

Effects of triple therapy with octreotide, galanin and serotonin on a human colon cancer cell line implanted in mice: comparison between different routes of administration

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Summary. A human colon cancer cell line was implanted subcutaneously in nude mice. After 7 days, the animals were divided into four groups. The first group received an intraperitoneal (i.p.) continuous infusion by an osmotic pump, the second was given i.p. bolus injections, the third received continuous subcutaneous (s.c.) infusion by an osmotic pump and the fourth group was given bolus s.c. injections. Each group was divided into 2 subgroups. The first subgroup received triple treatment with octreotide, galanin, and serotonin, 40 µg/kg body weight/day of each. The second subgroup was given sterile saline solution. Treatment lasted for 14 days. The volume and wet weight of the tumours in all treated groups tended to decrease, but was statistically significant only in the group with continuous i.p. infusion. The number of viable cells tended to decrease in all the treated groups, but was not statistically significant. Proliferation index was significantly reduced in mice given triple therapy i.p. as bolus injection and as continuous infusion, as compared with their respective controls. The apoptotic index increased significantly in mice receiving triple therapy as continuous i.p. infusion as revealed by both the TUNEL method and by poly (ADP-ribose) polymerase (PARP) expression. The number of tumour blood vessels was significantly reduced in the mice given triple therapy as continuous i.p. infusion, as compared with controls. There was no statistical difference between animals treated by different routes, regarding proliferation or apoptosis of the cancer cells, or the number or mean luminal area of tumour blood vessels. The present investigation showed that regardless of the route of administration, triple therapy with octreotide, galanin and serotonin generally reduced the volumes, weights, viable cells, vascularization and

proliferation of the tumours, as well as inducing apoptosis. Continuous i.p. infusion appears, however, to be the most effective route of administration.

Key words: Cancer, Colon, Galanin, Octreotide, Serotonin

Introduction

Colorectal cancer (CRC) is the second most common cancer form in non-smokers in the Western world (after breast cancer in women and prostate cancer in men), representing about 12% of all cancer cases (Berger et al., 1973; Valone et al., 1987; Midgely and Kerr, 1999). Surgery is the primary and still the most effective treatment option for patients with CRC. However, about half of these patients either have unresectable metastases at presentation or develop them after resection of the primary tumour (Simmonds, 2002). The estimated 5-year survival rate of these patients is 5% (Primrose, 2002). Chemotherapy, even with new agents such as irinotecan and oxaliplatin, has a poor response rate and a large proportion of patients with liver metastasis are beyond the scope of liver resection (Primrose, 2002).

Somatostatin analogues have been found to induce apoptosis and to have anti-proliferative effects on colon carcinoma (Alonos et al., 1992; Quin et al., 1992; Melen-Mucha et al., 2000). Recently, it has been shown that triple therapy with octreotide (a somatostatin analogue), galanin and serotonin increased necrosis and apoptosis of rat colon cancer cells (El-Salhy and Sitohy, 2002; El-Salhy et al., 2003; Sitohy and El-Salhy, 2003). The increased necrosis appeared to be caused by reducing the number of and constricting of the tumour-feeding blood vessels (El-Salhy and Sitohy, 2002; El-Salhy et al., 2003; Sitohy and El-Salhy, 2003). This treatment has also been found to have the same effects and in addition reduced proliferation in human cancer

(El-Salhy and Starefeldt, 2003; El-Salhy, 2004; El-Salhy and Dennerqvist, 2004). It would appear that this treatment is without any apparent side effects (El-Salhy and Sitohy, 2002; Sitohy and El-Salhy, 2003). Whereas the treatment reduced the proliferation rate in human colon cancer cells (El-Salhy and Starefeldt, 2003; El-Salhy 2004; El-Salhy and Dennerqvist, 2004), proliferation was unaffected in rat colon cancer (El-Salhy and Sitohy, 2002; El-Salhy et al., 2003; Sitohy and El-Salhy, 2003). Triple therapy in studies made on rat colon cancer was given in the form of subcutaneous bolus injections, whereas those made on human colon cancer used continuous intraperitoneal (i.p.) infusion. Could the route of administration be of importance regarding the effects of these substances? On the other hand can observed differences depend upon species differences? The present investigation was undertaken to elucidate the impact of the route of administration of these substances on a human colon cancer.

