Regional therapy with DTA-H19 vector suppresses growth of colon adenocarcinoma metastases in the rat liver

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Abstract. Curative surgery is possible in barely 10% of patients with colorectal liver metastases and combined treatment modalities scarcely improve survival in this group of patients. Hence, investigations of new therapeutic modalities are crucial. Overexpression of the H19 gene in liver metastases points to H19 as a target for cancer gene therapy. Here we have evaluated the possibility of regional intra-arterial treatment of liver metastases with the DTA-H19 plasmid. Intra-arterial treatment of a total dose of 2.5 mg (repeated injections of 500 μg DTA-H19 plasmid each dose after the first injection of 1000 μg) caused a significant delay in the tumor growth compared to control group. All of the tumors treated with the control vector increased in size, whereas 35.7% of the tumors in the groups treated with a total amount of 2.5 mg DTA-H19 plasmid shrank in size. The present study showed that the DTA-H19 plasmid administered intra-arterially significantly delayed the tumor growth and even resulted in tumor regression in high percentage of the treated animals with liver metastases of colon cancer. Since human liver metastases demonstrated overexpression of the H19 gene, regional administration of the plasmid seems to be a promising therapeutic approach.

Introduction

Surgical resection is the treatment of choice for patients with isolated liver metastases with a 10-year survival of 20% (1). However, curative resection of liver metastases is possible in <10% of patients. Moreover, partial hepatectomy results in more rapid growth of misdiagnosed micrometastases in the remaining liver (2). Regional intra-arterial chemotherapy has been shown to be effective in down-staging initially inoperable colorectal liver metastases (3). However, side effects of cytotoxic drugs still restrict the doses of the agents. DNA based therapy seems to be a promising approach because of its targeted destructive effects on tumor cells with minimal treatment-related side effects.

H19 is a maternally expressed, oncofetal gene that encodes an RNA (with no protein product) acting as a 'riboregulator' (4), which is expressed at substantial levels in embryonic tissues, in different human tumor types, and marginally or not expressed in the corresponding tissue of the adult (5-7). It was shown in our previous studies that vectors carrying the diphtheria toxin A-chain gene under the control of H19 promoter selectively kill tumor cells and inhibit tumor growth *in vitro* and *in vivo* (8). The therapeutic potential of a toxin vector driving by the H19 regulatory sequences was successfully demonstrated in a metastatic model of rat CC531 colon carcinoma in liver following intratumoral injection of the vector (9). Here we present the results of regional via hepatic artery administration of the DTA-H19 plasmid for the treatment of liver metastases of colonic origin in the same orthotopic rat model.

Materials and methods

Seventy-four adult (20 weeks old) Wag/Rij male rats (260-300 g) were used in the study. All the protocols were approved by our University as stated in the Helsinki Declaration.

Model of liver metastases. The model of liver metastases was generated by direct subcapsular injection of 100 μl tumor cells suspension containing $5x10^4$ CC531 colon cancer cells in PBS. CC531 colon cancer cells were kindly provided by Dr Peter J.K. Kuppen (Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands).

Upper midline laparotomy was performed under general anesthesia which was achieved by intraperitoneal injection of Chloralhydrate (320 mg/kg). The tumor cell suspension was injected subcapsulary into the left lateral, right and left median liver lobes. After two weeks the rats underwent relaparotomy. The liver was exposed and dimensions of the developed tumors were taken *in vivo* by a caliper.

Isolated liver perfusion. We investigated intravenous and intra-arterial routes of plasmid administration using the model of isolated liver perfusion. Nine rats underwent isolated liver

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perfusion. Three of them with perfusion fluid (oxygenated KRB buffer) only, three with the DTA-H19 plasmid added into the system during the perfusion while in the other three the plasmid was injected into the hepatic artery during the isolated portal vein liver perfusion.

