Induction of Apoptosis in Neuroendocrine Tumors of the Digestive System During Treatment with Somatostatin Analogs

Hassan Imam, Barbro Eriksson, Agneta Lukinius, Eva T. Janson, Per-Gunnar Lindgren, Erik Wilander and Kjell Öberg

From the Departments of Medicine (H. Imam, B. Eriksson, E.T. Janson, K. Öberg), Pathology (A. Lukinius, E. Wilander) and Diagnostic Radiology (P.-G. Lindgren), University Hospital, S-751 85 Uppsala, Sweden

Correspondence to: Professor Kjell Öberg, Department of Internal Medicine, University Hospital, S-751 85 Uppsala, Sweden

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The extent of apoptosis identified by *in situ* DNA nick end labelling (TUNEL) on tissue samples obtained from patients with neuroendocrine tumors was correlated with the clinical outcome in patients treated with high-dose somatostatin analog (lanreotide 12 mg/day), n = 8, or other biotherapy including interferon-alpha (IFN-α), n = 4, low-dose somatostatin analog (octreotide or lanreotide), n = 3, or a combination of both, n = 1. Biopsies were obtained before the start of treatment and/or after 6 months and 12 months. After 6 months of treatment, 5 patients receiving high-dose somatostatin analog showed a biochemical response (decrease in different neuroendocrine tumor markers) and 4 of these showed an increase in apoptotic index (AI: percentage of apoptotic cells) by 1.94 ± 1.71%. At 12 months, AI was also increased in patients with a biochemical response (4.22 ± 3.93%). However, none showed a decrease in tumor size on computerized tomography (CT) and none of the patients treated with low-dose somatostatin analog or IFN-α showed any significant increase in AI during treatment. In an experimental model, nude mice were xenografted with the neuroendocrine cell line (BON-1). From the 2nd day of tumor implantation, they received treatment with either placebo, high-dose octreotide, IFN-α, or a combination of both, for 28 days. In mice receiving treatment with high-dose octreotide (300 μg/kg, t.i.d) there was a threefold increase in apoptotic cells as compared to the placebo group (p = 0.0084), while the combination group had few cells with ultra-structural changes indicating apoptosis and the IFN-α treated group showed no significant changes. However, tumor growth inhibition was more pronounced in the combination group (p = 0.0011). This probably denotes that tumor growth inhibition could be achieved more efficiently by blocking the cell cycle than by inducing apoptosis. We concluded that treatment with high-dose somatostatin analogs may induce apoptosis in neuroendocrine tumors, while this is not found during treatment with low-dose somatostatin analogs or IFN-α. We also found that an increase in AI during high-dose somatostatin analog treatment was correlated with the biochemical response, but not with the tumor size as detected by CT in patients or with the tumor mass in the experimental model.

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Neuroendocrine tumors of the digestive system are rare and slow growing, with a malignant potential (1). These tumors produce hormones giving rise to specific symptoms such as the carcinoid syndrome, the Zollinger-Ellison syndrome, etc. (2). Previously, treatment of gastrointestinal carcinoid tumors was directed towards symptomatic relief of clinical syndromes (3). Chemotherapeutic agents showed a limited effect on tumor progression but debilitating side effects (4). The long-acting somatostatin analog, Octreotide, SMS 201-995, causes reduction in circulating levels of hormones and amines and may inhibit tumor growth in some patients (5). IFN-α may improve symptoms of a carcinoid syndrome by reducing circulating hormone levels and inhibiting tumor growth (6). For the past few years the treatment of patients with neuroendocrine tumors with somatostatin analogs in combination with IFN-α has yielded improved results (7).

Apoptosis refers to cell death with specific morphological changes which are different from necrosis (8, 9). It is assumed to be one of the regulating processes of tumor progression (10) and many drugs induce apoptosis while exerting antitumor action (11).

We have evaluated the event of apoptosis in the antitumor action of octreotide and IFN-α on neuroendocrine tumors using BON-1, a human serotonin secreting pancreatic endocrine tumor cell line (12) xenografted into nude mice. The experimental data were then compared with the results from a clinical trial where patients were treated with a new somatostatin analog, lanreotide, at a high-dose or with conventional doses of octreotide, low-doses of

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lanreotide (300–500 μg/d), IFN-α or a combination of IFN-α and octreotide. Apoptotic indices were evaluated by the TUNEL method and correlated to the therapeutic results. Electron microscopy was used to confirm the findings by the TUNEL method.

