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5-FU Uptake in Peritoneal Metastases after Pretreatment with Radioimmunotherapy or Vasoconstriction: an Autoradiographic Study in the Rat

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Abstract. *This study was conducted to test if tumour drug uptake could be increased in experimental colorectal cancer peritoneal metastases, by using pretreatment with peritoneal vasoconstriction or radioimmunotherapy. A total of 29 nude rats with peritoneal metastases were injected intraperitoneally (i.p.) with ^{14}C-labelled 5-FU. The animals were randomly allocated to 5 groups. Six days prior to 5-FU, group I (control) received i.p. NaCl, group II was subjected to i.p. radioimmunotherapy (RIT) ^{131}I-labelled anti-CEA monoclonal antibody (150 MBq) and group III received i.p. Norbormide 10 minutes before 5-FU. Two days prior to 5-FU group IV and V received i.p. NaCl (control) and RIT, respectively. 5-FU uptake was visualised with autoradiography and quantified by computer-based image analysis. Tumours in group III showed a higher uptake (mean±SD, 21.4±17) than in group I (11.8±10, p=0.04). This was also true when the analysis was restricted to larger tumours (≥ median 627 pixels) group III (23.2±19) vs. group I (11.8±7, p=0.002). Peritoneal tumours in group II were of smaller size (median area 308 pixels) than in group I (619 pixels), in group III (901 pixels), in group IV (769 pixels) and in group V (808 pixels). RIT decreased the tumour size whereas it did not affect 5-FU uptake. The uptake of 5-FU was potentiated by pretreating the animals with Norbormide. These results demonstrate that 5-FU uptake in experimental peritoneal metastases is increased when the peritoneal absorbtion of the drug is blocked using pretreatment with a vasoconstrictive agent. This principle may also be relevant when treating patients with colorectal cancer peritoneal metastases.*

Peritoneal spread of colorectal cancer implies a poor prognosis (1-4) and most patients are treated with cytotoxic agents. 5-Fluorouracil (5-FU), alone or in combination with other drugs, is most commonly used in order to achieve a regression of the tumour (5-9). Resistance to cytotoxic drugs is an important obstacle to successful treatment (10-12). Furthermore, in order to exert its effect, the drug must gain entrance into the peritoneal tumour. Poor vascular supply (13) and elevated interstitial fluid pressure (14) might prevent the efficient uptake of chemotherapeutic agents into bulky peritoneal tumour tissue. Radioimmunotherapy (RIT), directed against antigen-expressing tumour cells (15), is one possible way to enhance drug uptake in peritoneal metastases since RIT may cause vascular permeability changes (16). Another interesting way to promote drug uptake into tumour tissue would be to increase tumour exposure to the drug. If peritoneal resorbtion of 5-FU is reduced, uptake in peritoneal metastases would be increased by prolonged retention of high 5-FU levels. U/V Norbormide is highly toxic to rats when administered orally or parenterally, but almost non-toxic to other species. This agent produces a rodent-specific, extreme irreversible peritoneal vasoconstriction due to an a-adrenergic effect (17).

The aim of this experimental study was to determine if the uptake of 5-FU in peritoneal metastases, induced in a rat tumour model, is influenced by pretreatment with RIT or by blocking the peritoneal absorption with Norbormide.

Materials and Methods

Animals. Thirty-two nude rats (Rowett nu/nu, the Wallenberg Laboratory, Lund, Sweden), 17 females and 15 males, weighing 151-341 g, (mean 205 g) were used. They had free access to a

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standard laboratory diet and water. A 10-day period of acclimatisation preceded the first surgical procedure. The regional Ethics Committee for Animal Research, Uppsala, Sweden, approved the experiments.