Transverse incision of abdominal wall provided good approach to the vascular and biliary structures of the liver. The vessels were dissected and vascular catheters were inserted into the vessels (portal vein, hepatic vein and bile duct). The liver was perfused with oxygenated KRB buffer using the system for isolated liver perfusion which maintains the viability of hepatocytes for at least 3 h. The excretion of bile from the common bile duct was a control of liver function. The perfusion was performed for 2 h and samples of the perfusion buffer were collected from the system every 30 min. At the end of the perfusion, samples of the liver tissue and the tumors were excised and snap frozen. The corresponding samples underwent histological examination for evaluation of the changes in the tumor and liver tissue after the perfusion. The level of DTA-H19 plasmid in the perfusion fluid, liver tissue, and in the tumors was determined by PCR analyses.

Model for intra-arterial treatment. Intra-arterial administration was performed using a catheter inserted into the gastroduodenal artery (GDA). We used intravascular catheter MRE-0.25 (Micro-renathane®, Braintree Scientific, Inc., France) which was inserted into the GDA under microscope. The laparotomy was made through the upper midline incision. After measurement of exposed tumors, common hepatic, proper hepatic and gastroduodenal arteries were dissected. Proximal and distal control of the GDA was achieved by ligation of the distal part of the artery and clamping of the common hepatic artery. The catheter was inserted into the GDA through arteriotomy and fixated by ligation in the proximal part of GDA. This model provided repeated injections of the plasmid through the port connected to the catheter and placed in the subcutaneous tissue. Heparin was administered after every injection of the plasmid for prevention of the catheter thrombosis.

Animal groups. Initially, 28 rats were treated with single and repeated intra-arterial injections (4 injections with intervals of 3 days) of the plasmid. They were divided into 5 groups according to the applied dose. A first group received 200 μg (6 rats), a second group received 300 μg (4 rats), a third group (8 rats) received 500 μg at each injection. The fourth group received 1000 μg as first injection, followed by 3 additional injections of 500 μg (6 rats), and the fifth group was treated by 1000 μg in every injection (4 rats). In every group half of the group received DTA-H19 plasmid while another half a plasmid carrying a reporter gene as a control (Luc-H19).

An additional group (6 rats) was treated with the same protocol as the group four, but the plasmid was previously complexed with PEI (polyethylenimine). When PEI was used, complexes with a ratio PEI nitrogen to DNA phosphate of 6 were prepared in a solution of 5% w/v glucose.

Twenty-four hours after the last treatment the rats were sacrificed. The size of the tumor was measured with the caliper. The samples of liver near and far from the tumor were taken for histological examination and for investigation of levels of H19 RNA and the plasmid. An additional experiment including 20 rats using the protocol treatment of 1000 μg first injection following by 500 μg DTA-H19 DNA plasmid as described above was performed.

DTA biodistribution and expression in tissues after hepatic artery infusion. Twelve rats underwent intra-arterial injection of 1000 μg of the plasmid. They were divided in three groups and sacrificed after 1, 24 and 48 h respectively. Biodistribution of the DT-A plasmid in tumors, liver and internal organs were evaluated using quantitative real-time PCR (Q-PCR) analysis.

The levels of DTA-H19 plasmid in blood after its infusion through the hepatic artery were evaluated by Q-PCR analyses. DTA-H19 (1 mg) was injected into the hepatic artery. Total DNA was isolated from the blood samples collected at 20, 40 and 60 min post injection. Serum (100 μl) was used each time for purification by using The HiYield™ Gel/PCR DNA Extraction Kit (Real Biotech Corp., Taipei, Taiwan), according to the manufacturer's instructions, in a final elution volume of 30 μl. Genomic DNA from tissue samples was extracted using Jetquick tissue DNA extraction kit (Genomed, Lohne, Germany). Samples were analyzed using an ABI PRISM 7900HT sequence detection system, and the appropriate software (SDS2.2) according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). For plasmid determination, either 2 μl of the extracted gDNA, or 3 μl of the purified serum was amplified in a mixture of 20 μl containing 0.5 μM primers for determination of the DTA-H19 plasmid levels in blood and tissue (5'-GACTCTGTCCTGCGGAAACC-3') upstream (5'-CCGATTCCCATCCAGTTGAC-3') downstream, and 0.15 μM fluorescent probe (6-FAM-TGACG AGCACAAGCT-MGB). Amplification samples were carried out in steps of 50˚C for 2 min, 95˚C for 10 min, 40 cycles of 95˚C for 15 sec, and 60˚C for 60 sec.