MATERIAL AND METHODS

Patients

Sixteen patients with liver metastasis of histologically verified neuroendocrine tumors (13 midgut carcinoids; 3 endocrine pancreatic tumors {(EPT), 1 non-functioning, 1 glucagonoma, 1 gastrinoma}) with a median age of 65 years (range 52–76 years) were included in this study. Eight patients were given lanreotide (Somatuline®, Ipsen Biotech, Paris, France) at increasing doses up to 12 mg/d, s.c. in four divided doses for 6 weeks followed by the maximum tolerable dose (9–12 mg/d). Three of these patients were recently diagnosed as EPTs and five diagnosed as midgut carcinoid had a median duration of disease of 70 months (range 1–120 months). Four patients were given IFN-α, 3–5 MU × III-V/week (IntronA®, Schering Plough, NJ, USA) having a median duration of disease of 43 months (range 1–75 months), 2 were given lanreotide (30 mg every 2nd week, slow release i.m.), 1 was given octreotide (Sandostatin® SMS 201-995, Sandoz, Basel, Switzerland, 300–500 μg/d in 2–3 divided doses) and 1 patient received a combination of IFN-α and octreotide. All four patients were recently dignosed. Ultrasound-guided needle biopsies from liver metastases were taken before starting the treatment and after 6 and 12 months, or two biopsies were taken at 6-month intervals after treatment was begun.

Clinical therapeutic response criteria

Evaluation of clinical therapeutic response was measured both as changes in the levels of the tumor markers (5-hydroxy-indoleacetic acid; gastrin; glucagon; human chorionic gonadotropin-α subunit; chromogranin A) which are cited as biochemical response and as changes in tumor size measured as the product of the maximum and its perpendicular diameter on a CT scan mentioned as radiological changes. Patients were classified as responders (objective response-OR) when they had a >50% reduction of the principal tumor markers and/or tumor size. Biochemically stable disease (SD) was defined as a reduction of tumor markers by <50%. Radiologically stable disease was defined as no increase in tumor size and no appearance of new metastases. Progressive disease (PD) was defined as an increase in tumor markers or mass >25%.

Cell culture

BON-1, a human serotonin-secreting pancreatic endocrine tumor cell line, and a generous gift from Dr J. C. Thompson, Galveston, Texas, USA, was maintained in Dulbecco's Modified Eagle medium (Seromed, Biochrom KG, Berlin, Germany) and F 12 K (Nord Cell, Sweden) in a 1:1 ratio supplemented with 5% fetal calf serum (Seromed) and incubated in a humidified atmosphere of 5% CO_2-enriched air at 37°C. Single cell suspensions of 1×10^7 cells were injected to athymic nude mice.

Animals

Male athymic nude mice (BALB/c, 20–25 g, 4–5 weeks of age) were kept in a pathogen-free, temperature-controlled isolation unit with a 12 h light and dark cycle. The mice were fed a standard chow and water, given ad libitum.

Experimental design

BON-1 cell suspensions (1×10^7 cells/injection) were inoculated subcutaneously in five nude mice to establish tumors. When the tumors were approximately 1 cm^3 in volume, the mice were sacrificed by administration of an overdose of thiopental sodium, i.p. and the tumors were minced into 2–3 mm^2 pieces, two of which were implanted into the right flank of the nude mice. After transplantation of tumor pieces, 32 mice were randomly allocated to four different groups to receive saline (0.9% NaCl, 0.1 ml s.c. every morning), IFN-α (1 MU s.c. every morning), octreotide (300 μg/kg body weight t.i.d.) or a combination of IFN-α and octreotide at the same dose as single therapy. Treatments were started on the second day of tumor transplantation and continued for 28 days.

The mice were weighed weekly by the same observer. The two greatest perpendicular diameters of tumors were measured. The volume of the tumors was calculated using the equation $V = 4/3\pi r^3$, where, V = volume, r = radius, π = constant, expressed in cubic centimeters. Final volume (V_f) was measured using the equation $V_f = (V_1 + V_2)/2$. After removal of the tumors, paraffin blocks were made for TUNEL and the rest of the tissues were kept at −70°C until assayed for DNA.

