Effects of triple treatment with octreotide, galanin and serotonin on a human pancreas cancer cell line in xenografts

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Summary. Human pancreas cancer cells were implanted s.c. in nude mice. After 11 days, the mice were divided into two groups of 13. The first group received sterile saline solution and the second received triple therapy containing octreotide, galanin and serotonin, 40 µg/kg/day as a continuous i.p. infusion via an implanted osmotic pump for 14 days. Triple therapy prolonged the survival rate of the mice bearing human pancreatic carcinoma. Both the volume and weight of tumours in mice given triple therapy were less than in controls (not statistically significant). The proliferation index and the labelling index for epidermal growth factor (EGF) increased significantly in mice given triple therapy vis-á-vis controls. There was no statistically significant difference between control and treated tumours as regards, apoptotic index, necrosis, or number of tumour blood vessels. The increased survival rate was attributed to the reduced tumour load, since both weight and volume were reduced. It is most probable that octreotide was the responsible agent. Further investigation with single and double combinations of octreotide, galanin and serotonin are needed to identify the cause of increased cell proliferation in tumours subjected to these bioactive substances. Identifying the agent(s) inducing pancreatic cancer cell proliferation may be useful in combining a new treatment, as antagonists to these bioactive substances are available.

Key words: Adenocarcinoma, Galanin, Pancreas, Serotonin, Somatostatin

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Introduction

Adenocarcinoma of the pancreas is the fifth leading cause of cancer death in the western world with over 40000 deaths annually in Europe and nearly 30000 deaths in the USA. The incidence of pancreatic cancer has risen in many countries as their inhabitants adopt a westernised way of living with a peak around 10^{-12} per 10^5 population (Neoptolemos et al., 2003). This incidence is exceeded only by lung, colorectal, prostatic and breast cancer (Schnall and Macdonalds, 1996). Pancreatic carcinoma patients have a poor 5-year survival rate of less than 5%, and untreated patients with pancreatic cancer have a mean survival of approximately 4-6 months (Shankar and Russel, 2001; Korc, 2003). Surgery is the only potentially curative treatment, but patients often present with advanced disease, about half of them having distant metastases, 40% local spread and only 10% with tumours confined to the pancreas (Schnall and Macdonalds, 1996; Gibbs et al., 2003). Thus about 80% of patients with pancreatic cancer have unresectable tumour at presentation (Schnall and Macdonalds, s1996). Chemotherapy for these patients plays only a palliative role and is a limited option because of the premorbid condition of the patients (Schnall and Macdonalds, 1996; McKenna and Eatock, 2003).

It has recently been shown that triple therapy with octreotide (a somatostatin analogue), galanin and serotonin increased necrosis and apoptosis of rat colon cancer cells. The increased necrosis appeared to be caused by reducing the number of and constricting 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 increase apoptosis, while reducing cancer cell proliferation, and also reducing the number of tumour blood vessels supplying human colon cancer (El-Salhy and Starefeldt, 2003; El-Salhy, 2004). Comparison of triple therapy and standard treatment vs. 5-fluorouracil/leucovorin (Fu/Lv) showed

that triple therapy has the same anti-tumour effect as Fu/Lv but better therapeutic efficacy (El-Salhy, 2004). It seems that this form of treatment is free from side effects (El-Salhy and Sitohy, 2002; Sitohy and El-Salhy, 2003).

As the chemotherapy of colorectal carcinoma is similar to that of pancreatic cancer in several aspects and as both are gastrointestinal adenocarcinoma (Primrose, 2002; McKenna and Eatock, 2003), the present study was undertaken to ascertain whether the effects of triple treatment with octreotide, galanin and serotonin on human pancreatic adenocarcinoma are similar to the results obtained on colorectal carcinoma.

