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Intratumor treatment of C3H mouse mammary carcinoma with 5-fluorouracil adsorbed on activated charcoal particles

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A dosage form comprising 5-fluorouracil (5-FU, 25 mg/ml) adsorbed on a suspension of micronized charcoal (100 mg/ml) 2–5 μm in diameter, adsorbing 5-FU in aqueous solution was studied for intratumor treatment of mammary carcinoma in animal experiments. An *in vitro* desorption method is described to determine the amount of 5-FU adsorbed on activated charcoal particles which would be released once the drug concentration decreased around the charcoal. *In vivo* results indicate that an intratumor injection of 5-FU adsorbed on activated charcoal particles is a highly effective method for achieving tumor regression without increasing toxicity.

Key words: Charcoal suspension, drug delivery system, 5-fluorouracil, local chemotherapy.

Introduction

Since its introduction in the clinic 5-fluorouracil (5-FU) has proven to be one of the most important agents for treatment of advanced breast cancer.[1] In order to enhance the therapeutic efficacy the combination of an anticancer agent (5-FU) and a suspension of activated charcoal particles (5-FU-CH) was investigated.

An *in vitro* study was designed to determine the adsorbed amount of 5-FU released from activated charcoal particles. We also studied the effect of injecting 5-FU-CH directly into tumors in C3H mouse mammary adenocarcinoma.

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Materials and methods

Preparation of 5-FU adsorbed on activated charcoal

Vials from the same batch of 5-FU were obtained from Produits Roche SA (Neuilly sur Seine, France). Charcoal was obtained from Norit (Le Blanc Mesnil, France).

The activated charcoal was first micronized (Jet o' Mizer 0202 Lab service Macon).[2] The suspension of activated charcoal (100 mg/ml) in aqueous solution was sterilized at 120°C for 20 min, then 25 mg/ml of 5-FU was added and the suspension shaken at 120 oscillations/s for 30 min.

The pH was measured with a pH meter (Microcomputer Solution Analyseur Consort). The sterility test used was according to the US Pharmacopeia method[3] and the concentration of bacterial endotoxin was determined using limulus amebocyte lysate (LAL).[4]

Counts of particles were measured using a Coulter Counter model TA II (Coultronics® France SA, Margency, France). Each sample was analyzed twice.

The concentration of non-adsorbed, free 5-FU was determined in triplicate. An *in vitro* study of

5-FU released from activated charcoal was investigated.

Measurement of the 5-FU concentration

Concentrations of 5-FU were measured using high performance liquid chromatography (Schimadzu® LC 6A) with a UV spectrophotometric detector (Schimadzu® CR 5A). The wavelength was set at 265 nm. Chromatography was performed on a reserved-phase C18 analytical column (Kromasil, 5 μm, 250 × 4.6 mm inside diameter). The mobile phase, 0.05 M phosphate buffer, pH 5, was delivered at 1 ml/min.

The concentration of 5-FU in experimental samples was calculated from the equation of the standard curve and the peak area ratio of the sample. All subsequent concentrations were expressed as percentages of the initial concentration.

Desorption method

An *in vitro* desorption method was investigated. A volume (0.8 ml) of 5-FU adsorbed on activated charcoal particles was filtered through a 0.22 μm membrane filter (Millex-GS, Millipore SA®, Molsheim, France). After filtration the filter was connected to a 2 ml delivery catheter and to a disposable plastic syringe placed in an electrical pump (Vial Medical SE 200, Becton-Dickinson) programmed at a rate of 1.6 ml/min. The syringe was filled with 10 ml of a phosphate buffer (pH 7.0) so that 8 ml of the filtrate was collected in a glass tube after 5 min.

This desorption procedure (aimed at collecting the 5-FU adsorbed on the charcoal particle), using the same amount of phosphate buffer, was repeated to obtain a maximum desorption effect. Desorption curves were obtained by measuring the concentration of 5-FU dissolved in the filtrate at 5, 10, 15, 20, 25, 30, 35, 40 and 45 min.

