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Aerosol delivery of chemotherapy in an orthotopic model of lung cancer

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ABSTRACT: The aim of this study was to evaluate the effect on tumour growth of gemcitabine delivered by aerosol in an orthotopic model of lung carcinoma.

Large cell carcinoma (NCI-H460) cells were implanted intrabronchially in 24 male BALB/c nude mice on day (d) 0. Aerosols were delivered once a week from d1 to d29 using an endotracheal sprayer. Altogether, 16 animals received gemcitabine at 8 (n=8) and 12 mg·kg^{-1} (n=8), and eight received a vehicle aerosol. Animals were sacrificed on d36 for histological examination.

All animals in the vehicle group developed a large infiltrating carcinoma. Comparatively, four of 13 (31%) animals treated with gemcitabine had no visible tumour and nine of 13 (69%) had a smaller carcinoma with a mean±SEM largest tumour diameter of 2.05±0.7 *versus* 5±0.3 mm in the vehicle group. Gemcitabine was well tolerated at 8 mg·kg^{-1}. At 12 mg·kg^{-1}, three cases of fatal pulmonary oedema were observed, prompting a dose reduction to 8 mg·kg^{-1} in the remaining animals. A dose effect was observed, with more marked tumour growth inhibition in the animals treated at 12 mg·kg^{-1} on d1 and d8.

In conclusion, in this study, an animal model of aerosolised chemotherapy in lung cancer was developed and demonstrated inhibition of orthotopic tumour growth by aerosol delivery of gemcitabine.

KEYWORDS: Aerosol, chemotherapy, gemcitabine, lung cancer, orthotopic model

Regional chemotherapy has been proposed as a treatment modality in a number of situations in oncology in order to increase exposure of the tumour to the drug, while minimising systemic side-effects. Administration of drugs directly to the lungs *via* inhalation allows regional drug delivery to the lungs and airways with smaller doses and fewer systemic effects [1]. There is now increasing evidence to support the role of inhaled therapeutics in the treatment of various lung diseases. In lung cancer, regional chemotherapy could be useful in: unresectable bronchioloalveolar carcinoma or main bronchus carcinoma with limited invasion; endobronchial tumour relapse after surgery; *in situ* carcinoma or synchronous; or metachronous lesions in patients where a lesion has already been detected. Aerosol delivery of chemotherapy could be considered alone or in combination with other treatment modalities, such as radiotherapy. However, few studies have documented the feasibility of inhalation delivery of anticancer agents [2–5].

In a recent study [6], the current authors demonstrated that gemcitabine could be administered *via* endotracheal spray in rats without marked toxicity, with a maximum tolerated dose of 4–6 mg·kg^{-1} once a week for nine consecutive weeks. Under these conditions, procedure-related mortality was 1.2%, with no chemotherapy-related deaths and no clinical, histological or haematological signs of toxicity, apart from a slight decrease in platelet and red blood cell counts with no clinical consequence. Pulmonary deposition of gemcitabine, as assessed by scintigraphic imaging, was confirmed in 98% of spray administrations with a homogeneous pattern of deposition. The lung gemcitabine concentration after spray administration of 4 mg·kg^{-1} was estimated to be 50-fold higher than that obtained after *i.v.* administration of 10 mg·kg^{-1} [7].

Several orthotopic models have been developed to study human lung cancer. The intrabronchial orthotopic model in nude mice was found to closely mimic the natural progression pattern of human lung cancer [8, 9]. Therefore, the current authors considered that it could be relevant for *in vivo* testing of the efficacy of regional administration of chemotherapy.

The aim of this study was to develop an orthotopic model of human lung carcinoma in nude mice, and to evaluate the safety and

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efficacy on tumour growth of gemcitabine administered by endotracheal spray.

MATERIALS AND METHODS

Human lung tumour cell line

The NCI-H460 human large-cell lung carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were routinely maintained at 37°C in a humidified atmosphere of 5% CO_2 in air and cultured in recommended medium. For endobronchial implantation, cells were harvested by trypsinisation and then resuspended in RPMI 1640 medium at a final concentration of 1×10^5 cells per 10 μL. Cell count was performed with a haemocytometer using Trypan Blue exclusion (0.2%) to assess cell viability. All the experiments were carried out with cultures containing >95% of viable cells.

