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A STUDY OF Na^+, K^+-ATPase IN THE RAT SUBMANDIBULAR GLAND TUMOR INDUCED BY DMBA*

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In attempts to evaluate carcinogenic chemical effect on Na^+, K^+-ATPase activity in the rat submandibular gland tumor induced by DMBA, pellet from DMBA powder was inserted into the right submandibular gland. Both right and left submandibular gland were excised and weighed following the period of experiment. Excised glands were observed microscopically and estimated biochemically.

The results were obtained as follows.

1. *Swelling and nodular mass in the right submandibular gland region ould be found at 11th week post-implantation with DMBA.*
2. *The weight and size of the right submandibular gland was markedly increased following the period of the experiment.*
3. *Epithelial dysplasia and invasive epidermoid carcinoma could be observed at 7th and 11th week after implantation of DMBA, respectively.*
4. *The rate of tumor induction in the right submandibular gland was about 76% at 17th week following implantation of DMBA.*
5. *DMBA caused markedly depressed Na^+, K^+-ATPase activity as well as the activity ratio in the rgiht submandibular gland following the period of experiment.*

I. INTRODUCTION

Since the first successful production of experimental salivary gland tumor was attempted by Löwenstein in 1910, varieties of confliction data have emerged.

In 1962 Shafer[14] implanted wafers of a variety of carcinogens into the submandibular gland of rat and was able to study both carcinoma and sarcoma. But the latter variety was less frequently found and carcinomas were of epidermoid variety. Adenocarcinoma was not observed. DMBA was found to have the shortest induction time.

Cataldo et al.[3] using a technique of implanting pellets of DMBA into the submandibular glands of rats, have produced epidermoid carcinomas arising in epithelial cysts and adenocarcinomas. Only one case of sarcoma occurred in this series, and malignant mixed tumors were not observed.

Experimentally induced tumor by DMBA in rat submandibular glands usually appear as well-advanced epidermoid carcinomas of ductal origin[15]. The submandibular gland of rodents contains a segment of duct interposed between the intercalated and the striated duct, referred to as the granular convoluted

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tubule or granular duct[20]. It has been proposed that the homologous cell of experimental carcinomas in the submandibular glands are probably granular convoluted tubule cells which occupy over 40% of the area of the gland[18]. The enzyme $Na^+,K^+-ATPase$ is a heterodimeric surface membrane protein complex and abundant in the basolateral plasmalemma of epithelial cells in fluid and ion transport.

In the salivary gland $Na^+,K^+-ATPase$ acts to decrease the osmolarity of saliva and shifts the electrolyte concentration from high Na^+-low $K^+$to low Na^+-high K^+[9]. This activity is thought to be driven largely by the duct cell $Na^+,K^+-ATPase$ pump. The distribution of $Na^+,K^+-ATPase$ in salivary gland has been investigated by using the p - nitrophenylphosphatase method[10], an autoradiographic method[17] and employing ultrastructural immunochemistry[6].

A primary site of $Na^+,K^+-ATPase$ activity would be the excretory duct system on the basis of above studies, most of which dealt with only one major salivary gland type in a single species.

The present study aimed at determining the effect on $Na^+,K^+-ATPase$ activity in the rat submandibular gland tumor induced by DMBA.

II. MATERIAL AND METHOD

1. Experimental animal

One hundred rats of Sprague - Dawley strain, each weighing about 100 grams, and consisting of fifty males and fifty females, were used for experiment. The animal were separated according to sex, placed five in a cage, and fed a standard laboratory diet (Cheil Co.).

2. Carcinogen

Carcinogenic agent was 7, 12 - dimethylbenzanthracene (DMBA : Sigma Chem. Co.).

3. DMBA pellet formation

Using the method of Cataldo & Shklar[2], pellets from DMBA powder were made. A 15 - gauge syringe needle was shortened so as to leave only 4mm of the shaft of the needle extending the hub. A plunger of the proper diameter to fit a 15 - gauge needle was placed in the shortened needle so as to leave a 4mm space between the end of the needle and the tip of the inserted plunger. Holding the hub of the needle and the plunger with pencil grip and using an up and down stroke, powdered DMBA was tamped into 4mm space between the tip of the needle and the end of the plunger. When the up and down motion could no longer force the powdered DMBA into the needle, the flat needle tip was placed against a flat surface and a sharp blow was delivered to the protruding end of the plunger. The shortened needle was inserted into an intact 15 gauge needle, and the pellet was extruded by the plunger from the shortened needle to the shaft of the intact needle, and the pellet was extruded until it was just visible at the pointed end of the needle. The pellet weighed approximately 5mg.

