LH-RH receptors in human colorectal cancers: Unexpected molecular targets for experimental therapy

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Abstract. Since the efficacy of chemotherapy can be enhanced by targeting to specific receptors on tumors, we investigated the expression of LH-RH receptors in 5 human colon cancer lines and the effects of cytotoxic LH-RH analogs on these tumors. Nude mice bearing HT-29, HCT-116, HCT-15, LoVo and Colo-320DM cancers were treated with cytotoxic LH-RH analogs AN-152 and AN-207 or their respective cytotoxic radicals doxorubicin (DOX) and 2-pyrrolino-DOX (AN-201). The reduction in tumor growth was evaluated, and cell proliferation characteristics as well as apoptosis were analyzed by histological methods. LH-RH receptors on the tumors were investigated by radioligand binding assays and their mRNA expression by reverse transcriptase-polymerase chain reaction (RT-PCR). All 5 colorectal cancer lines expressed high affinity binding sites for LH-RH, and mRNA for the LH-RH receptors. Both cytotoxic LH-RH analogs AN-152 and AN-207 powerfully inhibited growth of all colon cancers. AN-207 had the strongest effect on HT-29 and HCT-116 tumors, and AN-152 was the most effective on Colo-320DM cancers. Cytotoxic radicals AN-201 and DOX were less effective on these 3 tumors, but had effects similar to AN-152 and AN-207 on HCT-15 and LoVo carcinomas. The four cytotoxic compounds also differently affected apoptosis and proliferation rate of the various tumor lines. Our findings suggest that cytotoxic LH-RH analogs should be considered for the therapy of patients with advanced colorectal carcinoma.

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Introduction

Colorectal cancer is the third most frequent cancer in the Western world (1-4). Over 145,000 new cases of colorectal cancer were diagnosed in 2005 in the US with an estimated 56,000 deaths (1). In Europe, about 660,000 patients suffer from colon carcinoma and approximately 180,000 die yearly of this malignancy (2-4). The incidence of colorectal cancer is also increasing rapidly in Japan, a country once considered to have a very low occurrence of the disease (5). In patients diagnosed at an early stage of colorectal cancer, surgical excision followed by adjuvant radiation or chemotherapy leads to a high degree of response and improves the survival rates (6). However, therapeutic options for advanced or disseminated cases are limited, and the responses to treatment are generally temporary (7,8). Thus there is an urgent need for the development of new, more efficient therapeutic modalities.

With increased understanding of molecular alterations that lead to malignant transformation of normal cells, more and more potential therapeutic targets are being discovered in tumors. Recently important advances in targeted therapy were made (9-13). Thus Willett *et al* have shown that Bevacizumab (Avastin), a humanized monoclonal antibody directed against VEGF, inhibits angiogenesis and has antivascular effects in human rectal cancers (9). Bevacizumab given in combination with conventional chemotherapy significantly improved survival of patients with metastatic colorectal cancer (10). Another targeted therapy for colorectal cancer is based on Cetuximab (Erbitux) a monoclonal antibody against extracellular domain of EGF receptor. Cetuximab used alone or in combination with irinotecan induced tumor regression in patients with colorectal cancer (11). We demonstrated earlier that receptors for certain peptide hormones which are highly expressed in colon cancers, can also serve as therapeutic targets (12). Thus, the treatment with a cytotoxic somatostatin analog powerfully inhibited the growth of human experimental colon cancers that expressed somatostatin receptors (13). These results encouraged us to seek other peptide hormone receptors in colorectal carcinoma for targeted chemotherapy.

The hypothalamic hormone LH-RH is the primary regulator of gonadal functions and reproduction in vertebrates (14). Receptors for LH-RH have been demonstrated in healthy sex organs, as well as in breast, ovarian, endometrial and prostate

cancers (4,12,14-16). On the basis of the presence of receptors for LH-RH on these tumors (12,16), we developed a new class of targeted antitumor agents by linking cytotoxic radicals to LH-RH agonists (12). Thus, we coupled DOX-14-*O*-hemiglutarate to [D-Lys^6]LH-RH to form targeted cytotoxic analog AN-152. An even more potent hybrid molecule (AN-207) was synthesized by conjugating 2-pyrrolino-DOX (AN-201), a daunosamine-modified derivative of DOX, which is 500-1,000 times more active *in vitro* than its parent compound, conjugated to the same carrier (14,16). These cytotoxic analogs AN-152 and AN-207 powerfully inhibited the growth of various experimental tumors expressing LH-RH receptors (16). Colorectal carcinoma is not closely related to tumors of the genital or endocrine system, but several studies suggest that the occurrence and progression of colon cancers is affected by sex hormones (4,15,16). The incidence rate of colorectal cancer is lower among women than men, particularly in the age group under 59 years and mortality rates are also slightly worse in men than in women (17). The presence of sex steroid receptors in human colorectal cancers was shown a long time ago (18,19) and confirmed by more recent studies (20,22). Some studies suggest that oral contraceptives and hormonal replacement therapy may decrease the risk of colorectal cancer (23,24), but there are conflicting reports whether estrogen receptors and estrogens have a protective effect against colon carcinoma or a stimulatory action (22,25).