Animals and animal care

The study was conducted in 24 male BALB/c nude mice (Charles River, Lyon, France) at 4 weeks of age, which were divided into three groups of eight animals. One group (group G8) received gemcitabine by endotracheal spray at a dosage of 8 $mg \cdot kg^{-1}$. In a validation study the authors found that gemcitabine could be administered *via* endotracheal spray in BALB/c nude mice without any sign of toxicity at a dosage of 8 $mg \cdot kg^{-1}$ for five consecutive weeks. In order to evaluate a dose effect, a second group (group G12) received gemcitabine at a dosage of 12 $mg \cdot kg^{-1}$. This dosage in mice corresponds to the upper limit of the maximum tolerated dose of 4–6 $mg \cdot kg^{-1}$ determined in rats [6, 10]. A plan was determined to reduce the dose to 8 $mg \cdot kg^{-1}$ in the case of severe toxicity in this group. A third group (vehicle group) received spray administration of the 0.9% saline vehicle solution. Mice, acclimatised for 2 weeks before entering study protocols, were maintained under specific pathogen-free conditions, housed in sterilised filter-topped cages, and were fed autoclaved food and water *ad libitum*. Animals were handled and cared for in accordance with the guide for the care and use of laboratory animals (National Research Council 1996) and European directives EEC 86/809. Protocols were conducted under the supervision of an authorised investigator with the approval of the institutional ethics committee (CNRS, Orléans, France).

Intrabronchial tumour cell implantation

The intrabronchial tumour cell implantation procedure was derived from that described by McLemore and co-workers [8, 9]. Animals were anesthetised by an *i.p.* injection of 100 $mg \cdot kg^{-1}$ of ketamine and 15 $mg \cdot kg^{-1}$ of xelazine. A 0.5-cm ventral incision was made over the region of the trachea superior to the supraclavicular notch. The trachea was then exposed and punctured with the bevel of a 23-gauge needle. A 1.9 Fr × 50 cm catheter (Infusion Therapy Systems; Becton Dickinson, Sandy, UT, USA) was blunt-ended, inserted into the trachea and advanced into the right or left main bronchus. Prior to cell implantation, an anteroposterior chest radiograph was performed in order to monitor the position of the catheter using a high-resolution, low-energy radiography system (MX-20, Faxitron X-ray Corp., Wheeling, IL, USA).

Finally, 25 μL of the $\times 10^5$ tumour cells per 10 μL inoculum were injected into a caudal lobe.

Drug preparation and radioisotope labelling procedures

Clinical grade gemcitabine (Gemzar; Eli Lilly and Co. Inc., Indianapolis, IN, USA) was supplied in vials containing 200 mg of freeze-dried powder for injection. A ^{99m}Tc-labelled tin colloid solution was used as tracer of the aqueous phase of the spray formulation in order to verify and quantify pulmonary deposition, as previously described [6]. Gemcitabine solution for spray administration was obtained by reconstituting the freeze-dried powder in the ^{99m}Tc-labelled colloid, and dilution with 0.9% saline solution to obtain the two dosages in a constant volume of 50 μL. The vehicle solution for spray administration was composed of the ^{99m}Tc-labelled colloid solution diluted with 0.9% saline to a volume of 50 μL.

Endotracheal spray administration

Endotracheal spray administrations were performed using a IA-1C Microsprayer™ (Penn-Century, Inc., PA, USA) connected to a FMJ-250 high-pressure syringe (Penn-Century, Inc.) containing 50 μL of the radiolabelled solution. After *i.p.* anaesthesia with ketamine/xelazine, the tip of the microsprayer was introduced into the animal's trachea using an especially designed laryngoscope. Spray administrations were performed under an especially devised fume hood to avoid exposure to the operator.

