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RESEARCH ARTICLE

Radio-responsive recA promoter significantly increases TNFα production in recombinant clostridia after 2 Gy irradiation

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One of the major problems with gene therapy today is the lack of tumour specificity. The use of anaerobic apathogenic clostridia as a gene transfer system can target anoxic areas within the tumour. These bacteria can be genetically modified to express therapeutic proteins such as TNFα locally in the tumour. As shown in our results, ionising irradiation can be used in clostridia to activate genes encoding cytotoxic agents under control of a radiation-inducible promoter. A 44% significant increase ($P < 0.05$) in TNFα secretion was seen 3.5 h after a single dose of 2 Gy. A second dose of 2 Gy was also capable of repeating gene activation and gave a significant increase of TNFα production of 42% ($P < 0.05$). These results provide evidence that spatial and temporal control of gene expression can be achieved using a radio-inducible promoter. Repetitive gene activation was feasible with a second dose of 2 Gy, indicating that fractionated radiotherapy could lead to repeated gene induction resulting in prolonged and enhanced protein expression. Gene targeting by ionising radiation could thus provide a new means of increasing the therapeutic ratio in cancer treatment. Gene Therapy (2001) **8,** 1197–1201.

Keywords: *Clostridium; radio-inducible promoter; radiotherapy; recA; TNFα*

Introduction

Approximately 30% of cancer-related deaths are caused by local tumour failure suggesting that improving local control has the potential to improve the survival of one-third of all cancer patients. Many strategies to improve local tumour control are currently under investigation. Promising strategies seem to be those that combine existing therapeutic modalities with new developments including combining ionising irradiation with gene therapy.[1–9]

This combination appears particularly promising as therapeutic genes can be chosen which have a radiosensitizing effect, thereby improving local tumour eradication. An example of this is the gene encoding the cytokine TNFα. This protein has direct cytotoxic effects on some tumour cells,[10] can activate a cellular immune response[11] and can cause destruction of tumour microvasculature.[12] Moreover, TNFα shows synergistic or additive cell killing in combination with radiation.[13]

A major obstacle in gene therapy is the specific targeting of therapeutic products to the tumour cell while concurrently leaving normal tissue unaffected. Tumour-specific gene expression can be achieved either by targeting the delivery system (transductional targeting) or by limiting expression of the gene to the tumour cells (transcriptional targeting). One way to address the latter strategy is the use of radiation-inducible promoters to spatially and temporally target gene expression.[1–9] For example, when the radio-responsive *Egr1* promoter was used to regulate expression of TNFα in human xenografts, an increase in tumour cure was noted without increasing normal tissue toxicity.[2,3]

The combination of gene therapy with radiotherapy thus appears to be a promising approach to increase the therapeutic ratio of cancer therapy. However, an important limitation in this strategy remains the lack of specificity of the vectors used to deliver the therapeutic gene to the tumour.

In our laboratory, we use strictly anaerobic apathogenic *Clostridium* spp. to deliver the therapeutic proteins to the tumour. When *Clostridium* spores are injected intravenously into a tumour-bearing animal, a selective colonisation of hypoxic/necrotic tissues, with more than 10^8 CFU/g tumour is obtained.[14] Moreover, the bacteria can be genetically modified to produce and secrete therapeutic proteins.[14–18] To further increase the specificity of cytotoxic protein delivery and to achieve temporal control of protein expression, we have investigated the use of prokaryotic radio-induced promoters to control gene expression in *Clostridium*.[19,20] Via the use of a reporter gene, we demonstrated that the *recA* gene, belonging to the SOS-repair system in bacteria, is induced by ionising irradiation.