Material and methods

Cell line and culture conditions

The cell line studied was a human colon cancer (SW 620) purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The medium used was Leibovitz's-L15 supplemented with 10% (v/v) foetal bovine serum (ATCC). The cells were kept at 37 °C in a humidified 5% CO₂ environment. Cell sub-culturing was done 1:2 or 1:4 every fourth day, using trypsin-EDTA detachment.

Animals and tumour implantation

Female 10-week-old nude mice (C57BL/6JBom-nu, Taconic M& B A/S, Bomholtvej, Denmark) with a body weight of 20.6±2.4 g (mean±SD) were used. Four mice were kept in each cage in a laminar-filtered airflow cabinet under pathogen-free conditions. They were fed on a standard pellet diet (R 34, Lactamin, Vadstena, Stockholm) and had free access to water. The animals were kept at a constant temperature (22±2 °C), relative humidity (55±5%) with a 12/12h dark/light cycle. All experiments were performed using aseptic techniques under laminar airflow. The animals were inspected twice daily and any sign of pain or discomfort was recorded. The local committee on animal ethics approved the investigation.

Eighty-four mice were injected subcutaneously (s.c.) in the right flank with a 100 µl of a suspension containing 2.5x10⁶ viable cells. After 7 days, 72 animals had developed a palpable tumour. These 72 were divided into four groups of 18. Group 1 (i.p. continuous infusion) was divided into two equal subgroups, the first (control) subgroup received intraperitoneal infusion with 100 ml saline solution (0.25 µl/h), and the second with 100µl saline solution containing 40 µg/kg body weight/day octreotide (Sandostatin®, Novartis), galanin

(synthetic human galanin, Sigma) and serotonin (5-hydroxytryptamine, oxalate salt, Sigma). Infusion continued for 14 days via an i.p. implanted -ALZAT osmotic pump (type 1002, B&K Universal AB, Sollentuna, Sweden). Group 2 (i.p. bolus injection) was also subdivided into two subgroups: controls injected with saline solution and a subgroup injected with the previously mentioned bioactive substances in the same dosage as in group 1. In this group the saline solution and bioactive substances were administered as a bolus injection i.p. once daily for 14 days. Group 3 (continuous s.c. infusion) was subdivided into a control subgroup given saline solution and a treated subgroup given the same dose of bioactive substances as group 1. The saline and bioactive substances were administered s.c. by implanting an ALZET osmotic pump in the left flank. Group 4 (bolus s.c. injection): the control subgroup was injected with 100 µl saline solution in the left flank, and the second subgroup had the same dose of bioactive substances as group 2.

In order to implant the osmotic pump i.p. and s.c., the animals were anaesthetized with a mixture of 1 ml midazolam (Dormicum®, Roche, Switzerland), 1 ml fentanyl/fluanson (Hypnorm®, Janssen, The Netherlands) and 2 ml distilled water. Each mouse was injected i.p with 0.3 ml of this mixture. For i.p. implantation, the pump was placed inside the abdominal cavity through a median abdominal incision. For s.c. implantation, a minimal incision was made in the left flank and the pump was placed directly beneath the skin. The abdominal wall and skin were closed with sutures and the animals were allowed to recover.

At the end of the experiments, the animals were weighed and then killed by decapitation. The animals were dissected and the abdomen and thorax were exposed. They were then inspected for possible occurrence of metastasis. Each tumour was carefully excised and the greatest and least diameters and the tumour weight were measured. The volume of the tumours was calculated with the use of the following formula:

$$\text{Volume} = (\text{mean diameter})^3 \times \pi/6$$

The tumours were fixed overnight in 4% buffered paraformaldehyde, embedded in paraffin and cut at 5 µm. The slides were then coded so that the performer of the subsequent quantification was not aware of the identity of the sections.