For plasmid expression detection either 2 or 4 μl of the prepared cDNA was amplified using (5'-GGCGTGGTCA AAGTGACGTA-3')upstreamprimer,(5'-CTTGCTCCATCAA CGGTTCA-3') downstream primer, and (6-FAM-CCAG GACTGACGAA GGTTCTCGCACT-TAMRA) probe.

To estimate the sensitivity of the quantitative RT-PCR procedure, plasmid DNA control was used with 10-fold serial dilutions of known quantities. For measuring the plasmid distribution in different tissues the plasmid DNA control starting from a concentration of 2 ng to 0.6 fg was used ($1.6x10^8$ copies down to <50 copies). These experiments were performed in duplicate.

RNA isolation and cDNA synthesis. Total RNA was extracted from the tumor, surrounding liver tissue and different internal organs tissue using the RNA STAT-60 Total RNA/mRNA isolation reagent (Tel-Test, Inc., Friendswood, TX, USA), according to the manufacturer's instructions. The RNA was treated with RNase-free DNase I (Roche Diagnostics GmbH, Mannheim, Germany) to eliminate any contaminating genomic DNA. The cDNA was synthesized from 2 μg total RNA in 20 μl reaction volume as described (10).

Determination H19 RNA level. Samples of liver and tumor from each rat were taken for determination of the H19 RNA level. PCR reactions were carried out in 25 μl volumes in the presence of 6 ng/μl of each of the forward and the reverse primers of the H19 cDNAs using 0.05 U/μl of Taq polymerase (Takara

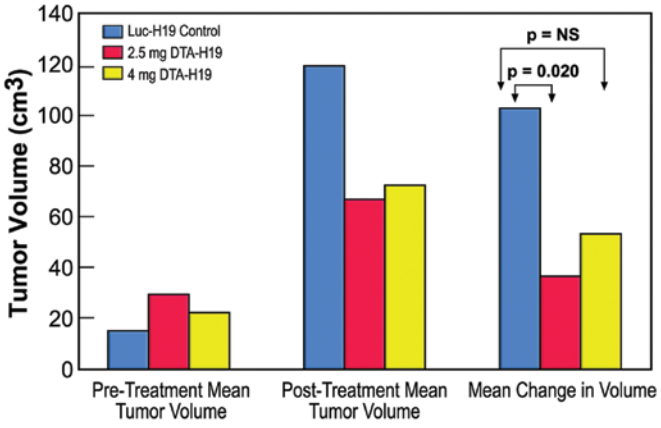

Figure 1. Effect of intra-arterial administration of the DTA-H19 plasmid on tumor growth. Retardation in tumor growth after treatment with the plasmid compared to control group.

Biomedicals, Japan) according to the manufacturer's instructions. The primer sequences used to amplify the rat H19 transcript were: (5'-ACTGGAGACTAGGGAGGTCTCTAGCA) upstream and (5'-GCTGTGTGGGTCTGCTCTTTCAAGATG) downstream. The PCR analysis was carried out for 30 cycles (98˚C for 15 sec, 58˚C for 30 sec, and 72˚C for 40 sec) and finally 72˚C for 5 min. The integrity of the cDNA was assayed by PCR analysis of the ubiquitous, cell cycle independent, histone variant, H3.3 (8). The products of the PCR reaction were run on 2% agarose in TAE electrophoresis running buffer (40 mM Tris-acetate and 2 mM EDTA, pH 8.5) stained by ethidium bromide and visualized by UV.

Statistical analysis. For statistical analysis of the differences between means, we used the Mann-Whitney U test, and differences were considered significant at $p<0.05$.

Results

Effect of intra-arterial administration of DTA-H19 on hepatic metastases. The therapeutic potential of DTA-H19 delivered through the hepatic artery was tested in the rat CC531 colon carcinoma liver metastases model described above.