In this report, we investigated whether ionising

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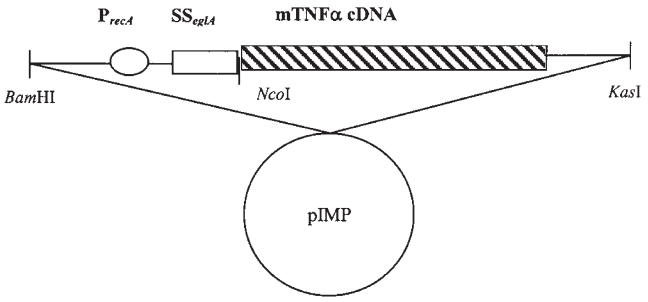


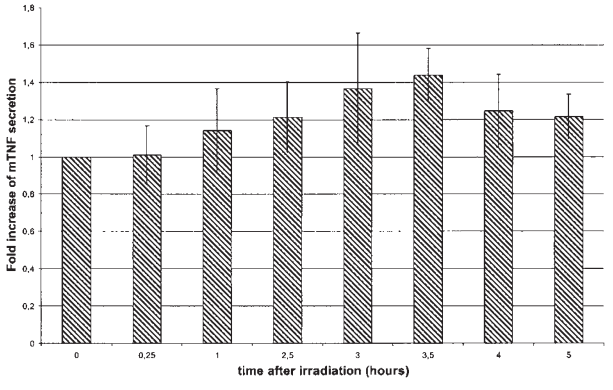
Figure 1 *The shuttle vector pIMP contains mTNFα under regulation of the recA promoter. Schematic representation of the pIMP-recA-mTNFα shuttle vector. P_{recA}, promoter region of recA; SS_{eglA}, signal sequence of eglA. Restriction sites are indicated.*

irradiation could increase the production of TNFα in *Clostridium* in the case of TNFα-cDNA placed under the control of the *recA* promoter. We measured the amount of TNFα production after single-dose irradiation and tested if gene activation could be repeated with a second radiation dose.

Results

ELISA analysis was used to quantify TNFα secretion by recombinant clostridia containing mTNFα-cDNA on the shuttle plasmid pIMP (Figure 1). Figure 2 shows the induction of TNFα production in recombinant clostridia containing the pIMP-*recA-TNFα* vector after a single dose of 2 Gy. The data are expressed as fold increase in secretion compared with unirradiated bacteria containing the same plasmid (see Materials and methods).

A single dose of 2 Gy showed a 1.44 (± 0.15 s.d.) fold increase of TNFα production compared with unirradiated bacteria ($P < 0.05$, Student's t test, two-paired samples for means). This maximum induction of 44% was observed 3.5 h after irradiation and declined thereafter. At earlier time intervals, no significant increase in TNFα secretion was seen, but there was a trend to increase secretion reaching a significant level after 3.5 h. These data demonstrate that the *recA* promoter can give a significant increase of TNFα production by recombinant clostridia after a single dose of 2 Gy.



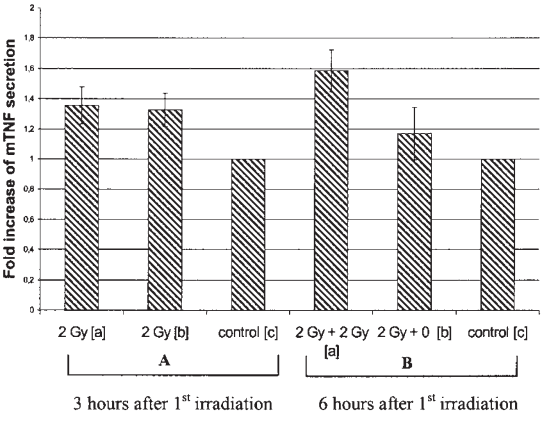
Induction factor ± standard deviation	1.00 ±0.00	1.01 ±0.16	1.14 ±0.22	1.21 ±0.19	1.37 ±0.30	1.44 ±0.15	1.25 ±0.19	1.21 ±0.12

Figure 2 *Induction of mTNFα in recombinant clostridia after a single dose of 2 Gy. Fold increase of mTNFα secretion in Clostridium acetobutylicum DSM792 pIMP-recA-mTNFα 15 min, 1 h, 2.5 h, 3 h, 3.5 h, 4 h and 5 h after a single dose of 2 Gy. The bars represent data from three independent experiments. Vertical bars represent standard deviation. Induction factors and standard deviations are represented in the table.*

