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DIURNAL VARIATION IN CELL POPULATION KINETICS OF NORMAL HAMSTER CHEEK POUCH AND HAMSTERS WITH SQUAMOUS CELL CARCINOMA†

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After twelve weeks of regular topical application of 0.5% 9,10-dimethyl-1,2-benzanthracene (DMBA), a marked diurnal variation was found in the DNA synthesis of squamous cell carcinoma in hamster cheek pouches. Such rhythms were barely found in the control group.

A statistically significant difference occurred between the high values and low values of labeling and clustering indexes of the experimental group. The labelling and clustering indexes in the experimental group were 9.6 times greater than that of the control group. The experimental group also showed non-random distribution of labeled tumor cells.

The experimental group of this study showed approximately 1.7 times more cells in DNA synthesis than were found in the experimental groups of our previous study. The control group had approximately 2.7 times less labelled basal cells in DNA synthesis than that of the previous study. This tends to confirm that in the previous experiment the control group was indirectly affected by a painted experimental side. These findings prove that the longer DMBA was used, the more epithelial cells in clusters entered DNA synthesis.

The rhythmic pattern of tumor cell clustering provides evidence of "symphokinesis". It is suggested that diurnal variation in malignant tumors may provide opportunities for improving treatment.

Key words: DMBA, diurnal variation, cluster

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Since Drooglerer and Leijden[1] first reported on the Mitotic index rhythms in animals, many related studies have found that circadian fluctuations in epidermal cell proliferation were regulated at the mitotic division; Lin, Goepp & Sewell[6] reported a strong diurnal DNA synthesis with the degree of variation dependent on the age of the experimental groups; Lin & Goepp[7] also demonstrated diurnal rhythms and non-random distribution of DNA synthesis and mitotic events in hamster cheek-pouch epithelium which had been treated with DMBA for periods of four and seven weeks. A similar phenomenon has also been reported by Glass & Goepp[3,4] in the mouse tongue after radiation injury and in the stratified squamous epithelium of wounded tympanic membranes[11]. The study of epithelial population kinetics has not only been done in growing animals and tissues recovering from injury but has also been undertaken in malignant tumors, such as Lewis lung carcinoma[12], Elrich ascites tumor[8,13], skin tumors[5], Muriane sarcoma[14], human solid tumors[15], renal adenocarcinoma[16] and brain tumors[17]. At

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present, studies of possible diurnal variation in cellular population kinetics during malignant periods of oral squamous cell carcinoma have not been done.

Squamous cell carcinoma is the most common malignant neoplasm occurring in the oral cavity. The tumor kinetics of such a cancer involves daily oscillations in cellular activity which may differ from normal. Cameron *et al*[18] reported statistical support for non-randomness in the spatial distributions of both mitotic figures and labeled cells in normal mouse esophageal epithelium.

The possibility of diurnal variation in the radiation sensitivity of tissues has been studied by Pizzarello *et al*[19] and Rugh *et al*[9]. They found that mortality rates varied in irradiated rats in relation to the time of day at which the radiation was given.

In chronotherapy on human solid tumors, Focan[20] reported that in man the peak cellular DNA synthesis occurs during the morning. Therefore, it was suggested that such a rhythm be considered when choosing the hours and sequence of administration of antineoplastic drugs[21,22]. In the selective timing of cyclophamide administration for tumors in mice, Cardoso *et al*[23] reported that the frequency of cures were the highest at 0900 hr. and lowest at 1800 hr. with difference in rate by 4 times.

The possible dependence of malignant oral tumors on circadian rhythms has not yet been reported. Thus, purpose of this present study is to examine the chemical carcinogen, DMBA, induced squamous cell carcinomas in hamster cheek pouch for possible diurnal variation, spatial patterns, and degree of synchrony of neoplastic cells in DNA synthesis.