Tumour cells. A human colonic adenocarcinoma cell line, LS 174T (18), was grown as a monolayer culture in a nutrient mixture composed of Ham's F10 medium (Flow Laboratories Swedish AB, Stockholm, Sweden) supplemented with 10% fetal calf serum, 2mM L-glutamine, penicillin (20 U/ml) and streptomycin (20 μg/ml). Tumour cells were removed from the culture dishes by means of a scraper (Cell Lifter, Costar Corporation, Cambridge, MA 02139, USA). The preparation was suspended and centrifuged at 1200 rpm for 5 min. The pellet was resuspended in Ham's F10 medium. This cell preparation has previously been shown to comprise 40% single cells and 60% tumour emboli, up to 200 μm in diameter, each containing an average of 200 cells (19). To determine the number of cells per culture disc, selected discs were trypsinised and the cells counted with a cell counter (Coulter Electronics Ltd., Harpenden, Herts, England).

Antibodies, radiolabelling and radioimmunotherapy. The previously studied anti-CEA MAb 38S1 (20-22) was supplied from Pharmacia AB (Uppsala, Sweden). ^{131}I-38S1 was labelled by the Chloramine-T method (23). Briefly, 3.5 mg MAb 38S1 was mixed with 1.5 GBq of a highly concentrated ^{131}I-preparation (Amersham International, Little Chalfont, Buckinghamshire, England) and 150 mg chloramine-T in 750 μl phosphate buffer pH 7.4 was added. After 10-min incubation at 0˚C, the reaction was terminated by adding 300 mg sodium metabisulfite in 50 μl 50 mM phosphate buffer pH 7.4. Labelled MAb was separated from unreacted ^{131}I by gel filtration on a PD 10 column (Pharmacia AB) eluted with 50 mM PBS pH 7.4. The specific activity of the ^{131}I-MAb preparations was approximately 0.3 MBq/μg, and more than 94% of the radioactivity in the final preparation was protein bound as determined by 10% TCA precipitation.

U/V Norbormide. ^{3}H-norbormide (cis-endo-2-U/V racemat) was a gift from the R.W Johnson Pharmaceutical Research Institute, Pennsylvania, USA. The structure of Norbormide, with its 4 elements of dissymmetry and 5 asymmetric carbon atoms, give rise to 16 possible isomers or 8 racemates (24). The racemate cis-endo-2-U/V, containing the most toxic isomer (cis-endo-V), was isolated by fractional crystallization (24). The crude compound was dissolved in an excess of hot methanol (65˚C) and recrystallized overnight at 5˚C. The precipitate was then recrystallized several times in acetone, *i.e.* dissolution in acetone at 56˚C followed by crystallization overnight at 5˚C. The final precipitate consisted of the Norbormide racemate cis-endo-2-U/V (U/V Norbormide) according to thin-layer chromatography on 0.2 mm silica gel 60 F-254 on alumina (Merck, Darmstadt, Germany), *i.e.* silica gel plates eluted with acetic acid/ethyl acetate (5:95 v/v) and silica gel plates eluted with chloroform/ethyl acetate (3:7 v/v), respectively (24).

Surgical procedures and experimental groups. Chloral hydrate 36 mg/ml in a mixture of sodium pentobarbital 0.97 mg/ml and manganese oxide 2.1 mg/ml in distilled water was administered *i.p.* at a dose of 3.3 ml/kg body weight for anaesthesia. The abdomen was opened by an 1.5 cm midline incision and the right lateral abdominal peritoneum (1 cm^2) was abraded with a scalpel and inoculated with 1x10^7 LS 174T cells. The muscle layer and skin were closed with 4.0 Ethilon sutures. Two weeks later, the animals were randomly allocated to 5 groups (Table I). Animals in group I (n=5) and in group IV (n=4) received 2 ml NaCl *i.p.* and served as controls, whereas animals in group II (n=6) and V (n=8) received 150 MBq ^{131}I-MAb 38S1 *i.p.* Six days (groups I, II and III) or 2 days (groups IV and V) later, a second laparatomy was performed and the animals were examined for macroscopic tumour growth in the abdomen. All 32 animals had a macroscopic tumour growth. One rat in group I died immediately after the second laparatomy and was excluded from further analysis. Two rats, one from group IV and one from group V, underwent tumour dry-weight analysis. For the remaining 29 animals, a 1.2-mm hollow needle was inserted through the abdominal wall as a guide to introduce an *i.p.* plastic catheter (Polyethylene Tubing, PP 380, Swevet AB, Stockholm, Sweden). After introduction of the catheter, the needle was withdrawn and the abdominal wall was closed. A dose of 17 μCi 5-fluro[2-^{14}C]uracil (Amersham), specific activity 54 mCi/mmol, radiochemical purity 97-98%, was dissolved in 2 ml 0.9% NaCl (37˚C) and injected *i.p.* in each rat. Animals in group III (n=6) received 2.5 mg/kg U/V Norbormide *i.p.* 10 minutes prior to labelled 5-FU injection. In all groups, the animals were killed in a CO_2 chamber 2 hours after 5-FU injection.