Scintigraphic assessment of spray deposition

Immediately after spray administration, a 30-s ventral static image was acquired using a high-resolution planar gamma camera combining a 4-mm NaI thick crystal with a 5-inch diameter position sensitive Hamamatsu R3292 photomultiplier (Gamma imager, Biospace Mesures, France). Acquisitions were obtained using a 35-mm parallel-hole collimator with 1.3-mm diameter holes. Data were acquired using a 20% window centred on the 140-keV photopeak of ^{99m}Tc in a 128 × 128 matrix, and recorded on a dedicated computer system for digital display and analysis. For standardisation of scintigraphic determinations, a model of tissue ^{99m}Tc γ-ray absorption was performed, using a cylindrical phantom (with a diameter similar to that of the animal's thorax) filled with water mixed with the same dose of radiolabelled solution used for spray administration. The activity of four regions of interest corresponding to the whole animal, the total lung surface, and the right and left lungs respectively, were calculated from spray administration scintigraphic images. Right lung and left lung activities were compared. Total lung deposition was expressed as a percentage of the delivered dose and was then calculated as microgram equivalent of gemcitabine.

Protocol

In order to evaluate a preventive effect of regional chemotherapy on tumour development, spray administrations were planned to be initiated precociously after tumour cells implantation. Intrabronchial implantations were performed on day (d) 0. The protocol then comprised five spray administrations separated by 1-week intervals (d1, d8, d15, d22 and d29). Each animal's clinical state was evaluated throughout the study to detect any signs of toxicity. Each animal's weight was assessed before each spray administration. On d36, animals were sacrificed and heart–lung blocks were removed. Lungs were fixed by tracheal perfusion with 10% buffered formalin, placed in a container of the same solution for 48 h and processed for histological examination.

Histopathological study

After fixation, lungs were totally included in 1-mm horizontal sections. Two block samples were prepared from each mouse. Lung tissues were embedded in paraffin and sections of 5 μm thickness were stained with haematoxylin–eosin–saffran. Three sections per block were evaluated for the presence and dimensions of infiltrating carcinoma (largest diameter in mm determined with an ocular micrometer) and histopathological signs of toxicity related to chemotherapy.

All histopathological studies were performed by the same observer without knowledge of the treatment groups.

Statistical analysis

All values were summarised by descriptive statistics and expressed as mean±SEM. When multiple comparisons were made between groups, significant inter-group variability was established with the Kruskal-Wallis test. The Mann-Whitney U-test was used for comparisons between two groups. A p-value <0.05 was considered significant.

RESULTS

Tumour cell implantation and endotracheal spray administrations

Altogether, 24 intrabronchial tumour cell implantations were performed on d0. One death occurred during the procedure (procedure-related mortality=4.2%). As assessed by chest radiograph examinations, tumour cells were implanted in the right caudal lobe in 19 of 23 cases and in the left caudal lobe in four of 23 cases. In total, 23 animals started spray administrations on d1 (seven in the vehicle group, eight in group G8 and eight in group G12).

Three additional deaths occurred on d8, several hours after spray administration in group G12. Macroscopic examination of the animals' lungs showed signs of pulmonary oedema with no visible tumour in these three cases. Consequently, the dosage of gemcitabine was reduced from 12 to 8 $mg \cdot kg^{-1}$ for subsequent spray administrations in the five remaining animals of group G12; good safety procedures were observed throughout.

Scintigraphic imaging confirmed pulmonary deposition in 100% of spray administrations, with a homogeneous pattern of deposition (fig. 1). On average, right lung activity was 24% higher than left lung activity. Total lung deposition was 89±10% of the delivered dose. The corresponding dose of gemcitabine deposited in the lungs was estimated to be 85 equivalent μg for a spray administration of 8 $mg \cdot kg^{-1}$ and 125 equivalent μg for a spray administration of 12 $mg \cdot kg^{-1}$.

