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Treatment of experimental liver metastases in the rat by continuous intraportal infusion of 5-fluorouracil and heparin: a pilot study

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Continuous portal venous infusion of 5-fluorouracil (5-FU) and/or heparin immediately following injection of tumor cells into the portal system in unrestrained rats has been assessed as a pilot study to examine whether the incidence of liver metastases could be reduced and survival thereby improved. Thirty-six rats were divided into four groups according to the treatment administered for 7 days by portal infusion: heparin, 5-FU, 5-FU + heparin and physiological saline solution as a control. After a 90-day follow-up, metastases were observed in every rat injected with tumor cells with the exception of one rat in the heparin group. The 90-day actuarial survival rates for the treatment groups of heparin, 5-FU, 5-FU + heparin and control were 44% (4/9), 11% (1/9), 33% (3/9) and 22% (2/9) respectively, with the survival curves not differing significantly. The apparently improved survival rate in the heparin group may be due to the insufficient period of observation. The conclusions of this study may be only preliminary owing to both the insufficient period of observation and the small number of rats, but the inhibitory effect of 5-FU on the development of liver metastases was disappointing as a result of the large number of tumor cells injected into the portal system. The technique used in this study, which enabled continuous portal venous infusion of drugs to be performed in unrestrained rats, may provide a useful model for the study of continuous infusion in the rat.

Key words: Colorectal cancer, 5-fluorouracil, heparin, infusion, liver metastasis, portal vein.

Introduction

The mortality due to colon cancer remains elevated, even after surgical resection with a curative goal.[1] Death is due to development of metachronous metastases, predominantly located in the liver.[2] It is generally accepted that liver metastases result from the spread of malignant cells from colon primary tumors via the portal venous system;[3,4] at the beginning of liver metastatic colonization, the vascularization of cancer foci is mostly supplied by portal tributaries.[5] Metastatic spread of cancer cells can occur before surgery but tumor foci may not be visible during operation. Furthermore, malignant cells have been detected in the portal blood after colic tumor manipulation by the surgeon;[3,6,7] thus Turnbull *et al.*[8] advocated the no-touch isolation technique in the hope of reducing peroperative dissemination of cancer cells to the liver; however, there are some controversies about the benefit of this no-touch isolation technique in long-term survival.[9]

Whatever the exact mechanisms of metastatic formation are, many efforts have been made to destroy the liver metastatic cells at an early stage after curative resection of the primary colic tumor; systemic adjuvant chemotherapy with 5-fluorouracil (5-FU) alone[10,11] or combined with other antimitotic drugs has met with little success,[12–14] partly because of insufficient drug concentration in the liver.

Regional infusion chemotherapy has been advocated to improve the drug effectiveness and to reduce the systemic toxicity. Taylor *et al.* reported a randomized study of 5-FU and heparin given postoperatively by the portal vein in patients with resectable colorectal tumors. 5-FU (1000 mg/day) and heparin (5000 units/day) were continuously perfused through the repermeabilized umbilical vein for 7 days immediately following operation. After a median follow-up of 4 years, incidence of liver metastasis was strongly reduced in the treated group (5/117) compared with the control group

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(22/127); the number of deaths was also diminished in treated patients (26 versus 54). Morbidity of intraportal infusion appeared weak but one death was undoubtedly related to the procedure.[15]

Although several clinical trials are in progress to ascertain the encouraging results of Taylor *et al.*, their follow-up has yet to start.[16] The present experiments were carried out as a pilot study in an attempt to report the feasibility of the technique for the continuous portal infusion in a newly described model, the tolerance of rats on both 5-FU and heparin, and the preliminary results of this therapy in the rat.

Materials and methods

Animals

Inbred 4- to 5-month-old male BDIX rats, weighing 280–350 g, were used. They have been bred in our laboratory by single-line brother–sister mating since 1971.