High levels of receptors for LH-RH have been demonstrated in sex steroid-dependent tumors such as breast and prostate cancers, and also in malignancies that are not directly influenced by the pituitary-gonadal axis. These include pancreatic cancers, renal cell carcinomas, hepatic cancers, nervous system tumors, oral and laryngeal carcinomas and melanomas (4,16,26). In fact, a recent study indicated that xenografts of colon carcinoma express binding sites for LH-RH, and a bacterial exotoxin-containing LH-RH hybrid inhibited growth of colorectal tumors in nude mice (27). Consequently, we investigated the expression of LH-RH receptors in five human colon cancer lines xenografted into nude mice and the antitumor effects of our cytotoxic LH-RH analogs AN-152 consisting of doxorubicin (DOX) linked to [D-Lys^6]LH-RH and AN-207 containing a highly potent derivative of DOX, 2-pyrrolino-DOX (AN-201) coupled to the same peptide carrier (12,14,16,26,28,29).

Materials and methods

Chemicals. LH-RH agonist [D-Lys^6]LH-RH (pyroGlu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2) carrier was synthesized in our laboratory by solid-phase method. DOX x HCl salt was purchased from Chemex Export-Import GmbH (Vienna, Austria). Cytotoxic LH-RH conjugates AN-152 (DOX-14-*O*-hemiglutarate coupled to [D-Lys^6]LH-RH), and AN-207 (DOX derivative AN-201 (2-pyrrolino-DOX) coupled to the same carrier) were synthesized as described (30). Cytotoxic somatostatin analog AN-238 was prepared by coupling one molecule of AN-201 to somatostatin analog RC-121 (D-Phe-Cys-D-Trp-Lys-Val-Cys-Thr-NH_2) (31). Chemicals, unless stated otherwise were purchased from Sigma (St. Louis, MO). For treatment, the cytotoxic compounds were dissolved in 0.01 M aqueous acetic acid and diluted with 6% (w/v) aqueous D-mannitol, and 0.2 ml/20 g body weight was injected of this solution.

Animals and tumors. Male athymic nude mice (Ncr nu/nu) were obtained from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD) and maintained under pathogen-limited conditions. HT-29, HCT-116, HCT-15, LoVo and Colo-320DM human colon cancer lines were purchased from the American Type Culture Collection (ATCC) (Manassas, VA) and maintained in culture as recommended by ATCC (13). Colo-320DM cells were cultured in RPMI-640 medium containing 10% heat-inactivated fetal bovine serum. To obtain tumor donor animals, 10^6 tumor cells/mouse were injected s.c. into groups of three nude mice. The resulting tumors were dissected, minced and 2 mm^3 pieces of tumor tissue were transplanted s.c. to both flanks of the experimental animals. When the tumors became measurable, groups were formed with about equal average tumor sizes and control and treatment groups were selected at random. All experiments were carried out in accordance with the institutional ethical guidelines for animal care.

Experimental protocol. In Experiment 1, the treatment of mice bearing HT-29 cancers was started 14 days after transplantation of tumors and was continued for 55 days. Groups of 9 mice were treated as follows: 1. Control, vehicle only; 2. AN-207, 200 nmol/kg; 3. AN-201, 100 nmol/kg; 4. AN-152, 20.7 μmol/kg; 5. DOX, 20.7 μmol/kg (13.6 mg/kg). The compounds were given i.p. on days 1 and 28, but DOX was injected only once on day 1. The dose of AN-201 was reduced because of its previously determined i.p. toxicity.

In Experiment 2, the mice with HT-29 tumors were treated i.v. with 150 nmol/kg AN-207, AN-201 or AN-238 on days 1, 15, 36 and 57. The expression of somatostatin receptors in HT-29 cancers was demonstrated earlier (13). In the present study we compared the effects of a cytotoxic somatostatin and LH-RH analog in the same experiment. The controls received vehicle. The groups consisted of 7 mice and the treatment was started 20 days after transplantation of tumors. The experiment was terminated on day 62.