Animal experimentation

C3H female mice aged 6–8 weeks were used for the study and kept under standard conditions (day–night cycle 12 h, specifically pathogen free, conventional caging, and feeding with commercial pellet rations and water *ad libitum*). The studies were carried out on a total of 29 animals weighing 20–25 g, 21 days after implantation of tumor cells of C3H mouse mammary adenocarcinoma into the hind leg. The tumors were obtained from the animal experimentation department of Dr Gosse (Institut Gustave Roussy). This is a syngenetic implantable tumor obtained from solid tissue transplants that is inoculated subcutaneously into receiving mice. The 29 animals were divided into three groups.

A volume (0.1 ml) of each dosage form was injected intratumorally with a 30 gauge hypodermic needle. 5-FU (25 mg/ml) adsorbed on 100 mg of the activated charcoal suspension in injectable water (5-FU-CH) was injected into the first group (nine mice).

A suspension of 100 mg of activated charcoal in injectable water (CH) was injected into the second group (10 mice). The control group (10 mice) had an injection of injectable water.

The animals were sacrificed by cervical dislocation on day 10 after injection and were autopsied. The tumor, liver, lung, heart, kidney and spleen were removed and fixed in formalin for histological examination.

Caliper measurements were obtained before and after injection of the dosage form. Tumor growth was monitored by calculating the tumor volume from caliper measurements before and 10 days after injection. Tumor volume (V) was calculated according to the formula:

$$V = \frac{(\text{length} \times \text{width})^2}{2}$$

the data were expressed as means and standard errors, and compared using student's *t*-test.

Results

Suspensions

The micronized activated charcoal particles had a specific surface area of 600 m^2/g, determined by BET adsorption. For the two suspensions (5-FU-CH and CH) the mean diameter of the charcoal particles was 6.5 μm and the particles did not exceed 10 μm. Twenty percent of the particles were less than 2 μm in diameter. The preparations were sterile and pyrogen free. The pH was 5.38 ± 0.03.

Measurement of the 5-FU concentration

The relative retention time of 5-FU was 2.5 min. The relationship between the peak area and 5-FU

concentration over the concentration was linear and the equation of the least squares regression line was:

$$y = 302\ x + 56.6,\ r^2 = 0.999.$$

There was 5 mg/ml of 5-FU in a free state, so the micronized activated charcoal was able to adsorb 80% of 5-FU even before shaking. Agitation (30 min) produced no significant increase in the adsorption of 5-FU.

Desorption method

Desorption curves of the 5-FU solution and 5-FU-CH suspensions after initial filtration and rinsing with the phosphate buffer are shown in Figure 1. A significantly faster rate of desorption was observed in the 5-FU solution compared with the 5-FU-CH.

The 5-FU adsorbed on micronized charcoal particles was released at a constant rate over 45 min without degradation and the desorption rate was 105 ± 2% in the phosphate buffer.

Animal experimentation

No significant difference was found in the mean volume of the tumors (measured by a caliper) of the 5-FU-CH group, before and after treatment. However, tumors in the control and charcoal suspension groups were 3.5 times their original volume after injection. It should be noted, however, that no difference existed in the tumor volume between the three groups before injections.

All animals, except for one in the 5-FU-CH group and two in the control group, survived. After treatment, body weight was lighter in the 5-FU-CH group compared with that of the control group and the CH group.