Cells and culture conditions

DHD/K12/PRO cell line was established in our laboratory from a transplantable colon adenocarcinoma induced by 1,2-dimethylhydrazine in syngeneic BDIX rats.[17,18] HT 29 cell line was established in culture from a human colon adenocarcinoma by Fogh and Trempe.[19] These cells were cultivated on monolayers in complete medium which consisted of Ham's F10 medium (Microbiological Associates, Walkersville, MD) supplemented with 40 mg/l gentamycin (Gentalline, Unilabo, Levallois, France) and 10% fetal calf serum (Gibco, Paisley, UK). The cells were detached for experiments by a sequential treatment with 1% ethylene diamine tetracetic acid salt (EDTA, Microbiological Associates) and 0.25% trypsin (Gibco) in Hank's medium without Ca^{2+} and Mg^{2+}. Detached cells were washed with complete medium, counted and prepared in Ham's F10 medium. Cell viability was checked by trypan blue exclusion and was always superior to 95%.

Catheterization into portal venous system

Each operative procedure was carried out under clean conditions. Anesthesia was induced and maintained using ether after an intramuscular injection of atropine at a concentration of 0.001 mg/kg. Abdominal exposure was carried out through a midline incision. The tip of the polyethylene catheter (0.70 mm diameter, Biotrol Pharma, Paris) was inserted into the cecal vein, a branch of the ileocecocolic vein, via a longitudinal venotomy, and secured by 6-0 Vicryl (Ethicon, Neuilly) (Figure 1). The free end of the catheter was passed subcutaneously from the abdominal wound to the head of the rat. An L-shaped needle was fixed to the head of the rat and attached to the end of a sheath which prevented twisting of the catheter. The catheter (about 2.5 m in length) was passed through both the needle and sheath (about 1.5 m in length) and was connected to an infusion pump (Razel Scientific Instruments, Inc., Stamford) (Figure 2). This technique enabled a rat to move

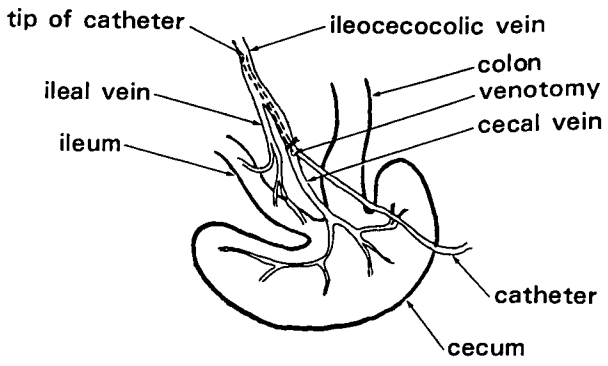

Figure 1. Veins of ileocecal region in the rat. A catheter was inserted into the cecal vein with its tip in the ileocecocolic vein.

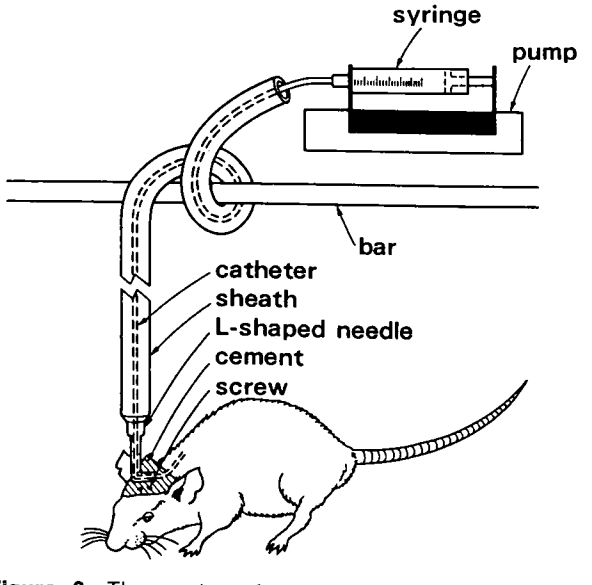

Figure 2. The system for continuous infusion in an unrestrained rat.

freely with continuous drug infusion into the portal system.