Material and methods

Animals

Female nude mice (C57BL/6JBom-nu, Taconic M& B A/S, Bomholtvej, Denmark), 10 weeks old and with a mean body weight of 24.6 g (range 22-29.3 g) were used. The animals were kept, 5 mice to each cage, in a laminar filtered airflow cabinet under pathogen-free conditions. They were housed at constant temperature (22+2°C), a relative humidity of 55+5% and with a 12/12h light/dark cycle. They had free access to a standard pellet diet (R34, Lactamin, Vadstena, Stockholm) and tap water. All experiments were performed using aseptic techniques under laminar airflow. The animals were inspected twice and were weighed once daily. Any sign of pain, discomfort or change in their behaviour was recorded. The local committee on animal ethics approved the investigation.

Cell line culture

The human pancreas cancer cell line (SW 1990) was obtained from The American Type Culture Collection (ATCC) (Manassas, Va, USA). The cells were grown in Leibovitz's-L15 medium supplemented with 10% (v/v) foetal bovine serum (ATCC) in a humidified atmosphere at 37°C without CO_2. Cells were routinely passed by removing the medium and overlaying the cell monolayer with 0.25% trypsin and 0.1% EDTA.

Tumour inoculation and treatment

Thirty mice were injected subcutaneously in the right flank with a 100 µl cell suspension containing 10×10^6 viable cells. Viable cells were counted on with a haemocytometer using trypan blue. After 11 days, 26 animals had developed a palpable tumour, and were randomly allocated to either of the two groups, 13 in each.

The first group received continuous i.p. infusion of sterile saline solution, while the second group was given octreotide, galanin and serotonin. The dosage was 40 µg/kg body weight of octreotide (Sandostatin®, Novartis), galanin (synthetic human galanin, Sigma, Stockholm) and serotonin (5-hydroxytryptamine oxalate

salt, Sigma). This dosage has been reported in earlier studies to be optimal (El-Salhy and Sitohy, 2002). Intraperitoneal infusion was effected by implanting an ALZET osmotic pump (type 1002, Durect Co., Cupertino, Calif, USA). In order to implant the pump i.p. the mice 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 was injected i.p. with 0.3 ml of this mixture. The pump was inserted into the abdominal cavity through a median abdominal incision. The abdominal wall and the skin were closed with sutures and the animal was allowed to recover.

At the end of the experiment, the mice were weighed and then decapitated. Each mouse was dissected and the abdomen and thorax were exposed. An inspection was made for possible presence of metastases. Tumours were carefully excised and the greatest and least diameters of each tumour and its weight were measured. Mean diameter was calculated as (the greatest + least diameter)/2. Tumour volume was calculated using the formula:

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

Tumours were fixed overnight in 4%-buffered paraformaldehyde, embedded in paraffin wax and cut at 5 µm. The slides were coded to avoid any performer bias during quantification.

Proliferation

Sections of tumour tissue from all mice were immunostained with the avidin-biotin-complex (ABC) method (DakoCytomation, Glostrup, Denmark), as described elsewhere in detail (El-Salhy et al., 1998). The primary antibodies used were proliferating cell nuclear antigen (PCNA) (monoclonal, code number M0879, dilution 1:50, DakoCytomation). Peroxidase was detected by immersing the sections in 50ml Tris-buffer containing 25 mg diaminobenzidine tetrahydrochloride (DAB) and 10µl of 30% H_2O_2, followed by counter-staining with methyl green. Specificity controls included both 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 by computer image analysis as described previously (Kerr et al., 1999). The image processing and analysis system 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 mm, respectively, the frames (field) representing areas of 5436 and 2340 mm^2,

respectively. An automated standard sequence analysis operation created by QUIPS was used, in which the labelled and unlabelled nuclei were pointed out with the computer "mouse"; when the mouse was clicked, a series of red (labelled nuclei) and yellow (unlabelled nuclei) dots appeared. The percentage of labelled cells of all the cells counted in each field and in all fields was calculated automatically. Quantification was done in ten randomly chosen fields from each tumour, where the X40 objective was used. The total number of cells counted for each tumour varied between 1600 and 3000.

