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Characteristics of Alpha-Adrenoceptors in Two Human Colorectal Cancer Cell Lines

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The DiFi and HT-29 human colorectal cancer cell lines were characterized and compared with respect to binding properties of alpha adrenoceptors present on the cell surface. Both cell lines possessed alpha-1 and alpha-2 adrenoceptors of high affinity; however, DiFi cells were rich in alpha-1 adrenoceptors, whereas HT-29 cells were rich in alpha-2 adrenoceptors. In each cell line, specificity of radioligand binding to alpha-1 or alpha-2 adrenoceptors was proved via competition studies using non-tritiated drugs. We believe this to be the first characterization of alpha-1 adrenoceptors in cell line HT-29 and of alpha-1 and alpha-2 adrenoceptors in DiFi cells. Differences between these cell lines in alpha adrenoceptor expression are discussed in relation to colon carcinogenesis. The high level of alpha-1 adrenoceptors seen in DiFi cells should make this cell line useful in studies of the function and regulation of this adrenoceptor subtype.

Adrenergic factors have been found to influence crypt cell proliferation in the large intestine in animal model studies (reviewed in 1). Alpha-1 adrenergic agents have an inhibitory effect on cell proliferation in the normal colonic crypt of mice (2), and alpha-2 adrenoceptors mediate a stimulatory effect on cell proliferation in the normal murine crypt (3). In premalignant states of colonic cell proliferation, alpha-1 and alpha-2 adrenergic agents have inhibitory effects (2). In colon carcinomas, alpha-2 factors produce growth inhibition and alpha-1 factors produce growth stimulation (2). These studies in mice

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suggest that modulation of colonic cell growth by adrenergic factors is disrupted in the neoplastic state in animal models.

Understanding of the role of adrenergic agents in colonic cell proliferation in humans is limited. The human colon carcinoma cell line HT-29 has been shown to possess two classes of sites (high and low affinity) for the alpha-2 adrenergic receptor (4). The number of alpha-2 adrenergic binding sites was found to increase when HT-29 cells were stimulated to proliferate.

A number of studies have reported that the various adrenergic receptor types can co-exist in a given tissue (5) or on a single cell (6), and both alpha and beta adrenergic receptors are present in various cancer cell lines. HT-29 human colon adenocarcinoma cells contain well-characterized alpha-2 adrenergic receptors that mediate a decrease in the production of the intracellular second messenger, cyclic AMP (cAMP;4,7). In other secretory epithelial cells, such as those in salivary glands, beta-adrenergic receptor stimulation is linked to increases in intracellular cAMP levels that regulate the secretion of mucin and other substances (8,9,10). In colonic carcinomas and adenomas induced in rodents, alpha-adrenergic receptor stimulation has important effects on proliferation of colonic epithelial cells (2,3); however, the characteristics of these receptors on mammalian colon cancer cells are not known.

Therefore, in this study we have characterized and compared the binding properties (density and affinity) of alpha adrenoceptors present in the HT-29 and DiFi human colorectal cancer cell lines.

MATERIALS AND METHODS

Cells

We studied two cell lines: the HT-29 cell line (American Type Culture Collection # HTB 38) and the DiFi cell line (11). Cells were seeded in plastic petri dishes and incubated at 37°C in a

humidified atmosphere of 5% CO_2:95% air. The RPMI 1640 cell culture medium was supplemented with 10% fetal bovine serum (FBS; GIBCO, Grand Island, NY) and contained glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 ug/ml) (all from GIBCO).

Drugs

Radioligands [^{3}H]prazosin (specific activity 70-80 Ci/mmol) and [^{3}H]rauwolscine (specific activity 80-90 Ci/mmol) were purchased from N.E.N.(New England Nuclear, Boston, MA). All the radioligands were checked for purity using thin-layer chromatography. Drugs not tritium-labeled were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Preparation and Rationale for Approach Finally Taken

Initially, the identification and characterization of specific binding of the radioligands, [^{3}H]prazosin and [^{3}H]rauwolscine, was carried out on the purified membranes of both HT-29 cells and DiFi cells. These cells were broken by Polytron homogenization (sonication) for 20 sec. Membranes were prepared by a differential centrifugation procedure detailed in (5). The specific binding (as defined by the binding of the radioligand displaceable by 10 uM phentolamine; see ref. 5) of both radioligands in the purified membrane preparation ranged from 15-30%. Furthermore, there was a specific binding of the radioligands to various cellular fractions (heavy nuclear fraction, mitochondria, microsomes), which may be due to their contamination with plasma membranes. Moreover, there was a large variation in the data between experiments, indicating a very poor signal-to-noise ratio in the system. We then checked the specific binding of [^{3}H]prazosin and [^{3}H]rauwolscine in the whole-cell preparation of both HT-29 cells and DiFi cells. The specific binding of the radioligands in these cell types was as follows:

HT-29 Cells	[^{3}H]Prazosin	35-40%
	[^{3}H]Rauwolscine	80-95%
DiFi Cells	[^{3}H]Prazosin	45-50%
	[^{3}H]Rauwolscine	25-30%

The specific binding in these whole-cell preparations was substantially better than that seen in purified membranes (except in the case of [^{3}H]Rauwolscine binding in DiFi cells), and the data from one experiment to another were reproducible. Therefore, a detailed study for the identification and characterization of alpha-1 and alpha-2 adrenoceptors in HT-29 and DiFi cells was done using the whole-cell preparation. Using the whole-cell preparation also permitted the number of receptors on each cell to be estimated.