Induction of liver metastatis

The procedure has been discussed previously.[20] In brief, 2×10^7 viable tumor cells in 1 ml of culture medium after trypsinization were injected in liver through the intraportal catheter and flushed by 0.5 ml of saline solution. This procedure resulted in a precedent study in 100% of liver metastasis 4 weeks after injection.

Continuous portal venous infusion

After catheterization, 36 rats were randomly divided into 4 groups according to the treatment administered for 7 days by portal infusion: heparin (group A), 5-fluorouracil (5-FU) (Roche, Neuilly-sur-Seine, France) (group B), 5-FU + heparin (group C) and physiological saline solution as a control (group D). 5-FU was given at a dose of 20 (mg/kg)/day, and heparin (Roche) at a dose of 320 (units/kg)/day in physiological saline solution given by continuous infusion over 24 h each day for 7 days. Physiological saline solution was given to the rats of the control group for 7 days. Eight rats, two rats from each group, were injected with the same lot of tumor cells and perfused for the same period. Continuous portal infusion was started using an infusion pump with a flow rate of 0.60 ml/h immediately following injection of tumor cells via the catheter. White blood cell counts were performed with a drop of blood taken from the coccygeal vein on days 0, 4, 7, and 11 after the injection of tumor cells. After the 7-day infusion, the catheters were cut just above the heads of the rats and the animals were replaced in normal cages.

Evaluation of antitumor effects

Each animal was carefully followed up postoperatively to determine survival times. The rats which died spontaneously were autopsied and, 90 days after injection of tumor cells, the surviving animals were killed. Complete autopsy examinations were performed for each rat. Livers were removed and weighed. Metastatic tumors in the livers were separated, weighed and fixed in Gendelman's and embedded in paraffin in order to confirm microscopically the presence of tumor.

In vitro cytotoxicity assay

In order to examine the sensitivity of tumor cells (PRO and HT 29) to 5-FU, *in vitro* cytotoxicity assay was performed according to a previously described colorimetric test.[21]

Anticoagulant effect of heparin

In preliminary experiments, rats received a continuous portal infusion of heparin at various doses (0, 320, 800, 1200, 1700 and 2000 (units/kg)/day) for 2 days. At the end of the experiment, the activated partial thromboplastin time (APTT) was determined at each dose on the blood of 3 rats collected by cardiac puncture.

Statistical analysis

The survival curves were compared by the logarithmic rank test. Comparisons of changes in white blood cell counts and of body weights, liver weights and liver tumor weights were made using Student's t test. Comparisons between groups with respect to the distribution of metastases were made by a χ^2 test of 3×2 contingency tables.

Results

Feasibility of the technique

The duration of the manipulation for preparing an unrestrained rat with an intraportal catheter ranged from 30 to 60 min with an average of 45 min. The position of the venotomy for inserting the catheter and that of the tip of the catheter are very important to allow a long-term survival of the rats. In our preliminary manipulation, making a venotomy on the ileocecocolic vein and putting the tip of the catheter in the portal vein brought about a high mortality rate (50%) because of the ischemic damage to intestines resulting from the portal vein thrombosis. A venotomy on the cecal vein with the tip of the catheter in the ileocecocolic vein (Figure 1) resulted in a reduced postoperative mortality rate (20%). The mortality rate of 20% was due to ischemic damage to cecum (12%), strangulation ileus (4%) and peroperative death (4%) resulting from profound anesthesia or hemorrhage. There was no problem with the obstruction of the catheter during 7-day infusion and, after the 7-day infusion,

Table 1. Observed and expected numbers of deaths

Treatment group	Number of rats	Observed number of deaths (*O*)	Expected number of deaths (*E*)	Relative death rate (*O*/*E*)	*P*
Heparin (A)	9	5	6.75	0.74	0.2
5-FU (B)	9	8	6.75	1.18	0.9
5-FU + heparin (C)	9	6	6.75	0.89	0.3
Control (D)	9	7	6.75	1.03	

no problem occurred in the rats with the catheter left in place.