MATERIALS AND METHODS

A total of seventy-two adult male golden Syrian hamsters were evenly divided into two groups. In the experimental group, the right cheek pouch of each hamster was painted with a No. 4 camel brush dipped in a 0.5% solution of 9,10-dimethyl-1,2-benzanthracene (DMBA) in heavy mineral oil. Each pouch was painted twice a week at 10:00 A.M. for twelve consecutive weeks. The pouches of the control group animals were not painted. After twelve weeks of painting, the hamsters of both the experimental and control groups were killed by ether one hour after each hamster received an injection of 100 μCi tritiated thymidine (3HTdR, specific activity of 3.0 Ci/mM). For a period of 24 hours at intervals of 2 hrs., 3 hamsters were killed. The actual time of day for collecting right side pouch specimens were 1300, 1500, 1700, 1900, 2100, 2300, 0100, 0300, 0500, 0700, 0900, and 1100 hrs..

The right side pouches of each hamster were excised, cleaned with sterile water and placed in 10% buffered formalin for a 24 hr. fixation period. Then tumor bearing areas of pouches were selected and horizontal sections were embedded in rosineskar paraffin. Six sections were then cut at a thickness of 5 microns and placed evenly on three glass slides. The first set of slides were stained with H & E., and the remaining slides were stained with Feulgen reaction and prepared for autoradiography[24]. 2000 basal cells per control pouch and 2400 tumor cells per experimental pouch were counted. Microscopic counting of labeled nuclei was done with the aid of 0.5 mm reticular eyepiece. Only cell nuclei which were covered by five or more grains within the nuclear outline were counted as labeled.

The definition of clusters and the measurement of labeling index, cluster index and cluster factor are based on Glass & Goepp[3], Lin *et al*[6]. Namely, the cluster is a labeled cell group which is consisted of two or more contiguous related labeled cells with more than two non-labeled contiguous cells. The labeling index is measured by the counted labeled cells times 100 and divided by the total number of counted cells in the basal layer or tumor area. The cluster index equals the number of clusters divided by a total number of counted cells times 100 and the cluster factor equals the number of clusters divided by the total number of labeled cells. To analyze the distribution of labeled cells within each specimen, each specimen was divided into a statistical grid.

Chi-square analysis[25] included 23 degrees of freedom in the experimental group and 49 degrees of freedom in the control group, and a 0.05 probability level was selected as the maximum to establish non-

randomness.

RESULTS

After 12 weeks of DMBA painting by visual observation, it was found that almost all the right pouches of experimental hamsters revealed large irregularly shaped tumor-like swellings with severe surface changes. Some pouches evidenced multiple small tumors with abscess formation. The size of tumors ranged from 0.2 centimeters to 2.5 centimeters in diameter, and they were reddish to yellowish-brown in color, and were soft in consistency. Cut surfaces revealed tumor mass projections with subsequent destruction of surface of epithelium and underlying connective tissue. Microscopic examination demonstrated squamous cell carcinoma in tumor-bearing pouch specimens.

Preliminary study of the autoradiographs of DNA synthesis under low power revealed apparent spatial grouping, clustering, and varying densities of distributions of labeled basal cells and tumor cells in both groups throughout the different time periods of a day. These findings were similar to those previously described by Glass and Goepp[3,4] and Lin and Geopp[7]. All samples were counted and the labeling index, cluster index and cluster factor were determined. The results are shown in Figures 1 through 3.

After determination of the labeling index and cluster index of the tumor cells in the experimental group and in the basal cells of the control groups, data was divided into the previously described statistical grids. Probability values were determined for all samples. These are indicated in Table 1.