Intra-arterial treatment in a total dose of 2.5 mg (repeated injections of 500 μg DTA-H19 plasmid each dose after the first injection of 1000 μg) caused a significant delay in the tumor growth compared to control group ($p<0.05$) (Fig. 1).

As shown in Table I all of the tumors treated with the control vector increased in size, whereas, 35.7% of the tumors in the groups treated with a total amount of 2.5 mg DTA-H19 plasmid shrank in size. The tumors in rats treated with 2.5 mg of DTA-H19 DNA plasmid showed also significantly smaller mean *in vivo* volumes ($p=0.02$), *ex vivo* volumes, and total weight than those treated with the control plasmid expressing the reporter gene, even though the baseline mean tumor volumes in the groups treated with DTA-H19 were larger than those observed in the Luc-H19 control group. Apparently, administration of a total dose of 4 mg of DTA-H19 (4 injections of 1 mg) did not show any improvement in the reduction of the tumor growth (Fig. 1) compared to 2.5 mg total dose.

Table I. Effect of DTA H19 administered via the hepatic artery on the growth of colon adenocarcinoma metastases in the rat liver.

	Luc H19 control	DTA H19 2.5 mg	DTA H19 4 mg
No. of rats	13	12	2
No. of tumors	27	24	6
Mean tumors/rat	2.1	2	3
Pre-treatment Mean tumor volume (mm^3)	15.8±11.5	30.0±12.4	22.9±10.8
Post treatment Mean change *in vivo* tumor volume (mm^3)	119.7±98.7	67.9±49.7	72.8±53
Increase in size (no.)	27 (100%)	15 (62.5%)	4 (66.6%)
Decrease in size (no.)	0 (0%)	9 (37.5%)	2 (33.3%)
Mean fold increase	7.6	2.3	3.2
Mean tumor weight (mg)	173.6±120.3	93.5±50.8	124.3±72.1

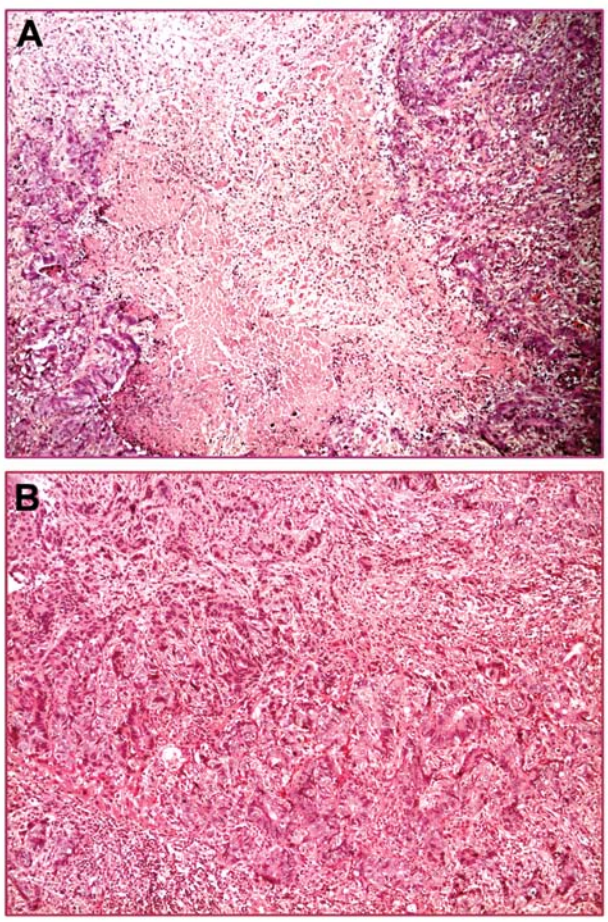

Figure 2. Histological pictures of tumor after intra-arterial treatment. (A) Large necrosis in tumor after administration of the DTA-H19 plasmid. (B) Tumor tissue after administration of the Luc-H19 plasmid.