Tumors: macroscopically

Black stains from the charcoal were found inside tumors in all cases or at the periphery of the tumors in the 5-FU-CH and CH groups. Stains were dispersed around the injection site. At the periphery

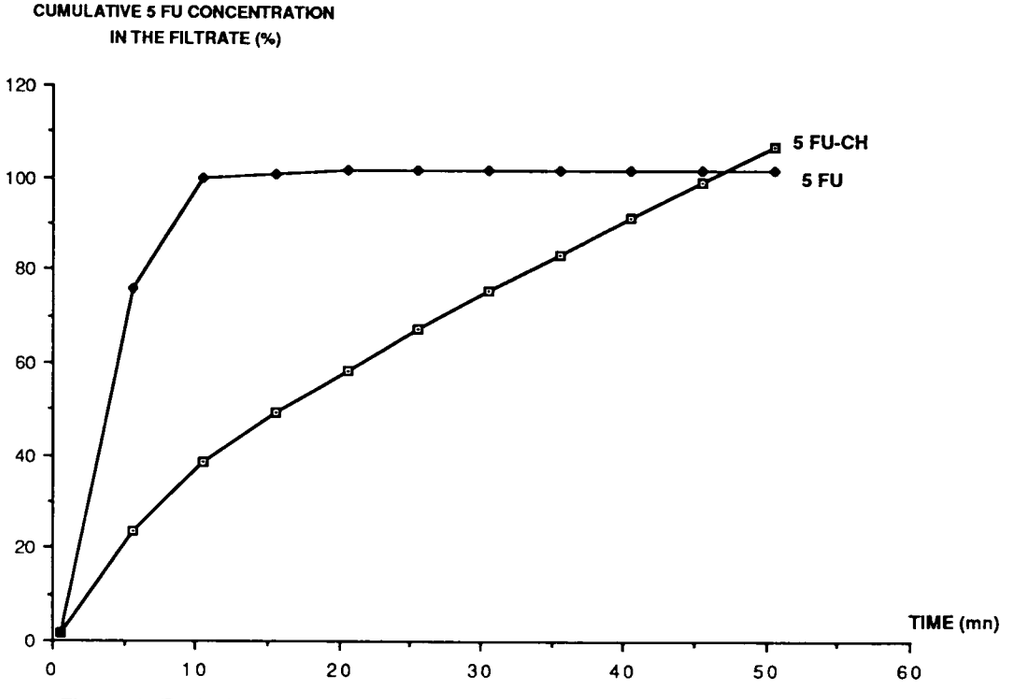

Figure 1. Typical results obtained from one set of measurements using the desorption method and describing the cumulative 5-FU concentration in the filtrate expressed in a percentage as a function of time.

