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EFFECTS OF DIMETHYLBENZ[*a*]ANTHRACENE-INDUCED MALIGNANCY ON THE SUBCELLULAR DISTRIBUTION OF PERIPHERAL BENZODIAZEPINE RECEPTORS IN SUBMANDIBULAR GLANDS OF RATS

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Abstract—The binding of [^{3}H]Ro 5-4864 to peripheral benzodiazepine receptors (PBRs) was studied in normal and malignant submandibular glands of rats. The carcinoma was induced by implantation of 7,12-dimethylbenz[*a*]anthracene (DMBA) into the glands. [^{3}H]Ro 5-4864 binding to normal and malignant submandibular glands indicated one population of binding sites with high affinity (K_D of 3.4 and 4.4 nM for normal and malignant respectively) and saturability (B_{max}) of 487 and 321 pmol/g tissue for normal and malignant respectively). Subcellular localization of PBRs indicates that mitochondria was the primary locale of the receptor in both cases and the decrease in B_{max} was due primarily to a decrease in the binding capacity of PBRs in mitochondria.

Benzodiazepines (BZs) have been used as anticonvulsants, muscle relaxants, and anxiolytic and sedative or hypnotic drugs [1, 2]. These effects of BZs are thought to be mediated through high-affinity, stereospecific receptors localized in the central nervous system [3–5]. However, several BZs bind with high affinity to a "peripheral-type" receptor found in many non-neutral tissues [6–11] as well as in several cultured cell lines [12–14] whose specificity differs physically [7, 15] and pharmacologically [9, 13, 16] from that of central BZ receptors.

Although the exact physiological function of peripheral benzodiazepine receptors (PBRs) is not known, recent reports suggest that drugs selective for PBRs produce a pleiotropic spectrum of pharmacological actions, including stimulation or inhibition of cell proliferation and differentiation [12, 17–20], chemotaxis [12], alteration of immune function and protooncogene expression [12, 21, 22], inhibition of mitogenesis in fibroblasts and growth of thymoma cells [17, 18]. Furthermore, since PBRs are located primarily in mitochondria and nuclei [10, 11, 23–27], it is possible that PBRs may play a role in nuclear events and mitochondrial functions. It has been suggested recently that PBRs are involved in mitochondrial cholesterol metabolism [28].

Since drugs specific for PBRs alter immune function [12, 21] and it is known that cell-mediated immunity plays an important role in the suppression of neoplastic diseases [29], it is possible that there exists a link between cell immunity, PBRs and development of neoplastic diseases. It has been reported recently that glial tumors have a high density of PBRs compared with nonmalignant tissues, and that PBR ligands may be used in the diagnosis of glial tumors [30].

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7,12-Dimethylbenz[*a*]anthracene (DMBA) is known to induce carcinogenesis in submandibular glands [31]. The present investigation was aimed at finding out whether DMBA-induced carcinoma in submandibular glands causes any modulation in the subcellular distribution of BZ receptors.

MATERIALS AND METHODS

Materials. All biochemicals were purchased from the Sigma Chemical Co., St. Louis, MO [^{3}H]Ro 5-4864 (86.4 Ci/mmol) was purchased from New England Nuclear, Boston, MA. Non-radioactive Ro 5-4864 was donated by Dr Peter Sorter, Hoffmann–LaRoche, Nutley, NJ.

Animals. Male Wistar rats were purchased from Camm Laboratories, Wayne, NJ. Sixteen male rats weighing approximately 100 g were randomly placed into two equal groups, designated A and B, and were kept in a temperature- and light-controlled animal facility. Food and water were given *ad lib.* The animals in group B were anesthetized with diethyl ether. A 3-cm incision was made on the ventral surface of the neck overlying the block of salivary glands. A 5-mg pellet of powdered DMBA was implanted into each submandibular gland through a cannula. Animals in group A were treated in the same manner as those of group B but without DMBA and served as controls. All animals were killed after 10 weeks. The glands were surgically removed and examined, and two from each group were fixed in 10% formalin, sectioned in paraffin, and stained with hematoxylin and eosin. Then the stained sections were examined under a light microscope.