In patient treatments, radiotherapy is typically given in multiple fractionated doses. Therefore, we examined if the radio-inducible promoter could be reactivated with a second irradiation. The data presented in Figure 3A represent the induction of TNFα 3 h after a first dose of 2 Gy. A 1.33 to 1.36 (± 0.11–0.12 s.d.)-fold increase was seen, which corresponds with the 37% increase in TNFα production seen in Figure 2. TNFα induction 3 h after a second dose of 2 Gy for sample [a] and a mock irradiation for sample [b] is shown in Figure 3B. The higher level of TNFα production seen after 2 × 2 Gy fractions in comparison to 1 × 2 Gy, indicated that the promoter can be reactivated at a clinically relevant dose. Overall, two fractions of 2 Gy resulted in a 1.59 (± 0.14 s.d.)-fold increase in TNFα production which was significant, compared with no irradiation ($P < 0.02$, Student's t test) and with a single dose of irradiation (1.17 (±0.18 s.d.)-fold increase), ($P < 0.05$, Student's t test). The 17% increase in sample [b] (Figure 3B) is the remaining induction effect from the first irradiation indicating that at this time interval, an induction of TNFα secretion as a result of the first irradiation remains. These data indicate that the *recA* promoter in *Clostridium* can be reactivated by a second dose of radiotherapy.

Discussion

Several previous studies have employed radiotherapy to spatially and temporally control gene expression using the *Egr1* promoter in almost each case.[1–9] However, in



Induction factor ± standard deviation	1.36 ±0.12	1.33 ±0.11	1.00 ±0.00	1.59 ±0.14	1.17 ±0.18	1.00 ±0.00

Figure 3 *Induction of mTNFα in recombinant clostridia after 2 × 2 Gy. (A) Fold increase of mTNFα secreted by C. acetobutylicum 3 h after a first dose of 2 Gy ([a] and [b]). (B) Fold increase of mTNFα secreted by C. acetobutylicum after a second dose of 2 Gy ([a]) in comparison with one dose of 2 Gy ([b]), 6 h after the first irradiation, 3 h after the second. Vertical bars represent standard deviation. Induction factors and standard deviations are represented in the table. All data are the result of three independent experiments.*

most studies induction of gene expression is achieved using total doses of 20–50 Gy sometimes using fractions up to 5 Gy.[3,12] Since a dose of 5 Gy is not used in a curative treatment setting, this high dose to achieve induction can be a limiting factor. Since the eukaryotic *Egr1* promoter is not functional in bacteria, we investigated the use of a prokaryotic radio-inducible promoter. In our study, significant induction of the *recA* promoter is achieved at only 2 Gy, which makes it more relevant for clinical use. A single dose of 2 Gy gives an increase of TNFα secretion of 44% 3.5 h after radiotherapy (Figure 2).

However, patients are not treated with a single dose of irradiation but with fractionated radiotherapy. This daily repeated small irradiation dose results in better tumour control for a given level of normal tissue toxicity than a single large dose. If fractionated radiotherapy is used, the first dose produces an increase of TNFα secretion of 33 to 36% 3 h after 2 Gy and an increase of 59% 3 h after a second dose of 2 Gy. Both inductions are statistically significant ($P < 0.05$ and $P < 0.02$, respectively). If we look at sample [b] which received a first dose of 2 Gy but did not receive a second irradiation, we see that at the second sample point, 6 h after the first dose (Figure 3B) there is still a 17% increase of extracellular TNFα activity, although not significant. This implies that of the 59% induction we measured after the second irradiation (sample [a]), 42% is the actual result of repeated gene activation and the residual 17% is still the effect of the first irradiation. This 42% induction after a second dose is in the range of induction values of 33–36% (± 11–12%) obtained after the first dose.

These data provide the proof of principle that the *recA* promoter in *Clostridium* can be efficiently reactivated by a second dose of radiotherapy and that the degree of induction remains more or less the same as compared with single dose irradiation.