The highest value of the labeling index

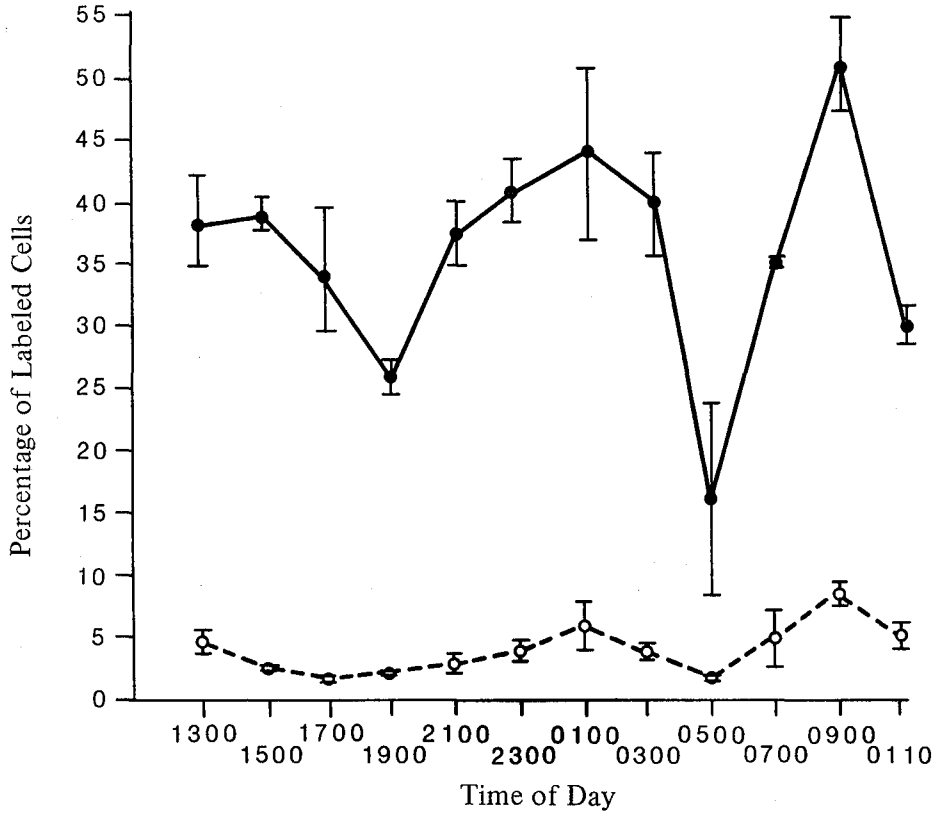

Fig. 1. Percentage of labeled tumor cells (solid lines) and control basal cells (dashed lines) per pouch sample after administration of ^{3}HTdR given at intervals of two hours during a 24-hour period.

Table 1. The Probability Values for All Samples

Time	Sample	Chi-square probability			
		Experimental		Control	
		Labeled cells	Clusters	Labeled cells	Clusters
1300	A	0.100	1.975	0.100	0.500
1300	B	0.005	1.500	0.900	0.900
1300	C	*	*	0.500	0.900
1500	A	0.500	1.750	0.500	0.900
1500	B	0.005	1.150	0.500	0.100
1500	C	*	*	*	*
1700	A	0.500	0.975	0.100	0.900
1700	B	0.001	0.975	0.500	0.900
1700	C	0.0001	0.995	0.500	0.500
1900	A	0.001	0.975	0.900	0.500
1900	B	0.0005	0.975	0.500	0.500
1900	C	0.100	0.995	*	*
2100	A	0.005	0.975	0.900	0.900
2100	B	0.005	0.995	0.900	0.900
2100	C	0.005	0.900	*	*
2300	A	0.500	1.150	0.975	0.900
2300	B	0.005	1.500	0.005	0.975
2300	C	0.005	0.995	*	*
0100	A	0.005	0.900	0.005	0.900
0100	B	0.010	1.200	0.005	0.005
0100	C	0.025	1.150	0.005	0.500
0300	A	0.005	0.975	0.005	0.900
0300	B	0.010	0.995	0.900	0.500
0300	C	0.050	1.500	*	*
0500	A	0.005	0.975	0.050	0.900
0500	B	0.005	0.900	0.500	0.995
0500	C	*	*	*	*
0700	A	0.050	0.900	0.900	0.025
0700	B	0.001	1.050	0.005	0.500
0700	C	*	*	*	*
0900	A	0.001	0.995	0.500	0.900
0900	B	0.500	1.750	0.010	0.900
0900	C	0.500	0.995	0.005	0.900
1100	A	0.900	0.995	0.500	0.900
1100	B	0.250	0.995	0.900	0.500
1100	C	0.500	0.990	*	*

*No animal has been used

(50.6%) in the experimental group occurred at 0900 hr. in the morning, and the lowest value (15%) was at the 0500 hr. in the early morning.

In the control group, high and low values of labeling and clustering indexes also demonstrated some statistically significant differences as shown in the data presented in Figures 1 & 2 and Table 1.