Subcellular fractionation. The submandibular glands (5 MG) from both control and treated groups were excised quickly, washed in ice-cold saline (0.9% NaCl), blotted dry, and weighted after removing fat or any other attached tissue. The SMG were minced with scissors, homogenized in 10 vol. of 0.25 M

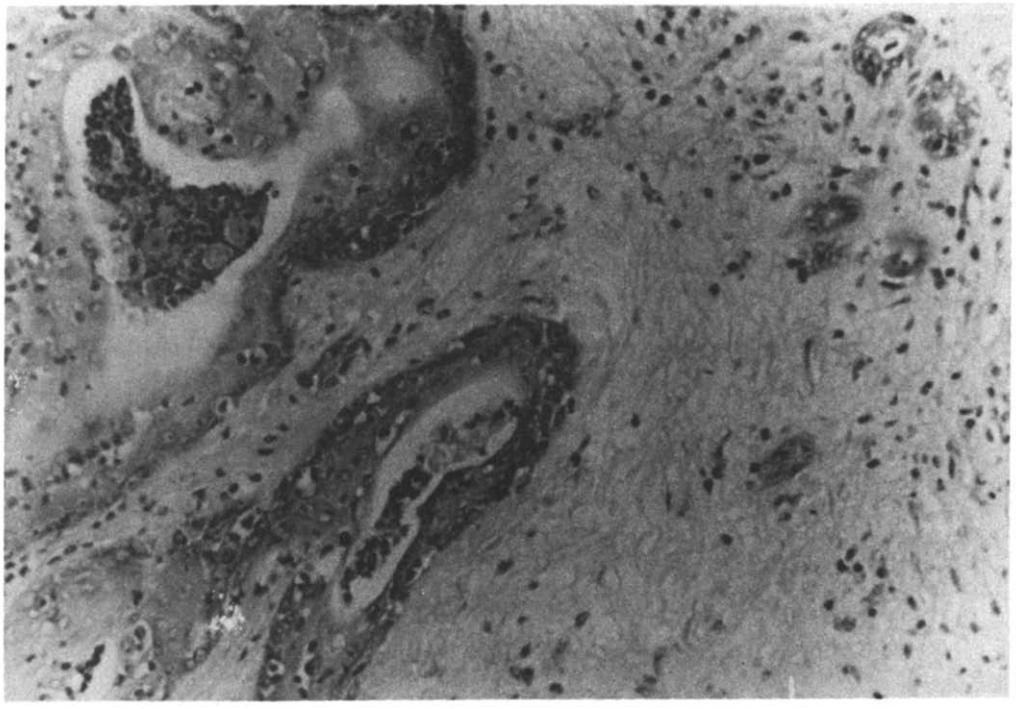

Fig. 1. Light microscopic structure of DMBA-implanted submandibular gland (×100, hematoxylin and eosin). Metaplastic epithelium shows pronounced malignant changes.

sucrose, 1 mM EDTA (pH 7.4) in a Potter–Elvehjem homogenizer, and then passed through a cheesecloth. The homogenate was centrifuged at 200 *g* for 10 min in a refrigerated Beckman TJ-6 centrifuge to remove any tissue debris and divided into two parts, one for the isolation of whole tissue membrane and the other for the subcellular fractionation.

For the isolation of membrane, the 200 *g* supernatant fraction was homogenized by using a Brinkmann polytron (setting 6–7, 15 sec) and centrifuged at 40,000 rpm using a 70 Ti rotor. For the subcellular fractionation, the supernatant fraction was recentrifuged at 600 *g* for 10 min. The nuclear pellet was washed with homogenizing buffer. The supernatant fractions were combined and centrifuged at 10,000 *g* in a refrigerated Sorvall RC-5 centrifuge using an SS-34 rotor for 10 min to obtain the mitochondrial fraction. The supernatant fraction was centrifuged in a Beckman L8-M ultracentrifuge at 105,000 *g* for 60 min using a 70 Ti rotor to obtain the microsomal and cytosolic fractions. Nuclear, mitochondrial and microsomal fractions were washed twice successively with 1.15% KCl and 0.1 M Tris–HCl, 1 mM EDTA (pH 7.4) and then suspended in a known volume of the buffer. All fractions were stored at −20° until analysis. The protein content of all fractions was determined by the procedure of Lowry *et al.* [32].