4. Pellet implantation

In order to insert the DMBA pellet into the rat submandibular gland, a median incision about 2cm long was made in the cervical skin under the pentothal sodium anesthesia (i.p., 25mg/Kg). The right submandibular gland was exposed by blunt dissection. The needle containing of the pellet was inserted the body of the glandular tissue and, the pellet was extruded by the plunger into the tissue. The needle was removed and the incision closed with 3 - 0 black silk.

5. Weigh and Preparation of the submandibular glands

Animals were sacrificed at immediately, 1st, 3rd, 5th, 7th, 9th, 11th, 13th, 15th and 17th week following implantation with pellet of DMBA and then, both right and left submandibular gland were excised, trimmed of adhering tissue remnants, rinsed several times with ice - cold normal saline to remove blood, and weighed after enucleation of pellet in right submandibular gland. A small portion of glandular tissue was prepared to specimen for routine histologic examination by H - E staining.

6. Protein assay

The excised submandibular glands were cut into small pieces, rinsed several times to remove saliva. The mince was then placed in 9 volumes of 0.25 M sucrose, 50mM imidazole and 0.1mM ATP, at pH 7.5. The suspension was subjected to a Virtis "45" homogenizer for 5 minutes and then, homogenized further in a glass vessel with Teflon pestle with 10 up - and - down movement. By the technique of Bradford[1], the homogenate containing 10 to 100μg protein was pipped into test tubes. The volume in the test tube was adjusted to 0.1ml with distilled water. 5ml of protein reagent was added to the test tube and the contents mixed either by inversion or vortexing. Using bovine serum albumin as the standard, the absorbance at 595 nm was measured after 2 minutes as compared with standard assay. Protein reagent was prepared as follow. 100mg of Coomassie Brilliant Blue R - 250 was dissolved in 50ml of 95% ethanol. To this solution 100ml of 85%(w/v) phosphoric acid was added. The resulting solution was diluted with distilled water to a final volume of 1 liter.

7. Determination of Na^+,K^+-ATPase activity

ATPase activity was determined by expression of micromoles of inorganic phosphate released per hour per miligram of protein by the method of Fiske and Subbarow[4]. Total volume of the reaction mixture was 1ml and contained, in final concentration, 5mM $MgCl_2$, 3mM ATP, 100mM NaCl, 10mM KCl, 1mM EDTA, 50mM Tris - buffer at pH 7.5 and then, 0.1ml of the enzymatic preparation was added. This mixture was incubated at 37°C for an hour. In this reaction mixture not included NaCl and KCl, Mg^{++}-ATPase activity was determined in the presence of 0.1mM ouabain by the technique of Jørgensen[7]. Na^+,K^+-ATPase activity was defined as the difference between total ATPase activity and Mg^{++}-ATPase activity by the method of Schwartz and Moore[13].

III. RESULT

1. Gross observation

Beginning at 11th week post - implantation swelling was visualized, and nodular masses palpated in the right submandibular gland region of all animals(Fig. 1). The weight and size of right submandibular gland was markedly increased following the experimental period(Table 1).

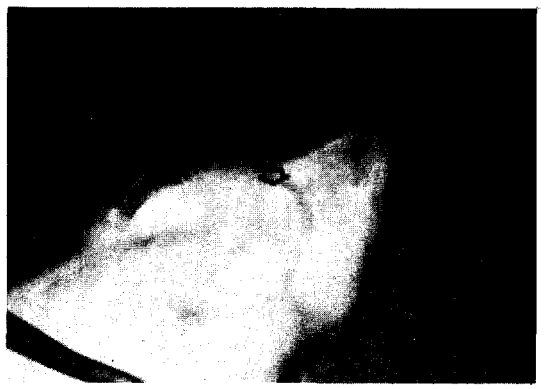

Fig. 1. Animal with moderate swelling and palpable mass on right side of neck at 11th week after DMBA implantation.

