

Anticancer Activity of a Lectin-rich Mistletoe Extract Injected Intratumorally into Human Pancreatic Cancer Xenografts

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Abstract. *Background:* In single case observations, tumour remissions after intratumoral injections of mistletoe extracts have been described. *Materials and Methods:* We investigated the antitumour activity of intratumorally (*i.t.*)-injected lectin-rich mistletoe extract at different dosages and *i.t.*-injected mistletoe lectin I in comparison to intravenous (*i.v.*) Gemcitabine and *i.t.* treatment with placebo in a human pancreatic cancer xenograft. *Results:* In a preliminary dose-response experiment, the most marked tumour inhibition was induced when mistletoe extract was given at 8 mg/kg body weight (BW) and mistletoe lectin I at 5.3 µg/kg BW. In a second experiment, bi-weekly *i.t.* injections of mistletoe extract over 8 weeks resulted in a very high antitumour activity with an optimal T/C value (=median relative tumour volume of the test group vs. the control) of 0.4% combined with 3/8 partial and 3/8 complete remissions. Gemcitabine was less active with 2/8 partial and 1/8 complete remissions and an optimal T/C of 4.6%. *Conclusion:* *I.t.*-injected lectin-rich mistletoe extract should be further evaluated in patients with inoperable locally advanced pancreatic cancer.

Pancreatic cancer is the cancer with the worst prognosis of all gastrointestinal malignancies, with approximately 28,200 deaths per year in the United States and 50,000 deaths per year in Europe. Because of local infiltration and early generalization, only 5 – 28% of all pancreatic carcinomas

are surgically resectable. In addition, local recurrence is observed in up to 86% of patients who undergo surgery. The median survival of patients with inoperable pancreatic cancer is about six months, ranging from three to fourteen months depending on performance status and extent of the disease (1).

Gemcitabine has been established as the standard chemotherapeutic treatment for advanced pancreatic cancer. Though it improves the quality of life in cancer patients, its effects on tumour remission and survival are only marginal (2). Many ongoing randomized trials are investigating whether combination therapies with Gemcitabine together with other chemotherapeutic agents can improve the clinical response and overall survival of pancreatic cancer patients (3). However, to date, the therapeutic options for patients with progressive pancreatic cancer remain disappointing.

Mistletoe extracts have been used in the complementary treatment of cancer patients for more than 80 years. Many preclinical and clinical investigations have shown immunomodulatory effects of mistletoe extracts applied subcutaneously (*s.c.*) (4). Two ingredients of mistletoe extracts – viscotoxins and especially mistletoe lectins – are known cytotoxic agents. Mistletoe lectins are 50 to 63 kDa glycoproteins, which belong to a group of type II ribosome-inactivating proteins. The lectin B subunit mediates cellular uptake of the disulfide-bonded hololectin by endocytosis. The cytotoxic A subunit enzymatically inhibits protein biosynthesis at ribosomal RNA. Depending on the concentration of the mistletoe lectins, this results in necrosis or in apoptosis (5-7).

Several published single cases describe tumour remission of gastrointestinal cancers by intratumoral (*i.t.*) injections of mistletoe extracts. For example, an inoperable recurrence of duodenal cancer, measuring 8 x 7 x 4 cm with contact to the aorta abdominalis and V. cava, was treated by *i.t.* injections of

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the lectin-rich mistletoe extract ABNOBAviscum Quercus® in high concentrations, followed by *s.c.* injections. After eight months of treatment, complete remission was diagnosed and reconfirmed by CT-scan six and twelve months later (8).

In previous investigations in our laboratory, we tested the antitumoral activity of this mistletoe extract on different human cancer xenografts. At doses of 4 and 8 mg/kg BW injected *i.t.*, a high antitumoral activity was seen in both a small cell lung cancer (LXFS 538) and a breast cancer model (MAXF 449). A transient no change was observed in the melanoma MEXF 695 cancer model (9).