Altogether, 20 animals (83%) were alive and in a good clinical condition on d36 (seven in the vehicle group, eight in group G8 and five in group G12). None of the 13 animals treated with gemcitabine presented any signs of chemotherapy toxicity. Mean weight gain between d0 and d36 was 4.5±0.5 g in the vehicle group *versus* 3.8±0.2 g in group G8 and 3.5±1.4 g in group G12 (nonsignificant).

Histological examination of the lungs and tracheobronchial epithelium showed no signs of inflammation or fibrosis suggestive of chemotherapy toxicity in the 13 animals treated with gemcitabine.

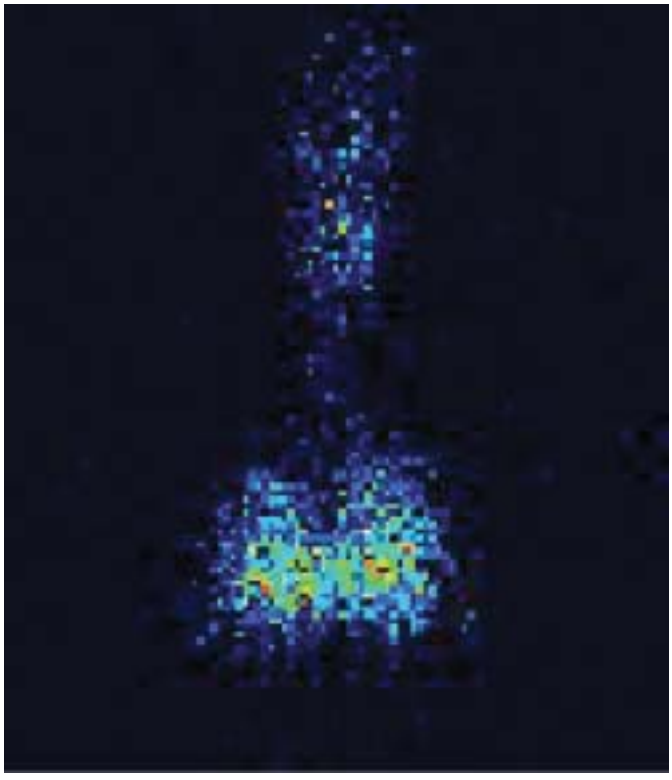

FIGURE 1. Ventral scintigraphic imaging after endotracheal spray administration showing a homogeneous pattern of pulmonary deposition.

Tumour growth

Histopathological studies were performed on the 20 animals that completed the whole protocol. In the vehicle group, seven of seven (100%) animals presented tumour development with a single, very large infiltrating carcinoma in a caudal lobe. Comparatively, four of 13 (31%) animals treated with gemcitabine showed no signs of tumour development and nine of 13 (69%) presented a smaller infiltrating carcinoma than in the vehicle group. In the animals in which infiltrating carcinoma was detected, the mean±SEM largest tumour diameter was 2.05±0.7 mm in animals treated with gemcitabine *versus* 5±0.3 mm in the vehicle group (p=0.008; table 1). Comparison between groups (fig. 2) showed a dose effect of regional chemotherapy, with a significant intergroup variability in tumour size (p=0.008). Mean largest tumour diameter was 2.9±0.8 mm in group G8 (p=0.042 *versus* control group)

TABLE 1 Comparison of tumour development in the vehicle group and in the animals treated with endotracheal spray of gemcitabine

	Vehicle	Gemcitabine
Group size	7	13
Presence of infiltrating cancer	7 (100)	9 (69)
Largest tumour diameter mm	5±0.3	2.05±0.7#

Data presented as n, n (%), or mean±SEM. #: p=0.008, gemcitabine *versus* vehicle.