Survival

The numbers of the rats which died of cancer within 90 days after injection of tumor cells for the treatment groups of heparin, 5-FU, 5-FU + heparin and control were 5, 8, 6 and 7 respectively. 7 out of 18 rats in the groups with heparin treatment (groups A and C) survived 90 days versus 3 out of 18 rats in the groups without heparin treatment (groups B and D). We have observed metastases in every rat with the exception of one rat in the heparin group. The 90-day actuarial survival of the rats in the four treatment groups is shown in Figure 3. The 90-day survival rates for the treatment groups of heparin, 5-FU, 5-FU + heparin and control were 44% (4/9), 11% (1/9), 33% (3/9) and 22% (2/9) respectively. Although in every analysis there was a slight advantage for the groups with heparin treatment (groups A and C), there was no difference statistically in life table analysis among the four groups; the *P* values (logarithmic rank test) for the groups of heparin, 5-FU, 5-FU + heparin versus the control group were 0.2, 0.9 and 0.3 respectively (Table 1). There was no significant difference in the mean liver weights and the mean liver tumor weights at autopsy among the four groups (data not shown). This finding should be interpreted as indicating a relatively slower development of the liver tumors in the heparin-treated animals (groups A and C) than in the non-heparin-treated ones (groups B and D), because there was no difference in the mean liver tumor weights between the two treatment groups (A, C versus B, D) in spite of the numerical superiority of the rats which survived 90 days in the heparin-treated groups (7/18) over those in the non-heparin-treated groups (3/18). Extrahepatic metastases were observed in different organs. Metastases in the liver, lung and kidney were examined in each group and counted (Table 2). Although there was a slight tendency to observe more extrahepatic metastases in the heparin-treated animals, there was no statistical difference among the four groups.

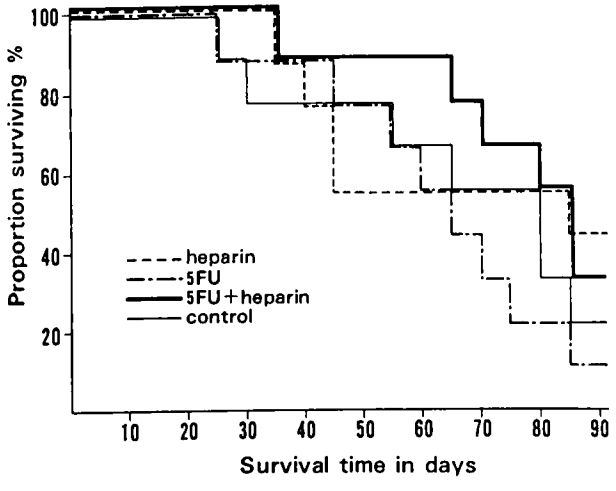

Figure 3. The actuarial survival curves for the treatment groups of heparin, 5-FU, 5-FU + heparin and control are shown. There was no difference among the four groups by the logarithmic rank test ($P = 0.2$, 0.9 and 0.3 respectively for the groups of heparin, 5-FU, 5-FU + heparin versus the control group).

Toxicity of 5-FU

In our preliminary study, signs of toxicity from 5-FU were observed in a dose of 30 (mg/kg)/day; 44% (4/9) of rats died of diarrhea and/or infection

Table 2. Distribution of metastases

Treatment group	Per cent metastases (incidence)		
	Liver	Lung	Kidney
Heparin	88.9 (8/9)	77.8 (7/9)	44.4 (4/9)
5-FU	88.9 (8/9)	55.6 (5/9)	44.4 (4/9)
5-FU + heparin	77.8 (7/9)	66.7 (6/9)	44.4 (4/9)
Control	100 (9/9)	33.3 (3/9)	44.4 (4/9)