For a comparsion of labeling index and clustering index of both groups, data was plotted in a histogram shown in Figure 4. This displays the labeling difference between the tumor cells of the experimental group and the basal cells of the control group, to be approximately 9.6 times in the difference. The difference between the tumor clusters, experimental group, and basal cell clusters, control group, was also approximately 9.6 times.

DISCUSSION

Preliminary examination of the tritiated thymidine (3HTdR) labeled specimens at different times of a day revealed that the percentage curves of the tumor cells and the cluster curves were parallel to each other (Figs. 1, 2). That is, the more labeling of tumor cells present, the more the clusters. The distribution of tumor cells and clusters in the DNA synthesis at different times of the day were both biphasic curves. Low points were found in the experimental group of labeled tumor cells at 1900 hr. and 0500 hr. The high points were at 0100 hr., 0900 hr. and 1300 hr. respectively. The difference between the highest and the lowest points of both the tumor cells and tumor clusters was approximately 2.5 times ($p < 0.05$).

The number of labeled cells present had

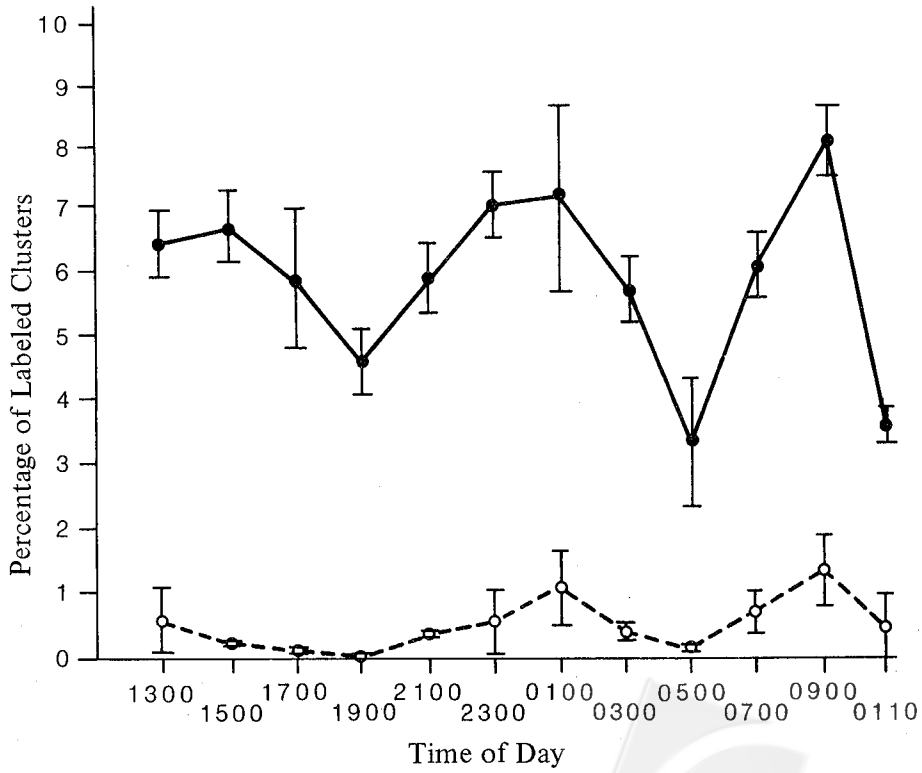

Fig. 2. Percentage of labeled clusters of tumor cells (solid lines) and control basal cells (dashed lines) per pouch sample after administration of ^{3}HTdR given at intervals of two hours during a 24-hour period.