In the present study, we tested the antitumoral activity of mistletoe extract in pancreatic cancer xenografts developed from patient tumours. We used ABNOBAviscum Fraxini-2®, because this extract has the highest known content of mistletoe lectin (10). We wanted to investigate whether complete remission in pancreatic cancer xenografts can be achieved by *i.t.* injections of this lectin-rich mistletoe extract and to compare the effect with that of Gemcitabine intravenously (*i.v.*), the current standard treatment for pancreatic cancer.

Materials and Methods

Tumours. The human pancreatic carcinoma PAXF 736, established in our laboratory through serial passage in nude mice, was used as the donor tumour. PAXF 736 is a poorly-differentiated pancreatic adenocarcinoma, derived from a 65-year-old patient.

Nude mice. Athymic nude mice (NMRI nu/nu) of Oncotests breeding colony were used in the experiments. The animals were maintained under specific pathogen-free conditions and had free access to food and acidified water. The drinking water was supplemented with potassium sorbate to prevent bacterial or fungal contamination. In the first study, mice received tumour implants subcutaneously (*s.c.*) in both flanks. In the second study, mice received the tumour implants in one flank only. Treatment began after randomisation, when the diameter of the tumour implants measured approximately 8 mm and weighed 200-400 mg.

The experiments were performed under the project licence number G-97/30 following German Animal Licence regulations, which closely adhere to the recently published United Kingdom Coordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia (11).

Drugs. The mistletoe extract ABNOBAviscum Fraxini-2® was provided by ABNOBA Heilmittel GmbH, Pforzheim, Germany. A 1 ml ampoule contained 15 mg mistletoe extract. The mistletoe lectin (ML) content was 9.9 µg lectin/ml; 90 – 95 % of which was in the form of mistletoe lectin I (ML-I). The Viscotoxin content was 96.6 µg/ml.

Mistletoe extract (ME) was administered in dosages of 4 mg/kg, 8 mg/kg (5.3 µg ML/kg) and 16 mg/kg body weight (BW). In relation to tumour volume, these dosages were similar to those used in the human duodenal cancer case study described in the introduction (8). Isolated mistletoe lectin I was given at a dosage equivalent to the ML content in ME at 8 mg/kg BW, *i.e.* 5.3 µg ML-I /kg BW.

Table I. *Experimental design of study 1.*

| Treatment | Dosage (/kg BW) | Schedule | Route |
|-----------------|-----------------|-------------------------|-------------|
| ME | 4 mg* | 2 x weekly x 4 weeks | <i>i.t.</i> |
| ME | 8 mg* | 2 x weekly x 4 weeks | <i>i.t.</i> |
| ME | 16 mg* | 2 x weekly x 4 weeks | <i>i.t.</i> |
| ML-I | 5.3 Bg* | 2 x weekly x 4 weeks | <i>i.t.</i> |
| Vehicle control | * | 2 x weekly x 4 weeks | <i>i.t.</i> |

ME, Mistletoe Extract; ML-I, Mistletoe Lectin I.
*(dissolved in) inert ascorbate-phosphate buffer.
Volume of each *i.t.* injection was 100 µl.

Table II. *Experimental design of study 2.*

| Treatment | Dosage (/kg BW) | Schedule | Route |
|-----------------|-----------------|-------------------------|-------------|
| ME | 8 mg* | 2 x weekly x 8 weeks | <i>i.t.</i> |
| Gemcitabine | 300 mg | days 1, 8, 15 | <i>i.v.</i> |
| Vehicle control | * | 2 x weekly x 8 weeks | <i>i.t.</i> |

ME, Mistletoe Extract; ML-I, Mistletoe Lectin I.
*(dissolved in) inert ascorbate-phosphate buffer.
Volume of each *i.t.* injection was 100 µl.

Gemcitabine was given *i.v.* at a dosage of 300 mg/kg BW on days 1, 8 and 15, which corresponds to the maximum tolerated dose (MTD) as determined in previous experiments in our laboratory (12).