Apoptosis

Apoptosis was detected with two parameters, namely DNA fragmentation and poly (ADP-ribose) polymerase (PARP) expression. DNA fragmentation was revealed by the TUNEL method, using a TA100 kit (R&D Systems Inc, Minneapolis, Minn., USA), according to the manufacturer's instructions. Controls included nuclease-generated positive controls; unlabelled sample controls and labelled untreated sections from normal tissue (mouse liver). Detection of PARP expression was by the ABC method as described previously, except that the sections were pre-incubated with proteinase K for 15 min at room temperature prior to incubating the primary antibody. 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 prlCE (proteinase resembling1b-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 necrosis

A slice, 2 mm thick was cut from the middle of each tumour. It was cut out with a razor blade controlled by a rat brain matrix (RBM-4000, ASI Instruments, Inc. USA). The slices were soaked for 10 min in a 2% solution of 2,3,5-triphenyltetrazolium hydrochloride (TTC) in 0.1 mol/L PBS (pH 7.4) in a small Petri dish, maintained at 37°C for 10 min. The slices were stirred gently to ensure even exposure of the surfaces to staining. The relative size of the tumour necrosis was measured using SigmaScan Pro version 5 (SPSS, Inc) using an automatic threshold of 40% in the green spectrum in a similar manner as described elsewhere (Bederson et al., 1986; Goldlust et al., 1996).

Tumour blood vessels

The sections were stained with the ABC method, using anti-human CD31 monoclonal antibodies diluted 1:20 (DakoCytomation, Clone JC70A). The sections were pre-incubated with proteinase K for 15 min at room temperature. Specificity controls were the same as described earlier. To determine the number of blood vessels/field, an automated standard sequence analysis operation created by QUIPS was applied, in which the blood vessels was pointed out with the computer "mouse". The number of blood vessels/field in each field was summed automatically. Ten fields from samples from each tumour were randomly selected and analysed with the X20 objective.

Epidermal growth factor (EGF) expression and receptors

Sections of tumour tissue from all mice were immunostained with ABC. The primary antibodies used were anti-human EGF (monoclonal, code number E2520, dilution 1:1000, Sigma) and anti-human EGF receptors (monoclonal, code number M 7239, dilution 1:100, DakoCytomation).

The labelling index of the EGF expressing cells vs. all cells counted was measured in the same way as for the proliferation index. The data from each field were tabulated, computed and statistically analysed automatically (El-Salhy et al., 1997). The number of cells, and the area of labelled cell membrane after immunostaining for EGF-receptors were determined, using an automated standard sequence analysis operation create by QUIPS, as described in detail earlier (El-Salhy et al., 1997). Briefly, the number of cells was counted using field measurements. The areas of the labelled cell membrane were measured using a threshold setting. The data from each field were tabulated, computed and statistically analysed automatically.

Statistical analysis

Comparison between controls and treated mice was performed with Wilcoxon's non-parametric and Fisher's exact test. P-values below 0.05 were considered significant.

Results

The mice appeared well throughout the experiment. All recovered after the intraperitoneal implantation of the osmotic pump. In the control group, 3 mice and one mouse died, 13 and 23 days respectively after tumour inoculation, (Fig. 1). At the end of the experiment, the body weight of the control group was 26.4±0.7 g (mean ± SE), vis-á-vis 27.1±0.5 for the treated group. There was no statistical difference between controls and treated animals regarding body weight (P=0.4). Dissection of the mice revealed that all the animals had regional lymph node metastasis. In addition, abdominal carcinosis was found in 3. At the end of the experiment, dissection of the animals revealed that 3 mice in the treated group had abdominal carcinosis, while one had regional lymph node metastasis. In the control group, one mouse disclosed both abdominal carcinosis and regional lymph node metastasis. There was no

statistically significant difference between controls and treated mice regarding the incidence of metastasis (P=0.6).

