Novel Therapy for the Treatment of Human Carcinoid

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Development of effective treatment for patients with carcinoid tumors has been hampered by lack of an experimental model. The authors have established the only long-term cell line of a functioning human pancreatic carcinoid tumor (BON) that produces tumors in nude mice. In this study the authors examined the effect of three agents, α-interferon (IFN), a somatostatin analog, SMS 201-995 (SMS), and an inhibitor of polyamine biosynthesis, α-difluoromethylornithine (DFMO), on the growth of BON tumors. BON was implanted bilaterally as 3-mm^2 pieces (subcutaneously [sc]) into male BALB/c nude mice. In the first study, 23 mice were randomized to four groups: control, IFN (1 × 10^6 units, sc, four times a day), IFN + SMS (300 μg/kg, intraperitoneally, three times a day), and IFN + 3% DFMO in drinking water. Treatments were initiated on day of tumor implantation. In the second study, mice were randomized to six groups: control, IFN, SMS, DFMO, IFN + SMS, IFN + DFMO, and IFN + SMS + DFMO. Treatments were started on day 15 after tumor implantation. Tumor area and body weights were measured weekly. In both studies mice were killed on day 28 after BON implantation and tumors removed, weighed, and analyzed for DNA and RNA content. In the first study, IFN either alone or in combination with SMS or DFMO suppressed BON tumor growth. When treatment was initiated after established tumor growth (study 2), however, the only effective treatments for suppression of growth of BON were IFN + DFMO and IFN + DFMO + SMS. It is concluded that combinations of these agents may offer an effective and relatively nontoxic approach for treatment of carcinoid tumors. This unique tumor will provide an excellent model to study effects of various treatment strategies.

DEVELOPMENT OF AN effective chemotherapeutic strategy for carcinoid tumors of the gut has been hampered by the relative rarity of the disease and lack of an *in vivo* model to evaluate tumor inhibition. In the past the treatment of carcinoid tumors of the gut has largely been directed toward symptomatic relief of the devastating sequelae of the carcinoid syndrome.[1-6] Combinations of various chemotherapeutic agents, evaluated in limited clinical trials, have been ineffective in slowing the growth of these tumors, and, in addition, treatment with these toxic agents is fraught with numerous debilitating side effects.[1,7-9]

We have been interested for years in the experimental investigation and development of novel and nontoxic agents for the treatment of gastrointestinal (GI) cancers. We have demonstrated that somatostatin, a peptide that blocks the release of growth hormone and all known GI hormones, and α-difluoromethylornithine (DFMO), an inhibitor of polyamine biosynthesis, can inhibit growth of animal and human gut cancers.[10-14] Recently we have shown that the long-acting somatostatin analog, SMS 201-995 (SMS), and DFMO suppress the growth of human pancreatic carcinoid (BON) xenografts in nude mice.[15]

Interferon-α (IFN) is a naturally occurring protein produced by leukocytes in response to viral infections.[16] Administration of large doses of IFN has been shown to inhibit growth of a wide range of cancers.[17-21] Recent studies have suggested that administration of IFN can suppress the growth of carcinoid tumors and ameliorate symptoms associated with carcinoid syndrome.[22-26] In addition, IFN alone appears to be less toxic and more effective in producing subjective relief of symptoms and tumor inhibition than the combination of streptozocin and 5-fluorouracil (5-FU).[27]

The purpose of the present study was to evaluate the effect of IFN, either alone or in combination with SMS or DFMO, on the growth of BON, a human pancreatic

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Presented at the 102nd Annual Scientific Session of the Southern Surgical Association, Boca Raton, Florida, December 3-6, 1990.

Supported by grants from the National Institutes of Health (PO1 DK 35608, 5R37 DK 15241) and by a grant from the American Cancer Society (PDT-220).

Dr. Evers is a recipient of an American Surgical Association Foundation Fellowship Award.