TUNEL

The method was performed as described before (13), which is based on the specific binding of Tdt to 3'-OH ends of DNA breakage. After deparaffinization and rehydration of paraffin sections, tissues were digested with 20 mg/ml proteinase K (Boehringer Mannheim Scandinavia AB, Bromma, Sweden) at room temperature (RT) for 15 min and frozen sections were digested for 10 min. Digestion was stopped by washing in distilled water (DW). The sections were treated with 3% H_2O_2 for 5 min at RT and washed with DW. TdT buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) containing Tdt (0.3 e.u./μl; Gibco BRL, Gaithersburg, MD) and biotin dUTP (Boehringer Mannheim Scandinavia AB) added to cover the sections and incubated in a

Table 1

Summary of in situ identification of apoptotic cells and relation to the clinical progression of patients treated with high-dose lanreotide

No.	Type of tumor	Duration of disease (months)	Apoptotic index (%) 0 months	6 months	12 months	Clinical progress 6 months	12 months
1.	Carcinoid	120	4.2 ± 0.8	3.65 ± 0.21	3.95 ± 0.38	SD-bio SD-radio	PD-bio PD-radio
2.	EPT (non-function)	0	3.92 ± 0.21	4.15 ± 0.08	5.44 ± 0.23	OR-bio SD-radio	OR-bio SD-radio
3.	EPT (gastrinoma)	0	6 ± 0.28	6.87 ± 0.18	16.05 ± 3.15	OR-bio SD-radio	OR-bio SD-radio
4.	EPT (glucagonoma)	0	2.94 ± 0.37	5.63 ± 1.13	6 ± 0.08	OR-bio SD-radio	OR-bio SD-radio
5.	Carcinoid	1	5.9 ± 0.66	4.92 ± 1.61	2.82 ± 0.24	OR-bio SD-radio	PD-bio SD-radio
6.	Carcinoid	103	2.75 ± 0.07	ND	5.01 ± 0.27	ND	OR-bio SD-radio
7.	Carcinoid	11	8.2 ± 0.15	12.19 ± 1.52	ND	OR-bio SD-radio	ND
8.	Carcinoid	70	5.32 ± 0.42	4.09 ± 0.43	ND	PD-bio SD-radio	ND

Abbreviations: PD = progressive disease, SD = stable disease, OR = objective response, EPT = endocrine pancreatic tumor, bio = biochemical, radio = radiological, ND = not done.

humid atmosphere at 37°C for 60 min. The slides were transferred to TB buffer (300 mM sodium chloride, 30 mM sodium citrate) to terminate the reaction and kept for 15 min at RT and in PBS for 5 min. After blocking with 2% BSA (in PBS) for 10 min at RT, the sections were subsequently incubated with Extra-avidin Peroxidase (Sigma Chemical Co.) at 37°C for 30 min and stained with 3-amino-9-ethyl-carbazole (AEC) for about 30 min at 37°C, washed with PBS, then counter-stained with hematoxylin.

Counting of apoptotic cells

Cells were counted per tumor area with the aid of a 1 cm^2 grid divided into 100 small squares. The grid was kept at the eye piece and the sections were viewed at a magnification of ×400. Cells were counted at at least three areas over tumor tissue and the observers were blinded of the clinical outcome or other factors. Positive apoptotic cells were calculated as a percentage [apoptotic index (AI)].

DNA extraction and agarose gel electrophoresis

DNA was isolated from frozen tissue using a DNA isolation kit according to the protocol provided by the supplier (Invitrogen, San Diego, CA.); 20 μg DNA was mixed with 0.25% bromophenol blue and 30% glycerol in water and electrophoresed in 1% agarose gel, which was stained with 0.5 μg/ml ethidium bromide. The DNA was made visible by UV light and photographed.

Electron microscopical examination

Confluent monolayers of BON-1 cells were cultured in petri dishes and incubated for 72 h with IFN-α (1500 IU/ml medium), octreotide (50 ng/ml medium) or a combination of IFN-α and octreotide. An untreated control cell culture was also included. After rinsing in PBS, the cells were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer with 0.1 M sucrose (pH 7.2) for 3 h, postfixed in 1% osmium tetroxide in the same buffer for 1.5 h, dehydrated in graded ethanol, immersed in propylene oxide and embedded in Agar 100 (an epoxy resin, Agar Scientific, Standsted, Essex, UK). During the propylene immersion, the cells were loosened from the petri dishes and pelleted (2 000 g for 10 min). Ultrathin sections, placed on copper grids were contrasted with uranyl acetate and lead citrate. Examination was performed in a Philips 201 electron microscope.