MTT assay

A part of the tumours was cut into as a small piece as possible and put in sterile tubes containing 5 ml of Leibovitz's-L15 medium with 10% fetal bovine serum containing 6 mg trypsin, 2 mg DNA and 25 mg collagenase type 1. The tubes were incubated in a water bath at 37 °C under continuous stirring for 60 min. After filtering the tissue debris, the tubes were centrifuged at 300xg for 10 min. The supernatant was discarded and the cells were suspended in 5 ml of Leibovitz's-L15

Experimental colon cancer treatment

medium with 10% foetal bovine serum. The number of viable cells was measured using the MTT cells assay kit (ATCC). The assay is based on conversion of a tetrazolium salt into a blue formazan product by mitochondria during cell respiration. From the cell suspensions, 100 μ l in triplicate of the undiluted suspension or of 1:2 and 1:3 diluted suspensions were plated out in the wells of the microtiter plate, including 3 wells of the medium as blanks. The cells were incubated for 24 h to recover and 10 ml of tetrazolium salt was added to each well, and the plate was incubated for another 4 h. After adding 100 μ l of the detergent solution to each well, the plate was left overnight in the dark at room temperature. The absorbance in each well was read by using an ELISA plate reader (LabSystems Multiskan MS, Stockholm, Sweden) at dual wavelengths of 650- and 570 nm. The number of viable cells was calculated with a standard curve prepared from serial dilutions from 10×10^6 to 0.5×10^6 cells/ml.

Proliferation

To detect proliferation, the tumour tissue sections from all the animals were immunostained with the avidin-biotin-complex (ABC) method (Dakopatts, Glostrup, Denmark), as described in detail earlier (El-Salhy et al., 1998). Briefly, the sections were incubated overnight with the primary antibody at room temperature. The antibody used was proliferating cell nuclear antigen (PCNA) (monoclonal, code no. M0879, dilution 1:50, Dakopatts). Incubation with the secondary antibody, biotinylated swine anti-mouse IgG, diluted 1:200, was carried out for 30 min at room temperature and for another 30 min with the avidin-biotin-peroxidase complex, diluted 1:200. Peroxidase was detected by immersing the sections in 50 μ l Tris-buffer containing 25 mg diaminobenzidine tetrahydrochloride (DAB) and 10 μ l of 30% H_2O_2 followed by counter-staining with methyl green. Specificity controls included negative controls and positive controls. As a negative control, the sections were incubated with non-immune serum instead of the primary antibodies. Positive controls included immunostaining of sections from human colon adenocarcinoma.

The proliferating index was determined as described previously (Kerr et al., 1999) as adapted for computer image analysis. The image processing and analysis system used was Quantimet 600MC (Leica, Cambridge, England). The program used in this system was QWIN (Leica's Windows-based image analysis tool kit, version 2.6). In addition, the system included QUIPS (version 2.6), an interactive programming system. When using x20 and x40 objectives, each pixel in the computer monitor corresponds to 0.173 and 0.86 μ m, respectively, and the frame (field) represents areas of 5436 and 2340 μ m², respectively. An automated standard sequence analysis operation was created by QUIPS, in which both labelled and unlabelled nuclei were identified by clicking the computer "mouse", causing series of red and yellow dots to appear. The percentage of labelled cells of

all the cells counted in each field was calculated automatically. Ten fields from each tumour were randomly chosen and the x40 objective was used. The total number of counted cells for each tumour varied between 1800 and 3000.

