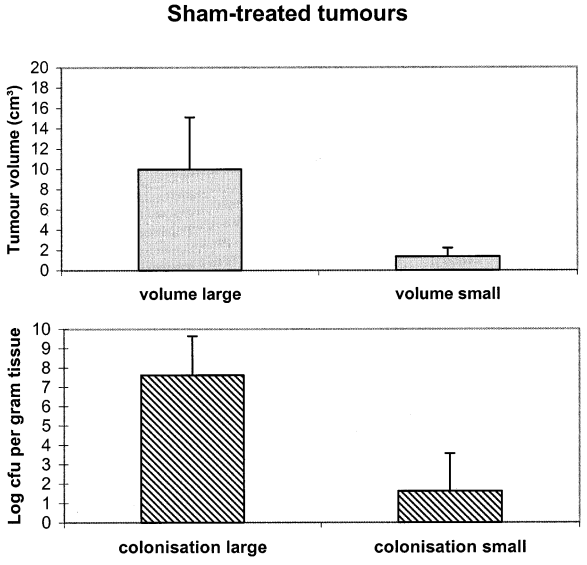

Fig. 1. The number of cfu measured in large (> 3 cm^2) (left panels) and small (0.2–3 cm^2) tumours (right panels). Bars represent mean ± S.E.M. The difference between colonisation 'large' and colonisation 'small' is very significant ($P < 0.0001$).

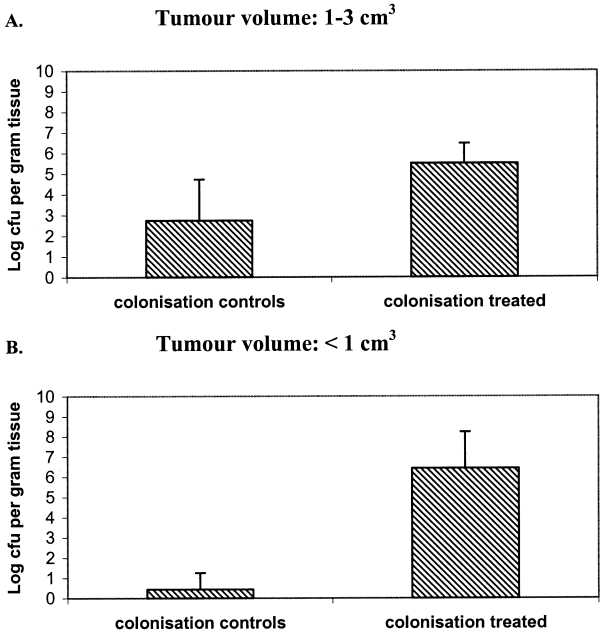

Fig. 2. The number of cfu measured in rhabdomyosarcomas with volumes between 1 and 3 cm^2 (A) and with volumes less than 1 cm^2 (B) for sham-treated (left panels) and combreAp-treated (right panels) rats. Colonisation is significantly higher in the combreAp-treated tumours ($P < 0.001$ and $P < 0.0001$, respectively). Bars represent mean ± S.E.M.

Gross examination of excised sham-treated small tumours always revealed a firm, viable and well-vascularised tissue at transsection. Histopathological examination demonstrated few, scattered foci of necrosis only in some tumours of 1–3 cm^3, whereas this was not observed in tumours < 1 cm^3. In tumours treated with combreAp alone, a large core of necrosis with a rim of viable tumour tissue was observed at 4–5 days post-injection (the standard time-interval for colonisation measurements). When *Clostridium* spores were injected 4 h prior to the combreAp treatment, most of the tumours became soft on palpation during the follow-up period. When transsected for the colonisation study, these tumours showed some degree of liquid necrosis. Also in these tumours, viable tissue was a rim surrounding the necrotic centre.

3.2. Removal of Clostridium from the tumour by antibiotic treatment

Experiments were designed to determine the effectiveness of an antibiotic treatment specific for anaerobes in order to stop tumour colonisation, if desired. After treatment with metronidazole, a decreasing number of cfu was measured in the tumour as a function of treatment dura-

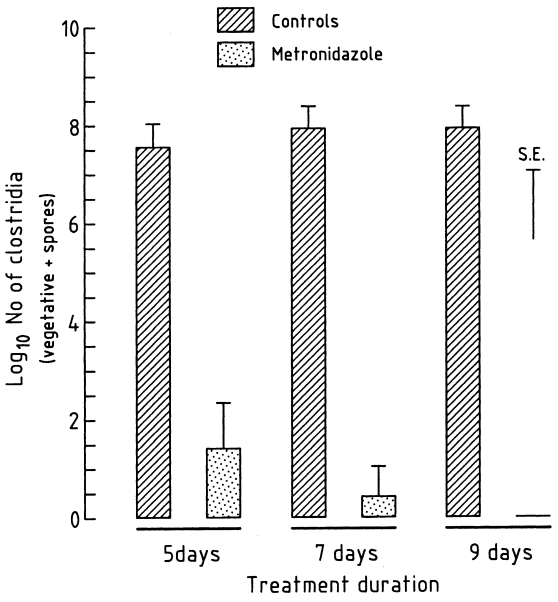

Fig. 3. Effect on the number of cfu in tumours after 5, 7 or 9 days of treatment with metronidazole. The number of bacteria with and without metronidazole treatment are indicated. After treatment with metronidazole, a decreasing number of cfu was measured in the tumour.

tion. In these treated animals, the number of cells before and after heating was not significantly different (data not shown), which means that all the vegetative cells were killed. After 9 days no bacterial growth nor presence of spores was detected in the tumours of metronidazole-treated animals (Fig. 3). During this period, colonisation in tumours of animals that were not treated with antibiotics remained high. These results clearly indicate that metronidazole eradicates *Clostridium*.