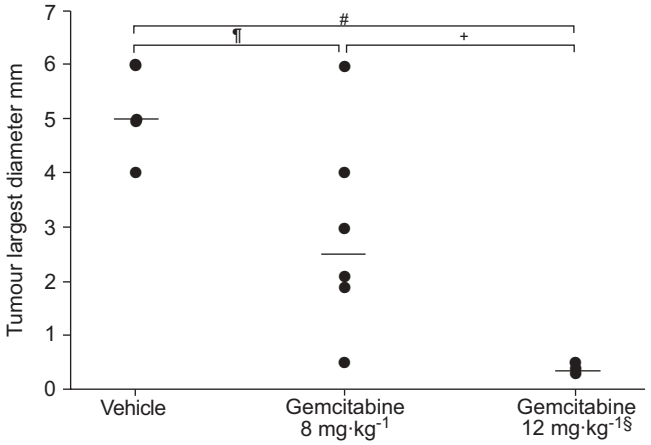

FIGURE 2. Median and individual values of largest tumour diameter in the various groups. #: p=0.014; ¶: p=0.042; +: p=0.02; §: animals received 12 mg·kg^{-1} of gemcitabine on day (d)1 and d8 followed by 8 mg·kg^{-1} on d15, d22 and d29.

and 0.3±0.03 mm in group G12 (p=0.014 *versus* control group and 0.02 *versus* group G8).

DISCUSSION

Few studies have documented the feasibility of delivering chemotherapy by inhalation. TATSUMURA *et al*. [2] demonstrated that 5-fluorouracil (5-FU) administered by inhalation accumulated in the trachea, bronchi and, interestingly, in the regional lymph nodes of patients treated 2 h before thoracic surgery for lung cancer. They also investigated the antitumour effect of 5-FU administered by inhalation in 10 selected patients with unresectable lung cancer, and obtained four partial responses and two complete responses without stomatitis or any other notable side-effects. HERSHEY *et al*. [3] treated dogs with advanced stages of primary lung cancer or lung metastases with paclitaxel or doxorubicin aerosols administered twice weekly. Tumour regression was achieved in 25% of dogs with measurable tumours, without the side-effects normally associated with systemic administration of these drugs and without pulmonary toxicity in the dogs treated with paclitaxel. Recently, a phase-I study demonstrated the feasibility and safety of aerosol administration of 9-nitrocamptothecin in a liposomal formulation in 25 patients with primary or metastatic lung cancer [4]. Partial remissions and stabilisations were observed in two and three patients, respectively.

Gemcitabine (difluorodeoxycytidine) is a chemotherapy molecule belonging to the nucleoside analogue family. It has been demonstrated to be effective in the treatment of nonsmall cell lung cancer both as monotherapy and in combination with other drugs [11]. It is a pro-drug, which is inactive in the extracellular compartment. It only becomes cytotoxic after reaching a nucleated cell in which it undergoes several phosphorylations [12]. The gemcitabine formulation does not contain any chemical ingredients incompatible with aerosol delivery. These advantages, combined with the absence of irritant properties, make gemcitabine an attractive candidate for local administration.

In a recent study, the current authors demonstrated that gemcitabine could be administered by aerosol in rats at a maximum tolerated dose of 4–6 mg·kg^{-1} [6]. At this dosage, administered once a week for nine consecutive weeks, no chemotherapy-related deaths and no clinical or histological signs of toxicity were observed. This study also demonstrated the safety and efficacy of endotracheal spray as an aerosol delivery procedure in rodents. Endotracheal administration bypasses the upper respiratory tract, depositing the spray directly into the lower respiratory tract. Deposition in the nasal passages is avoided, facilitating intrapulmonary delivery of a coarse aerosol with droplet size (mean mass diameter 18±3 μm) that would not enter the lungs with a conventional aerosol delivery device. The endotracheal sprayer is also a small, closed device, facilitating its use for the study of potentially toxic and/or radioactive substances.