Table I. *Number of rats and (peritoneal metastases) in the experimental groups. The number of tumours larger or smaller than 627 pixels (median tumour area) are shown separately.*

Group	I	II	III	IV	V
	5 (58)	6 (70)	6 (50)	4 (19)	8 (46)
Small tumours (< 627 pixels)	30	54	15	8	15
Large tumours (≥ 627 pixels)	28	16	35	11	31

Group I = Treated with NaCl, 6 days prior to 5-FU injection.
Group II = Treated with ^{131}I-MAB 38S1, 6 days prior to 5-FU injection.
Group III = Treated with Norbormide, 10 min prior to 5-FU injection.
Group IV = Treated with NaCl, 2 days prior to 5-FU injection.
Group V = Treated with ^{131}I-MAB 38S1, 2 days prior to 5-FU injection.

Autoradiography and distribution analysis. After sacrifice, the rats were immediately frozen in ethanol, cooled with dry ice to -78˚C for 10 minutes. The frozen rats were then mounted in an aqueous gel of carboxymethyl cellulose, which was rapidly frozen around the animals. Sagittal whole-body sections, 20 μm thick, were attached onto a tape (No. 810, Minnesota Mining & Manufacturing Co., USA). The sectioning was performed at -20˚C with a cryomicrotome (PMV Co, Stockholm, Sweden), as previously described (25, 26). The sections were freeze-dried and covered by X-ray film (Agfa Structurix D7, Agfa-Geavert, Belgium) for 8-week. However, rats in group II, which were injected with ^{131}I, were initially exposed for 1 week, followed by 8-week exposure allowing decay of ^{131}I for visualisation of ^{14}C-originated radioactivity. For subsequent quantification of autoradiograms, ^{131}I and ^{14}C standard staircases (Autoradiographic ^{14}C-Micro-Scales,