In Experiments 3, 4, 5 and 6 five groups were formed: 1. Control, vehicle only; 2. AN-207; 3. AN-201; both given at 150 nmol/kg dose; 4. AN-152; 5. DOX; both given at 6.9 μmol/kg dose i.v. In Experiments 3, 4, and 5 the groups were composed of 7 mice. In Experiment 6, we used 6 mice per group. In Experiment 3, the treatment of the mice bearing HCT-116 cancers was initiated 21 days after transplantation. The mice were injected with AN-207 or AN-201 on days 1, 11, 28, 49 and AN-152 or DOX on days 1, 7, 11, 15, 22, 28, 49. The mice were sacrificed on day 70. In Experiment 4, 24 days after transplantation of HCT-15 tumors, the mice were grouped and treated according to the same schedule as in Experiment 3. The animals were sacrificed on day 61.

In Experiment 5, the treatment of mice bearing LoVo cancers was initiated 23 days after transplantation. The groups had 7 mice and were treated with AN-207 and AN-201 on days 1, 15, 28 and 49, or with AN-152 and DOX on days 1, 8, 13, 18, 22, 28 and 49. The experiment was terminated on day 56.

The nude mice with Colo-320DM cancers (Experiment 6) were grouped 25 days after transplantation and treated as those in Experiment 3. The experiment ended on day 63. Body weights and tumor sizes were measured weekly and tumor volume was determined as length x width x height x 0.5236. Tumor growth reduction was calculated with the formula: TGR % = 100-100X (T-t)/(C-c), where t and c = the volume of treated and control tumors, respectively, at the beginning of therapy, and T and C = volume of the same tumors at the end of the experiment. The mice were sacrificed by decapitation under Metofane (Methoxyflurane, Shering-Plough, Union, NJ) anesthesia.

Histological analysis. Tumor specimens were fixed in 10% buffered formalin and were embedded in paraffin. Mitotic and apoptotic cells were identified by their morphologic appearance. They were counted in sections stained with hematoxylin-eosin and their numbers per 1,000 cells were accepted as the mitotic and apoptotic indices, respectively. Nucleolar organizer regions (NOR) in tumor cell nuclei were demonstrated with the AgNOR method as described (32). The number of AgNOR granules is an indicator of cell proliferation. The silver-stained black dots in 50 cells of each tumor were counted, and the AgNOR number per cell was calculated.

Receptor assay. For the characterization of membrane receptors for LH-RH, ligand competition assays were used by analyzing the binding of radiolabeled [D-Trp^6]LH-RH to tumor membrane homogenates. The preparation of membrane fractions, the radioiodination of [D-Trp^6]LH-RH and measurement of protein concentration of the homogenates have been described (33,34). The LIGAND PC computerized curve-fitting program was used to determine the type of receptor binding, the maximal binding capacity and the dissociation constant of the receptors (34).

Radioimmunoassay (RIA). LH was determined by RIA using material provided by the National Hormone and Pituitary Program (NHPP, Rockville, MD) (rat LH-RP-3/AFP-71871B/rat LH-I-9/AFP-10250C, anti-rat LH-RIA-11/AFP C 697071P). Serum testosterone levels were determined by Coat-A-Count RIA kit from Diagnostic Products Corp. (Los Angeles, CA, USA). Interassay and intraassay coefficients of variations were less than 15% and 10% respectively for both assays. GH was determined by using materials provided by the NHPP (GH-RP-2/AFP-3190B, rat GH-I-6/AFP-5676B, and anti-rat GH-RIA-5/AFP-411S).

RNA isolation and reverse transcription (RT)-PCR. RNA was extracted using the MicroRNA Isolation Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions and samples were reverse-transcribed to cDNA using the GeneAmp RNA Core Kit (Perkin-Elmer, Foster City, CA) according to instructions. The samples were PCR-amplified for ß-actin and human LH-RH receptor using the GeneAmp RNA Core Kit. Specific primers for ß-actin and human LH-RH receptor, as well as PCR conditions were described previously (33-35). PCR products were then subjected to electrophoresis on a 1.8% agarose gel, stained with ethidium bromide, visualized under UV light and quantified using Kodak EDAS 290 Imaging System with Kodak 1D Image analysis Software.