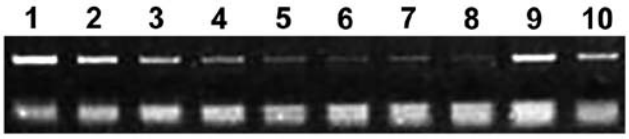

Figure 3. Level of the DTA-H19 plasmid in the liver and tumor tissue determined by PCR analysis. The PCR analysis of the liver and tumor samples after injection of the plasmid into the portal vein shows higher levels of the DTA-H19 plasmid in the liver tissue (lanes 1-4 and 9) than in the tumor (lanes 5-8). Lane 10, DTA plasmid as a positive control.

The histopathological study of the tumors showed the presence of multiple large necroses in the tumors after the treatment with the plasmid DTA-H19 as compared to the tumors from the control group (Fig. 2).

Administration of the DTA-H19 plasmid in combination with PEI. In order to improve delivery of the plasmid into the cells, the plasmid was complexed with the transfection enhancer reagent polyethylenimine (PEI). The group which received the plasmid in combination with the transfection reagent PEI showed multiple large necroses in the liver tissue at sacrifice in all the rats, which might be a result of the toxicity effect of the PEI. Thus no conclusions could be obtained regarding the effect on the tumor growth (data not shown).

Pharmacokinetics and biodistribution study of the plasmid during isolated liver perfusion. The distribution of the plasmid DTA-H19 in the normal liver tissue and in the liver tumors after injection of the plasmid into the portal vein or into the hepatic artery during isolated liver perfusion was investigated. The level of DTA-H19 plasmid in the perfusion buffer, liver tissue, and in the tumors was determined by PCR. Samples of the perfusion buffer evaluated during isolated liver perfusion revealed high levels of the DTA-H19 plasmid during the initial 30 min of the perfusion. This was followed by significant decrease in the plasmid concentration till 60 min from the start of the perfusion while the DTA-H19 plasmid was still detected. No traces of the plasmid were found after 90 min.

The PCR analysis of the liver and tumor samples from the rats in which the plasmid was injected into the portal vein showed higher levels of the DTA-H19 plasmid in the liver tissue than in the tumor (Fig. 3). In contrast, injection of DTA-H19 into the hepatic artery during isolated liver perfusion resulted in higher levels of plasmid DNA in the tumor tissue as compared to normal liver tissue (Fig. 4).

Thus, delivery of DTA-H19 via the hepatic artery may permit higher localized plasmid concentration to be delivered to the liver metastases as compared to the normal liver, which may increase response rates reducing any potential local and possibly systemic toxicity.

The expression of the DTA-H19 plasmid in organs and tissues 24 and 48 h following intra-arterial injection. The expression of the plasmid was evaluated using QPCR analysis with 18s rRNA and β-actin as a positive control. Tables II and III demonstrate high expression of the plasmid in the tumors and low or no expression in the liver tissue far from the tumors and in the other organs.

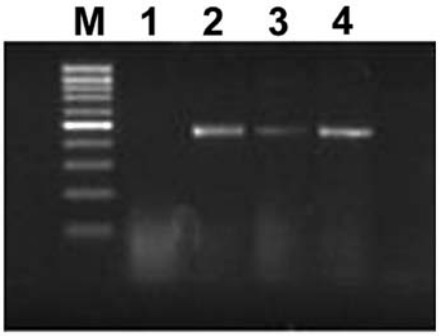

Figure 4. Levels of plasmid DNA in the tumor tissue and normal liver tissue after injection of DTA-H19 into the hepatic artery during isolated liver perfusion. The PCR analysis shows higher levels of plasmid DNA in the tumor tissue (lanes 2 and 4) as compared to normal liver tissue (lanes 1 and 3).

Table II. Expression of the DTA in tumor and different organs after intrahepatic delivery of DTA-H19 plasmid.

Organs	Average expression after 24 h (copies/1 μg cDNA)	Average expression after 48 h (copies/1 μg cDNA)
Tumor	897	273
Liver near tumor	230	230
Liver far of tumor	290	70
Kidney	100	No expression
Lung	170	70

DTA cDNA was determined by QRT-PCR in tumors and in different organs 24 and 48 h after intra-arterial injection of DTA-H19 plasmid (18s rRNA FAM TAMRA control kit was used as positive reference).