Statistical analysis

Results are expressed as the percentage and the mean ± standard deviation. The test of significance was carried out by means of the StatView 4.01 program using an unpaired *t*-test. P-values less than 0.01 were considered significant.

RESULTS

Evaluation of biopsies from patients

In the 8 patients treated with high-dose lanreotide, the pre-treatment AI mean was 4.83 ± 1.86%. At 6 months, 7 patients could be evaluated for AI and clinical response. Of these, 5 had an objective biochemical response and in 4 of them, where AI was possible to count, the mean increased by 1.94 ± 1.71%. One patient had SD and one had PD while AI remained unchanged or decreased by 1.23%, respectively. At 12 months 6 patients could be evaluated, 4 of whom had an objective biochemical response. The

Table 2

Relation of tumor progression after treatment and percentage of apoptotic cells identified by the TUNEL method in carcinoids of the gut

No.	Type of tumor	Duration of disease (months)	Treatment	Apoptotic index (%)		Clinical progress	
				1st biopsy	2nd biopsy	1st biopsy	2nd biopsy
1.	Carcinoid	1	IFN-α	7.56 ± 1.95	8.18 ± 2.29	OR-bio SD-radio	PD-bio SD-radio
2.	Carcinoid	75	IFN-α + chemo	4.69 ± 0.1 (untreated)	6.28 ± 0.25	PD-bio & radio	SD-bio & radio
3.	Carcinoid	43	IFN-α	2.01 ± 0.22	2.97 ± 1.25	PD-bio & radio	PD-bio SD-radio
4.	Carcinoid	7	IFN-α	2.87 ± 0.42	3.88 ± 0.49	PD-bio & radio	PD-bio & radio
5.	Carcinoid	1	IFN-α + octreotide	4.35 ± 0.78 (untreated)	3.97 ± 0.58	PD-bio & radio	OR-bio SD-radio
6.	Carcinoid	1	Octreotide	4.66 ± 1.97 (untreated)	4.91 ± 1.62	PD-bio & radio	PD-bio SD-radio
7.	Carcinoid	0	Lanreotide	5 ± 0.57 (untreated)	4.8 ± 1.41	PD-bio & radio	OR-bio SD-radio
8.	Carcinoid	0	Lanreotide	2.2 ± 0.57 (untreated)	2.2 ± 0.28	PD-bio & radio	OR-bio SD-radio

number of labelled cells increased by 4.22 ± 3.93% of the pre-treatment figure. At 12 months one patient showed biochemical and radiological PD while AI remained unchanged. Another patient, who had biochemical PD but no radiological change, showed a 3.08% decrease in apoptotic index (Table 1).

In the second part of the study, only two biopsies from another 8 patients were analyzed. Five patients had biopsies before treatment and after 6 months of treatment, while 3 had biopsies at 6-month intervals during treatment. Changes in AI in the second biopsies were less then 1% irrespective of treatment, and with the exception of one patient who was receiving IFN-α and chemotherapy, AI increased by 1.59% (Table 2).

Experimental tumors: Evaluation of in situ staining by TUNEL

In the octreotide-treated group of animals AI increased threefold (11.77 ± 2.77%) ($p = 0.0084$) as compared with the placebo group (4.02 ± 0.15%), while the increase in AI in the IFN-α (4.05 ± 0.33%) and the combination (5.27 ± 1.55%) groups was only marginal (Table 3) (Fig. 1).

Table 3

Summary of in situ identification of apoptotic cells in experimental neuroendocrine tumors after different treatments

No.	Groups	Apoptotic Index (%)
1.	Control	4.24 ± 0.27
2.	IFN-α treated	4.05 ± 0.33
3.	IFN-α + octreotide treated	5.27 ± 1.55
4.	Octreotide treated	11.77 ± 2.77*

* $p = 0.0084$ (group 1 vs. 4).

Growth variation

There were no significant differences in the final body weight between different treatment groups and the control group (data not shown). However, at day 28 the mean tumor volume was 4.07 ± 1.66 cm^2 in the control group. This was significantly different from the combination treatment group with a mean tumor volume of 0.74 ± 0.72 cm^2 ($p = 0.0011$). The mean tumor volume in the IFN-α treated group was 2.77 ± 1.02 cm^2 and 1.86 ± 0.98 cm^2 in the octreotide-treated group, which did not differ significantly from the control group (Fig. 2).