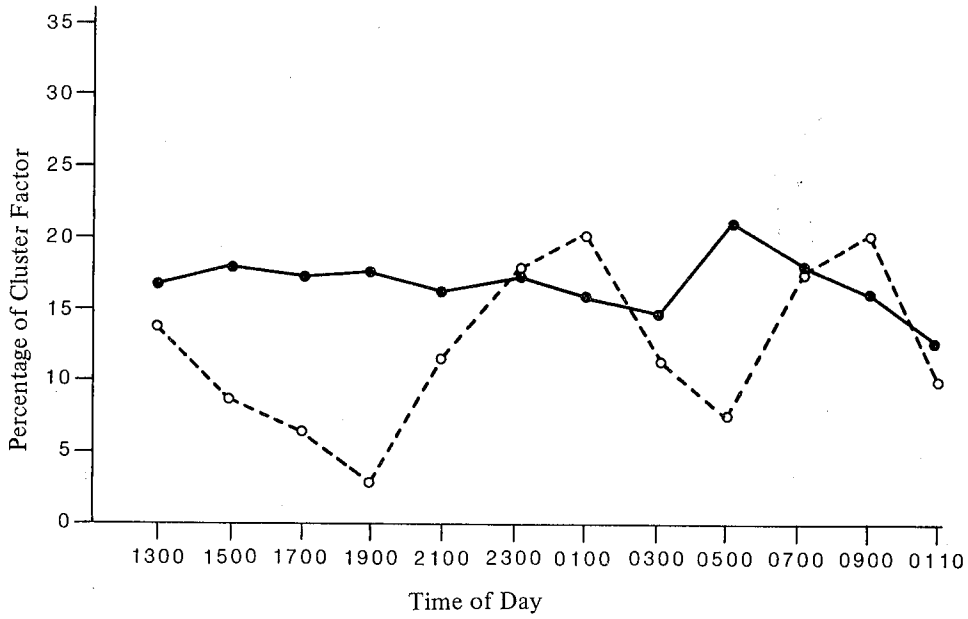

Fig. 3. Percentage of cluster factor of labeled tumor cells (solid lines) and control basal cells (dashed lines) per pouch sample after administration of ^{3}HTdR given at two hour intervals during a 24-hour period.

a high value at noon and then declined slightly in the afternoon, reaching a low point at 1900 hr. in the evening. Following this, the curve climbed slightly and peaked at midnight. After midnight, the curve again declined reaching a nadir at 0500 hr.. Then the curve climbed sharply to the higher level at 0900 hr..

Chi-square analysis of labeled tumor cells and their clusters in DNA synthesis revealed nonrandomness pattern was reported in a previous study in a premalignant model[7], and was found in two of the twelve time periods of the DMBA painted group which had been painted for four consecutive weeks. Nonrandomness was also found in four of the twelve time periods in the DMBA painted group which had been painted for seven consecutive weeks.

By comparing the results of a previous study[7] with the present study, it can be seen that a longer DMBA painting period is related to more labeled cells in nonrandom distribution. In addition, in a previous study[7], the label index and cluster index of experimental groups were two to two and a half times higher than that of the control group. However, in the present study's experimental group, this percentage is approximately 9.6 times higher than that of the control group.

Also in a previous study, Geopp *et al*[6] demonstrated a 100% identical reciprocal relationship between the label index value and cluster factor value in young mouse tongue epithelium at different times of the day. In the group painted with DMBA for four weeks, it was found that approximately 40% of the label index value and cluster factor value showed this "reciprocal phenomenon". In the group painted with DMBA for seven weeks, it was also found that 18% showed this relationship. In this present study, this kind of labeling index and cluster factor relationship occurred in only 8% of the diurnal cycle.

In general, the findings of this study showed that the labeling index, cluster index and cluster factor increased proportionately with the length of the painting time of the chemical carcinogen, DMBA.

In this experiment it was found that the higher the number of labeled cells, the higher

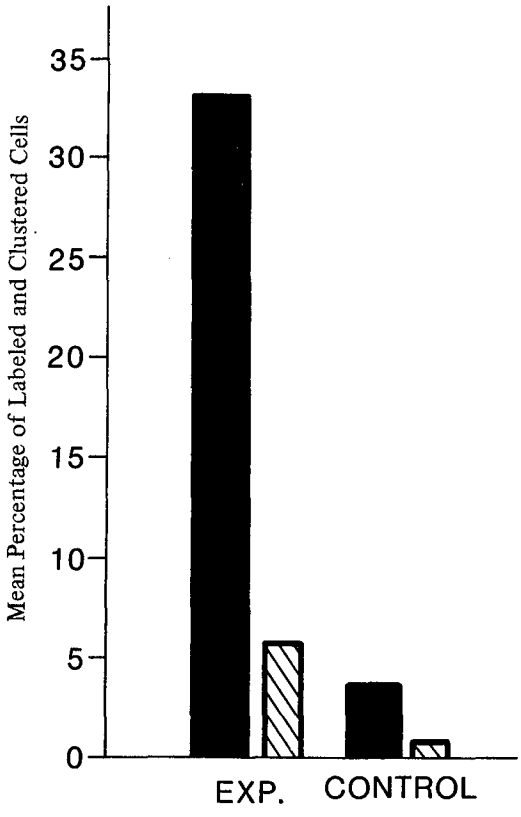

Fig. 4. Mean percentage of labeled cells (solid bar) and clusters (diagonal bar) in experimental and control groups of hamsters.