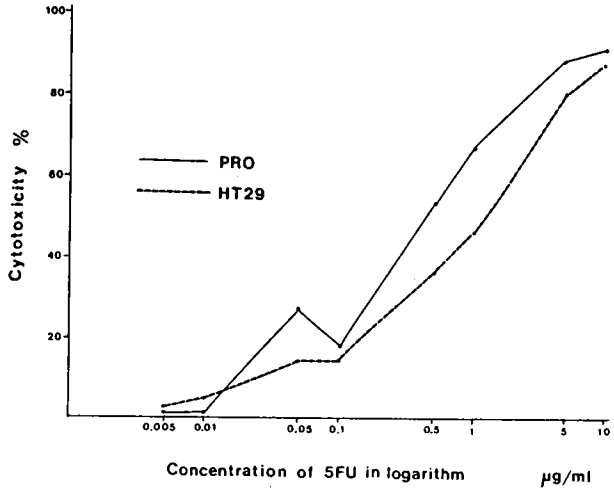

Figure 4. *In vitro* cytotoxicity of 5-FU against the rat tumor cells (PRO) and the human cells (HT 29) is shown. These tumor cells were cultivated with various concentrations of 5-FU for 7 days and cytotoxicity assay was performed according to a colorimetric test.

resulting from leukopenia. We have, therefore, administered 5-FU in a dose of 20 (mg/kg)/day. Even at this dose, 1 out of 15 died probably of the toxicity from 5-FU. The mean white blood cell counts in the 5-FU-treated animals fell significantly compared with those in the non-5-FU-treated ones between the fourth and seventh day of infusion ($p < 0.05$, data not shown). These results suggest that the dose of 5-FU of 20 (mg/kg)/day is maximum for continuous portal infusion in the BDIX rat.

In vitro cytotoxicity assay of 5-FU

Figure 4 represents *in vitro* cytotoxicity of 5-FU against the rat tumor cells (PRO) and the human ones (HT 29). The sensitivity of PRO to 5-FU was a little higher than that of HT 29; median lethal doses for PRO and HT 29 were 0.4 μg/ml and 1 μg/ml respectively.

Anticoagulant effect of heparin

The measurement of APTT after 2-day portal infusion of heparin at various doses was performed (Table 3). A systemic anticoagulant effect was not observed in the administration of heparin in a dose of 320 (units/kg)/day, which was the dose tried out in this study. In preliminary experiments, the administration of heparin in a dose of 1700 (units/kg)/day brought about a high mortality rate during 7-day infusion; 66.7% (8/12) of rats died of either intraperitoneal hemorrhage (6/8) or intestinal hemorrhage (2/8). The mortality rate in a dose of 800 (units/kg)/day of heparin was 9% (1/11); this single death was related to intestinal hemorrhage. Signs of hemorrhage were not observed in a dose of 320 (units/kg)/day of heparin.

Table 3. APTT after the 2-day portal infusion of heparin

Doses of heparin ((units/kg)/day)	APTT (s)
0	16.8 (1.8)
320	16.2 (3.2)
800	38.6 (4.5)
1200	58.3 (3.8)
1700	49.8 (5.3)
2000	134.3 (6.5)

Values are given as mean ± SD of 3 rats.

Discussion

Based on early reports of Taylor *et al.* who in 1977[22] and 1979[23] reported the advantage of continuous portal infusion chemotherapy, several clinically controlled adjuvant studies are in progress using portal infusion of 5-FU and heparin following radical surgery for colorectal cancer.[16] Their follow-up is, however, far from being completed. The present experiments were carried out as a pilot study in an attempt to report the feasibility of the technique for continuous portal infusion in a newly described model in the rat, the tolerance of rats on both 5-FU and heparin, and the preliminary results of this therapy in the rat. Animal experiments offer several advantages over a controlled randomized trial in man with respect to ethical problems, the homogeneity of conditions and rapid results with sufficient evaluation at little cost. The 7-day infusion was performed in unrestrained rats in an attempt to reduce the stress of rats, because stress may influence the development of metastatic tumors.[24,25] We administered 5-FU in a dose which was maximum for rats in a 7-day continuous portal infusion. The conditions in this study were comparable with those of Taylor *et al.* However, the number of tumor cells injected through the portal vein might have been too great for us to examine the effect of 5-FU and/or heparin on liver

metastasis, because 100% of liver metastasis was observed in the control group with the number of tumor cells used in this study. Taylor *et al.* reported that an apparent survival advantage by continuous portal infusion occurred only in patients with Dukes' B colon cancer.[15] Accordingly, a smaller number of tumor cells should be injected into the rat in order to be nearer to the condition of patients with Dukes' B colon cancer.