Apoptosis

Apoptosis was detected by the TUNEL method and by the expression of poly (ADP-ribose) polymerase (PARP) as revealed by immunocytochemistry. TUNEL detection was made using the TA100 kit (R&D Systems Inc, Minneapolis, MN, USA). This kit detects DNA fragmentation by incorporating modified nucleotides at the 3'OH ends of the fragments by using terminal deoxynucleotidyl transferase (TdT). The incorporated 5-bromodeoxyuridine (BrdU) was detected with a biotinylated anti-BrdU antibody. A streptavidin-conjugated horseradish peroxidase bound specifically to the biotinylated antibody. The peroxidase was detected with DAB. The sections were counter-stained with methyl green. The method was performed according to the manufacturer's instructions. Controls included nuclease-generated positive controls; unlabelled sample controls and labelled untreated sections from normal tissue (mouse liver). To detect PARP, the ABC method was applied as described previously, except that the sections were pre-incubated for 15 min with proteinase K at room temperature. The monoclonal anti-poly (ADP-ribose) polymerase (Sigma, St. Louis, Mo, USA) was used, diluted 1:50. This antibody recognizes a 116 kDa protein which corresponds to PARP, and the 85 kDa apoptosis-induced cleavage product of pRICE (proteinase resembling 1 β -converting enzyme) as well as CPP32 (cysteine protease). Specificity controls were the same as for proliferation. The apoptotic index was determined in the same way as the proliferation index.

Tumour blood vessels

The sections were stained both with haematoxylin-eosin and by the ABC method, using anti-human CD31 monoclonal antibody diluted 1:20 (Dako, Clone JC70A). The sections were pre-incubated for 15 min with proteinase K at room temperature. Specificity controls were the same as described earlier in proliferation. In order to determine the number of blood vessels/field and the mean blood vessel luminal area, an automated standard sequence analysis operation created by QUIPS was applied whereby the blood vessel lumen was drawn with the computer "mouse" and the number of blood vessels/field and the mean blood vessel luminal area in each field were calculated automatically. Ten fields in CD31-immunostained samples from each tumour were randomly chosen and the x20 objective was used.

Statistical analysis

A comparison between groups was performed with the Kruskal-Wallis non-parametric ANOVA test, with

Dunn’s multiple comparisons as post-test. P-values below 0.05 were considered significant.

Results

The animals were healthy during the experiments and at the end of the experiments there was no statistical difference in the animal weight between controls and treated mice (Fig. 1). Dissection of all mice showed no sign of metastasis. On removing the subcutaneously-implanted ALZAT pumps, they were found to be encapsulated with transparent membrane.

Tumor volume and weight

The volume and weight of the tumours in all treated groups tended to decrease, but it was statistically significant (P=0.03 and 0.02, respectively) only in the group with intraperitoneally- implanted pumps (Figs. 2, 3).

Viable cells

The number of viable cells tended to decrease in all

the treated groups (Fig. 4). This decrease, however, was not statistically significant.

Proliferation and apoptotic indices

The proliferation index differed significantly between different groups (P<0.001) (Figs. 5, 8). The proliferation index was significantly reduced in mice given both triple therapy as an i.p. bolus injection and as a continuous infusion when compared with their corresponding controls (P<0.5 and <0.01, respectively). There was no statistical difference between animals treated by different routes.

Regarding the apoptotic index (Figs. 6, 8), a statistical difference was observed between different groups of mice as revealed by both the TUNEL method and by PARP expression (P<0.0001 and =0.0007, respectively). The apoptotic index increased significantly in mice given triple therapy as a continuous i.p. infusion by both markers of apoptosis (P<0.001 and <0.05, respectively). Again, there was no statistical difference between animals treated by different routes.

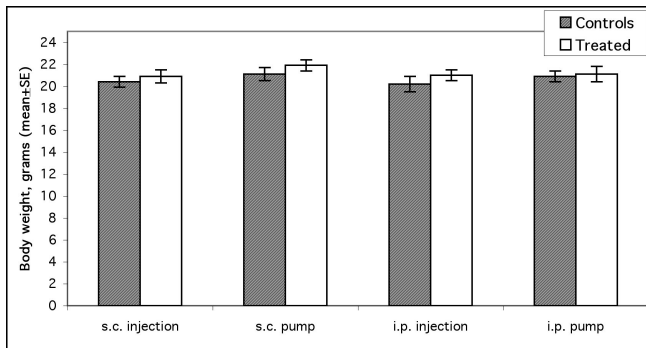


Fig. 1. Body weight of mice in the various treatment groups. Tumor weight and pump weight (in groups with implanted pumps) are subtracted.