Receptor binding assay. For the receptor binding studies all the pellets were suspended in 50 mM Tris–HCl (pH 7.4) and homogenized by using a Brinkmann Polytron (setting 6–7, 15 sec) [11]. The binding of [^{3}H]Ro 5-4864 to different fractions was determined in a volume of 1 mL containing: 0.1 mL membrane protein (50–60 μg), 0.1 mL radioligand (0.5 to 16 nM), 0.1 mL Ro 5-4864 (5 μM) (or buffer) and 0.7 mL assay buffer (50 mM Tris–HCl, pH 7.4). The reaction was initiated by the addition of tissue and incubated at 0–4° for 60 min. Then the reaction was terminated by rapid filtration over 0.3% polyethylenimine-treated Whatman GF/B strips [11, 33] using a Millipore filtering manifold, with two 5-mL washes of ice-cold buffer. The specific binding of [^{3}H]Ro 5-4864 was defined as the difference in binding obtained in the presence and absence of Ro 5-4864 (5 μM). The radioactivity retained by the filters was measured in a Beckman LS 355 liquid scintillation spectrometer, using 8 mL hydrofluor (National Diagnostics, Somerville, NJ) as a scintillant. Scatchard analysis, Hill plot and determination of binding constants were performed according to Bennet [34].

Biochemical assay. Succinate dehydrogenase, a mitochondrial marker, and NADPH–cytochrome *c* reductase, a microsomal marker, were assayed as described by Possmayer *et al.* [35]. 5′-Nucleotidase for plasma membrane was determined by the method of Parry and Pedersen [36]. The DNA content of all fractions was determined as described by Burton [37].

RESULTS AND DISCUSSION

At the end of week 10 of DMBA implantation, the submandibular glands showed extensive metaplasia of the ductal epithelium in the central zone outlined by proliferating islands of ductal epithelium. Some of the islands of proliferating ductal epithelium showed severe squamous metaplasia. The central

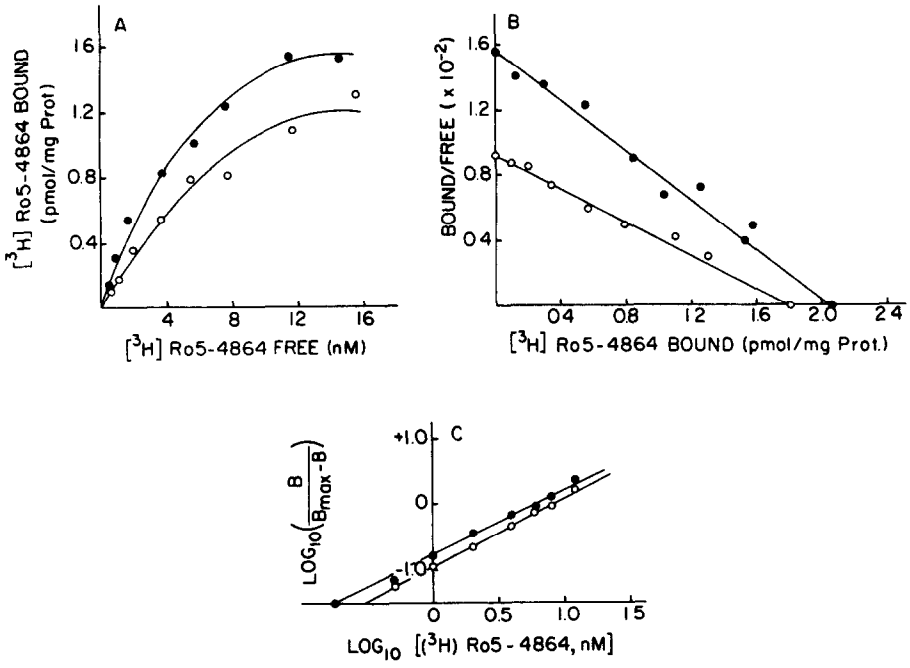

Fig. 2. Saturation isotherm (A), Scatchard analysis (B) and Hill plot (C) of [^{3}H]Ro 5-4864 binding to normal (●– – –●) and malignant (○– – –○) submandibular gland membranes of rats. For A and B, data shown are representative of five independent experiments. Bound = picomoles of specifically bound [^{3}H]Ro 5-4864 per milligram of protein. Free = total concentration of [^{3}H]Ro 5-4864 added in the incubation medium—specifically bound [^{3}H]Ro 5-4864 per milligram of protein. The regression line (γ = 0.98 for both control and malignant) indicates a K_D of 3.13 nM and a B_{max} of 2.13 pmol/mg protein for control, whereas a K_D of 4.76 nM and a B_{max} of 1.79 pmol/mg protein for malignant membranes. The Hill plot (C) represents mean data of three experiments. [^{3}H]Ro 5-4864, nM = concentration of [^{3}H]Ro 5-4864, 0.5 to 16 nM. B = picomoles of specifically bound [^{3}H]Ro 5-4864 per milligram of protein for each concentration of [^{3}H]Ro 5-4864. The regression line (γ = 0.98) gives a Hill slope of 1.05 for control and 1.07 for malignant glands.