Gel electrophoresis of DNA

DNA laddering is one of the cardinal features of apoptosis and Fig. 3 indicates that the octreotide group had a more distinct laddering pattern than the other groups (Fig. 3).

Electron microscopy of BON-1 cells

Control BON-1 cells (Fig. 4a) showed a normal cancer cell morphology. Undifferentiated secretory granules were seen in the cytoplasm and surplus granules were eliminated by autophagy in secondary lysosomes (14). IFN-α treated cells (Fig. 4b) showed a morphology similar to that of the control group, i.e., an unaffected nucleus, undifferentiated secretory granules and granules under decomposition in secondary lysosomes. A convoluted cellular surface was seen in some cells. In many of the octreotide-treated cells (Fig. 4c) a typical apoptotic cellular morphology was seen with compaction of chromatin into sharply delineated masses lying close to the nuclear membrane and nuclear fragmentation. Condensation of cytoplasmic organelles and packaging into apoptotic bodies were not seen in many cells. Decomposed cells were often seen in this

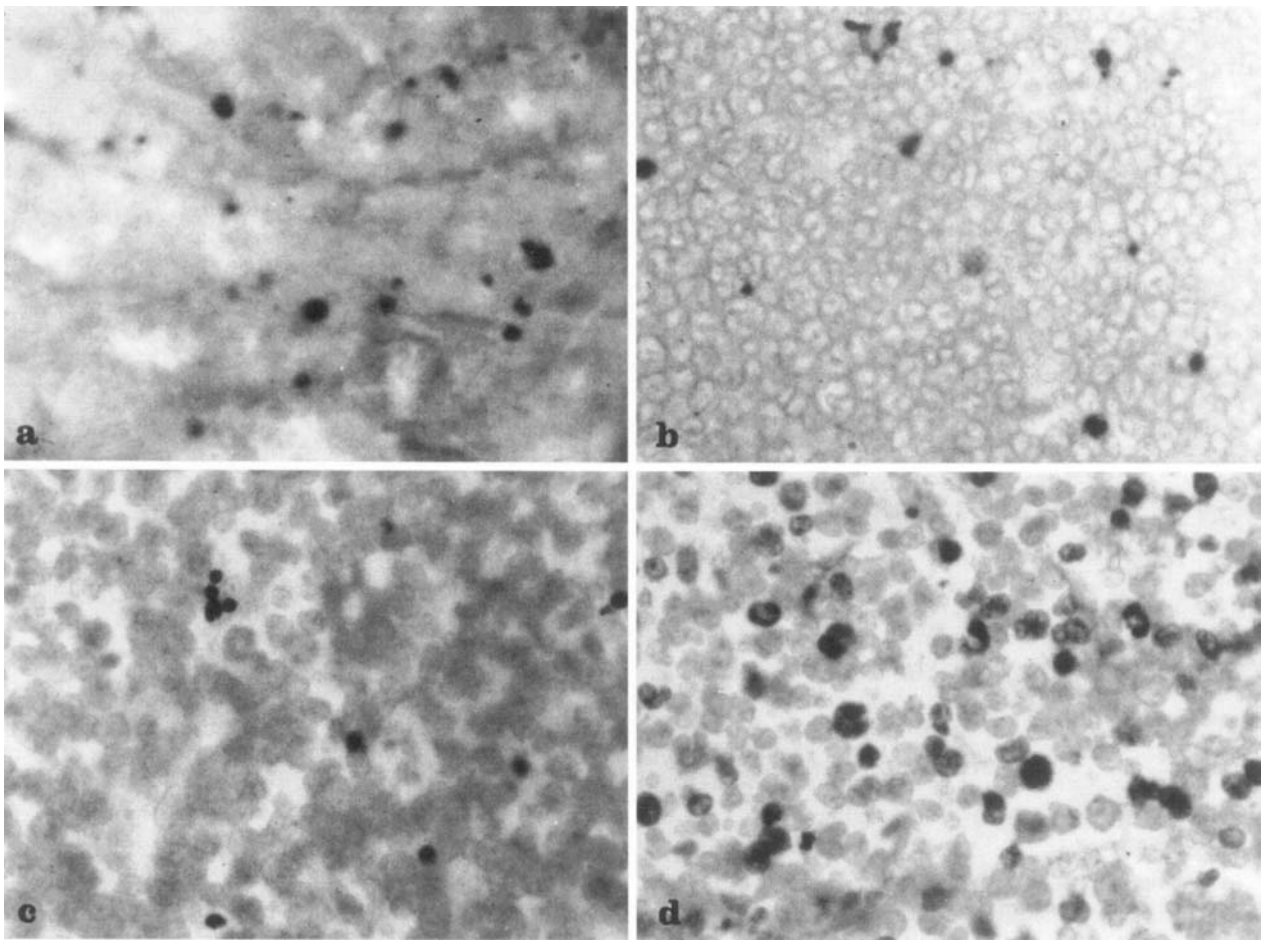

Fig. 1. In situ staining of experimental tumor tissues by the TUNEL method, showing increased number of apoptotic cells in octreotide-treated tumor. a) control group. b) IFN-α-treated group. c) IFN-α and octreotide-treated group. d) Octreotide-treated group. ×400.

experiment. BON-1 cells treated with IFN-α and octreotide (Fig. 4d) showed an apoptotic morphology as well. However, a typical apoptotic morphology was not as pronounced as in cells treated with octreotide alone.

DISCUSSION

Normal homeostasis of the tissue is maintained by the balance between cell proliferation and cell elimination. In tumors, the normal homeostasis rule is altered, which indicates either increased cell proliferation or decreased cell death (10). Perturbed apoptosis might be one of the factors involved in carcinogenesis. In the treatment of neuroendocrine tumors, particularly carcinoids, IFN-α and octreotide have demonstrated significant