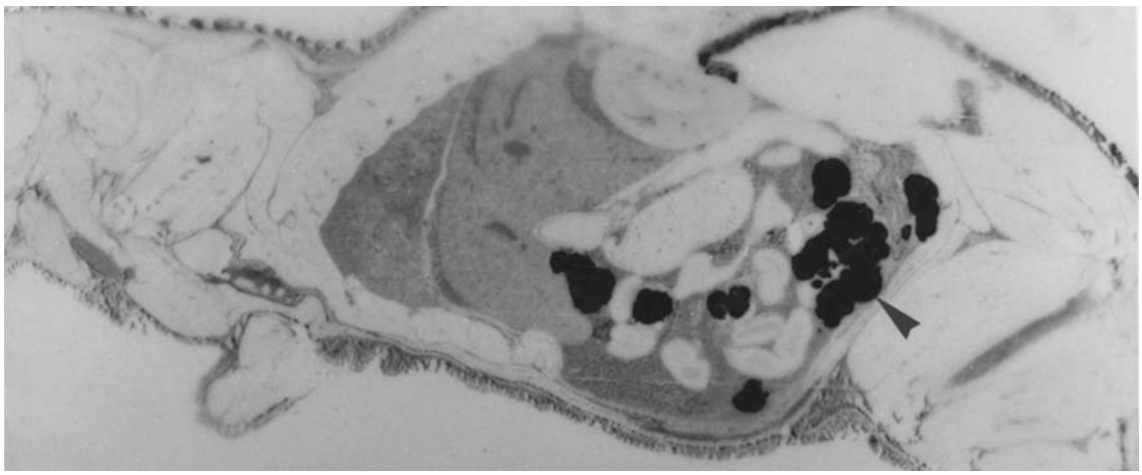

Figure 1. *Autoradiographs of whole body section after 1-week exposure showing ^{131}I-originated radioactivity in peritoneal metastases (arrow).*

Amersham, UK) were co-exposed with the sections. An objective evaluation of the sections was performed by computer-based image analysis (27). The sections were coded, *i.e.* the observer was not aware of which group the sections belonged to. Briefly, autoradiogram films were transilluminated, captured by a video camera, and stored in a computer. Regions of interest were analysed from the digitised picture presented on the image display system. Uptake was expressed as density and the area as pixels.

Statistical methods. The results are expressed as mean±standard deviations (SD). A *two-factor repeated measures analysis of variance* was performed to determine if the mean concentration of radioactivity differed significantly among groups. In this analysis, the group and rat are treated as the "between" factors and the tumour is treated as the "within" factor. *Dunnett's test* (28) was used to compare treated groups with the control group. Where appropriate, the *Bonferroni* (28) correction was used to controll the overall *type I error rate* at 0.05. A two-tailed *p* value of less than 0.05 was considered statistically significant.

Results

Metastatic growth and ^{131}I uptake. A total of 243 peritoneal metastases were observed (Table I). After 1-week exposure, group II showed a high uptake of ^{131}I in peritoneal tumours and skin relative to the other organs (Figure 1). The concentrations (kBq/g tissue) in tumours and skin were 213 (53) and 171 (89), respectively. The corresponding concentrations in liver and intestine were 30 (13) and 16 (9), respectively.

Patterns of 5-FU metastatic uptake. After 8-week exposure, the tumour uptake of 5-FU was higher in group III than in group I (Figure 2, Table II, $p=0.04$). However, there was no difference between group II and I. When the analysis was stratified by tumour size into small tumours (<627 pixels) and large tumours (≥627 pixels), where 627 was the median tumour area, large tumours in group III had a higher radioactivity concentration compared with group I (Table II, $p=0.002$). However, this was not the case for small tumours. When comparing group V with group IV, the mean 5-FU uptake did not differ (Table II). The tumour size was reduced in group II compared with group I (Table III, $p<0.007$), whereas the size did not differ appreciably between groups IV and V (Table III, $p>0.70$). The drug concentration was higher in the tumour periphery compared with tumour centre, particularly in group III, although this difference did not reach statistical significance (Table III, $p=0.10$). There was no difference in the tumour fluid content between group IV (79%) and group V (77%).

Discussion

This experimental study was designed to investigate if the uptake of 5-FU in peritoneal metastases improves after pretreating with radioimmunotherapy or by an agent, which blocks the peritoneal resorption of 5-FU. An interesting finding in this peritoneal metastases induced in a rat tumour model study was that tumours which have been treated by RIT 6 days prior to sacrifice showed a general decline in tumour volume in comparison to the control group. Furthermore, the uptake of 5-FU in peritoneal metastases improved after pretreating the animals with a local vasconstrictive agent.