Control mice received the solvent of ME and ML-I (isotonic, isohydric ascorbate-phosphate buffer) in the same volume following the same schedule as the test groups.

Experimental design. Study 1: In the first study, five treatment groups of eight nude mice (n=40) were used. Tumours (pancreatic adenocarcinoma PAXF 736) were implanted *s.c.* in both flanks of each animal. The tumour on the right side was selected for *i.t.* injections, whereas that on the left side served for evaluation of systemic effects. The experimental design is illustrated in Table I.

Study 2: In the second study, three treatment groups of eight nude mice (n=24) were used. Tumours (pancreatic adenocarcinoma PAXF 736) were implanted only in one flank of the nude mice. (This allowed a longer observation period). The experimental design is illustrated in Table II.

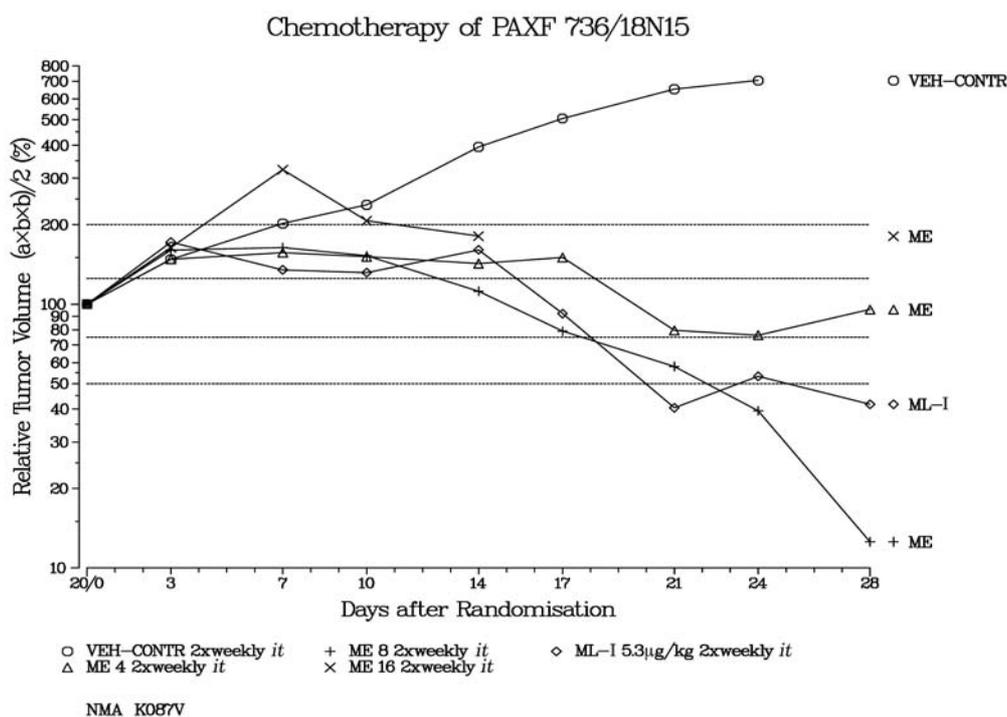


Figure 1. Chemotherapy of human pancreatic carcinoma PAXF736 with Mistletoe extract and Mistletoe lectin I.

In both studies tumour growth was assessed twice weekly by caliper measurements in two dimensions. When the tumours had reached a median diameter of approximately 8 mm, mice were randomised into different treatment groups and therapy was started.