Binding Assay

Binding experiments for the characterization of alpha-1 and alpha-2 adrenoceptors were performed using fresh membranes. In the binding assay, TRIS buffer (50 mM TRIS, 10 mM $MgCl_2$, pH 7.4) was used in the incubation medium. [^{3}H]prazosin was used to label alpha-1 adrenoceptors, and [^{3}H]rauwolscine was used to label alpha-2 adrenoceptors. These radioligands were freshly diluted with cold TRIS buffer. The purity of the radioligands was checked routinely. The incubation medium contained 100 ul of TRIS buffer with or without unlabeled drug and 50 ul of diluted radioligand. The reaction was started by adding 100 ul of membrane suspension to make a final volume of 250 ul. Incubation was carried out in a shaking water bath at 25^{o}C for 40 min (apparent equilibrium conditions for the binding of the radioligand to the receptor site

were established in these cells). There was no degradation of the radioligand in the presence of membranes or whole cells during incubation. The binding reaction was terminated by adding 4.0 ml of cold TRIS buffer to the entire incubation mixture. The radioligand bound to the receptor site was separated and washed with cold TRIS buffer by rapid filtration over Whatman Glass Fiber GF/C filters. Scintillation cocktail (Aquassure, N.E.N.) was added to the dried filters and counted in a liquid scintillation counter (Tracor Analytic, Model 6892, Chicago, IL). Quench correction in the counting of tritium was made by using the external standard ratio method, as described previously (5,12). The specific binding of [^{3}H]prazosin and [^{3}H]rauwolscine was defined as the radioactivity displaceable by 10 uM phentolamine. Justification of the use of 10 uM phentolamine to define specific binding is based upon our previous experience (5).

Specificity of [3H]Prazosin Binding in DiFi Cells and [3H]Rauwolscine Binding in HT-29 Cells

This was examined by using several structurally dissimilar non-radiolabeled drugs (agonists and antagonists) selective for alpha-1 and alpha-2 adrenoceptors. These drugs (prazosin, yohimbine, methoxamine, & B-HT 933 in both cell lines, plus l-norepinephrine in HT-29) were allowed to compete for the specific binding of the radioligand in concentrations ranging between 10^{-10} M and 10^{-4} M. Competition curves were plotted to calculate the affinity of the unlabeled compounds for the receptor sites.

Protein Content in Membrane Fractions

This was determined by the method of Lowry et al. (13) using bovine serum albumin as the standard. The specific binding of the radioligand to the membranes was normalized in terms of protein content.

Analysis of the Data

Saturation ligand binding and competition curves were analyzed using the IBM computer program (EBDA and LIGAND, 14) for single or multiple binding sites. From this program, the values for the maximum binding capacity (B_{max}), an apparent equilibrium dissociation constant (K_D) and the K_i (inhibition constant) values for the unlabeled compounds were obtained. All the values for B_{max}, K_D, and K_i are reported as the mean $\pm$ SEM (standard error of the mean) of separate experiments.

RESULTS

Saturation radioligand binding curves yielded the characteristics of [^{3}H]prazosin and [3H]rauwolscine binding in HT-29 and DiFi whole-cell preparations, as well as an estimate of the number of alpha-adrenoceptor subtypes per cell (Table 1). These data suggest that in HT-29 cells both alpha-1 and alpha-2 adrenoceptors of high affinity are present. However, the density

Table 1. Characteristics of [^{3}H]prazosin and [^{3}H]rauwolscine binding in HT-29 and DiFi cells

	[^{3}H]prazosin[1] binding	[^{3}H]rauwolscine[2] binding
HT-29		
K_D	1.70 ± 0.52 nM (*n=3)	2.12 ± 0.28 nM (n=4)
B_{max}	45.5 ± 9.5 fmol/mg protein (n=3)	248.5 ± 66.7 fmol/mg protein (n=4)
or B_{max}	2740 ± 57 receptors/cell	14,965 ± 4,020 receptors/cell
DiFi		
K_D	1.89 ± 0.54 nM (n=5)	3.36 ± 0.77 nM (n=6)
B_{max}	102 ± 20 fmol/mg protein (n=5)	26.3 ± 3.1 fmol/mg protein (n=6)
or B_{max}	6,142 ± 1,185 receptors/cell	1584 ± 187 receptors/cell

1 Selectively labels alpha$_1$-adrenoceptors.
2 Selectively labels alpha$_2$-adrenoceptors.
* n represents the number of separate binding experiments.

of alpha-2 adrenoceptors per cell is about five-fold higher than the density of alpha-1 adrenoceptors. DiFi cells also express both alpha-1 and alpha-2 adrenoceptors on their cell surface. In contrast to the HT-29 cells, alpha-1 adrenoceptors on DiFi cells were about four-fold higher than the alpha-2 adrenoceptors.