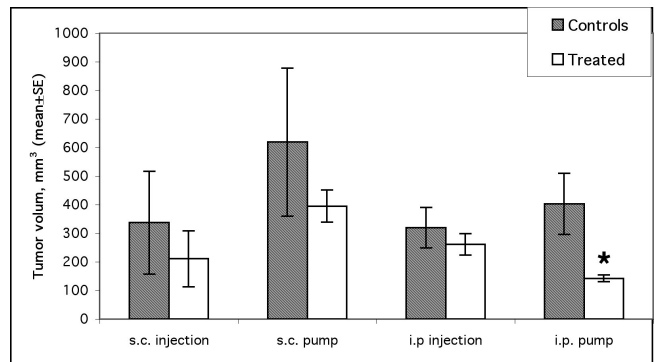


Fig. 2. Tumor volume in the different groups of mice after treatment. *: P=0.03.

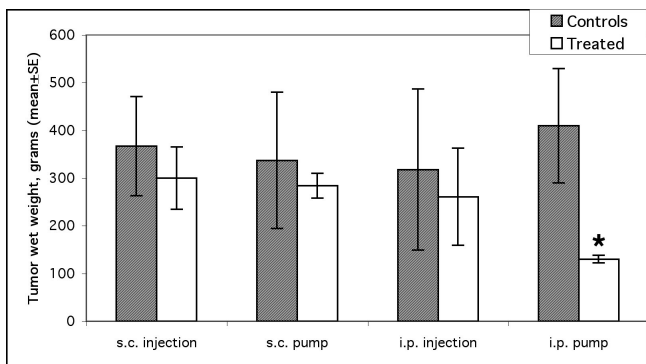


Fig. 3. Weight of the tumors of controls and treated mice. *: P=0.02.

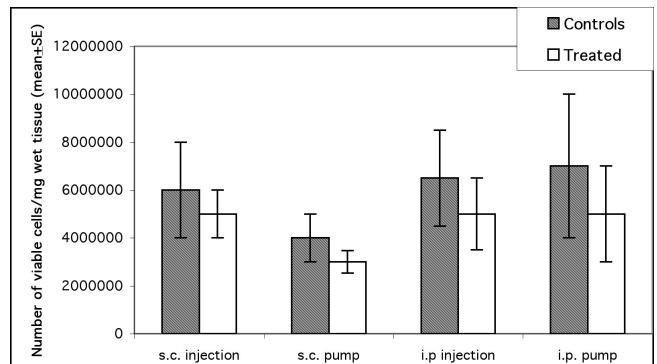


Fig. 4. Number of viable cells as detected by MTT-assay in controls and treated animals.

Experimental colon cancer treatment

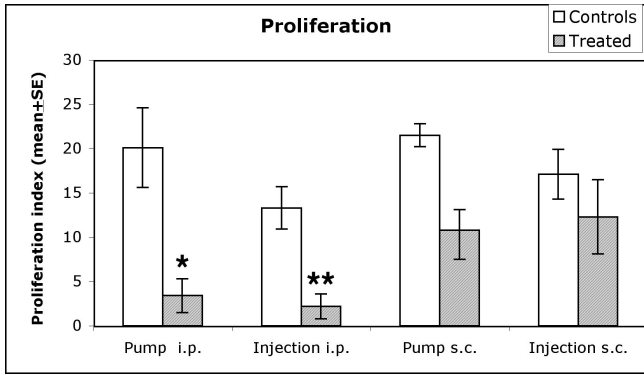


Fig. 5. Proliferation index in controls and animals treated by different routes. *: P=0.02, **: P=0.008.