Evaluation. Tumour volume was calculated using the formula $0.5 \times \text{length} \times \text{width}^2$. Relative tumour volume (RTV) was calculated for each individual tumour by dividing the tumour volume at day X (a specific day after the start of treatment) by the tumour volume at day 0 (start of treatment) multiplied by 100 %.

$$\text{RTV} = \frac{T_x (\text{tumour volume at day X})}{T_0 (\text{tumour volume at day 0})} \times 100\%$$

Tumour response was defined as follows:

| | |
|--------------------------|------------------|
| complete remission (CR): | RTV \leq 10% |
| partial remission (PR): | 10% < RTV < 50% |
| minor remission (MR): | 50% < RTV < 75% |
| no change (NC): | 75% < RTV < 125% |
| progression (P): | RTV > 125% |

Optimal tumour growth inhibition (T/C%) was calculated from the ratio of median RTV values of treated (T) versus control (C) groups:

$$\text{T/C \%} = \frac{\text{RTV treatment group}}{\text{RTV control group}} \times 100\%$$

Activity rating: A T/C value of >50% means that the test drug is inactive (-). A T/C value of <50% is defined as the standard criterion for antitumour activity:

| | | |
|------|-----------------------|--------------------------------|
| + | (tumour inhibition): | 25% < T/C < 50% |
| ++ | (tumour stasis): | T/C < 25% and 75% < RTV < 125% |
| +++ | (partial regression): | T/C < 25% and 10% < RTV < 75% |
| ++++ | (complete remission): | T/C < 25% and RTV < 10% (13) |

Toxicity: The definition of maximum tolerated dosage (MTD) allows a body weight loss of less than 20 % and a lethality of less than 25 % for treatment duration of <3 weeks. (12)

Pathological investigations. At the end of the second study, animals were investigated histopathologically and tumour sites were tested for viable tumour cells, necrosis and inflammatory reactions in H.E.-stained sections. The proliferation of viable tumour cells was defined as the percentage of Ki-67-positive cells (for details see Table V).

Results

In the first study, *i.t.*-injected mistletoe extract (ME) demonstrated high *in vivo* activity against pancreatic cancer xenografts in the two lower dosages tested (see Figure 1 and Table III). At 4 mg/kg BW ME, an optimal T/C value of 10.8% at day 24 and one partial remission was achieved. With *i.t.* application of 8 mg/kg BW ME, the optimal T/C value was 1.8% at day 28; one partial remission (PR) and one complete remission (CR) were obtained. The *i.t.* application of 16 mg/kg BW ME resulted in a body weight

Table III. Study 1: Antitumour activity against pancreatic adeno-Ca-xenograft PAXF 736/22N19 (short treatment period: 28 days).

| Groups*1 (n=8) | Dosage (/kg BW) | Schedule | Route | Toxicity | | Antitumour activity | | | |
|-------------------|--------------------|------------|--------------|----------------------------|-----------------------------|---------------------|--------------|----------------------|--------------------|
| | | | | Lethality n=(until day) | Body weight loss % (day) | Optimal T/C (%) | T/C (day) | Clinical response | Activity rating |
| Mistletoe extract | 4 mg | 2 x weekly | <i>i.t.</i> | 0/8 | -2.9 (28) | 0 | 24 | 1 PR | + + |
| | | | <i>syst.</i> | | | 0 | 7 | - | - |
| Mistletoe extract | 8 mg | 2 x weekly | <i>i.t.</i> | 1/8 (14) | ± 0 (28) | 1 | 28 | 1 CR, 2 PR | + + + |
| | | | <i>syst.</i> | | | 74 | 7 | - | - |
| Mistletoe extract | 16 mg | 2 x weekly | <i>i.t.</i> | 3/8 (14)*2 | -21.5 (14)*2 | 45.8* | 14 | - | + |
| | | | <i>syst.</i> | | | 58 | 7 | - | - |
| Mistletoe lectin | 5.3 Bg*3 | 2 x weekly | <i>i.t.</i> | 1/8 (20) | -6 (28) | 5 | 28 | 3 PR | + + + |
| | | | <i>syst.</i> | | | 54 | 14 | - | - |
| Vehicle control | | 2 x weekly | <i>i.t.</i> | 1/8 (11) | -13.6 (21) | | | - | - |

*1each group consisted of 8 mice bearing bilateral tumours on each flank; *2Discontinued on day 14 due to toxicity;

*3 ~ 8 mg/kg BW mistletoe extract.