Tumour volume and weight

The weights and volumes of tumours at the end of the experiment from mice treated with triple therapy and controls are reported in Fig. 2. The tumour weights and volumes in treated were reduced, though not statistically significantly (P=0.3 and 0.3, respectively).

Proliferation and apoptotic indices and tumour necrosis

The proliferation index was significantly increased (Figs. 3, 7) in the mice treated with triple therapy, as compared with controls (P=0.03). The apoptotic index in both treated and control mice are illustrated in Figs. 4

and 7. The index did not differ statistically between the treated and control animals, both by the TUNEL method and with PARP-expression (P=0.1 and 0.2, respectively). There was no statistical difference between control and treated mice regarding the necrotic index of the tumours (P=0.09) (Figs. 5, 7).

Tumour blood vessels

The number of tumour blood vessels (Fig. 6) did not differ significantly between the treated and control groups (P=0.2).

EGF expression and EGF-receptors

The labelling index of EGF was significantly increased (P=0.02) in mice treated with triple therapy, as compared with controls (Figs. 8, 9). There was no statistical difference between controls and treated tumours as regarding the labelled area/cell of EGF-receptors (P=0.2) (Figs. 8, 9).

Discussion

Of the untreated mice 23 and 8% died after 13 and 23 days after the tumour inoculation, respectively. When comparing lifespan of mice and humans, one day of a mouse life corresponds to approximately one month in humans. Bearing in mind that the mean survival of patients with untreated pancreatic cancer is 2-6 months, the cause of death in untreated mice appears to be the implanted pancreas carcinoma. This is supported by the post-mortem examination, where no other cause of death could be found. Thus, triple therapy with octreotide, galanin and serotonin appears to improve the survival rate of the mice bearing human pancreatic carcinoma. One must bear in mind in the following discussion that the comparison is made between the treated and the
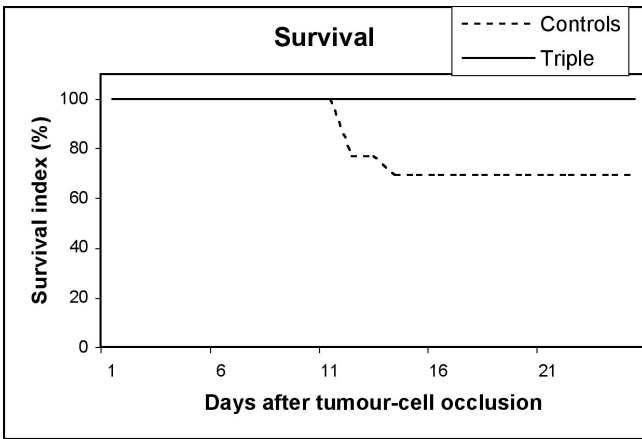

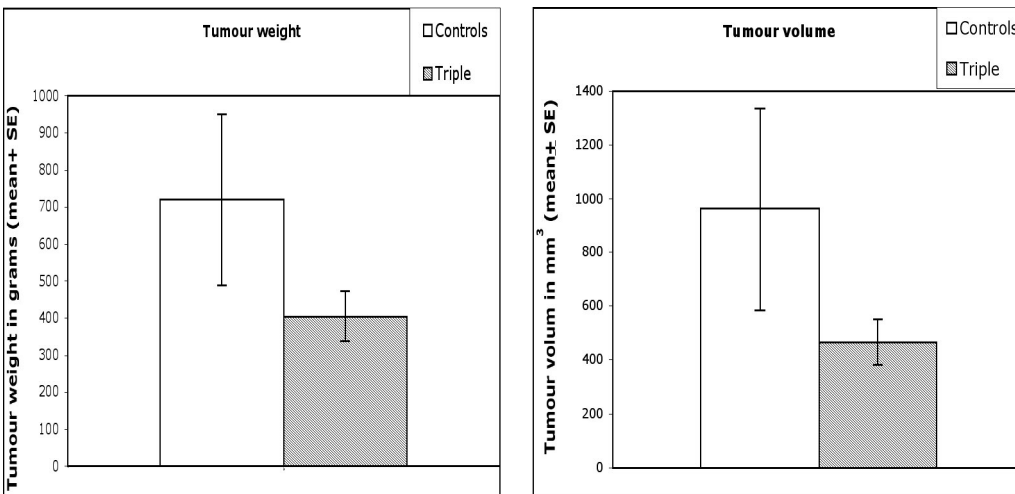**Fig. 1.** Survival curve of the mice bearing the human pancreatic cancer.