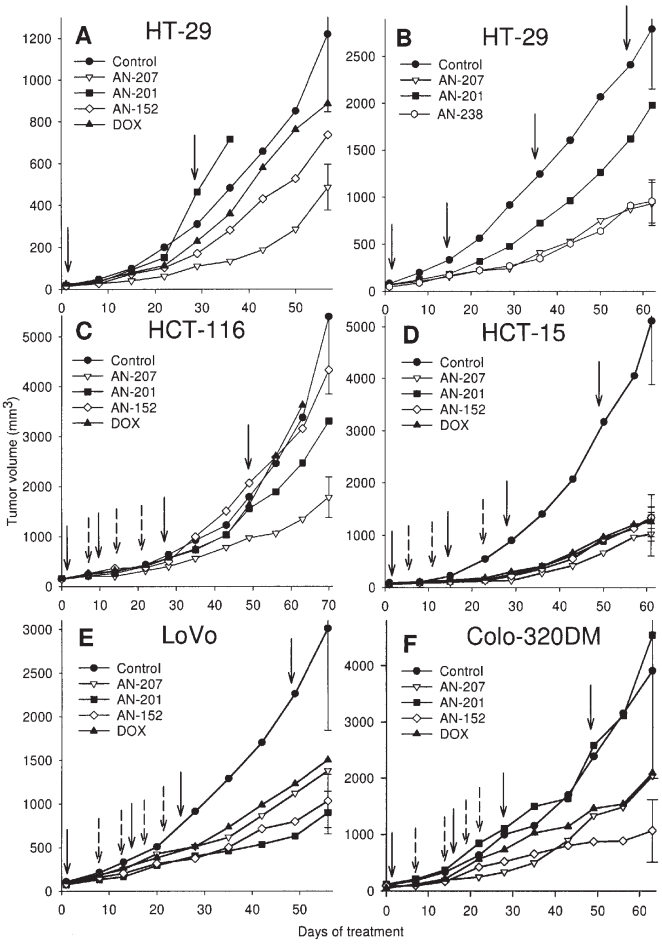

Figure 1. Effect of treatment with cytotoxic LH-RH analogs AN-207 and AN-152, as well as their respective cytotoxic radicals AN-201 and doxorubicin (DOX) on growth of various human colon cancers in nude mice. In experiment 2 (B) cytotoxic somatostatin analog AN-238 was given for comparison. The vertical bars show SE. Vertical arrow, treatment with all compounds; Vertical dotted arrow, treatment with AN-152 and DOX.

Relative mRNA levels of hLH-RH were normalized versus the corresponding levels of human ß-actin.

Statistical analysis. The SigmaStat software (Jandel Scientific, San Raphael, CA) was used for the statistical evaluation of the data. Differences between mean values were analyzed by one-way ANOVA, and the groups were compared using Dunnett's method.

Results

Effect of treatment on tumor growth. In Experiment 1, AN-207, given i.p. at 200 nmol/kg dose on days 1 and 28, significantly inhibited growth of HT-29 cancers as shown by the reduction in tumor volume and tumor weights (Fig. 1A, Table I). The first injection of AN-152 resulted in a moderate growth reduction, but the second dose was practically ineffective. The cytotoxic radicals AN-201 and DOX had no effect (Fig. 1A, Table I). Among the histologic characteristics analyzed, mitotic and apoptotic indices were not changed by the treatments, but AgNOR numbers were decreased in the groups receiving AN-207, AN-152 or DOX.

In Experiment 2, treatment with 4x150 nmol/kg AN-207 significantly suppressed growth of HT-29 cancers but AN-201

Table I. Effect of treatment with cytotoxic LH-RH analogs AN-207 and AN-152, as well as their respective cytotoxic radicals AN-201 and doxorubicin (DOX) on growth characteristics of various colon cancers in nude mice.