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Accepted for publication January 2, 1991.

carcinoid tumor that has been xenografted into nude mice. In the second part of this study, we evaluated these agents alone or in various combinations in the treatment of established BON tumor.

Materials and Methods

Human Carcinoid Tumor Line

A specimen of a metastatic carcinoid tumor of the pancreas was obtained 5 years ago from a 28-year-old man at the time of exploratory laparotomy. We successfully established a cell line, which we call BON, in tissue culture. BON is currently maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) and F12K (Gibco) in a 1:1 ratio supplemented with 10% (vol/vol) fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT); it is passed at a 1:2 ratio when cells reach 80% confluence. Cell cultures are monitored routinely for mycoplasma contamination using a fluorescent stain (Hoeschst #33258), and no mycoplasma growth has been detected. BON has retained its original histologic appearance, which is that of a poorly differentiated neuroendocrine tumor. BON cells possess gastrin and somatostatin receptors and synthesize serotonin and chromogranin A. Single-cell suspensions (1×10^7 cells) of BON cells will reliably produce discrete encapsulated tumors when injected subcutaneously into athymic nude mice.

Animals

Male athymic nude mice (BALB/c, 20 to 25 g, 3 to 4 weeks of age, Life Science, St. Petersburg, FL) were housed under pathogen-free conditions in a temperature-controlled isolation unit with 12-hour light and dark cycles in accordance with the National Research Council's Guide for the Care and Use of the Nude Mouse in Biomedical Research.[28] The mice were fed a standard chow (Autoclavable Rodent Chow #5010, Ralston Purina, St. Louis, MO) and sterile water, both given *ad libitum*.

Experimental Design

In both experiments, dispersed BON cells (passage 8) from tissue culture were inoculated subcutaneously (sc) in four to eight nude mice (1×10^7 cells/injection) to establish tumors. When tumors became approximately 10 cm^2 in area, the mice were killed and tumors minced into 3-mm^2 pieces, which were then implanted bilaterally into the flanks of nude mice.

In the first experiment, 23 mice were randomly allocated to receive either saline (0.1 mL, intraperitoneally [ip], three times a day, and 0.1 mL, sc, every morning), IFN (recombinant interferon-α-2a, 1×10^6 units, sc, every morning) alone or in combination with SMS (300 μg/kg, ip, three times a day), or 3% (wt/vol) DFMO (a gift from W. J. Hudak, Ph.D., Manager of Research Information at the Merrell Research Center, Cincinnati, OH) in drinking water. Dosages for SMS and DFMO were determined from previous studies.[13-15] The dosage of IFN was determined from a pilot study that showed this to be the maximally effective and nontoxic dose (data not shown). All treatments were started on the day of tumor implantation and continued until killing. Water bottles were covered to prevent light degradation. Drinking water was renewed every 2 days. SMS, a gift of Sandoz Research Institute, Hanover, NJ, and IFN, a gift of Hoffman La Roche Laboratories, Nutley, NJ, were diluted to the required concentration with saline.

The mice were weighed weekly; the tumors were measured twice weekly by the same observer with Vernier calipers (Mitutoyo Corporation, Tokyo, Japan) that were accurate to 0.5 mm. Surface areas of the tumors were calculated as the product of the two greatest perpendicular tumor diameters, and were expressed in square millimeters. Mice were killed on day 28 by cervical dislocation, and tumors were removed, weighed, and frozen at −70 C until assayed for DNA and RNA content.

In the second experiment, BON tumor chunks were implanted bilaterally into the flanks of 40 athymic nude mice. On day 14, all mice were weighed and tumors measured. The mice were then randomized to seven treatment groups: control (saline, 0.1 mL, ip, three times a day, and 0.1 mL sc, every morning), IFN (1×10^6 units, sc, every morning), SMS (300 μg/kg, ip, three times a day), 3% DFMO, IFN + SMS, IFN + 3% DFMO, or the combination of all three agents. Treatment was started on day 15 and continued until killing. Body weights and tumor areas were measured twice weekly. Mice were killed on day 28 after tumor implantation. Tumors were removed, weighed, and frozen at −70 C until analysis.

