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Original article

New application of carbon nanotubes in haemostatic dressing filled with anticancer substance

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ABSTRACT

The drug-carrier system used as innovative haemostatic dressing with oncostatic action is studied. It is obtained from CDDP (cisplatin) doped SWCNT (single walled carbon nanotubes), modified and purified by H_2O_2 in hydrothermal treatment process. In the *in vivo* nephron sparing surgery (NSS) study we used 35 BALB/c nude mice with induced renal cancer using adenocarcinoma 786-o cells. Animals were divided into four groups: CDDP(M−), CDDP(M+), CONTROL(M−) and CONTROL(M+). In CDDP(M−) and CDDP(M+) groups we used, intraoperatively, carbon nanotubes filled with cisplatin (CDDP). In CONTROL(M−) and CONTROL(M+) groups carbon nanotubes were used alone. During NSS free margin (M−) or positive margin (M+) was performed. In the CDDP(M−) group, we do not observe local tumor recurrences. In Group CDDP(M+) only one animal was diagnosed with tumor recurrence. In control groups the recurrent tumor formation was observed. In our study, it is shown that CDDP filled SWCNT inhibit cancer recurrence in animal model NSS study, and can be successfully applied as haemostatic dressings for local chemoprevention.

1. Introduction

The global number of organ sparing surgeries is still growing [1,2]. This situation is correlated with important need for a searching of the new solutions for the potential most emerging problems related with the clinical choice of this kind of treatment. One of the major problems of such a type of treatment (for example, breast conserving therapy (BCT) or a method to which we refer to in this paper, *i.e.* nephron sparing surgery (NSS)) is a potential risk of local cancer recurrence [3–5]. In this field, currently undertaken solutions refer mainly to the activities suggested by the international and national scientific associations in systematically published official guidelines and recommendations. In our opinion existing solutions are still insufficient, but we are sure that they have clinical relevance [6,7].

Following this, in the presented study we use, for the first time, a new carbon-nanotube-based haemostatic dressing filled with anticancer substance. This idea is the result of searching of solution of two mainly correlated problems occurring in organ sparing surgeries, that is intraoperative bleeding and possibility of local tumor recurrence. It was shown previously that haemostatic dressings can be successfully applied as bio-active matrices for targeted implementation of selected cytostatics for local chemoprevention [8]. Thus we use, for the first time, carbon nanotubes filled with cisplatin. The homeostatic properties of carbon nanotubes are well confirmed in the literature [9–11]. RCC cancer model, applied in this study, is also sensitive for CDDP both *in vitro* and *in vivo* [12,13].

Cispaltin (cis-diamminedichloroplatinum(II) – CDDP) was developed in 1965 and clinically tested in 1972. It shows antineoplastic activity against different types of cancer. However, this drug has acute and cumulative renal toxicity [14,15]; thus, new platinum based drugs are still discovered [16–18]. There are different attempts to use small CDDP doses to avoid different side

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effects and inherent toxicities. Also, other new CDDP conjugates and delivery systems for anticancer therapy have been described in literature [19–23].

We estimate the usefulness of carbon nanotubes combined with CDDP as a new experimental concept of innovative hemostatic dressings preventing local recurrence after nephron sparing surgery (NSS) of the kidney cancer on the xenografted murine model. In this way a new concept of tumor recurrence prevention is proposed. The drug-carrier system is obtained using single-walled carbon nanotubes, modified and purified by H_2O_2 hydrothermal treatment [24]. As it was recently shown by us, this system has very good anticancer properties [24]. The nanotube sample having relatively small burn-off but high concentration of surface oxygen groups is chosen for the preparation of CDDP-containing drug delivery system. Z-contrast TEM, EDX and kinetic data are reported to explain the properties of CDDP/nanotube-loaded systems, and the rate of CDDP delivery. Next, to check the efficiency in *in vivo* tests studied drug delivery systems are tested using model of organ sparing surgery for renal cancer.

2. Materials and methods

2.1. Nanotubes, drug deposition, microscopy analysis and kinetics

Commercial, high purity opened single walled nanotubes from Nanostructured & Amorphous Materials (NanoAmor, Houston, TX, USA) were investigated (labeled as A0-o). They were hydrothermally purified and oxidized in conc. 30% H_2O_2 at 493 K (the labeling of the sample is A0-o-493).

To introduce CDDP (Sigma–Aldrich, 99.9% pure), A0-o-493 sample (30 mg) was dispersed (by sonification) in a CDDP (60 mg) water solution (10 ml) and vigorously stirred for about 20 h at room temperature. Next, the solvent was removed by the evaporation process, in vacuum chamber at 323 K. Obtained sample is labeled as A0-o-493-CDDP-H_2O.