Tumor	Treatment	Final tumor volume (mm^3)	TGR %	Tumor weight (mg)	No. of dead	Mitotic index	Apoptotic index	Ratio of apoptotic to mitotic indices	No. of AgNORs
1. HT-29	Control	1219±372	0	1236±374	2	10.8±0.9	7.9±1.2	0.79±0.12	6.05±0.10
	AN-207	485±109[a]	61	568±111[a]	6	9.0±2.3	8.0±1.2	1.21±0.49	4.90±0.18[a]
Exp. 1	AN-201				8				
	AN-152	735±158	41	944±218	0	9.4±0.6	8.7±0.8	1.03±0.16	5.20±0.10[a]
	DOX	885±201	28	1095±296	6	6.5±1.3	10.0±1.1	1.82±0.53	5.08±0.17
2. HT-29	Control	2793±643	0	3112±543	2	14.9±1.4	13.8±0.7	1.02±0.11	6.22±0.17
	AN-207	927±228[a]	68	1187±304[a]	2	13.1±1.8	17.4±1.3[a]	1.64±0.28[a]	5.69±0.08[a]
Exp. 2	AN-201	1976±559	29	1972±586	1	15.8±1.3	13.9±0.6	0.94±0.09	5.78±0.11[a]
	AN-238	952±228[a]	67	1111±201[a]	3	10.5±0.9	18.0±1.1[a]	1.85±0.24[a]	5.60±0.14[a]
3. HCT-116	Control	5398±1555	0	4131±1191	4	8.4±0.9	6.7±0.9	0.85±0.13	7.10±0.05
	AN-207	1781±403[a]	69	2138±475	2	10.1±1.4	13.0±1.5[a]	1.64±0.42	6.30±0.09[a]
Exp. 3	AN-201	3301±737	40	3836±1110	2	10.0±1.5	8.4±1.3	1.26±0.40	6.79±0.17
	AN-152	4327±1122	20	6274±1608	2	9.7±1.1	6.7±1.1	0.80±0.19	6.89±0.16
	DOX				6	11.5±6.0	12.5±3.0	1.31±0.42	6.75±0.65
4. HCT-15	Control	5104±1221	0	3224±1588	3	8.2±1.4	3.9±1.0	0.51±0.11	6.38±0.10
	AN-207	1022±414[a]	81	427±218[a]	2	8.5±1.1	10.3±1.6[a]	1.40±0.33[a]	5.70±0.10[a]
Exp. 4	AN-201	1332±197	75	1291±248	1	11.6±1.8	7.7±0.6	0.78±0.13	5.61±0.12[a]
	AN-152	1334±443	75	1540±427	2	7.9±1.0	6.1±0.8	0.87±0.15	5.50±0.13[a]
	DOX	1255±283	76	1693±1062	5	7.8±2.3	9.8±0.3	1.39±0.44	6.05±0.25
5. LoVo	Control	3005±1166	0	2789±1767	3	16.1±0.8	13.9±1.1	0.87±0.07	5.38±0.32
	AN-207	1378±390	55	1068±121	4	15.0±1.7	13.8±1.2	0.97±0.13	4.80±0.16
Exp. 5	AN-201	897±244[a]	72	641±183	2	21.1±2.8	19.1±2.0	0.92±0.05	4.76±0.20
	AN-152	1032±304[a]	67	1293±346	0	21.2±2.4	18.6±2.4	0.97±0.15	4.45±0.09[a]
	DOX	1054±448	52	2484±703	2	17.9±2.7	15.1±1.1	0.98±0.18	4.79±0.16
6. Colo-320DM	Control	3906±631	0	5763±2769	3	9.7±0.3	9.2±1.6	0.95±0.18	4.87±0.09
	AN-207	2040±631	48	2701±1089	3	10.0±0.9	9.8±1.4	1.02±0.21	4.58±0.18
Exp. 6	AN-201	4538±953	-16	4601±1169	0	9.7±0.9	9.1±1.3	0.99±0.18	4.58±0.10
	AN-152	1064±555[a]	74	891±738[a]	4	4.2±0.7[a]	7.0±1.0	1.75±0.34	4.33±0.09[a]
	DOX	2091±373	46	2297±638	3	7.8±1.4	10.0±1.5	1.50±0.40	4.68±0.16

Values are means ± SE. TGR, tumor growth reduction. [a]$P<0.05$.

was ineffective. Tumor volume changes are shown in Fig. 1B and other data on growth are in Table I. AN-207 enhanced apoptosis in the tumors while AN-201 had no such effect. There was no change in mitotic indices, but both compounds decreased AgNOR scores in the tumors (Table I). The effects of cytotoxic somatostatin analog AN-238 on growth parameters were similar to those of AN-207. At the end of the experiment, body weights were not significantly different among the groups. Spleen weights were smaller in the treated groups, but the weights of other organs were not different from control (data not shown).

In Experiment 3, AN-207 significantly inhibited growth of HCT-116 cancers (Fig. 1C, Table I). The number of apoptotic cells was significantly increased and AgNOR scores decreased in the tumors treated with AN-207. The other three compounds had no effect on growth of HCT-116 tumors.

AN-207 likewise powerfully inhibited the growth of HCT-15 cancers as shown by a significant reduction in tumor volume and weight (Fig. 1D, Table I). Histologically, an enhancement of apoptosis and a decrease in proliferation was seen in the tumors treated with AN-207. The effects of AN-201, AN-152 and DOX were similar to those of AN-207.

The growth of LoVo cancers in Experiment 5 (Fig. 1E) was significantly inhibited by AN-201 and AN-152. AN-207 and DOX were less effective, but still caused growth reduction >50%. Among the histologic characteristics, apoptosis was not affected by the treatments, but AgNOR numbers were reduced in the tumors treated with AN-152.

Table II. Binding characteristics of LH-RH receptors in human colon carcinomas growing in nude mice.

Tumor	K_d (nM)	B_{max} (fmol/mg protein)
HT-29	4.17±0.2	334.6±36.8
HCT-116	1.23±0.1	381.4±17.4
HCT-15	4.58±0.2	260.5±15.5
LoVo	1.21±0.1	491.0±8.0
Colo-320DM	8.43±0.3	496.0±9.1

The data are means ± SE. Two-three binding experiments were performed in duplicate or triplicate. K_d, dissociation constant; B_{max}, maximal binding capacity.

Table III. mRNA levels of LH-RH receptors in HT-29 colon cancers.