Fig. 2. Tumour weights and volumes of controls and mice given triple therapy with octreotide, galanin and serotonin.
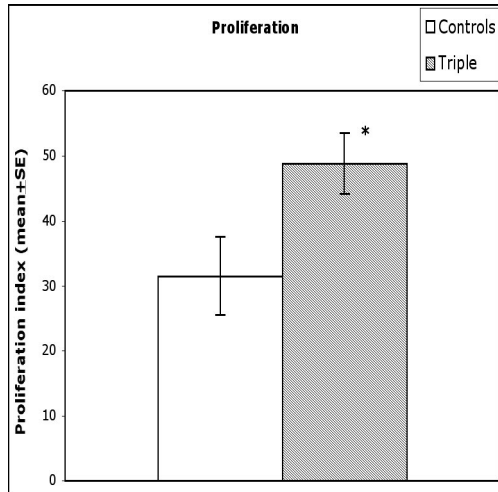
surviving untreated controls and that about one-third of the untreated mice, which were assumed to have died of the tumour are not included. The increased survival rate could be attributed partly to the reduced tumour load, as both the weight and volume were reduced (though not statistically significantly) after triple therapy.

Instead of reducing the cancer cell proliferation as seen earlier in colon cancer (El-Salhy and Starefeldt, 2003; El-Salhy, 2004), triple therapy with octreotide, galanin and serotonin increased pancreatic cancer cell proliferation. Further investigation with single and double combinations of octreotide, galanin and serotonin are needed to identify what causes this aggrandizement. Identifying the agent(s) inducing pancreatic cancer cell proliferation and expression of EGF may be useful in combing new treatment, as antagonists to these bioactive substances are available. Pancreatic cancer cell lines are usually resistant to apoptosis mediated by chemotherapeutic agents or by apoptosis-inducing receptors such as Fas or TNF-R (Korc, 2003). The present findings that triple therapy had no effect on pancreas cancer cell apoptosis, in contrast to previous observations in colon carcinoma (El-Salhy and

Fig. 3. Proliferation index (%) in controls and mice given triple therapy with octreotide, galanin and serotonin. *: $P<0.05$.
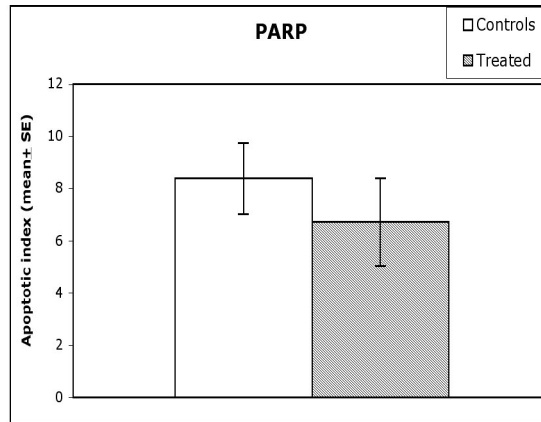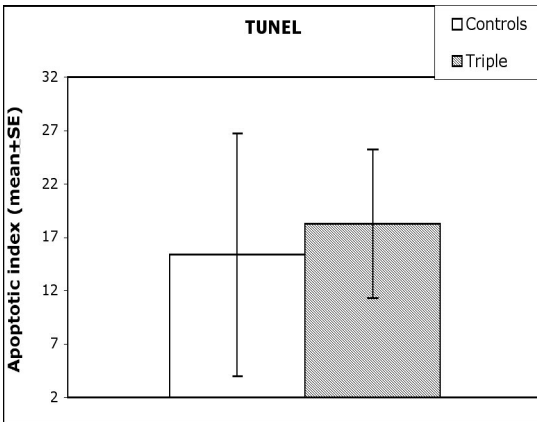