Tumour blood vessels

The number of tumour blood vessels differed significantly in different groups (P=0.01). It was reduced in the mice given triple therapy as continuous i.p. infusion, compared with controls (P<0.05). The mean luminal area of these blood vessels did not differ (P=0.07) between different groups (Figs. 3, 4). There was no statistical difference between animals treated by different routes as regards both the number and the mean luminal area of tumour blood vessels.

Discussion

The present investigation showed that, regardless of

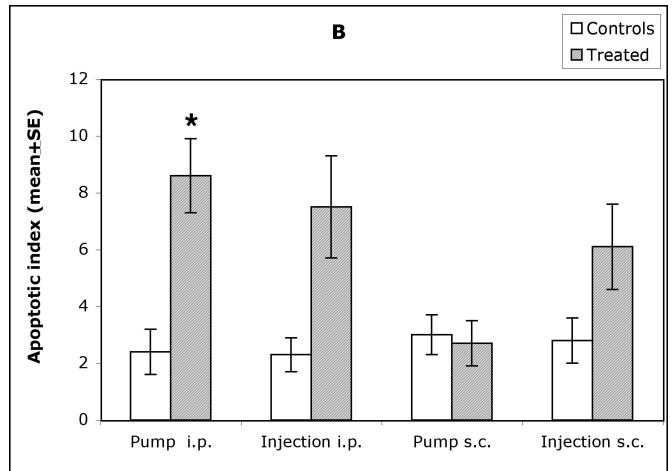
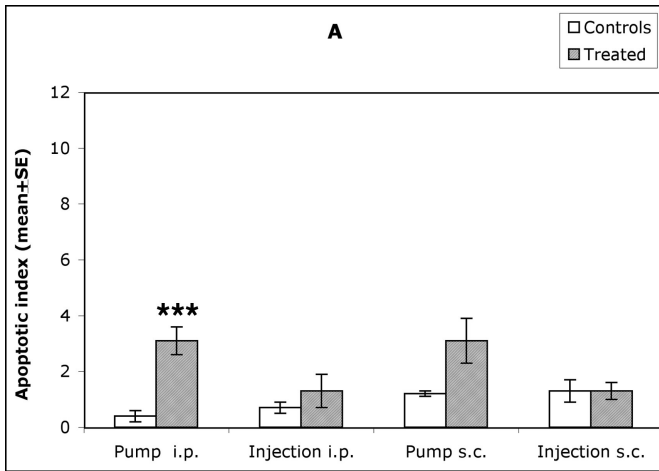


Fig. 6. Apoptotic index in controls and mice treated with TUNEL method (A) and as revealed by poly (ADP-ribose) polymerase (PARP) expression (B). ***: P=0.001 (A) and *: P=0.05 (B)

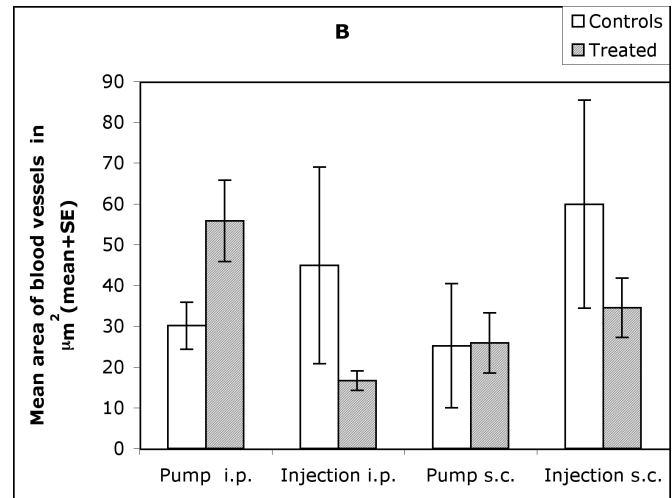
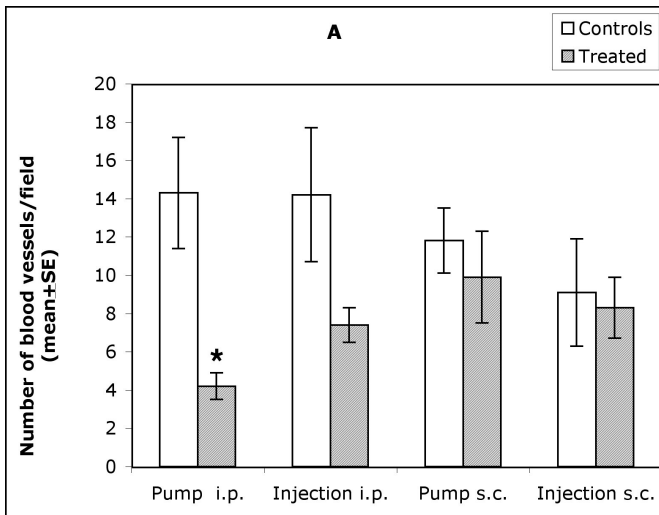