Tumor	Group	mRNA expression of LH-RH receptors relative to mRNA of ß-actin (arbitrary units)
HT-29	Control	6.87±1.39
	AN-207	4.45±2.45
	AN-152	5.45±0.55
	Other untreated HT-29 tumors	6.62±0.72

In Experiment 6 (Fig. 1F), AN-152 had the most powerful inhibitory effect among all compounds on Colo-320DM cancers. Treatment with AN-207 or DOX resulted in a moderate reduction of growth and AN-201 was ineffective. Histologically, apoptosis was not affected by the therapy, but AN-152 caused a decrease in cell proliferation rate as shown by lower mitotic indices and AgNOR numbers.

Systemic effects of treatment. To evaluate systemic effects and toxicity of the compounds, body and organ weights, as well as mortality were analyzed. Body weights were usually decreased by 5-10% within 7-10 days after treatment with any cytotoxic compound and returned to original levels at the time of the next injection. Final body weights were not significantly different among the groups, except in Experiment 1, in which higher doses were given i.p. to the mice. In all experiments, the weights of testes were significantly reduced after treatment with AN-152 or DOX. AN-207 caused a reduction in testis weights only in Experiments 2 and 3. Serum testosterone and LH were measured in Experiment 1. Testosterone levels were reduced to 0.17±0.01 ng/ml by AN-207, 0.48±0.17 ng/ml by AN-152 and 0.50±0.01 ng/ml by DOX from a 4.72±2.20 ng/ml value in the control group. However, these changes were not significant statistically because of the high varation among individual values. Mean serum LH was 475.3±184.1 pg/ml in the control animals and

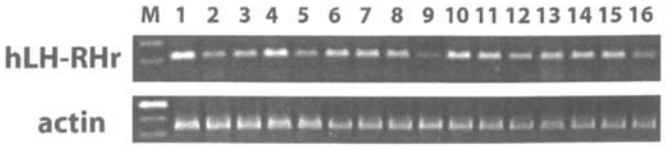

Figure 2. Agarose gel electrophoresis of reverse-transcribed and PCR-amplified mRNAs of human LH-RH receptors in human colon cancers grown in nude mice. The expected sizes of the PCR products were: 310 base pairs (bp) for human LH-RH receptor and 459 bp for ß-actin. Lanes: M, standard DNA size marker; 1-3, experiment 1 control group; 4-8, untreated HT-29 tumor samples from another experiment; 9 and 10, experiment 1 tumor treated with AN-207; 11-16, experiment 1 tumors treated with AN-152.

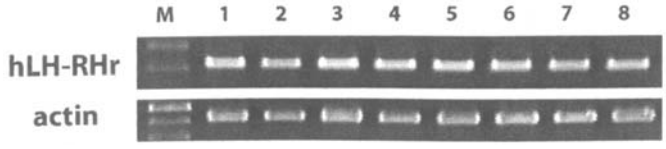

Figure 3. Agarose gel electrophoresis of reverse-transcribed and PCR-amplified mRNAs for human LH-RH receptors in four human colon cancer lines grown in nude mice (all untreated control samples). The expected sizes of the PCR products were: 310 base pairs (bp) for hLH-RHr and 459 bp for ß-actin. Lanes: M, standard DNA size marker; 1 and 2, HCT-116; 3 and 4, HCT-15; 5 and 6, Colo-320DM; 7 and 8, LoVo.

LH levels were decreased to 257.5±2.5, 345.6±46.5 and 172.0±76.0 pg/ml in groups treated with AN-207, AN-152 and DOX, respectively. The changes were not significant statistically. In some experiments, liver and spleen weights were decreased by various compounds. The death rate varied among the experiments. In Experiment 1 i.p. injections of AN-201 and AN-207, caused high mortality, though at half the dose AN-201 was more toxic than AN-207. The total number of the mice that died in Experiments 3-6 showed no significant differences among the groups being as follows: Control, 13; AN-207, 11; AN-201, 5; AN-152, 8 and DOX, 16.

Receptor-binding assays. Radiolabeled [D-Trp^6]LH-RH was bound to a single class of specific binding sites on all colon cancer samples. The concentrations of receptors for LH-RH were variable, but all 5 human cancer models investigated expressed high affinity binding sites for LH-RH. The binding data are shown in Table II.

mRNA analysis of LH-RH receptors in colon cancers. In Experiment 1 mRNA expression of receptors for LH-RH was analyzed in tumor samples from each group. The results are shown in Fig. 2. The levels of mRNA for LH-RH receptors were quantified by an image analyzer and normalized to the corresponding levels of human ß-actin. All HT-29 tumor samples investigated expressed mRNA for human LH-RH receptor. Treatment with AN-207 or AN-152 did not significantly change mRNA expression for LH-RH receptors (Table III).

mRNA expression for human LH-RH receptor was also analyzed in representative control samples of the HCT-116, HCT-15, Colo-320DM and LoVo colon tumors used in this study. Fig. 3 shows that all samples expressed mRNA for human LH-RH receptor.