BW, body weight; *i.t.*, intratumoral; *syst.*, systemic; T/C, relative tumour volume Test / Control; CR, complete remission; PR, partial remission.

Activity Rating: + (tumour inhibition) 25% < T/C < 50%;

+ + (tumour stasis): T/C < 25% and 75% < RTV < 125%

+ + + (partial regression): T/C < 25% and 10% < RTV < 75%

+ + + + (complete remission): T/C < 25% and RTV < 10%

Table IV. Study 2: Antitumour activity against pancreatic adeno-Ca-xenograft PAXF 736/22N19 (long treatment period: 57 days).

| Groups*1 (n=8) | Dosage (mg/kg/ injection) | Schedule | Route | Toxicity | | Antitumour activity | | | |
|-------------------|---------------------------------|----------------------|-------------|--------------------------|-----------------------------|---------------------|--------------|----------------------|--------------------|
| | | | | Lethality (until day) | Body weight loss % (day) | Optimal T/C (%) | T/C (day) | Clinical response | Activity rating |
| Gemcitabine | 300 | days 1, 8, 15 | <i>i.v.</i> | 3/8 (4,8,21) | gain | 4.6 | (32) | 1 CR, 2 PR | + + + |
| Mistletoe Extract | 8 | 2 x weekly x 8 weeks | <i>i.t.</i> | 2/8 (55,55) | -6.2% | 0.4% | (57) | 3 CR, 3 PR | + + + + |
| Vehicle control | | 2 x weekly x 8 weeks | <i>i.t.</i> | 1/8 (22) | -3.6% | | | | |

i.t., intratumoral; *i.v.*, intravenous; T/C, relative tumour volume Test / Control; CR, complete remission; PR, partial remission.

Activity Rating: + (tumour inhibition) 25% < T/C < 50%;

+ + (tumour stasis): T/C < 25% and 75% < RTV < 125% ;

+ + + (partial regression): T/C < 25% and 10% < RTV < 75%

+ + + + (complete remission): T/C < 25% and RTV < 10%

loss of 21.5% at day 14 and three out of eight animals died. The treatment was discontinued due to toxicity. Isolated mistletoe lectin I (ML-I), given at a dose equivalent to the ML-I content of 8 mg ME (5.3 µg/kg BW ML-I), resulted in an optimal T/C value of 5.9% at day 28 with 3/8 partial remissions (PR).

In all *i.t.* treatment groups, the specific growth delay (SGD) could not be determined because the median tumour volume of 200% or 400% was not reached within the observation period of 28 days. No antitumoral activity of ME and ML-I against the untreated tumour on the opposite side was observed.

In the second study, the antitumoral activity of *i.t.*-injected ME at a dosage of 8 mg/kg BW was tested against

placebo and also against *i.v.*-injected Gemcitabine at a dosage of 300 mg/kg BW on days 1, 8 and 15 (see Figure 2 and Table IV). Gemcitabine exhibited high activity against the tested pancreatic cancer xenograft, with an optimal T/C value of 4.6% on day 32 accompanied by one CR and two PRs. *I.t.*-injected ME showed a very high antitumoral activity superior to that of Gemcitabine. The optimal T/C value was 0.4% on day 57 with three PRs and three CRs. The SGD of Gemcitabine was 38, whereas that of *i.t.*-injected ME could not be determined because a double tumour volume was not obtained in this therapy group.