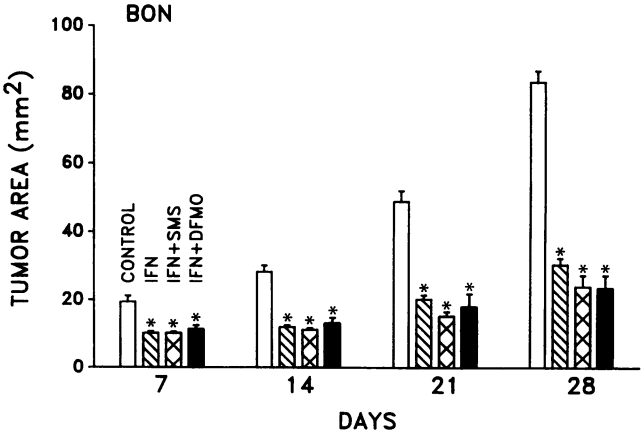

FIG. 1. BON tumor area (mm^2) in relation to time from implantation comparing interferon-α (FN) (1×10^6 units, sc, every morning; single-hatched bars, n = 12 tumors), IFN + SMS 201-995 (300 μg/kg, ip, tid; double-hatched bars, n = 12 tumors); and the combination of IFN + 3% DFMO (closed bars, n = 10 tumors) to control group (open bars, n = 12 tumors). (* = $p < 0.05$ *versus* control, one-way ANOVA).

DNA and RNA Analysis

Tumors were thawed, homogenized, extracted, and analyzed for DNA and RNA by methods that we have previously described.[29]

Statistical Analysis

Results are expressed as the mean ± standard error of the mean and were analyzed using one-way analysis of variance (ANOVA) at the 0.05 level of significance. Mean values were separated using Fisher's least significant difference procedure.