Fig. 7. Number of tumour blood vessels (A) and blood vessel luminal area (B) in controls and treated mice. *: P=0.05

Experimental colon cancer treatment

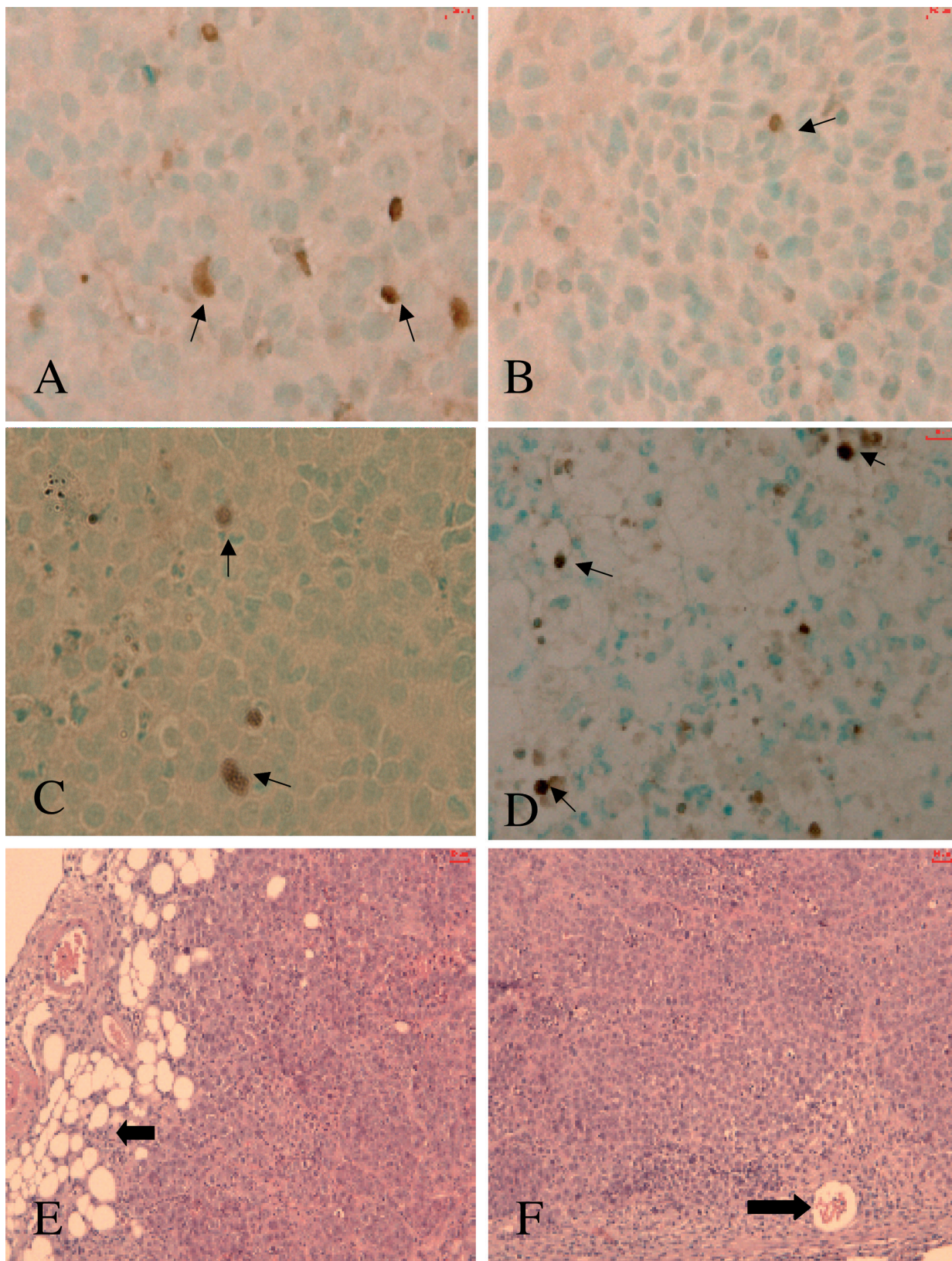


Fig. 8. Proliferation (**A and B**), apoptosis as revealed by the TUNEL method (**C and D**) and blood vessels (large arrows) as revealed by haematoxylin-eosin (**E and F**) in a tumour from a control mouse (**A, C and E**) and in a tumour from a mouse treated with continuous i.p. infusion of triple therapy (**B, D and F**). Proliferating and apoptotic nuclei stained brown (small arrows). A-D, x 350; E, F, x 100

Experimental colon cancer treatment

the route of administration, triple therapy with octreotide, galanin and serotonin generally reduced the volume, weight, number of viable cells and proliferation, induced apoptosis and reduced vascularization of a human colon cancer cell line implanted in mice. However, continuous intraperitoneal infusion appears to be the most effective route of administration. This could be explained by the fact that octreotide, galanin and serotonin have, like most of the neuroendocrine peptides/amines of the gut, a short half-life in the blood stream. Whereas intermittent administration of these substances gives a high concentration of short duration, continuous administration gives lower concentration but a lengthy duration. Subcutaneous infusion of the bioactive substances proved less effective than the i.p. route, possibly because i.p. infusion ensures rapid passage to the blood stream and consequently to the tumour. Dysfunction of the osmotic pump, implanted s.c., caused by the transparent