2. Histological examination

In right submandibular gland epithelial dysplasia could be observed at 7th week(Fig. 2) and invasive epidermoid carcinoma at 11th week post - implanta-

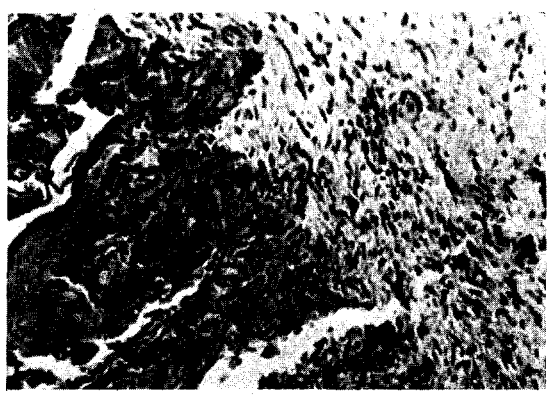

Fig. 2. Epithelial dysplasia, not invading to adjacent connective tissue at 7th week after implantation of DMBA (H-E×200).

Fig. 3. Invasive epidermoid carcinoma at 11the week following DMBA implantation (H-E×200).

tion(Fig. 3). The rate of tumor induction in the right submandibular gland was about 76%(76 of the 100 rats) at 17 weeks after implantation with DMBA.

3. ATPase activity

The control submandibular gland in left side revealed relatively steady active Na^+,K^+-ATPase enzyme system with normal growing pattern, but in the right submandibular gland DMBA caused the remarkable increase in size and mass, and markedly depressed Na^+,K^+-ATPase activity(Table 2) as well as the activity ratio to the left submandibular gland(Table 3) following the period of the experiment.

Table 1. Wet weight of submandibular gland (gram)

WEEK	RIGHT SIDE(experimental)	LEFT SIDE(control)
0	0.61± 0.15	0.60± 0.13
1	0.63± 0.21	0.61± 0.18
3	0.85± 0.32	0.72± 0.23
5	0.98± 0.21	0.78± 0.15
7	1.53± 0.43	0.81± 0.30
9	2.23± 0.58	0.85± 0.27
11	3.46± 0.75	0.86± 0.21
13	3.95± 0.83	0.89± 0.32
15	4.53± 0.98	0.92± 0.29
17	5.13± 1.03	0.94± 0.36

Table 2. ATPase activity of submandibalr gland (nMole/min/mg. protein)

	RIGHT(experimental)			LEFT(control)		
WEEK / ATPase	TOTAL	Mg^{++}	Na^+-K^+	TOTAL	Mg^{++}	Na^+-K^+
0	1,280± 156	758± 62	522± 45	1,315± 182	761± 58	554± 62
1	1,312± 162	785± 46	527± 56	1,298± 157	753± 46	545± 68
3	1,265± 159	732−74	533± 81	1,321± 195	762± 58	559± 64
5	1,146± 183	716± 85	430± 83	1,265± 201	746± 53	519± 83
7	1,126± 167	719± 93	407± 68	1,318± 203	759± 65	559± 62
9	1,068± 195	705± 88	363± 74	1,254± 165	716± 48	538± 54
11	1,053± 201	691± 71	362± 58	1,276± 146	735± 56	541± 63
13	989± 216	681± 65	308± 63	1,288+153	758± 60	530± 46
15	1,018± 253	701± 58	317± 74	1,317± 195	742± 56	575± 63
17	965± 219	698± 89	267± 48	1,342± 198	753± 65	589± 83

Table 3. Ratio of $Na^+,K^+-ATPase$ activity to the control group

WEEK	%	WEEK	%
0	94	9	67
1	97	11	67
3	95	13	58
5	83	15	55
7	73	17	45

Ⅳ. DISCUSSION

According to Standish[16] the process of development of tumor in submandibular glands of rat begins with squamous metaplasia and abnormal proliferation of the striated duct epithelium, sequentially proceeding to epidermal or keratinizing cyst formation, dyskeratosis of the cystic wall epithelium and epidermal cancer. The results of the present experiment revealed that epithelial dysplasia could be observed at 7th week after implantation, provided that the metaplastic epithelium was successively influenced by DMBA.