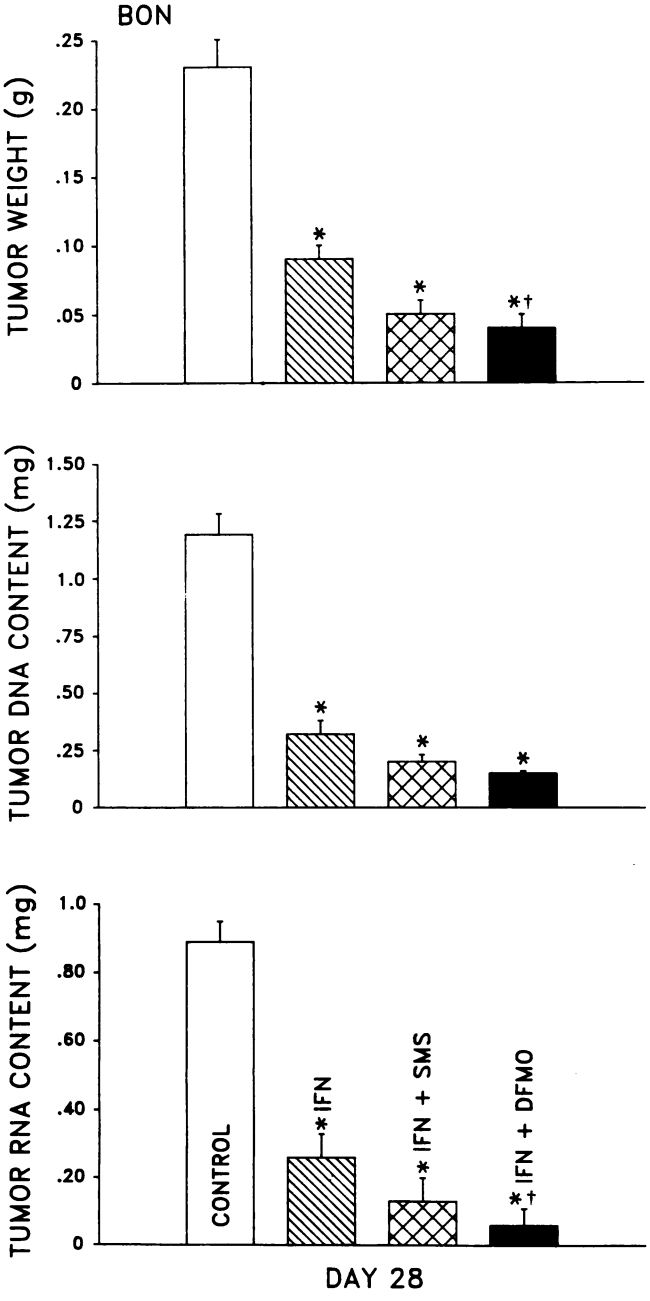

FIG. 2. Tumor weight (g), DNA and RNA content (mg) of BON tumors in control group (open bars, n = 12), IFN group (single-hatched bars, n = 12), IFN + SMS 201-995 (double-hatched bars, n = 12), IFN + DFMO (closed bars, n = 10) when killed (* = $p < 0.05$ *versus* control; + = $p < 0.05$ *versus* IFN; one-way ANOVA).

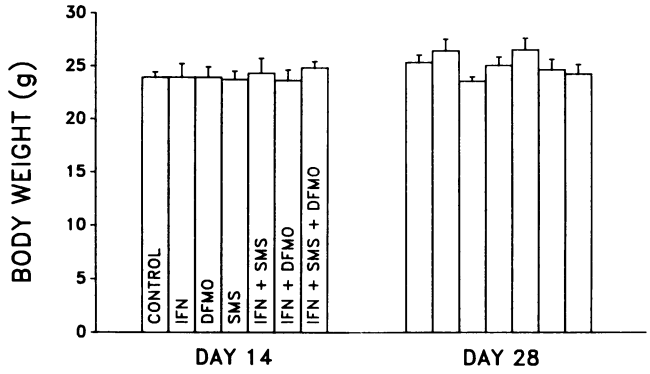

FIG. 3. Body weights (g) of mice at time of initiation of treatment (day 14) and final body weights when killed (day 28).

Results

Experiment 1

There were no significant differences in final body weight in treated mice compared with the control group. Food and water intake were monitored and there were no differences in consumption between groups (data not shown).

Figure 1 demonstrates BON tumor progression in square millimeters over the 28-day treatment period. IFN, administered alone or in combination with either SMS or DFMO, significantly inhibited tumor area by day 7; inhibition continued to the time of killing (day 28). Tumors treated with IFN + DFMO were significantly smaller than those treated with IFN alone. Tumor weight, DNA, and RNA contents are shown in Figure 2. The mean tumor weight of the IFN-treated group was 63% of that of the control group; DNA content was 73% and RNA content 71% of controls. Combining SMS with IFN produced no further decreases in tumor values compared with IFN alone; however, the combination of IFN + DFMO was statistically more effective in suppressing tumor weight and RNA content.

Experiment 2

Body weights of mice were similar at the time treatment was begun, and there were no differences in final body weights at time of killing (Fig. 3).

Tumor areas were similar in all groups before initiating treatment (Fig. 4). After 2 weeks of treatment, only 3% DFMO, as a single agent, was effective in inhibiting final tumor area. Inhibition of BON tumor area by IFN + DFMO and the combination of all three agents was similar to that of DFMO alone (Fig. 4).