The *i.t.* injection of ME resulted in inflammation and necrosis in the first phase of treatment (results of study 1; data not shown). The pathohistological investigation of the

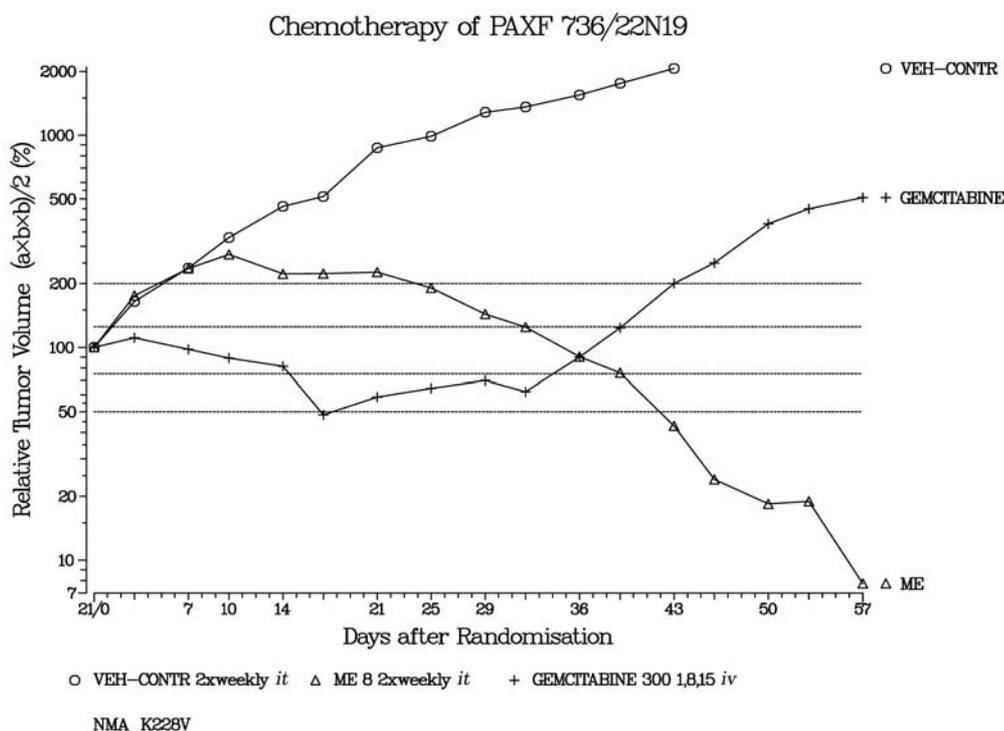


Figure 2. Chemotherapy of human pancreatic carcinoma PAXF736 with Mistletoe extract or Gemcitabine.

long-term effects after 8 weeks of treatment are shown in Table V: the inflammatory reaction and necrosis regressed and in four out of five investigated animals no viable tumour cells were found, as confirmed by evaluating the proliferation marker Ki-67.

Discussion

In both experiments, *i.t.*-injected ME showed antitumoral activity. We obtained the best results with a dosage of 8 mg/kg BW. Four mg/kg BW were less effective and 16 mg/kg BW were toxic. Earlier investigations in both a breast cancer and a NSCLC xenograft yielded similar results, also with an optimal dosage of 8 mg/kg BW (9).

The antitumoral activity of *i.t.*-injected ML-I was nearly comparable with that of the whole plant extract. This suggests that ML is essential for the observed cytotoxic effects. Intravesically applied ME (14) and recombinant ML-I (15) were also comparably effective in the local treatment in different murine and rat urinary bladder cancer models. Recombinant ML-I is currently undergoing clinical investigation in several clinical trials.

In contrast to the high local antitumoral activity observed, there was no systemic antitumoral effect on the untreated tumour located on the opposite flank of the nude mice. When interpreting these results, one should consider that

the nude mice used in this study were immunodeficient and did not have an intact T-cell system. Given systemically (usually by *s.c.* injection), the potential antitumoral effects of ME are probably mediated by the immune system (16, 17). This animal model is, therefore, not appropriate for investigating the systemic effects of ME.