We calculated that the concentration of 5-FU in a liver blood flow in a dose of 20 (mg/kg)/day was 0.20 μg/ml. This value was calculated from the liver blood flow value in the rat *in vivo* (70 (50–100) (ml/min)/kg body mass).[26] As shown in Figure 4, this concentration of 5-FU corresponds to an *in vitro* cytotoxicity of 30% against the tumor cells (PRO). Nevertheless, there was no inhibitory effect of 5-FU on the experimental liver metastasis *in vivo*. It could not be assumed that the ineffectiveness in continuous portal infusion of 5-FU in the rat may be due to a difference in the sensitivity to 5-FU between tumor cells in the rat and those in man, as the sensitivity of PRO to 5-FU was a little higher than that of a human colon cancer cell line (HT 29) (Figure 4). Possible explanations for the findings of ineffectiveness are as follows: (1) the concentration of 5-FU calculated in a liver blood flow may not always coincide with a real concentration because of the metabolism, the elimination, the accumulation etc. of 5-FU; (2) the concentration of 5-FU in the periphery of liver may not be sufficient to kill the tumor cells as 5-FU may principally flow in the central part of the liver; (3) the number of tumor cells for inducing liver metastases may be too great as discussed previously.

There have been conflicting reports in the literature about the effect of heparin on metastatic formation.[27–35] In this study, the rats in the heparin group appeared to have an improved 90-day actuarial survival rate. This improved rate, however, may be due to the use of a period not long enough to give a survival curve; four rats in the heparin group survived 90 days, but three of these four rats had metastases. It seems to be difficult at the moment to take into account the remaining tumor-free rat because of the small number of rats in this experiment. We administered a dose of heparin whose concentration was similar to or a little higher than that obtained in man by Taylor *et al.*[15] As shown in Table 3, the dose used in this study was, however, too low to obtain a systemic effect of anticoagulation in the rat. Taylor *et al.* reported that heparin was initially included to avoid the theoretical hazard of the portal vein thrombosis.[15] Accordingly, as for anticoagulation, the dose of heparin should be increased in order to confirm the effect of heparin on the incidence of liver metastasis. Another interesting finding on the effect of heparin is that there has been a slight tendency for extrahepatic metastases to increase in the heparin-treated animals. This result may be interpreted as an increased transhepatic passage of tumor cells caused by heparin treatment and several investigators have reported similar findings.[36,37]

The main interest of the present study is to report the feasibility of long-term continuous infusion of the rat liver for evaluating the effect of cytotoxic agents on hepatic metastases. Even if liver metastases are the most frequent localization of human colorectal carcinoma, there are only a few reports on the effects of chemotherapy on liver metastases of mouse or rat colon carcinoma.[38,39] In these reports cytotoxic drugs were administered by systemic intravenous discontinuous injections. We are aware of only two reports about chemotherapy of rat liver metastases through the intraportal way,[40,41] but, in the former report, the metastases resulted from a solitary implant of Walker carcinosarcoma and the treatment was limited to one or two bolus intraportal injections of doxorubicin whereas, in the latter report, isolation liver perfusion during 25 min was studied. The technique used in our study, which enabled us to perform continuous intraportal infusion of drugs in unrestrained rats, may provide a useful model for the study of continuous infusion in the rat.

Conclusions

Continuous portal venous infusion of 5-FU and/or heparin immediately following injection of tumor cells into the portal system in unrestrained rats has been performed as a pilot study. The conclusions of this study may be only preliminary. Heparin treatment was slightly more effective, although with no statistically significant difference, but the inhibitory effect of 5-FU on the development of liver metastases was not observed as a result of the large number of tumor cells injected into the portal system.

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