The most obvious progress in recent years in the effort to improve prognosis after resection of colorectal cancer has been observed following adjuvant chemotherapy given systemically (29, 30). Immunotherapy has also resulted in a reduced mortality when used as adjuvant therapy (31). Clinically, the prognosis in peritoneal dissemination of colorectal cancer is poor when treated with systemic chemotherapy, and most

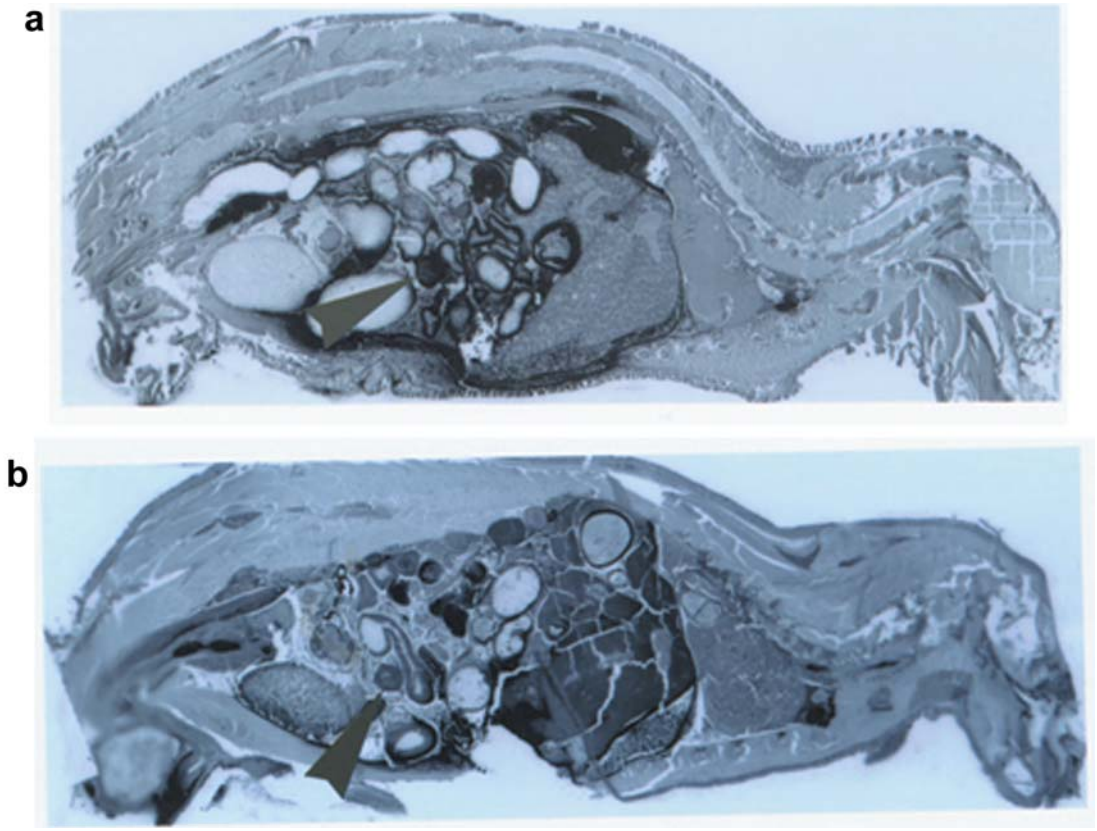

Figure 2. *Autoradiographs of whole body section of rats, after 8-week exposure showing uptake of ^{14}C-labelled 5-FU in peritoneal metastases (arrows). A rat treated with i.p. Norbormide 10 minutes prior to labelled 5-FU injection (group III) is seen in A and a rat treated with i.p. 5-FU only (group I) in B.*

patients with peritoneal metastases die within 6-12 months (5, 7, 32). However, there have been favourable reports on intraperitoneal chemotherapy alone or in a combination with cytoreduction (33, 34). Furthermore, encouraging results in patients with advanced colorectal cancer treated by RIT has been reported (35, 36). However, an effective treatment for peritoneal spread of colorectal cancer is still lacking.

The route of tumour spread and implantation in the present experimental model resembles the human situation, which may be advantageous when studying drug uptake that is dependent on the tumour blood supply. The assumption that there is a unique arrangement of the blood supply in each metastasis, as well as the fact that metastases in an individual rat share several characteristics specific to the individual rat, made us consider the use of a statistical method which takes into account "between" factors as well as "within" factors. The rationale for pretreatment with RIT for 2 or 6 days was an assumption that local irradiation with two defined durations may render the tumour periphery more disposed to 5-FU uptake through increased vascular and connective tissue permeability. The reason for testing Norbormide was to test the concept of peritoneal vasoconstriction and thereby to improve 5-FU uptake by prolonging tumour exposure to 5-FU.