In order to show the specificity of the radiochemical binding to alpha-1 or alpha-2 adrenoceptors, we performed competition studies with various non-radiolabeled drugs as described in the Materials and Methods section. Because the signal to noise ratio in the specific binding of [^{3}H]prazosin was higher in DiFi cells and that of [^{3}H]rauwolscine was higher in HT-29 cells, we characterized the specificity of the major binding site for each cell line (Table 2). Non-tritiated drugs (agonists and antagonists) were allowed to compete for the specific binding of [^{3}H]prazosin in DiFi cells or [^{3}H]rauwolscine in HT-29 cells.

Data from the competition studies (Table 2) suggest that in DiFi cells the specific binding of [^{3}H]prazosin is to the alpha-1 adrenoceptors, because prazosin, a selective alpha-1 adrenoceptor antagonist, was about 200-fold more potent than yohimbine, a selective antagonist to alpha-2 adrenoceptors. This is also

Table 2. Competition studies showing specificity of [^{3}H]prazosin for DiFi cells and of [^{3}H]rauwolscine for HT-29 cells

	DiFi Cells		HT-29 Cells	
Competitor	K_i for [^{3}H]prazosin specific binding		K_i for [^{3}H]rauwolscine specific binding	
Prazosin	1.32 ± 0.45 nM	([1]n=4)	24.6 ± 8.6 μM	(n=5)
Yohimbine	264 ± 47 nM	(n=5)	16.2 ± 3.8 nM	(n=6)
Methoxamine	412 ± 86 nM	(n=3)	25.8 ± 0.9 μM	(n=3)
B-HT 933	> 20 μM	(n=3)	0.64 ± 0.32 μM	(n=3)
1-Norepinephrine	[2](N.T.)		0.35 ± 0.07 μM	(n=7)

[1] Indicates the number of separate experiments performed.
[2] Not tested.

supported by the studies with the selective agonists, methoxamine and B-HT 933, where methoxamine (a selective agonist for alpha-1 adrenoceptors) was more effective than B-HT 933 (a selective agonist for alpha-2 adrenoceptors) at competing for the specific binding of [^{3}H]prazosin in DiFi cells. Thus, DiFi cells contain specific alpha-1 adrenoceptors on their cell surface.

In HT-29 cells, yohimbine, a selective antagonist for alpha-2 adrenoceptors, was about 1500-fold more potent than prazosin (a selective antagonist for alpha-1 adrenoceptors) at competing for the specific binding of rauwolscine. This suggests that the binding of [^{3}H]rauwolscine in HT-29 cells is specifically to alpha-2 adrenoceptors. This is also supported by the studies with selective agonists for alpha-1 adrenoceptors, methoxamine, and alpha-2 adrenoceptors, B-HT 933. B-HT 933 was about 40-fold more potent than methoxamine at competing for the specific binding of [^{3}H]rauwolscine in HT-29 cells.

DISCUSSION

In summary, there are both alpha-1 and alpha-2 adrenoceptors of high affinity in both the DiFi and HT-29 cell lines. However,

DiFi cells are rich in alpha-1 adrenoceptors and HT-29 cells are rich in alpha-2 adrenoceptors. Competition studies have proved the specificity of these receptors in both cell lines. We believe that this study offers the first characterization of alpha-1 adrenoceptors in cell line HT-29 and of alpha-1 and alpha-2 adrenoceptors in DiFi cells.

Differences observed in the expression of alpha adrenoceptors between DiFi cells and HT-29 cells could reflect differences in the way each cell line adapted to culture conditions during establishment, differences related to the site of origin of the original carcinoma from which each was derived (DiFi cells originated from a rectal tumor, HT-29, from a colonic one), or differences in genetic alterations that led to tumor initiation/progression.

The latter idea is attractive because it is known that DiFi cells originated in a patient with FAP (familial adenomatous polyposis; 11), whereas HT-29 cells did not. Initiation of FAP is believed to be a result of loss of wild-type genetic material from the long arm of chromosome 5 (at the FAP locus, 5q21-22)(15), not far from the gene for the alpha-1 (and beta-2) adrenoceptor (at 5q32-34, two genes separated by < 225 kilobases, 16). We have recently reported genetic changes (allelic deletions and rearrangements) at the beta-2 adrenoceptor locus in colorectal carcinomas from patients both with and without FAP (17); we have also observed such tumor-specific allelic deletions at the colony-stimulating factor-1 receptor (CSF-1R) locus (18), in the center of this region at 5q33.1 (19), as well as at the nearby glucocorticoid receptor (GRL) locus (20), recently reassigned from proximal to distal chromosome 5q (21). Thus, it seems possible that the elevation of alpha-1 adrenoceptors in DiFi cells could stem from such structural changes in 5q known to be associated with FAP.

With its high alpha-1 adrenoceptor count/cell, the DiFi cell line should prove useful in probing the mode of action and regulation of alpha-1 adrenoceptors, just as HT-29 cells have proved useful in similiar studies of alpha-2 adrenoceptors.

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