Fig. 4. Apoptotic index in controls and animals treated with triple therapy as detected with the TUNEL method and by the expression of poly (ADP-ribose) polymerase (PARP).
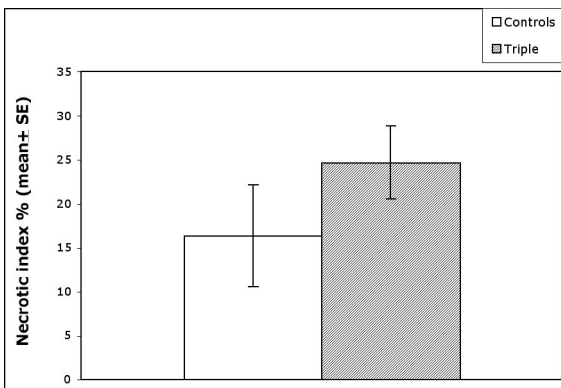

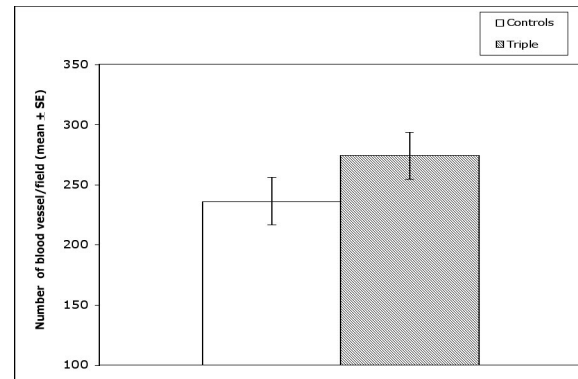Fig. 5. Necrotic index in controls and treated animals.