Tumor weights and DNA content are shown in Figure

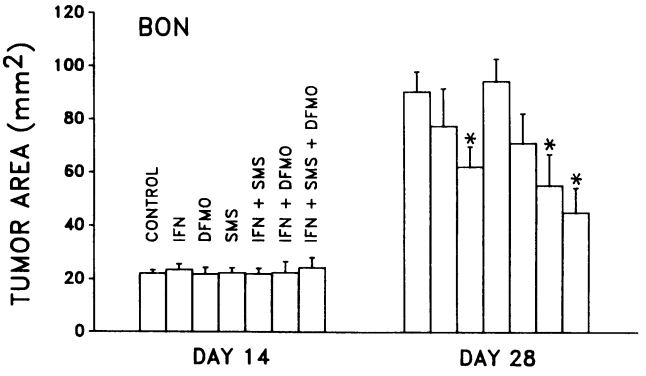

FIG. 4. BON tumor area (mm^2) at time of initiation of treatment (day 14) and final tumor area after 2 weeks treatment (day 28).

5. Similar to results of tumor area, neither IFN nor SMS were effective in suppressing tumor weight or DNA when administered as single agents. Although tumor area was suppressed by DFMO alone, weight and DNA content were not affected. IFN + DFMO and the combination of all three agents significantly inhibited BON weight and DNA content. Analysis of the seven groups by ANOVA demonstrated no significant differences in tumor RNA content ($p = 0.08$); however the same general trend in mean values exists as noted with area, weight, and DNA, even though statistical significance was not obtained (Table 1).

Discussion

The human pancreatic carcinoid cell line, BON, grows as discrete masses when injected subcutaneously into athymic nude mice, thus providing a unique opportunity to evaluate *in vivo* effects of various chemotherapeutic agents on the growth of a functioning human foregut carcinoid tumor.

Recently several clinical studies have evaluated the effect of IFN on the growth and symptomatic relief in patients with carcinoid tumors.[22–26] Significant subjective improvement in symptoms associated with decreases in 5-HIAA levels was reported, with median duration of response varying from 6 to 34 months. Tumor size remained stable or actually decreased in 40% to 80% of patients in these series. In addition, in a randomized controlled study of IFN compared with combination treatment of streptozocin and 5-FU, IFN treatment was judged superior based on a significantly higher proportion of patients who demonstrated stable disease and less side effects. Other reports of IFN therapy for carcinoid tumors have been less encouraging. Initial good results were short-lived in a series by Moertel and colleagues,[30] who noted subjective improvement in 65% of patients and a decrease in tumor size in 20%; however, the median duration of response was only 7 weeks. In our present study, IFN, administered as a single agent, inhibited growth of BON when treatment was initiated at time of tumor placement; however, when treatment was begun after established tumor growth, no significant inhibitory effects were noted.

We[31] recently reviewed the effects of somatostatin on growth of both solid and endocrine cancers. Antitumor effects of somatostatin appear to be mediated both by a direct action by specific somatostatin receptors and by an indirect effect by decreasing secretion of gut hormones and other growth factors. In contrast the action of DFMO is quite specific. α-Difluoromethylornithine blocks production of polyamines by inhibiting ornithine decarboxylase, the rate-limiting enzyme in polyamine biosynthesis.[32] Polyamines are the building blocks of proteins and are essential for rapidly dividing tissues. We have previously shown that the long-acting somatostatin analog, SMS, and DFMO were equally effective in inhibiting BON tumor growth when administered at time of tumor placement.[15] In the present study, only DFMO was effective as single-agent therapy in suppressing area of established BON tumors. Tumor weight, DNA, and RNA contents were not suppressed with any of the agents administered alone. From these results, it appears that these agents, at the doses administered, are not effective as single-agent therapy in suppressing established tumor growth.

The combination of chemotherapeutic agents that block tumor growth at different steps in the cell cycle are more effective than single-agent therapy. Similarly, in our pres-