Discussion

Although much progress has occurred in the systemic treatment of colorectal cancer which led to the decline in mortality rates (11), improved therapies are needed for patients with advanced disease. The development of targeted agents such as Bevacizumab and Cetuximab is a welcome addition to systemic chemotherapy (10,11).

Our approach is based on cytotoxic LH-RH analogs that are targeted to LH-RH receptors (12). LH-RH acts through binding to its G-protein-coupled receptors with 7 transmembrane domains. In vertebrates, three subtypes of LH-RH receptors have been identified (36,37). Type I receptors are characteristic of the pituitary and play the main role in the regulation of gonadotropin secretion (37). Type II LH-RH receptors are well preserved in many vertebrates, but they are vestigial and inactive in humans (38). However, some investigators assume that type II receptors can appear in tumors and that these receptors may have a role in tumor cell growth (39). Type I and II LH-RH receptors differ also functionally in their intracellular mechanisms, internalization etc. (40). Type III LH-RH receptors seem to be related to type II receptors, thus they may have common genetic roots in lower vertebrates (37).

LH-RH receptors have been demonstrated in the brain, testis, ovaries, prostate and placenta of various mammals. These receptors transmit the regulatory effects of LH-RH on steroidogenesis, cell proliferation, apoptosis and embryo implantation (41). In addition to normal tissues, LH-RH binding sites have been also found in various tumors such as pituitary adenomas and breast, ovarian, endometrial and prostatic carcinomas (14,15,26,37,41,42).

Although numerous publications reported the expression of LH-RH receptors in prostate, uterine, ovarian and mammary tumors (14,15,26,37,41), only scarce data about their occurrence in colorectal cancers are available. One study demonstrated LH-RH and LH-RH receptor immunoreactivity in the villous and glandular epithelium in small and large intestines of rats (43). Another group reported that binding sites for LH-RH are expressed on Colo-205 colon cancers, and that a chimeric protein consisting of a toxin and an analog of LH-RH inhibited the growth of these cancers in nude mice (27,44).

In our study, we demonstrated the presence of LH-RH receptors in all 5 colon cancer lines investigated. mRNA for LH-RH receptors was expressed in all tumor samples analyzed and the sequence detected corresponds to type I LH-RH receptor found in the pituitary. The analysis of the binding data indicated that all five colon cancer models displayed dissociation constant values (K_d) in the lower nanomolar concentration range and radiolabeled peptide was bound to a single class of binding sites. This range of K_d values (1-8 nM) clearly represents high affinity binding that is required for efficient chemotherapy based on targeting of cytotoxic LH-RH conjugates to their receptors on cancers. These results are in accord with previous findings demonstrating the presence of specific, high affinity binding sites for LH-RH in various human cancer lines and surgical specimens of human cancers. Our results are also in good agreement with previous observations on the binding affinity and capacity of receptors for LH-RH expressed on the pituitary.

The expression of LH-RH receptors in various cancers makes possible the use of LH-RH analogs as carrier vectors to deliver cytotoxic agents to these tumors. The toxic moieties of such hybrid compounds can be bacterial toxins (44), plant toxins (45), or, as in our studies, cytotoxic drugs (4,12,15,16, 26,28-30). Cytotoxic analogs first bind to LH-RH receptors on the cell surface with high affinity (33), and then after they enter the cells (12,46-48), the cytotoxic radicals interfere with vital molecules in the cytoplasm or nuclei.