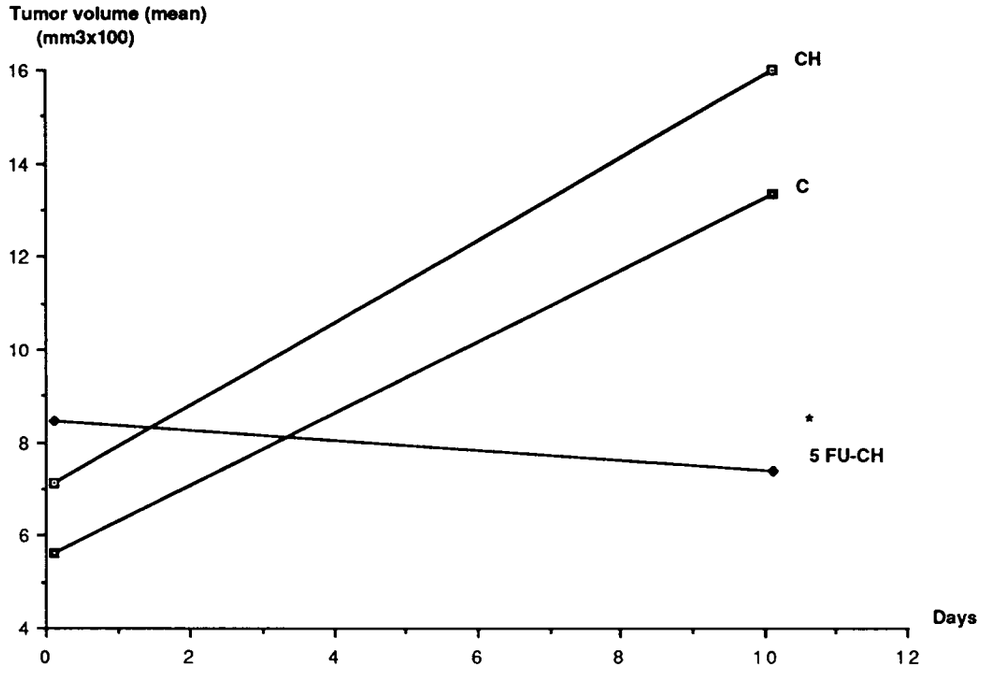

Figure 2. Mean tumor volume of each experimental group over time. Animals were treated on day 0. C, control. $^*p < 0.01$ versus control mice.

of the tumors, stains were like dense black spots (2–5 mm in diameter) in two out of eight cases for 5-FU-CH mice and three out of nine cases for CH mice. The tumor volume (after dissection) in the CH and control group was greater than that of tumors in the 5-FU-CH group.

Tumors: histologically

5-FU-CH mice. Inside the tumors, the distribution of necrosis was mainly around charcoal particles. The mean amount of tumor necrosis was 56% (median 55%; range 40–75%). Charcoal particles were dispersed within the necrosis. Charcoal particles were found in the cytoplasm of a few tumor cells. They were also found in a few histiocytes, but this was rare (Figure 3).

CH mice. Inside the tumor, charcoal was found inside the necrosis, the mean amount of tumor necrosis was 50% (median 50%; range 30–70%). The charcoal particles inside the necrosis were grouped together. No charcoal particles were observed inside cells (tumor cells or histiocytes) (Figure 4).

Control mice. The mean amount of necrosis was 50% (median 40%; 25–75%). Necrosis was present throughout the tumor in six out of eight cases, or two out of eight cases in only one part of the tumor.

The mean amount of necrosis seen after histological examination was the same in CH, 5-FU-CH and control mice.

Organs

5-FU-CH mice. Charcoal was found in sites other than the tumors in two out of seven cases: in alveolar macrophages of lung, in kupffer's cells of the liver, in macrophages of the spleen and in rare glomerular cells of the kidney. No charcoal was found in the heart. In the other cases, these organs were normal, without other diseases.

CH mice. Charcoal was found in sites other than the tumors in six out of nine cases. In two cases rare particles of charcoal were found in the lung and liver. In the other four cases charcoal was found in the lung, liver and spleen, in the same cells as in the 5-FU-CH mice. However, no charcoal was found in the kidney and heart in any of the cases.

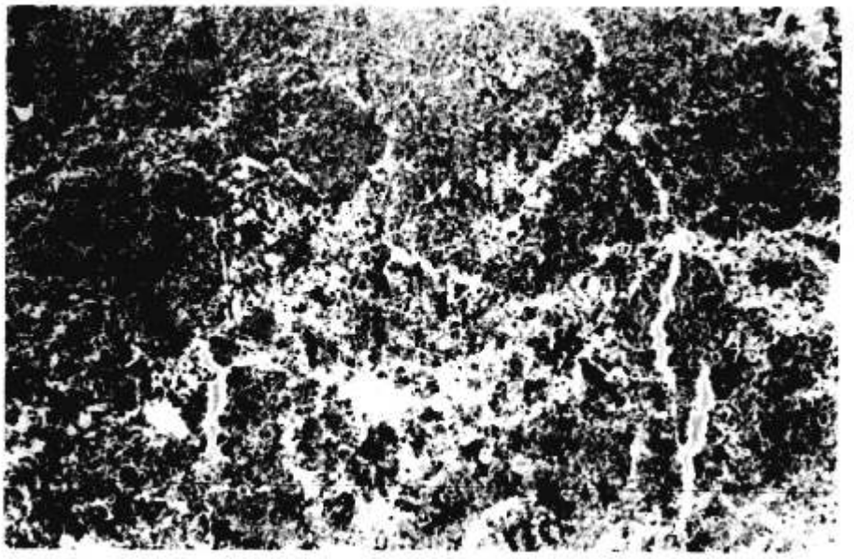

Figure 3. The arrows indicate charcoal particles dispersed within the necrosis in mice injected with a suspension of 5-FU adsorbed on charcoal (hematoxylin & eosin Saffon, × 125).