Table III. Expression of the DTA gene in tumor and different organs after intrahepatic delivery of DTA-H19 plasmid.

Organs	Average expression after 24 h (copies/1 μg cDNA)
Tumor	78
Liver near the tumor	16
Liver far from the tumor	No expression
Kidney	No expression
Lung	No expression

DTA cDNA was determined by QRT-PCR in tumors and in different organs 24 h after intra-arterial injection of DTA-H19 plasmid (β-actin control kit was used as positive reference).

The pharmacokinetics of DTA-H19 in rat blood following intra-arterial injection. The pharmacokinetics of DTA-H19 in rat blood following intra-arterial injection is shown in Fig. 5. The rat has ~20 ml of blood and about 10 ml of plasma. This means that the rat had at 20 min 225 μg of DNA in the total blood volume of the rat; this is 45% of the total amount of

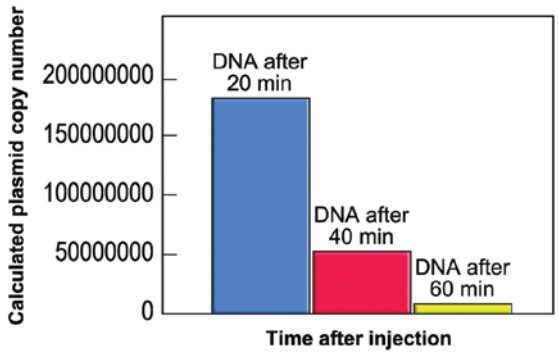

Figure 5. Pharmacokinetics of the DTA-H19 in blood. The concentration of genomic DNA in blood after hepatic artery infusion into rats was determined using Q-PCR method. Total DNA was isolated from the blood at 20, 40 and 60 min post injection and genomic DNA was extracted and analyzed for plasmid content obtaining the following levels: 22.5, 2.5 and to 0.87 ng/μl of plasma, respectively.

injected plasmid indicating that the half-life time of the plasmid in the blood is ~20 min. At 40 min, 25 μg of the plasmid was left in the total blood volume of the rat; this is 5% of the total injected plasmid. At 60 min 8.7 μg of the plasmid was found in the total blood volume of the rat, which is 0.17% of the injected plasmid.

Discussion

All the developed tumors in the orthotopic animal model for liver metastases of the colon showed high expression of H19 gene. The crucial role of this gene in the process of carcinogenesis and metastases has recently been demonstrated (11). Moreover, in hypoxic stress conditions, H19 is largely activated enabling survival of tumor cells under hypoxic conditions, which can promote tumor progression and resistance to therapy (11,12). Functional link was found between p53, HIF1-α and H19 which may play important role in tumor development (13). Studies of various tumors have demonstrated a re-expression or an over-expression of the H19 gene when compared to healthy tissue (14). In cancers of different etiologies and lineages, aberrant expression in allelic patterns was observed in some cases, suggesting that H19 may play a role in tumorigenesis. While H19 shows mono-allelic expression in most tissues throughout development, with the exception of germ cells at certain stages of maturation, and in extra-villous trophoblasts (15), bi-allelic expression of this gene, referred to as 'relaxation of imprinting' or loss of imprinting, have been found in an increasing number of cancers. Moreover, the H19 gene is expressed within both epithelial and stromal components of human invasive adenocarcinomas where high expression matches tumor invasion (16). On the contrary, H19 repression is accompanied by dedifferentiation-derived mesenchymal stem cells and its inability to form tumors (17).

H19 was found highly expressed and /or manifest aberrant allelic pattern of expression in over 30 types of cancer (14). Investigation of the expression of the H19 gene in hepatic metastases derived from a range of human carcinomas showed that H19 is highly expressed in the majority of hepatic metastases tested (18). Moreover, in the first report on the importance of the Twist gene product in tumor metastasis, the investigators showed that H19 is the highest differentially expressed gene in metastatic tumor cell lines investigated (19). Although the oncogenic mechanisms of H19 needs further investigation, the fact that H19 is highly expressed in many types of tumors in high percentage of cases points to H19 as a target for cancer gene therapy.