This will be important in achieving controlled high levels of therapeutic gene expression in the clinical setting. In patient treatments, daily radiation fractions would be capable of activating the radio-inducible promoter leading to a daily increase of TNFα of 44% locally in the tumour.

There is, however, a rapid decline in promoter activity. A maximum level of induction is achieved after 3.5 h, which drops back to half after 6 h. When we compared these kinetics with those of the *Egr1* promoter, the kinetics are quite similar.[4] Joki *et al*[4] measured a maximum induction 1–3 h after a single dose of 20 Gy, and this induction dropped to about 50% after 6 h, and to basal level after 12 h. Notwithstanding these rapid promoter kinetics, they proved a therapeutic benefit *in vitro* when using their radio-induced promoter.

Unirradiated clostridia produce TNFα because of basal activity of the *recA* promoter. Maximum levels of 6200 pg/ml TNFα were measured in untreated samples. This basal activity can be reduced by inserting an additional Cheo box in the promoter region.[21] After irradiation of the recombinant clostridia, concentrations of 8800 pg/ml of mTNFα can be achieved (data not shown). Previous studies have reported that doses of hTNFα ranging between 10 and 1000 U/ml are cytotoxic to human tumour cell lines.[13] Since mTNFα has a specific activity of about 10^8 U/mg (data not shown), the concentration of 8800 pg/ml corresponds to 880 U/ml. This concentration should be sufficiently high enough to result in a cytotoxic effect on human tumour cells indicating that *Clostridium* is capable of producing sufficient amounts of TNFα to have an antitumoral effect.

In vitro and *in vivo* experiments are planned to prove that an additional increase in TNFα of 44% can lead to an increase in cell kill. However, when we look at some dose–response curves for TNFα, we can expect that an increase in TNFα concentration of 44% can lead to an increase in cell kill.[5]

Spatial and temporal control of gene expression by ionising irradiation is a relatively new and promising concept. The system we describe exploits the benefits of a tumour-specific vector in combination with radiotherapy which triggers and increases gene expression. Since therapeutic agents such as TNFα can be highly toxic, temporal control of protein expression can be very beneficial. In patient treatments, physicians will know from what time-point TNFα will be present in the tumour. TNFα will be expressed locally in the tumour, mainly in the hypoxic regions. However, TNFα shows a reduced cytotoxicity to hypoxic cells, because the mechanism of direct cytotoxicity may involve hydroxyl radical production.[22] However, since TNFα is a small and compact molecule, it will diffuse and will also reach the more oxygenated cells. Moreover, in addition to a direct cytotoxic effect, TNFα also has an indirect antitumour effect. In combination with irradiation, TNFα causes occlusion of tumour microvessels, without significant normal tissue damage.[12] TNFα gene therapy targeted by ionising radiation results in an amplified bystander effect, resulting in extensive necrosis distal to thrombosed tumour vessels.

Fractionated radiotherapy can provide a method to repeat gene induction resulting in enhanced and prolonged gene expression. Since anaerobic bacteria like *Clostridium* may colonise other hypoxic/necrotic tissues besides tumoral, such as for instance abscesses and infarcted tissues, spatial control of gene expression would be advantageous. Further benefiting the treatment plan, *Clostridium* can be completely eradicated by the administration of antibiotics,[23] suggesting that complete control of gene expression can be obtained; radiation will switch it on, and, if necessary, antibiotics can switch it off.

The use of a prokaryotic radio-induced promoter to temporally and spatially control gene expression is not only limited to *Clostridium*-mediated therapy for cancer. Recently, genetically engineered *Salmonella*[24,25] and *Bifidobacterium longum*[26] have shown that these bacteria also have the desirable properties of an antitumour vector. Both strains provide selective colonisation of solid tumours and can express proteins. Since the SOS-repair system in bacteria is highly conserved, the *recA* promoter can probably act as a radio-induced promoter in other bacteria to control gene expression after radiotherapy. The combination of radiotherapy, which preferentially kills well-oxygenated cells, with *Clostridium* (or analogous)-mediated protein delivery, which targets the hypoxic cells, provides new possibilities for future cancer therapy.