beneficial value. IFN-α and octreotide both improve clinical symptoms and may have a growth inhibitory effect on tumors in clinical studies (5). However, it is important to point out that significant tumor shrinkage is a rare event. In the present study we have been able to demonstrate some of the tumor growth inhibitory effects of somatostatin analogs in the experimental settings and antitumor effect in patients. In patients treated with a high dose of somatostatin analog (lanreotide, 12 mg/d), apoptotic cell number increased during treatment, being highest at 12 months of therapy, and the increase in AI was correlated to the biochemical response. However, there was no significant reduction in tumor size in patients with increased apoptosis. Among the patients included in the study, all showed progressive disease on CT-scan prior to start of high-dose lanreotide. Therefore stabilization of the disease for 6–12 months was considered a therapeutic response. It has previously been shown that during treatment with IFN-α, there is a biochemical response which cannot be correlated with a parallel reduction in tumor size by computerized tomography. However, if a biopsy is taken from a liver metastasis after treatment, it shows that tumor cells are replaced by fibrous tissue (15). Thus, the actual number of tumor cells has been reduced even though the tumor size remains unchanged. A similar mechanism might operate in patients treated with a high dose of somatostatin analog. Regular doses of somatostatin analogs did not significantly increase the AI and did not induce any change in tumor size.

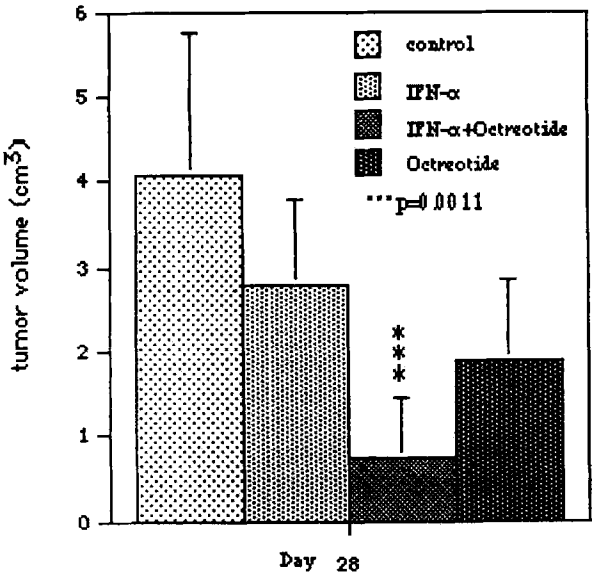

Fig. 2. Histogram showing mean tumor volume in mice of different treatment groups at day 28 of treatment. IFN-α and octreotide had an effect on growth inhibition but the effect was more pronounced in the combination group. ***p = 0.0011 (control vs. combination group)

Patients treated with IFN-α or a combination of IFN-α and octreotide did not show any significant increase in apoptosis.