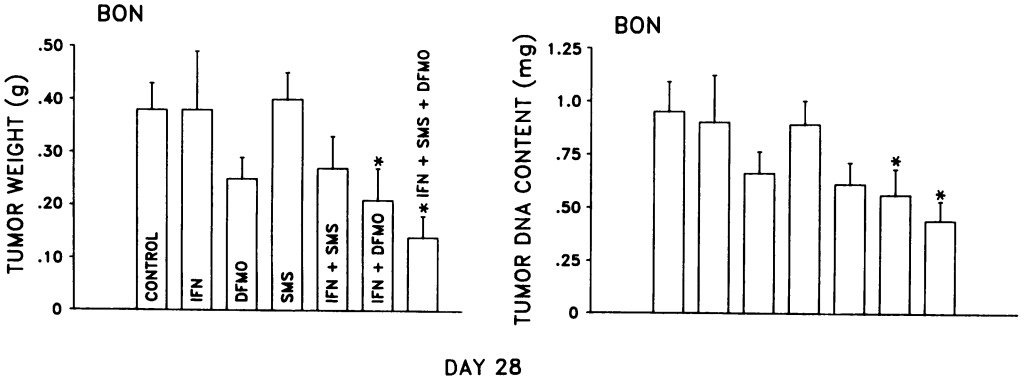

FIG. 5. BON tumor weight (g) and DNA content (mg) comparing interferon-α (IFN) (1×10^6 units, sc, every morning), SMS 201-995 (300 μg/kg, ip, tid), 3% DFMO, IFN + SMS, IFN + DFMO, and IFN + SMS + DFMO. (n = 10 to 12 tumors/group; * = $p < 0.05$ *versus* control, one-way ANOVA).

TABLE 1. *Effect of Therapy on BON Tumor RNA Content*

	Tumor RNA Content (mg)
Control	2.0 ± 0.3
IFN	2.1 ± 0.6
DFMO	1.5 ± 0.3
SMS	2.3 ± 0.3
IFN + SMS	1.5 ± 0.3
IFN + DFMO	1.4 ± 0.3
IFN + SMS + DFMO	0.9 ± 0.2

Values indicate the mean ± SEM (n = 10 to 12 tumors/group).

ent study, various combinations were found to be more effective in suppressing BON growth; however, unlike traditional chemotherapeutic regimens, there was not an associated increase in morbidity rate. Combining IFN with DFMO inhibited BON tumor weight and RNA content more than IFN alone when treatment was started at time of tumor implantation. The combination of IFN + DFMO and IFN + DFMO + SMS suppressed established BON tumor growth when compared with controls in the second study. Mean values of area, weight, and DNA content were decreased in those tumors treated with all three agents; however these did not represent significant differences compared with DFMO alone or IFN + SMS or DFMO. It is possible that a longer treatment period would have demonstrated a greater tumor suppression after administration of all three agents.

The molecular mechanisms responsible for the antitumor effect of IFN are not completely understood. α-Interferon induces a specific protein kinase that phosphorylates eukaryotic-initiation-factor 2 (eIF-2), which prevents subsequent translation[33,34] and, therefore, suppresses protein synthesis. An additional possible mechanism of IFN action could be a reduction of c-*myc* mRNA levels, which has been noted in correlation with a cessation of growth after IFN treatment; however these findings appear tissue specific and do not occur in all circumstances.[35]

The mechanism underlying the enhanced suppression of BON tumor with interferon in combination with DFMO is not clear. Sunkara and colleagues[36] noted a similar effect of IFN combined with DFMO in treating a melanoma cell line and a human lung cancer line. In their study, polyamine levels were decreased after DFMO but not IFN. We could postulate that tumor inhibition is attained at multiple levels (*e.g.*, inhibition of polyamine biosynthesis, protein translation, and oncogenes associated with cell growth), and, therefore, this therapy is more effective because of a block in multiple pathways necessary for cell growth.

In conclusion we found that IFN alone suppresses BON tumor growth when started at the time of initial tumor placement; however, there was no effect on established tumor growth when given alone. Combination of IFN with DFMO or IFN + DFMO + SMS produced significant suppression of established tumor growth without an associated increase in toxicity. Therapeutic use of these agents in combination may offer an effective and relatively nontoxic alternative in the treatment of carcinoid tumors. The tumor line BON should prove to be a useful model to determine the most effective treatment schedules and dosages.