In vivo testing of regional chemotherapy requires the growth of implanted tumours in a localised intrapulmonary environment. Several orthotopic models have been developed to study human lung cancer. Various techniques have been used to introduce tumour cells, including intrathoracic, intrapleural and intrabronchial implantations [8, 9, 13]. More extensive tumour growth was observed than with *s.c.* xenograph models. The histological characteristics of orthotopic tumour models were found to be consistent with the clinical tumour from which the cell lines were derived. Furthermore, when cells were implanted intrabronchially, tumours predominantly grew in the lung parenchyma in contrast with intrathoracically implanted tumours that were frequently located in the chest wall or pleural space. In view of these potential advantages, the present authors considered that the intrabronchial orthotopic model could be a relevant model for *in vivo* testing of aerosol chemotherapy. Using the intrabronchial propagation method described by MCLEMORE *et al.* [8, 9], the current authors developed an orthotopic model of large cell undifferentiated carcinoma in nude mice. The intrabronchial implantation procedure was safe with a low procedure-related mortality (4.2%). Radiographic procedures were used to optimise intrabronchial cell implantation. Tumour cells were implanted in the right caudal lobe in 83% cases. Implantation in the right lung is preferable to facilitate subsequent radiographical imaging of tumour development, as no other major anatomical structures (*e.g.* the heart) are located in this area [8]. All animals in the vehicle group presented tumour development 36 days after intrabronchial implantation with a single, large infiltrating carcinoma in a caudal lobe. Tumour sizes were homogeneous, with the largest diameters ranging 4–6 mm.

This is belived to be the first *in vivo* investigation of the antitumour effect of gemcitabine aerosol on orthotopic primary lung cancer. The ^{99m}Tc-labelling of gemcitabine formulation allowed verification and quantification with scintigraphic imaging of actual pulmonary deposition after each endotracheal spray delivery. Pulmonary deposition was confirmed in 100% of administrations with a homogeneous pattern and a slight superiority of right-to-left lung deposition. On average, 89% of the delivered dose was actually deposited in the lungs. An effect of regional chemotherapy was observed on tumour growth with prevention of tumour development in 31% cases and significant inhibition of tumour growth in the remaining cases. They also observed a dose effect of regional chemotherapy, with more marked tumour growth inhibition in animals that received 12 mg·kg^{-1} of gemcitabine on d1 and d8 than in animals treated

with 8 mg·kg^{-1} from d1 to d29. At the dosage of 8 mg·kg^{-1}, aerosolised gemcitabine was well tolerated with no clinical and histological signs of toxicity. Three cases of acute fatal pulmonary oedema were observed a few hours after endotracheal spray delivery of gemcitabine at the highest dosage, prompting a dose reduction for subsequent administrations. This acute toxicity was not observed in the control group, in the group G8 and in the remaining animals of the group G12 subsequently treated with 8 mg·kg^{-1}. Thus, an acute toxicity of aerosolised gemcitabine was suspected in these three animals and compared with that described in isolated cases in the literature after systemic infusion of gemcitabine [14, 15]. However, the current study was not designed to compare the pulmonary safety profile of gemcitabine *via* aerosol and *via* systemic administration. In a previous study from the same group in rats [6], no signs of pulmonary toxicity were observed on histological examination after nine weekly pulmonary administrations at 4 mg·kg^{-1}, corresponding to a lung gemcitabine level almost 50-times that previously estimated in rats after *i.v.* administration of 10 mg·kg^{-1} [16].

The results presented here support the potential value of aerosol delivery of chemotherapy. They are in agreement with those recently published by KOSHKINA *et al*. [17], who demonstrated that gemcitabine administered *via* aerosol inhibits the growth of lung metastasis in two osteosarcoma lung metastasis animal models. Aerosolised gemcitabine was also effective against primary tumour in this study. Conversely, intraperitoneal gemcitabine administration at similar dosage had no effect on lung metastasis. WATTENBERG *et al*. [18] also recently observed chemoprevention of upper respiratory tract cancer in the Syrian golden hamster model by aerosol administration of 5-fluorouracil, a chemotherapy molecule also belonging to the nucleoside analogue family.

In conclusion, a safe and reproducible animal model of aerosol delivery of chemotherapy in primary lung cancer has been developed. Preliminary results demonstrated inhibition of intrabronchial orthotopic tumour growth by aerosol delivery of gemcitabine. Further studies are required to determine the optimal dosage and frequency of aerosol administrations and to assess the antitumour effect of aerosolised chemotherapy on established tumours. Validation of noninvasive strategies is currently under progress to allow accurate periodic monitoring of orthotopic tumour development.

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