In the animal model using the BON-1 cell line, IFN-α and octreotide both showed growth inhibitory effects which were significantly enhanced by the combination of both the drugs. Somatostatin analogs are known to induce

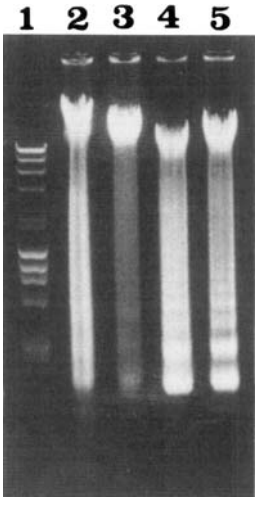

Fig. 3. Gel electrophoresis of DNA, extracted from experimental tumor tissue of mice showing laddering which is one of the hallmarks of apoptosis. Lane 1: DNA molecular weight marker. Lane 2: DNA from the control group. Lane 3: DNA from the IFN-α and octreotide-treated group. Lane 4: DNA from the IFN-α-treated group. Lane 5: DNA from the octreotide-treated group.

apoptosis (16). In this study, growth inhibition did not correlate with induction of apoptosis in animals receiving combination therapy. Animals receiving octreotide as a single drug showed tumor growth inhibition as well as increased AI. In our animal experiments, we have used a high dose of octreotide (300 μg/kg, t.i.d) compared with regular clinical total doses of 300–500 μg/d. This high dose is in the same range as high-dose lanreotide (12g/d) in the clinical study. Both somatostatin analogs bind to the somatostatin receptor subtypes 2 and 5 ($sstr_2$ and $sstr_5$) with high affinity (20). Recently, Srikant and co-workers have shown that apoptosis might be mediated through receptor subtype 3 ($sstr_3$) (pers. comm.). Octreotide and lanreotide are also binding to $sstr_3$ but have a tenfold lower affinity than $sstr_2$ and $sstr_5$ (20). This might explain the increased induction of apoptosis only with a high dose of octreotide but not with regular doses.

We did not observe any increase in induction of apoptosis by single IFN-α treatment in the tumor model and could not demonstrate any statistically significant tumor growth inhibition. The reason for this might be that IFN-α therapy was instituted 2 days after implantation of tumors in the mice. Similar results have been reported by Evers and co-workers (21), where they could demonstrate a growth inhibitory effect of IFN-α when therapy was started at the same time as tumor cells were inoculated but not when started after establishment of tumor.

Electron microscopic examination confirmed the results found by the TUNEL method. IFN-α-treated cells did not show any morphological difference from control cells. IFN-α plus octreotide-treated cells showed apoptotic changes but not such marked changes as those found in cells treated with octreotide alone. In octreotide-treated cells, most of them had typical morphological changes due to apoptosis. However, many cells were under decomposition, probably caused by too long an incubation in the same medium. Apoptosis is a quick process and morphological changes are early events on the way to cell death (22). It might explain why it is difficult to find typical features such as organelle compartmentation, membrane blebbing and apoptotic bodies in the apoptotic cells.

The results of this study show that tumors treated with octreotide had more apoptotic cells than controls, which indicates that octreotide induces apoptosis and is in agreement with the results of Szende and co-workers (16). There were fewer apoptotic cells in the combination group, while it had a more pronounced tumor growth inhibitory effect. There are reports that increased apoptosis does not correlate with tumor regression, but the patients presented a worse prognosis when tumor showed increased apoptosis (23, 24). This might indicate that a decrease in tumor mass is related more to cell growth inhibition than to cell death. Increased cell death could reflect increased cell proliferation without affecting the overall growth of the tumor (25). Apoptosis occurs in cells in the late G_1 and G_2 phases and