the number of clusters. These findings are consistent with Glass & Goepp's study[3,4] on mouse tongue after radiation injury.

Statistical analysis of labeled basal cells and clusters at different times of a day in the control group showed that the difference between high and low points was small. The curves tended to be smooth and flat. These findings are consistent with a previous study of Lin and Goepp's[6] where it was found that the degree of diurnal rhythm tends to diminish with the increasing age of the animal. With this in view, one can explain at least partially Goepp and Fitch's study[26] on oral radiation death. They found that the mortality rate decreased in the elder age groups of mice and they concluded that the mortality rate appeared not to be influenced by rhythmic cycles after the mouse reached an age of 16 weeks assuming that radiation sensitivity was related to cell cycle percentages.

The reciprocal relationship between the cluster factor and labeled basal cells in the control group also supported the findings of Lin, Goepp & Sewell's study[6] in diurnal variation in epithelial cell population kinetics of the young mouse tongue.

CONCLUSION

The purpose of this study was to determine whether or not the diurnal rhythm and spatial patterns were present in the basal cells of normal adult hamster cheek pouch epithelium and tumorous epithelium (squamous cell carcinoma) induced by 0.5% DMBA after 12 weeks of painting.

The DNA synthesis of the basal cells in the normal cheek pouch epithelium showed very little evidence of diurnal variation. However, there was strong diurnal rhythm found in DNA synthesis of tumor cells and related clusters in the experimental group. Therefore, malignant tumor formation in epithelium exhibits the same cell population synchrony phenomeon as benign tumor formation or tissue repair. The differences between these types were in degree and patterns of synchrony or symphokinesis. In addition, nonrandom distribution of tumor cells could be observed at most time periods in a day.

This study has demonstrated that abnormalities which occur in epithelial cell population kinetics of malignant cells are similar to changes seen in the formation of premalignant cells, except that the degree of change increased in malignant cell populations.

This study also suggests that certain times of the day when chosen according to these diurnal variation patterns may help to improve the results of non-surgical therapies.

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正常倉鼠頰囊袋與患類上皮癌袋中DNA合成期之表皮細胞及細胞叢集與一日間時辰節律變化之關係

林立民　Robert A. Goepp*

使用0.5%二苯甲蒽(9,10-dimethyl-1,2 benzanthracene, DMBA)於倉鼠(Golden Syrian hamster)頰囊袋定期塗抹(每週三次)12週後於實驗組中，絕大多數之倉鼠均產生類上皮癌，在實驗組與對照組中於DNA合成期之癌細胞(含細胞叢集)及基底細胞(含細胞叢集)均呈周日節律性狀況(diurnal variation)前者之幅度及頻率高而密，後者則偏於低且平坦緩慢，被標幟的細胞及細胞叢集之百分率在統計學上有顯著之差異，前者約爲後者之9.6倍，且於一天中之大部份時刻呈不隨意分佈，其間最高值與最低值有顯著差異。在進入DNA合成期之細胞數與細胞叢數之百分率比癌前期周日變化報告之實驗組增加1.7倍，然而比對照組之數目減少2.7倍。正合乎前實驗之推論，在DNA合成期之癌細胞呈周日節律性分佈及部份呈細胞叢狀，證實了「動力同部」(symphokinesis)之可能性，動力同部就是一群細胞在進入DNA合成期或細胞分裂期時呈叢狀且和諧狀的不隨意分佈，它不僅在幼鼠口腔中之底層細胞被報告過，且倉鼠癌前期之變性細胞中也被發現過。對於已確知對放射線敏感之癌細胞而論，本篇之發現強調了選擇時辰治療癌症之重要觀念。

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