Watanabe et al.[19] reported that tumors of the submandibular gland were recognized at 24 weeks in 11(55%) of 20 rats, while Shklar and Cataldo[16] and Kim[8] had produced 100% induction rate in all DMBA - implanted animals at 20 weeks. The result of the present investigation showed that the induction rate of epidermoid carcinoma in the right submandibular gland was 65% at 17 weeks after implantation with DMBA. This would indicate the difference of method for pellet formation and duration for experiment. The $Na^+,K^+-ATPase$ is the plasma membrane protein that couples the transmembrane transport of Na^+ outward and K^+ inward to the hydrolysis of cytoplasmic ATP[5]. This enzyme probably exists at some level in most types of cells, serving to maintain the high K^+ and low Na^+ activities essential to cell metabolism. The duct system of human and rat submandibular glands, composing of intercalated, striated, and excretory ducts, have well - developed striated ducts which are rich in $Na^+,K^+-ATPase$, and secrete a nearly hypotonic saliva[20]. Primary saliva formed by the acinar cells is near isotonic, with high Na^+ and $Cl^-$and low K^+. The high electrolyte content serves as the osmotic force to pull water into the lumen of the acinus. In most gland types, the electrolyte content of saliva is modified as it passes through the duct system. Na^+ and Cl^-decrease and K^+ increase with an overall drop in tonicity[22]. The morphology and the related ion transport capacity of duct system, as well as the flow rate of saliva, would be expected to dictate the degree of electrolyte modification. Winston et al.[21] suggested that parasympathetic stimulation somehow activates cellular ion transport mechanism, leading to production of a large flow of saliva in salivary gland. The removal of Na^+ and secretion of K^+ are probably related to both the length and the $Na^+,K^+-ATPase$ activity of the ducts. The induced hypertrophy of the rat submandibular gland involves the acinar cells, while the granular ducts decrease in number, become compressed and may even disappear after chronic administration of isoproterenol[15]. Since the granular ducts represent one of the main secretory elements[12,20], the almost complete loss of the action transport enzyme system, suggests that the major active electrolyte transport process occurs in the ductal system, rather than in the acinus. Schwartz and Moore[13] also reported that isoproterenol - induced hypertrophied glands revealed a $Na^+,K^+-ATPase$ with markedly depressed activity, but little effect on salivation. Schneyer[11]also suggested little difference in saliva volume per unit between the normal and isoproterenol - treated gland. It is thought to be possible because isoproterenol caused an increase acinar protein without a concomitant in $Na^+,K^+-ATPase$. The present study showed markedly depressed ATPase activity as well as activity ratio following implantation of DMBA. This findings lend carcinogenic chemical effect to the presence of active cation transport in rat submandibular gland.

Ⅴ. CONCLUSIONS

1. Swelling and nodular mass in the right submandi-

bular gland region could be found at 11th week after implantation with DMBA.

2. The weight and size of the right submandibular gland was markedly increased following the period of the experiment.
3. Epithelial dysplasia and invasive epidermoid carcinoma could be observed microscopically at 7th and 11th week after implantation with DMBA, respectively.
4. The rate of tumor induction in the right submandibular gland was about 76% at 17th week following implantation of DMBA.
5. DMBA caused markedly depressed Na^+,K^+−ATPase activity as well as the activity ratio in the right submandibular gland during the period of the experiment.

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백서의 DMBA 유도 악하선종양에서의 Na^+, K^+-ATPase 에 대한 연구*

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체중 100g 내외의 Sprague Dawley계 정상백서 암, 수 각 50마리씩을 실험동물로 하여 분말형태의 순수 DMBA를 소구(pellet)로 압착제작하여 우측 악하선에 매식후 즉시, 1,3,5,7,9,11,13,15,17주째 10마리씩 희생시켜 좌,우측악하선을 적출, 무게를 측정하고, 육안적, 조직학적, 생화학적으로 실험기간에 따라 비교분석하여 다음과 같은 결론을 얻었다.

1. 매식후 11주째부터 우측 경부 악하선부위의 종창을 관찰할 수 있었으며, 결절덩어리를 촉진할 수 있었다.
2. 우측악하선의 무게와 크기는 실험기간의 경과에 따라 괄목할만한 증가를 보였다(매식후 17주째는 좌측에 비해 약 5.5배의 무게를 나타냈다).
3. 매식후 7주와 11주째부터 조직학적으로 상피이형성과 침윤성 유표피암의 소견을 각각 관찰할 수 있었다.
4. 우측 악하선에서의 유표피암의 발생율은 매식후 17주째 76%이었다.
5. DMBA는 현저히 억제된 Na^+,K^+-ATPase 활성도를 야기하며 실험기간의 경과에 따라 활성도 비율도 현저히 저하시킨다.

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