High-resolution transmission electron microscopy (HRTEM) images were taken using the transmission electron microscope F20X-TWIN (FEI-Tecnai) operated at 200 kV.

Z-contrast imaging with a scanning TEM was obtained using Fischione HAADF (High Angle Annular Dark Field) detector operated at 200 kV.

Kinetic studies of CDDP desorption from the A0-o-493-CDDP-H_2O were performed by immersion of 10 mg of each sample in DMEM medium (see below 100 ml). The measurements were carried out at 310 K during 48 h, at pH = 7.4. At a certain period of time, the 0.2 ml of solution with released CDDP was analyzed. The CDDP concentration was measured with the use of atomic absorption spectroscopy (each measurement was repeated 5 times, and the data were averaged). Note that the total measurement time was 100 h, since after rapid release of CDDP a very slow process occurs.

2.2. Tumor model

We used 35 four-week-old male BALB/c-Nude (CAnN. Cg-Foxn1nu/Crl) mouse. This animal model was selected using the CORE (Collection of Oncology Research Experiments) library data and basing on our experience from preliminary trial tests.

All animals were derived from the Charles River strain and delivered by the Research Models and Service, Germany GmbH group. All animals were delivered healthy with detailed serological, bacteriological and parasitology control examination tests. All experiments conducted were approved by the relevant Local Ethical Committee (approval number# 38/2012) and the work has been carried out on the basis and rules of conducting animal experiments compatible with the EU guidelines (2010/63/EU) and other representative EU recommendations. Tumor was created using renal cell adenocarcinoma 786-o line (ATCC® CRL-1932™). Renal cell carcinoma xenografted model is lethal urologic malignancy [13,25–27].

786-o cells were cultured in RPMI-1640 medium containing 10% of fetal bovine serum (FBS), supplemented with 5 μg/ml amphotericin B, 100 μg/ml streptomycin and 100 U/ml penicillin. All cell lines were grown in 25-cm^2 Nunc T-flasks at 36 °C and 5% CO_2 and 95% humidity until 3rd passage. The precise and delicate cut and the preparation of skin and muscles layers on the left site (for easier anatomical access) in the flank localization under the arch rib were performed to expose kidney. The 5×10^6 786-o cells were injected under the kidney fibrous capsule, in the region of lower pole of kidney. After that all flaps were closed using standard surgical suture. All procedures were prepared using surgical op – loups 3,2-5,0x (Carl Zeiss – EyeMag PRO F).

2.3. Study groups

After 5th week of injection of a xenograft RCC cells, when tumors reached the operative size, the nephron sparing surgery was performed in all groups. The macroscopic average of induced tumor size was equal to 2 mm. Each resected tumor was taken as a specimen for the histopatological examination, to confirm the RCC character and margin of resection.

Animals were divided into four groups. Two of them were study groups and other two were control groups. The first, second and third group CDDP(M−), CDDP(M+) and CONTROL(M−) consisted of 10 mice, and the fourth group CONTROL(M+) consisted of 5 mice.

In CDDP(M−) and CONTROL(M−) the resection was performed with surgical parenchymal margin (margin negative M−) and in CDDP(M+) and CONTROL(M+) the margin was left (margin positive M+). The surgical margin (M−) was approximately in the cut size of 1 mm, and (M+) involves leaving about 1 mm of the tumor after resection (both margins were measured macroscopically).

In CDDP(M−) and CDDP(M+) we have used, intraoperatively, together with electrocoagulation, the innovative carbon nanotubes filled with CDDP as intraoperative hemostatic dressings with oncostatic action.

In CONTROL(M−) and CONTROL(M+) during NSS operation we have used only carbon nanotubes (without covering with oncostatic or other reactive chemical substances).

In both cases carbon nanotubes were imposed onto bleeding resection field. Before this they (1 mg) were suspended in the PBS (100 μl). This method was used to ensure that no additional factors affect the kinetics of cytostatic delivery.

All surgical procedures in all groups were always performed by the same operating team according to the same scheme and surgical techniques. All experimental interventions were done always at the same time of a day, step by step according to the strictly fixed study protocol.

After surgery the animals were separated in individual, previously sterilized cages and were kept in a standard laboratory rodent circadian rhythm time, in a 24 h light/dark cycle (LD 12:12). No specific and other medications were given during all the time of study. The animals were fed on with multi-composite, sterile and totally pathogen free Altromin© fodder (Altromin Spezialfutter GmbH & Co. KG) *ad libitum.*

All animals were sacrificed after 5 weeks of nephron sparing surgery to be sure for recurrence. The left kidneys were collected for morphological and histological examination, fixed in 4% buffered formaldehyde to be later embedded in paraffin. Five-micron-thick sections were stained with hematoxylin and eosin (H&E). To evaluate the data 640×480-pixel resolution was acquired from each specimen using a digital camera (Olympus,