Fig. 6. Number of tumour blood vessels detected with CD31 antibodies, in controls and treated animals.
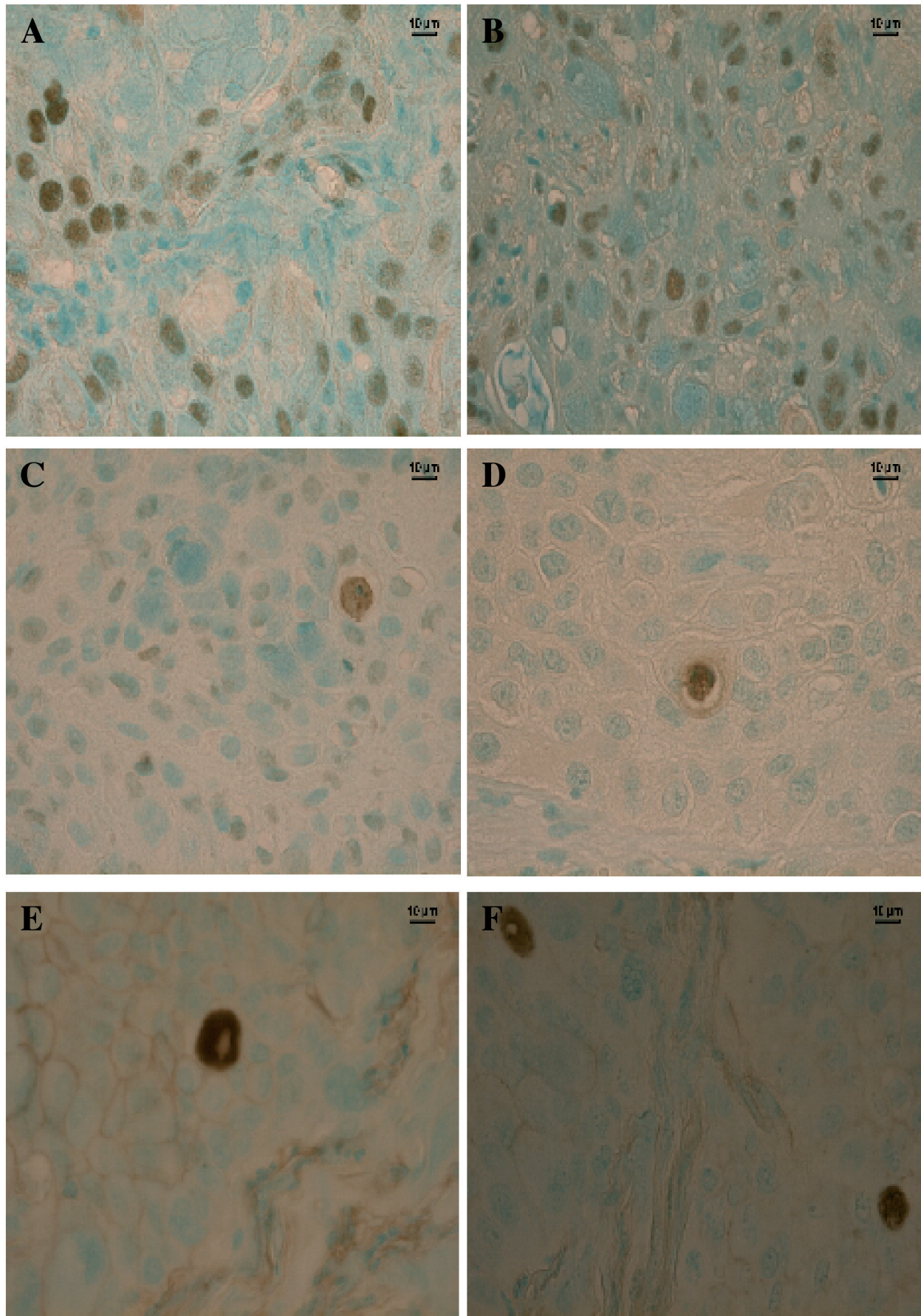
Fig. 7. Proliferation **(A)**, apoptosis **(C)** and EGF-immunoreactive cells **(E)** in a control tumour, and proliferation **(B)**, apoptosis **(D)** and EGF **(F)** in a tumour treated with triple therapy. The proliferating and apoptotic nuclei stained brown, as did the cytoplasm of EGF expressing cells.