3.3. Analysis of the occurrence of Clostridium-specific antibodies and effect on tumour colonisation

An ELISA assay was developed to quantify the amount of *Clostridium*-specific antibodies in serum of rats that were previously injected with *Clostridium* spores and treated with metronidazole before re-injection. A comparison was made both with animals receiving *Clostridium* spores only once and with animals that were not treated at all. To obtain a sensitive assay, optimum concentrations of primary serum antibody, HRP-labelled second antibody and antigen were determined. A maximum discrimination between the points in the standard curve could thus be obtained. After a single injection of *Clostridium* spores, an immune response was induced but only a small amount of *Clostridium*-specific antibodies could be detected (<50 AU). The majority (8/12) of animals that were injected twice with *Clostridium* spores could be labelled as 'non-responders': the amount of *Clostridium*-specific antibodies was not different compared to the animals that received only one injection of the spore suspension. In the other animals of this experimental group (4/12), an increase (up to 10-fold) of *Clostridium*-specific antibodies in the serum could be demonstrated. Interestingly, regardless of the immune response status, tumour colonisation did occur to the same extent in animals that were previously injected with a clostridial spore suspension compared to animals that were not treated before. Again, spores as well as vegetative cells were present in the tumour as concluded from the difference in cfu before and after heat treatment and confirmed by microscopical evaluation of the tumour suspensions. During the course of the experiments, no change in body temperatures or body weights could be detected.

4. Discussion

It is a well established phenomenon that certain species of *Clostridium* can selectively colonise hypoxic/necrotic regions of solid tumours [2,3]. It intrinsically means that if a recombinant *Clostridium* is administered to a tumour-bearing subject for therapeutic purposes, the metabolically active cells that produce the therapeutic proteins will be present only in the solid tumour. In addition to this tumour selectivity, a major advantage of using an anaerobic bacterial vector system is the fact that the therapeutic gene has not to be transduced into the genome of the tumour cell. Eventual problems herewith associated such as a low transduction efficiency or the risk of insertional mutagenesis can be avoided, since the anti-cancer gene will be expressed and secreted from the bacteria.

However, one of the disadvantages of such type of vector systems so far was that only larger tumours (>3 cm^3) could become colonised with *Clostridium*. The latter finding would hamper the overall advantage of the proposed *Clostridium* tumour targeting system being of low value when considering both small tumours or tumours not containing severe hypoxic/necrotic regions. The present investigations demonstrated that a significant improvement of tumour colonisation in very small tumours (<3 cm^3) could be obtained when the rats were injected with combreAp after the administration of clostridial spores. The results of the histopathological analysis as well as the gross appearance at the transsection of the tumour indicated the strong relationship between the increased presence of *Clostridium* and the level of necrosis induced by the combreAp.

Important considerations using 'classical' gene therapy vectors should be made regarding the safety issues. The concern that viral vectors could recombine with endogenous viruses remains. If the therapeutic gene would have unexpected major side-effects, the recombinant virus can not be removed from the body and gene expression can not be stopped. Furthermore, viruses are classified as hazardous agents class 2 in the European list of infectious agents. In contrast, the apathogenic bacteria used in our approach are classified as hazardous agents class 1 and sensitive to antibiotics. Moreover, as demonstrated in

our studies, complete removal of vegetative *Clostridium* cells, even from the tumour tissue, could be achieved after 9 days with metronidazole, a standard antibiotic for anaerobic infection,. Metronidazole and other electron-affinity drugs have also been used as hypoxic radiosensitisers in cancer treatment, which suggests that these compounds have the ability to reach the hypoxic, badly perfused tumour areas [15]. This indicates that their anti-bacterial effectiveness is not hindered by the absence of an adequate intratumoural vascular network.

Another important factor to be taken into consideration is an eventually induced host immune response after a single or repeated administration of *Clostridium* and its consequences on tumour colonisation. The results indicated the absence of such an induced immune response in most rats. Moreover, even in those animals that showed a 10-fold increase in *Clostridium*-specific antibodies in the serum following a second bacterial administration, colonisation of tumours was not affected. This is of great importance, since it implies that long-term production of the therapeutic proteins from the engineered *Clostridium* is possible in tumours.

Taken together, these findings illustrate the advantages of a vascular targeting approach for use in *Clostridium*-guided tumour targeting systems. Furthermore, this combined anti-cancer approach may also incorporate radiation therapy. Combining combreAp with single dose irradiation irradiation seems to result in an increased tumour-cell kill, as indicated by at least three research teams (Li et al. [16], Chaplin et al. [17] and Landuyt et al. [18]) using rodent tumours. These strategies may be exploited to improve both the selectivity to target the solid tumours, and the subsequent production of the therapeutic protein into the extracellular microenvironment of these tumours.

Acknowledgements

We are grateful to Prof. Ron Pero (Oxigene Inc., Lund, Sweden) for the delivery of Combretastatin A-4 phosphate for the present investigations. The 'Onderzoeksfonds K.U. Leuven', the 'Fonds voor Wetenschappelijk Onderzoek-Vlaanderen', and 'Verkennende Internationale Samenwerking' are acknowledged for financial support. J. Theys and S. Nuyts are research fellows of IWT ('Vlaams Instituut voor de Bevordering van het Wetenschappelijk-Technologisch Onderzoek in de Industrie').

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