capsule observed at the end of the experiments, could not be excluded, however. The i.p. route of administration has been reported to be equivalent to intravenous administration of other chemotherapeutic agents in colon cancer (Ekberg et al., 1988). Continuous intravenous infusion of the triple therapy seems therefore to be the most appropriate route of administration for clinical applications.

Whereas a marked reduction in cancer cell proliferation was observed in the present study of human colon cancer with both i.p. and s.c. as continuous infusion or as bolus injections, no such reduction was within the subcutaneous route of administration. The absence of effect on proliferation in rat colon cancer reported earlier (El-Salhy and Sitohy, 2002; El-Salhy et al., 2003; Sitohy and El-Salhy, 2003) could reflect a difference in colon carcinoma between human and rodent origin. It might even be due to a type II (B) statistical error, as the number of animals in previous studies on rat colon cancer was rather small.

Although the results of this investigation are encouraging and support earlier studies (El-Salhy and Sitohy, 2002; El-Salhy et al., 2003; Sitohy and El-Salhy, 2003; El-Salhy and Starefeldt, 2003; El-Salhy, 2004; El-Salhy and Dennerqvist, 2004) that triple therapy with octreotide, galanin and somatostatin is a promising form of treatment for colorectal cancer, it is not altogether certain that this treatment would be of use clinically. The differing response of various colorectal cancers and tolerability of the treatment in humans remain to be investigated.

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