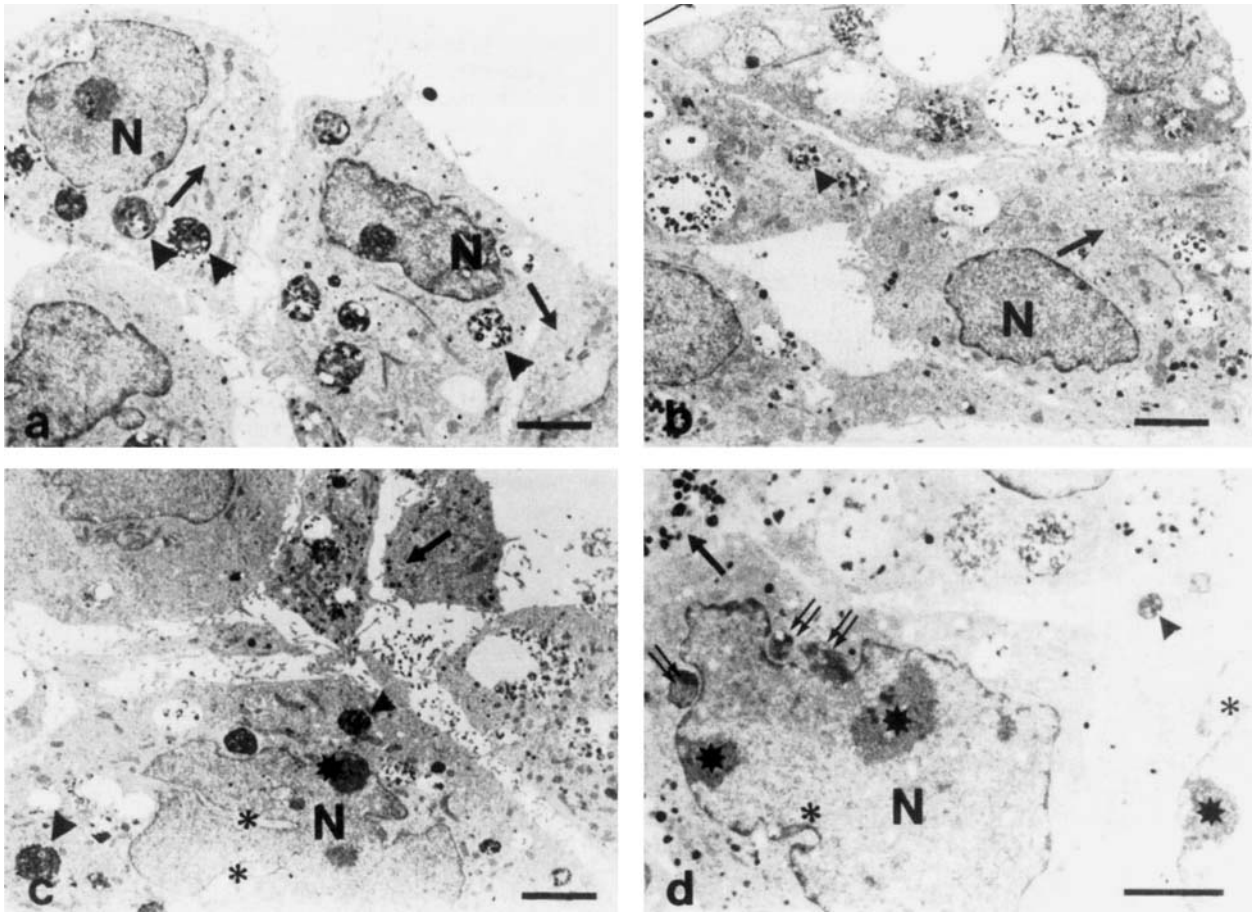

Fig. 4. Electron microscopic appearance of BON-1 cells. a) Control cells with normal appearance. × 3800. b) IFN-α-treated cells showing a morphology similar to the control cells. × 3800. c) Octreotide-treated cells with a typical apoptotic morphology. × 3800. d) Cells treated with IFN-α and octreotide have a clear apoptotic morphology but not to such a high degree as in cells treated with octreotide alone. × 5800. (→) = undifferentiated secretory granules, (▶) = autogranulophagy, (N) = nulceus, (✸) = condensed chromatin near the nuclear membrane, (⇉) = fragmented nucleus, (*) = deep invaginations into the nucleus. Bars = 2.5 mm.

not in those at G_0 and M (26). IFN-α binds to a specific receptor and causes cell-cycle arrest in G_0 or G_1 (27), so the cells remain at the resting phase without progressing to apoptosis. This might explain the unchanged number of apoptotic cells in the combination treatment and single therapy with IFN-α. In an in vitro study, it has been reported that IFN-α protects chronic lymphocytic leukemic cells from apoptosis (28). The enhanced growth inhibitory effect of the combination therapy may be related to other mechanisms, e.g. cell-cycle arrest, antiangiogenesis, reduction of growth factor/receptor expression or even differentiation, which has been described for IFN-α (29, 30). The role of apoptosis in the antitumor response is still obscure. High-dose somatostatin analog therapy needs to be further explored in clinical studies which are currently under clinical trials in patients with breast and pancreatic cancer.

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