The tumour size in group II was smaller than in the others groups, which is most likely a result of the β-radiation which reduces the tumour cell's capacity to proliferate, in accordance with previous reports on experimental models (37). The absence of anti-tumour effect in group IV is probably due to the shorter time period for the effect of local irradiation. RIT did not improve 5-FU uptake in peritoneal metastases when analysed after 2 or 6 days. RIT was thus not an effective method to promote peritoneal drug uptake. On the other hand, RIT may be used to eradicate residual tumour after cytoreductive surgery and *i.p.* chemotherapy. Theoretically, an oedema can be initiated by the radiation, thereby causing a higher intratumoral pressure. However, an analysis of the water content in tumours from groups IV and V did not show any difference in this respect.

One possible reason for failure of intraperitoneally administered drugs to cure larger tumour masses is the poor penetration into tumour tissue. In a previous study, the

Table II. *Concentration of ^{14}C-originated radioactivity in peritoneal metastases, in all tumours, in small (<627 pixels) and large (≥627 pixels) tumours, separately, as well as in liver and intestine (kBq/g tissue). Figures are mean (SD).*

	All tumours	n	Small tumours	n	Large tumours	Liver	Intestine
Group I	11.8 (11)	30	11.9 (13)	28	11.8 (7)	21.3 (27)	9.1 (5)
Group II	9.8 (11)	54	11.2 (12)	16	4.8 (2)	7.1 (4)	9.7 (10)
Group III	21.4 (17)	15	17.1 (11)	35	23.3 (19)	17.3 (15)	34.4 (33)
Group IV	12.1 (3)	8	12.1 (4)	11	12.1 (3)	15.6 (9)	11.4 (8)
Group V	10.1 (10)	15	10.0 (4)	31	10.2 (4)	16.2 (11)	11.6 (5)

Abbreviations, see Table I.

Table III. *Radioactivity concentration (kBq/g tissue) in the tumour periphery, tumour centre and tumour area (pixels). Figures are mean (SD).*

Group	I	II	III	IV	V
Tumour Periphery	16.8 (15)	13.1 (14)	33.1 (28)	12.8 (5)	11.7 (5)
Tumour centre	8.5 (9)	7.6 (11)	11.0 (10)	10.5 (4)	8.7 (4)
Tumour area	874 (732)	484 (511)	1375 (1027)	824 (937)	968 (973)

Abbreviations, see Table I.

concentration of 5-FU measured at a depth of 0.6 mm from the peritoneal surface was only 5% of that in the peritoneal fluid (38). Norbormide was introduced on the market as a specific raticide, but it is no longer used for various reasons. This drug has a strong local vasoconstrictive effect and in this study it improved the 5-FU uptake in peritoneal tumours, especially in "large tumours". This was probably due to reduced peritoneal absorption of 5-FU leading to increased 5-FU exposure time to the tumours, which promoted local diffusion of 5-FU. Although Norbormide is not suitable for use in humans, this study shows that the concept of peritoneal vasoconstriction is a valid method to increase 5-FU uptake in peritoneal tumours. Our results are consistent with a previous report where the anti-tumoral effect of *i.p.* chemotherapy was enhanced by an injection of *i.p.* epinephrine (39). However, the risk in systemic (mainly cardiovascular) and local (intestinal infarction) effect of epinephrine limits *i.p.* application in patients with peritoneal carcinomatosis. There are other vasoconstrictors, which may be convenient for clinical use, *e.g.* vasopressin (40).

In conclusion, our experimental study in the rat showed that peritoneal vasoconstriction is an interesting principle for increasing the uptake of 5-FU in peritoneal metastases. Radioimmunotherapy did not enhance 5-FU uptake but produced an anti-tumour effect.

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