Fig. 8. Labelling index of EGF and EGF-receptor area/cell in controls and treated animals. *: P<0.05.
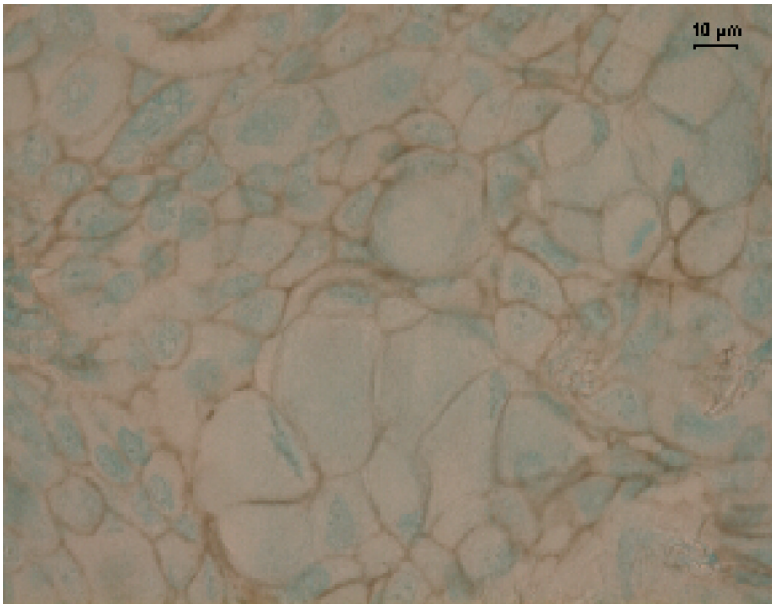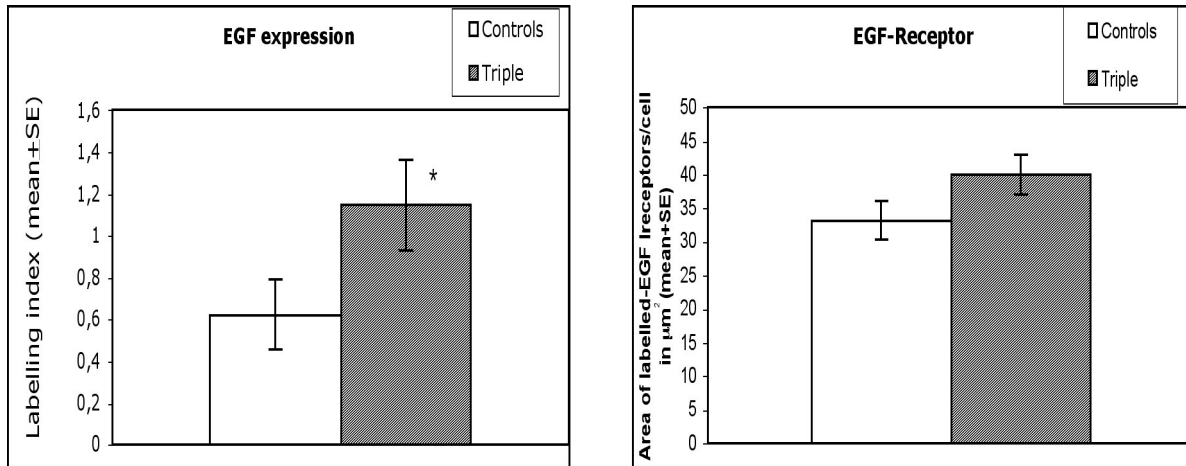

Fig. 9. EGF-receptors in an untreated tumour.

Starefeldt, 2003; El-Salhy, 2004) is therefore not surprising. Whereas triple therapy generally and galanin specifically reduced the tumour-feeding blood vessels of colon cancer (El-Salhy and Sitohy, 2002; El-Salhy et al., 2003; Sitohy and El-Salhy, 2003; El-Salhy and Starefeldt, 2003; El-Salhy, 2004), it had no effect on the vascularization of pancreas carcinoma as disclosed in the present investigation.

Whereas the effects of galanin and serotonin on pancreatic cancer are, to the best of our knowledge, unknown, the effects of somatostatin and its analogue have been elicited in several studies. Thus, octreotide has been found to inhibit proliferation of pancreatic cancer (Qin et al., 1995; Fisher et al., 1996; Kikutsuji et al., 2000). Furthermore, pancreatic cancer cells have

been reported to exhibit somatostatin receptors in some, though not all pancreatic tumours (Fisher et al., 1996, 1998; Kikutsuji et al., 2000). The effect of octreotide on pancreatic cancer growth seems to be dependent on the expression of somatostatin receptors (Fisher et al., 1996). Pancreatic cancer cells have been reported to express both EGF and EGF-receptors in about 50% of pancreatic tumours (Uegaki et al., 1997). The inhibition of pancreatic cancer growth seen after octreotide treatment has been suggested to take place via the cAMP pathway (Qin et al., 1995). Moreover, this inhibition has been suggested to be caused by reduction in EGF-receptor expression by the pancreatic cancer cells (Szepesházi et al., 1999). In the present study, the expression of EGF expression of EGF-receptors of

pancreatic cancer cells was not reduced. Instead the expression of EGF of pancreatic cancer cells was increased. It is possible that this finding is due to the difference in the concentration of octreotide used, as in the earlier studies a higher dose of octreotide was used. It can also be due to the effects of the synergistic effects of galanin and serotonin.

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