Because the tumour on the opposite side remained unaffected by treatment and reached a maximum volume, we had to stop the first experiment after only 28 days, in accordance with the German Animal Licence regulations. To enable a longer treatment and observation time in the second study, pancreatic adenocarcinoma was implanted in only one flank of each animal. The most effective dosage determined in the first study (8 mg/kg BW) was tested against placebo and *i.v.*-injected Gemcitabine, the current standard treatment for advanced pancreatic cancer. Gemcitabine showed a good antitumoral activity with an optimal T/C value of 4.6% at day 32 and one complete and two partial remissions. We had to discontinue Gemcitabine treatment after three infusions at weekly intervals because the MTD had been reached by day 21. After day 36, the tumour volume increased again.

As shown in Figure 2, the effect of the *i.t.*-injected ME was completely different from that of Gemcitabine: in the first 7 to 10 days of treatment, the tumour volume increased. Taken together with histopathological investigations, this was obviously caused by the inflammatory reaction of tumour

Table V. Study 2: Histopathological investigations.

| Group | Random-No. | Tumour (H.E.-stained) | Proliferation (Ki-67) | Necrosis (H.E.-stained) | Inflammation |
|-------------------------------|-------------------------|--------------------------|--------------------------|----------------------------|--------------|
| Control | 3749 | ++ | 20-40% | 0 | ++ |
| | 3758 | +++ | 30% | ++ | +++ |
| | 3759 | +++ | 60% | ++ | ++ |
| | 3761 | +++ | 40% | ++ | +++ |
| | 3762 | +++ | 250% | +++ | +++ |
| | 3765 | +++ | 40% | +++ | +++ |
| | 3768 | +++ | 50% | ++ | ++ |
| | Gemcitabine <i>i.v.</i> | 3742 | + | 10% | + |
| 3747 | | + | 30% | + | ++ |
| 3763 | | 0 | 0 | 0 | + |
| 3767 | | 0 | 0 | 0 | + |
| 3774 | | ++ | 20% | + | ++ |
| Mistletoe Extract <i>i.t.</i> | | 3743 | 0 | 0 | 0 |
| | 3751 | 0 | 0 | + | ++ |
| | 3769 | + | 0 | ++ | ++ |
| | 3771 | 0 | 0 | + | + |
| | 3776 | 0 | 0 | 0 | + |

Tumour (H.E.-stained): 0 = no viable tumour cells, + = small, ++ = medium, +++ = large tumour.

Proliferation (Ki-67): estimated percentage of Ki-67-positive cells in proliferation.

Necrosis (H.E.-stained): 0 = absent, + = small or questionable necrosis, ++ = obvious, moderately large necrosis, +++ = obvious large necrosis.

Inflammatory reaction: 0 = none, + = slight, ++ = moderate, +++ = strong.

tissue to the *i.t.*-injected ME. Prior to day 21, the median tumour volume remained constant, then, with continued bi-weekly injections, decreased progressively until day 57 with an optimal T/C value of 0.4% and three partial and three complete remissions. The histopathological investigation confirmed these results: no viable tumour cells were detected in preparations from animals that attained complete remission.

In addition to the patient with a local recurrence of duodenal cancer described above, there are additional cases of remission of gastrointestinal cancers treated with *i.t.*-injected ME which lend support to the clinical relevance of this treatment. Repeated endoscopic applications of an ME rich in ML-III resulted in remission of a cardia carcinoma (18). In patients with inoperable hepatocellular carcinoma and in those with liver metastases of colorectal cancer, tumour remission was reported subsequent to *i.t.* injections of another lectin-rich ME (19).

The results of our preclinical investigation demonstrate that intratumoural injections of a lectin-rich ME can effect complete remissions in a pancreatic cancer xenograft. The standard treatment Gemcitabine was less effective than *i.t.*-injected ME. Thus the effects of *i.t.*-injected lectin-rich ME should be clinically investigated in patients with inoperable locally advanced pancreatic cancer.

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