Materials and methods

Bacterial strains, media and culture conditions

Clostridium acetobutylicum DSM792 was grown in 2 × YT medium[27] at 37°C in an anaerobic incubator (model 1024;

Forma Scientific, Marietta, OH, USA) with 90% N_2 and 10% H_2 and palladium as the catalyst.

For primary vector constructions, *Escherichia coli* TG1[28] was used. This strain was grown in Luria-Bertani broth at 37°C. *E. coli* ER2275 was used for *in vivo* methylation of plasmid DNA before electroporation of *C. acetobutylicum*.[29] The *E. coli*/*C. acetobutylicum* shuttle plasmid pIMP was used as cloning vector.[30] Media were supplemented, when applicable, with erythromycin (25 μg/ml) or ampicillin (50 μg/ml).

Plasmid construction, transformation procedures

The *recA* promoter was isolated as previously described.[20] The mTNFα cDNA was available on plasmid pIG2mTNF (Innogenetics, Gent, Belgium). The signal sequence of the *eglA* promoter was used to obtain secretion of TNFα.[15]

The promoter and signal sequence were cloned upstream of the mTNFα cDNA after introducing unique *Nco*I and *Kas*I restriction sites in the pIMP vector using the 'Quickchange Site-directed Mutagenesis kit' (Stratagene, La Jolla, CA, USA) (Figure 1).

E. coli was transformed using chemically competent cells obtained with the RbCl method. Transformation of *C. acetobutylicum* DSM792 was carried out as recently published.[31]

All general DNA manipulations in *E. coli* were carried out as described by Sambrook *et al*.[28]

Irradiation

Bacteria were grown until early log phase (OD_{600nm} = ± 0.3). At this time-point cultures were divided into two sets, one of which was exposed to radiation while the other was mock-irradiated and used as a control. Bacteria were exposed to 2 Gy with a ^{60}Cobalt unit at a dose rate of 0.9 Gy/min. After irradiation, bacteria were incubated anaerobically at 37°C and samples were taken at different time intervals after exposure.

For repetitive gene activation, bacteria were grown to early log phase. Cultures were then divided into three flasks and exposed to 2 Gy (flasks [a] and [b]) or mock-irradiated (flask [c]). Three hours after radiation, the culture was centrifuged (10 min, 10000 r.p.m., 4°C) and supernatant was taken for analysis. An interval of 3 h after irradiation was chosen because at this time-point the highest degree of induction was seen. Because in batch culture bacteria already reach stationary phase after ± 5 h, repetitive gene activation could not be tested at greater time intervals. Therefore, we chose to resuspend the bacteria in fresh medium to have actively dividing bacteria. Similarly, in the clinical setting, bacteria gain a continuous supply of nutrients which will result in a continuous growth of viable cells. The pellet was resuspended in fresh 2 × YT medium and allowed to regrow for 40 min. Flask [a] was irradiated a second time with 2 Gy, while flasks [b] and [c] were mock-irradiated. Again, 3 h after irradiation and incubation at 37°C, cultures were centrifuged and supernatant was sampled for analysis.

Each experiment was independently repeated three times.

Analysis of mTNFα production

The amount of TNFα secreted by recombinant clostridia was quantified using ELISA. Supernatant taken from irradiated and non-irradiated cultures was diluted 10-fold in phosphate-buffered saline plus 7.5% bovine serum albumin and 100 μl aliquots were put in a 96-well microtiter plate. Further manipulations were done according to the manufacturer's protocol (DiaMed EuroGen, Tessenderlo, Belgium).

Concentrations of secreted mTNFα were calculated and compared for the irradiated and non-irradiated cultures. The level of radio-induced TNFα production was expressed as fold increase in mTNFα concentration of irradiated samples compared with the corresponding non-irradiated samples.

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