It has been shown that cytotoxic LH-RH analogs are internalized by human ovarian and endometrial cancer cells (29,46) and MCF-7 human breast cancer (47). Thus, using two-photon laser scanning microscopy, the AN-152 fluorophore conjugate could be observed as it interacted with LH-RH receptor positive MCF-7 breast cancer cells (47). The receptor-mediated entry of AN-152 into the cell cytoplasm was clearly demonstrated (47) (reviewed in ref. 29). Recently in collaboration we were able to show that internalization of cytotoxic LH-RH analog AN-152, monitored by confocal laser scanning microscopy, induces apoptosis in human endometrial and ovarian cancer cell lines (48). The mechanism of action of cytotoxic LH-RH analog AN-207 containing AN-201, is similar to that of AN-152. An accumulation of AN-207 in LH-RH receptor-positive tumors is followed by receptor-mediated internalization (28,29). Both doxorubicin and cytotoxic radical AN-201 are powerful DNA-intercalating agents that selectively kill rapidly proliferating cell types such as cancer cells (28,29). Thus the main effect of AN-152 and AN-207 is tumor eradication (28,29). To date, we do not have any conclusive explanation for the fall in serum LH and testosterone in mice after treatment with AN-152, DOX or AN-207. These phenomena could have occurred through a down-regulation of LH-RH receptors in the pituitary by the [D-Lys^6]LH-RH carrier, cytotoxic effect of AN-152, AN-207 or DOX itself on the pituitary or direct action of these agents on the testes. The effects of alterations in sex steroid levels on tumor growth must be further investigated in future studies with cytotoxic analogs AN-152 and AN-207 on sex hormone sensitive tumors such as prostate cancers and breast cancers. However, these changes in serum testosterone were not significant and unlikely to have contributed to inhibition of colon cancers. We have previously investigated and observed some cytotoxic effects of AN-152 and AN-207 on the pituitary and testes (29,49-52). In earlier studies AN-207 caused a selective but transient damage to the gonadotroph cells of the pituitary (50). AN-201 non-selectively damaged pituitary LH, GH and TSH function (50). Both AN-207 and the carrier [D-Lys^6]LH-RH decreased the levels of mRNA for LH-RH receptors in the rat pituitary (51). However, the pituitary function completely recovered 1-2 weeks later (50,51). The lack of toxicity of AN-207 on the pituitary cells, which are slowly proliferating, may be explained by the fact that the cytotoxic radical AN-201 more selectively affects rapidly proliferating cell types (29). Our data previously indicated that AN-152, doxorubicin and AN-207 can reduce the weight of testes (49,52) suggesting that this effect is not related to targeting and may be attributed to partial hydrolysis of DOX and AN-201 from the respective hybrid analogs by carboxylesterase enzymes in the circulation. Some of these findings have been previously reviewed (16,29). A decrease in tumoral

LH-RH receptor levels on the prostate cancers also has been described previously after treatment with cytotoxic LH-RH analog AN-152 (49).

In our present study, cytotoxic LH-RH analogs powerfully inhibited the growth of human colon cancers transplanted into nude mice. Although, the two cytotoxic analogs have the same LH-RH carrier, AN-207 was more effective than AN-152 on HT-29 and HCT-116 tumors, but AN-152 which was used at a dose 80 times higher had a greater effect than AN-207 on LoVo and Colo-320DM cancers. Thus, the activity of the cytotoxic compound seems to depend also on the type of the cytotoxic radical. Various tumor lines show different sensitivity to DOX and AN-201. Both cytotoxic radicals had very strong inhibitory effect on HCT-15 cancers, but had little effect on HT-29 and HCT-116 tumors. However, the effects of the cytotoxic analogs did not always show a close correlation with the effects of the corresponding radicals and AN-152 was not superior to DOX in HCT-116 and HCT-115 cancers. Likewise the effects were not strictly related to the receptor concentration in the tumors. Thus, other characteristics such as vascularity, blood supply, proliferation rate etc. may also affect the efficacy of therapy with cytotoxic LH-RH analogs. The carrier LH-RH analog alone was not tested in this study, because in several previous experiments LH-RH analogs administered at such low doses as in cytotoxic analog therapy had no inhibitory effects on tumors (12,26,29,52). Earlier we demonstrated that HT-29 tumors express receptors for somatostatin and cytotoxic somatostatin analog AN-238 powerfully inhibited growth of this tumor (13). The present study shows that the inhibitory effect of AN-207 on growth of HT-29 cancers is similar to that of cytotoxic somatostatin analog AN-238.

Histological growth characteristics were also differently affected by the two cytotoxic LH-RH analogs. AN-207 increased apoptosis and decreased cell proliferation in HT-29, HCT-116 and HCT-15 cancers, while AN-152 had no significant effect on apoptosis in LoVo and Colo-320DM tumors and only decreased the proliferation.

There were no significant differences in toxicity of the compounds, as reflected by overall body weight and mortality data. Body weights were 5-10% lower 7-10 days after treatment with any cytotoxic compound, but body weights returned to original values by the time of the next treatment. Final body weights were not significantly different among the groups, except for a reduction in body weights in all treated groups in Experiment 1, in which the compounds were administered i.p.

In conclusion, our study demonstrates that receptors for LH-RH are expressed in various human colon cancer lines transplanted into nude mice. Cytotoxic LH-RH analogs AN-207 and AN-152 powerfully inhibited *in vivo* growth of these colon cancers xenografted into nude mice and on some tumors their effects surpassed those of the corresponding cytotoxic radical. Cytotoxic LH-RH analog AN-152 is presently in clinical trials in Germany in women with gynecological cancers. On the basis of our results patients with advanced or metastatic colorectal carcinoma could be considered for inclusion in future clinical trials with cytotoxic LH-RH analogs, after establishing the presence of LH-RH receptors in biopsy samples.

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