Table 1. Kinetic parameters of [^{3}H]Ro 5-4864 binding to normal and malignant submandibular glands of rats

Group	K_D (nM)	B_{max} pmol/mg protein	B_{max} pmol/g tissue
Normal	3.41 ± 0.21	2.13 ± 0.02	487.3 ± 2.5
Malignant	4.42 ± 0.23	1.79 ± 0.03*	320.6 ± 7.4*

Values are means ± SE from five experiments.
* Binding capacity of PBRs in malignant tissue was significantly lower than that in normal tissue ($P < 0.001$, Student's t-test).

areas of metaplastic epithelium in several islands showed neoplastic changes in the form of cellular atypia, mitosis, dyskeratosis, extensive keratinization and epithelial pearl formation (Fig. 1). Submandibular glands from the control group did not show any change to indicate any inflammatory or neoplastic changes.

[^{3}H]Ro 5-4864 bound to both normal and malignant submandibular gland membranes in a saturable fashion (Fig. 2, A and B). On the basis of protein and wet tissue weight, the malignant gland had, respectively, 16 and 35% lower ligand binding capacity than normal (Table 1). Since the apparent binding affinity was not significantly different between the normal and malignant tissue, the larger effect on the basis of latter units (pmol/g tissue) could result if the malignant tissue was edematous. However, since the malignant tissue was not edematous (Fig. 1), the larger effect due to the latter units was due most probably to the increase in nuclear DNA content of the malignant tissue.

The Scatchard plot (Fig. 2B) indicated an apparently homogeneous population of binding sites in both cases. The saturation experiments resulted in a Hill coefficient of 1 (Fig. 2C), which is also indicative of binding site homogeneity and noncooperativity.

It is interesting to note that while we observed a reduction in the PBR density of submandibular

Table 2. Subcellular distribution of [^{3}H]Ro 5-4864 binding to peripheral benzodiazepine receptors in normal and malignant submandibular glands of rats

Subcellular fraction	Normal			Malignant		
	[^{3}H]Ro 5-4864 binding			[^{3}H]Ro 5-4864 binding		
	pmol/mg protein	pmol/g tissue	% Distribution	pmol/mg protein	pmol/g tissue	% Distribution
Homogenate	1.5 ± 0.1	358.5 ± 7.4		1.0 ± 0.1*	185.2 ± 10.7†	
200 *g* Pellet	1.0 ± 0.1	27.5 ± 1.3	7.7	0.6 ± 0.1	25.1 ± 6.5	13.6
Nuclei	1.1 ± 0.1	30.9 ± 1.6	8.6	1.0 ± 0.2	49.6 ± 10.5	26.8
Mitochondria	7.3 ± 0.8	217.3 ± 7.4	60.6	1.8 ± 0.2	72.0 ± 18.3†	38.9
Microsomes	1.7 ± 0.3	50.0 ± 3.4	13.9	1.1 ± 0.1	28.9 ± 8.6	15.6
Cytosol	0.1 ± 0.0	32.2 ± 0.7	9.0	0.1 ± 0.0	5.9 ± 2.2†	3.2

Values are means ± SEM of five experiments. Receptor binding assays were based on an 8 nM [^{3}H]Ro 5-4864 concentration. Statistical significance was determined by Student's *t*-test. Malignant tissue values without a superscript had a difference from normal tissue values of $P > 0.05$ which was not considered significant.

, † Binding capacity of subcellular fractions of malignant tissue was significantly different from that of normal tissue ($P < 0.02$; † < 0.001).

glands due to malignancy, Starosta-Rubinstein *et al.* [30] reported that glial tumors have a high density of these sites compared with nonmalignant tissues. Thus, it is possible that there is a cellular specificity as far as the effect of malignancy on PBRs is concerned. The submandibular gland consists of numerous cell types which may differ in their densities of PBRs. Alterations in PBR density may reflect different proportions of cell types in malignant tissue, rather than changes in any one cell type.