Figure 4. The arrows indicate charcoal particles grouped together inside necrosis for the charcoal group (CH); some viable cells (stars) persist around the necrosis (hematoxylin & eosin Saffon, × 125).

Control mice. The spleen, lung, kidney, heart and liver were normal.

Discussion

For effective anticancer chemotherapy, it is essential that tumor cells are exposed to a high concentration of drug for a long time. The systemic administration of drugs cannot increase the drug concentration within the tumor to an effective level.

A dosage form comprising an anticancer agent (5-FU) adsorbed on activated charcoal particles was investigated for intratumor treatment of mammary carcinoma in animal experiments. *In vitro* 5-FU-CH managed to prolong the release of 5-FU. When the

drug concentration decreased the charcoal particles released the adsorbed 5-FU so that 5-FU concentration in the free state was maintained at the same level. The 5-FU adsorbed on the charcoal particles remained in dynamic equilibrium with the concentration of non-adsorbed drug. Injection of 5-FU-CH directly into the tumor prevented progression in this experiment.

The charcoal particles consisted of micronized particles, 2–5 μm in diameter, which did not diffuse macroscopically into surrounding tissue; however, particles were sometimes found in distant organs in macrophages at histological examination. We do not know why there were charcoal particles inside macrophages at distant localizations. Intratumor injections may have damaged the endothelium of the blood vessels, thus allowing charcoal particles to be transported by the blood flow away from the tumor site. These particles may also have been displaced by the migration of macrophages which have phagocyted charcoal particles in the tumors.

Tumor cell and histiocyte uptake of charcoal particles in the 5-FU-CH group could be explained by the zeta potential of the charcoal particle. Tumor cells have a higher negative charge as compared with normal cells.[5] They are also able to accumulate more cationic substances than anionic substances as compared with normal cells.

We have previously shown that the degree of uptake of micronized charcoal particles by both tumor cells and histiocytes is higher when the excipient is sodium chloride than when injectable water is used.[6] In that previous report, the measured zeta potential of the charcoal particles was negative in water and zero in sodium chloride. In the present study, it is possible that the excipient employed with 5-FU, i.e. tromentamol, altered the particle charge of charcoal particles in a similar manner to sodium chloride. This might probably explain why charcoal particles were found more often in tumor cells and histiocytes in the 5-FU-CH group.

Necrosis was present in the three groups and intratumor injections clearly lead to local necrosis. Perhaps necrosis was found inside tumors in the 5-FU-CH group because mice were sacrificed 10 days after treatment; it might have been eliminated had the dissection occurred several days later. The charcoal particles within the tumor in the 5-FU-CH group were dispersed in the necrosis, whereas they were grouped together in the CH group. The difference in the degree of diffusion between these two groups could be explained by the osmotic effect of two different excipients.

The injection of a non-physiologic excipient (injectable water) can lead to local tissue destruction which induces particle accumulation and limits diffusion. The measured osmolarity of 5-FU-CH suspensions (232 mosm) was close to the isotonicity of physiological salines. This could explain the enhanced dispersion of charcoal particles within the tumor. Effective antitumor activity would indeed be improved if 5-FU adsorbed on charcoal particles is dispersed within the tumor.

Although charcoal particles are not biodegradable, they were well tolerated and no local or organ toxicities due to intratumor chemotherapy were observed.

These results are in agreement with studies which have reported a prolonged duration at a site of an anticancer agent adsorbed on small particles.[7,8] The gradual release of cytotoxic agents by activated charcoal particles should prove useful in the future design of protocols with parenteral drug administration.

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