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Discussion

DR. JOHN MCDONALD (Shreveport, Louisiana): It is not surprising that a somatostatin analogue and/or an inhibitor of polyamine synthesis will inhibit the implantation of tumor. There are data from others that suggest that growth factors and polyamine are required for metastases to become established. And I suppose the implantation of these tumor cells is more akin to the metastatic process than to *de novo* tumor growth, which probably explains why there was a less impressive effect on established tumor growth in their second experiment.

The effect of interferon is more perplexing, because, to my knowledge, the known pathways of interferon action involve primarily its effect on thymic-derived lymphocytes, of which the nude mice has none.

I would ask the investigators how they envision interferon producing its effect in this model. Second the paper suggests that this may be a means of treating carcinoid tumor. What evidence have we to believe that these cells, passed in culture many times and implanted into an immunologically incompetent animal, behave in any way like the original tumor? For example, does it metastasize? Does it grow to kill the animal, and so forth?

Finally will these agents affect other cell lines of human tumor or some of the many established animal tumors available for study? In other words, how specific or general is the effect produced here?

The concept of developing tumor treatment with nontoxic biologic agents seems to me to be very important, and these studies are encouraging steps in that direction.

DR. MARSHALL URIST (Birmingham, Alabama): The authors appear to have developed a very challenging model that, in many ways, repeats the properties of the naturally occurring tumor.

In the experiments that they have shown, treating the tumors at the time of implantation has been effective; however when established tumors are present, the challenge is much greater.

I, therefore, would like to ask what happens when treatment is continued beyond 2 weeks in those animals that have therapy established after the tumor has begun to grow? Are there any circumstances under which actual tumor regression can be observed?

The use of these agents in human trials initially appears to be rewarding; however in further trials the high response rates are found to be brief and, in fact, there has been no demonstration that treatment with these particular agents has any effect on overall patient survival.

Because these are functioning tumors, is there any evidence that the use of these different agents does, in fact, inhibit the proliferation of these different compounds from the tumors?

What is the mechanism of action in this situation, especially the combination of all three agents?

DR. PAUL JORDAN (Houston, Texas): I just wondered, Dr. Townsend, what the definition for a carcinoid was. I think you gave us that in the beginning, but I wonder—where does the microcarcinoidosis of the stomach that we are hearing about now fit? That is supposed to come from an ECL-like cell. Is it a different kind of carcinoid? Do you expect this method of treatment to work for all carcinoids, regardless of what hormone the tumor makes?

DR. MARK EVERS (Closing discussion): Dr. McDonald asked about the mechanism of action of α-interferon in our model. Specifically was this a direct antitumor effect or were the effects indirect as a result of enhancing the immunologic system? We believe that the effects of interferon were directly mediated. Studies have shown that α-interferon can directly inhibit protein synthesis at either the transcriptional or translational level. In addition we have treated BON cells *in vitro* with α-interferon and have noted a similar, dose-related inhibition of tumor growth.

The second question was regarding the characteristics of our tumor line compared with the original patient tumor. For all of our studies, we use BON cells in early passage (that is passages 8 to 12). Histologically, these tumors are identical to the original tumor. In addition, they produce serotonin, pancreastatin, neurotensin and chromogranin A as was noted from the original operative specimen. The BON tumors in nude mice grow as discrete tumors and do not metastasize.

The last question was whether we have examined the effect of somatostatin or DFMO on the growth of other tumors. Our laboratory has worked extensively with both agents and have found a wide range of inhibitory effects on pancreatic, stomach, and colon cancers.

Dr. Urist asked whether these agents decrease either the synthesis or release of secretory products. We have not studied the effects of either DFMO or α-interferon on secretion; however we have studied the effects of somatostatin and found a decrease in both the synthesis and release of serotonin.

Finally Dr. Jordan asked whether gastric carcinoid tumors arise from the same cell type. To our knowledge, carcinoid tumors have been described in almost every organ in the body and all arise from Kulschitzsky cells.