Data on the subcellular distribution of [^{3}H]Ro 5-4864 binding to peripheral benzodiazepine receptors in normal and malignant submandibular glands of rats are shown in Table 2. The mitochondria had the highest ligand binding capacity in both normal and malignant tissues. The authenticity of the subcellular fractions was established by determination of the subcellular marker enzymes (Fig. 3). The binding capacity of PBRs was decreased significantly in the mitochondria and cytosol of the malignant tissue. The reduction observed in the PBR density of the malignant tissue appeared to be due to a diminution in mitochondrial density of PBRs. The decrease in PBR density in the mitochondria may result from a decrease in the number of mitochondria as well as a down-regulation of receptor density.

The nuclei of the malignant tissue had a qualitatively higher distribution of PBR density (26.8%) than that of the normal tissue (8.6%). Even though this difference between the normal and malignant

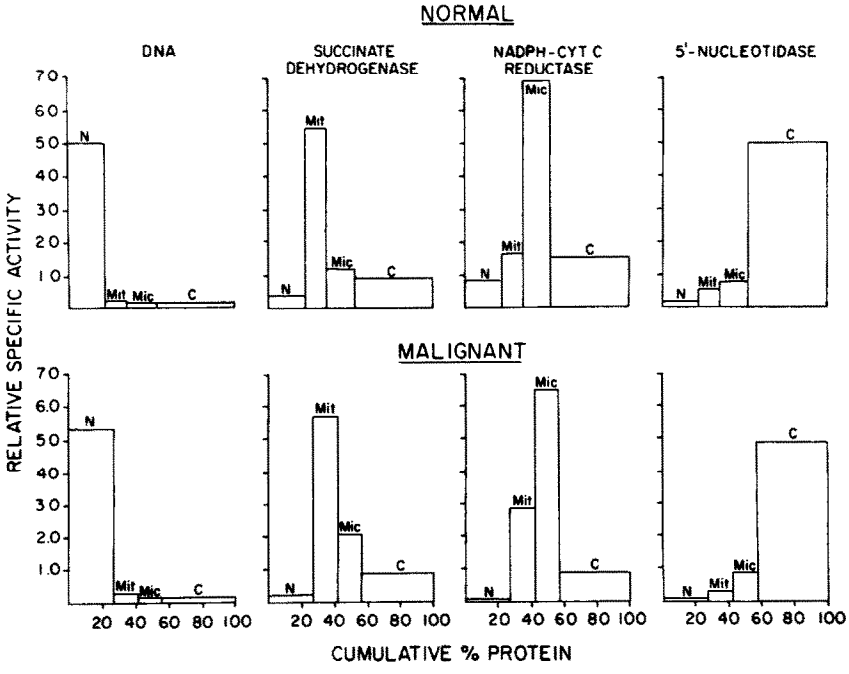

Fig. 3. Subcellular distribution pattern of DNA and marker enzymes of normal and malignant submandibular glands of rats. The relative concentration of DNA or relative specific activity of the marker enzymes (i.e. the concentration or specific activity in the subcellular fraction compared to that in the whole homogenate) is plotted as a function of cumulative percent protein. Key: N, nuclear fraction; Mit, mitochondrial fraction; Mic, microsomal fraction; and C, cytosolic fraction.

tissue was not statistically significant, the presence of PBRs in nuclei cannot be attributed to either mitochondrial or tissue debris contamination. If mitochondrial contamination was solely responsible for the nuclear PBR density, we would have expected a higher level of PBRs in the nuclei of the normal tissue because, in contrast to the nuclei from the malignant tissue, the nuclei of the normal tissue had a higher level of succinic dehydrogenase activity. Furthermore, the nuclei of the malignant tissue contained higher levels of DNA than that of the normal tissue. We recently confirmed that the nuclear localization of PBRs is authentic [27].

One might argue that the effect seen on the mitochondria in malignant tissue could be a direct effect of DMBA. However, the direct effect of DMBA is unlikely in view of the fact that addition of DMBA to the assay medium had no effect on the binding capacity of PBRs in the mitochondrial preparation. Further studies are needed to explore the significance of the modulation of PBRs in mitochondria of malignant tissue. It is possible that in malignancy, there is a modulation in the permeability of the mitochondrial membrane by alteration in the density of PBRs which have been shown recently to play a role in the metabolism of cholesterol in the mitochondria [28].

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