Recently, different anti-cancer therapies such as immunotherapy, and gene therapy were presented as an alternative to conventional chemotherapy protocols. Advantages of these methods including targeted treatment and low side effects determine the significance of their development. Various gene therapy strategies have been suggested for the treatment of advanced colorectal cancer (20-23). Some of them exploiting differences in gene expression levels between liver metastases and normal hepatic tissue for targeted treatment.

We have previously reported results of direct intratumoral injection of the DTA-H19 plasmid (9) which ensures high killing activity and has a great advantage in excluding toxicity to normal cells, since the released DT-A protein from the lysed cells will not be able to enter neighboring cells in the absence of the DT-B fragment (24). Besides, the use of these plasmid vectors avoids any immunological consequences which might result from the use of viral vectors.

Although direct intra-tumoral injections of the plasmid demonstrated clear effect on the tumor growth, this route of administration has restricted applications in practice because liver metastases are usually multiple. Therefore, regional treatment providing high levels of the drug in the tumors without affecting the normal tissue might be an optimal variant for the treatment of liver metastases.

Intra-arterial administration showed significant advantages providing high levels of the plasmid in the tumor, while low levels of the plasmid in hepatocytes were obtained following intra-portal injection (Figs. 4 and 5), reflecting blood supply to the hepatocytes and tumor tissue. Thus, the model of intra-arterial plasmid administration which allowed high levels of the plasmid in the tumor had been chosen for treatment of liver metastases. Intra-arterial treatment also provided high expression of the plasmid in the tumor (Tables II and III). Relatively high expression was also detected in liver tissue surrounding tumor corresponding to previous reports that showed high H19 gene expression at the liver tissue surrounding tumor (2). Rapid disappearance of the plasmid from the circulation during isolated liver perfusion and after intra-arterial injection affirms an entrance of the plasmid into the cells. Accumulation of the plasmid in the tumor cells following repeated administrations is, probably, important for enforcement of its effect. We evaluated the therapeutic effect of the plasmid following different doses of the plasmid administered as single dose or as repeated injections. The experiments have shown that low doses of the plasmid administered as single dose had no effect on the tumor growth (data not shown). Substantial effect of the treatment, determined as a shrinkage or significant delay in the growth of the tumor and appearance of multiple large necroses, was monitored only after elevation of the total dose to 2.5 mg administered in 4 injections. These results demonstrate that accumulation of the toxin vector in the tumor cells following it repeated injections improves the therapeutic effect. At the same time, while the described total dose of the plasmid (2.5 mg) showed a substantial effect on the tumors, there was

no additional improvement after following elevation of the total dose to 4 mg. The dose of 2.5 mg, probably, provided a saturation of the plasmid in the tumor and a further dose elevation did not have any additional effect.

Delivery of the plasmid into the cells has a crucial role in the effect of the treatment. Since significant improvement in the delivery of a plasmid into the cells when using the polycation PEI was previously reported (9), we tried to use the complex DNA/PEI in the treatment of liver metastases while delivered by intra-arterial injection. Extended liver necroses were obtained which made its use not suitable in this type of administration.

Obviously, the described distribution of the plasmid into the tumor showed high levels in the periphery of the tumor and low levels in the central part reflecting the pattern of blood supply of the tumors which is rich in the periphery of the tumor and very poor in the central part causing central necrosis in large tumors. This fact may probably explain the delay in the growth or shrinkage of the tumors under the treatment but not their disappearance. It seems that the DTA-H19 plasmid kills cells in periphery of the tumor while it cannot reach the tumor center.

A therapeutic effect of the DTA-H19 vector in treatment of liver metastases of colon carcinoma in rats based on the over-expression of the H19 gene in tumor cells was evaluated in regional administration. This study showed that repeated intra-arterial injections of the DTA-H19 plasmid significantly delayed the tumor growth and even resulted in tumor regression in high percentage of the treated animals with liver metastases of the colon cancer. Since human liver metastases demonstrated over-expression of the H19 gene, regional administration of the plasmid seems to be a promising therapeutic approach in their treatment.

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