

# Creatine kinase isoenzymes activity in serum and buccal pouch tissue of hamsters during DMBA-induced squamous cell carcinogenesis

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The sequential changes of creatine kinase (CK) isoenzyme activity in serum and hamster buccal pouch tissue (HBPT) of normal, premalignant, and malignant stages during 13 wk of DMBA induced hamster buccal pouch squamous cell carcinogenesis, were studied. We found that the total CK activity in both serum and HBPT of the DMBA painted groups increased during the premalignant period (3-7 wk), with the peak being at 7 wk, while declining in the malignant period (9-13 wk). No significant difference in the serum and HBPT CK-MM, CK-MB and CK-BB activity, in DMBA-induced carcinogenesis, could be established. However, the macro CK type 2 (MCK-2), a cathodically migrating atypical creatine kinase isoenzyme, initially appeared in the sera and tissues of the 3rd wk DMBA-treated group. Its activity gradually increased during the premalignant and malignant stages, paralleling the increasing number of DMBA applications. There was a correlation between the degree of differentiation, and the size and number of the induced tumors with MCK-2. No MCK-2 could be detected in any of the sera or tissues of the control, nor in the 1st wk DMBA-treated group. We propose that MCK-2 is a potential tumor marker in oral malignancy and its presence may provide valuable information for early diagnosis of a possibly premalignant transforming oral lesion.

**Key words:** creatine kinase isoenzymes; DMBA-carcinogenesis, oral; hamster; tumor marker.

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Creatine kinase, CK (ATP:creatine N-phosphotransferase, E.C.2.7.3.2.) catalyzes the reversible phosphorylation of creatine according to this equation: adenosine triphosphate (ATP)+creatine=adenosine diphosphate (ADP)+creatine phosphate (CP) in which ATP is the phosphate donor (1). There are 4 known CK isoenzymes distributed in the cytoplasm and mitochondria (2) of the cells. The cytoplasmic CK isoenzymes, namely, CK-MM (muscle type), CK-MB (myocardial type) and CK-BB (brain type), are composed of the dimer of two immunologically distinct M (muscle) and B (brain) polypeptide subunits (3, 4), which are so called because they have been isolated from skeletal muscle and brain tissue respectively (5). However, there are many reports of atypical forms of CK termed macro CK (6, 21-28, 42). These include macro CK

type 1, identified as CK-BB linked to IgG, which, during electrophoresis, migrates between the CK-MM and CK-MB bands (2) and macro CK type 2 (MCK-2), which is an aggregate of mitochondrial CK that migrates cathodically to CK-MM band during electrophoresis (2). Creatine kinase isoenzymes are present in various normal tissues and neoplasms (7). Normal serum and skeletal muscle contain almost exclusively CK-MM. CK-MB is found primarily in cardiac muscle, while CK-BB is in the brain, and the gastrointestinal and genitourinary tracts (8, 9). The basis for clinical use of CK isoenzymes stems from the fact that their abnormal appearance reflects damage to the tissue of origin (10). The increase in serum CK-MM activity is found in various muscle disorders and prolonged exercise (11). Serum CK-MB level is used as an indi-

cator of myocardial damage (12). As CK is an important enzyme in regulating cell energy reservoir (1), it should be expected to be greatly affected during tumor development (13). Abnormal CK-BB activity was reported in the serum of patients with lung (small cell type) (14, 15), prostate (16, 17), gastrointestinal (18, 19) and breast cancer (20). Recently, the MCK-2 has been investigated as a tumor marker in breast (21, 22), gastrointestinal (23-25) and hepatic carcinoma (25-28). A review of the literature shows there are few CK-isoenzyme studies in oral malignancies, particularly in animal model. The normal hamster buccal pouch tissue (HBPT) is composed of a thin squamous cell epithelial layer, connective tissue and a muscle layer and is one of the most commonly used animal models for oral carcinogenesis (29). The purpose



of this study was to determine the sequential comparative changes in CK isoenzyme activity in serum and HBPT during DMBA induced carcinogenesis. Furthermore, the use of MCK-2 as a potential tumor marker in oral malignancies was analysed.

## Material and methods

### Induction of carcinoma

Forty-eight non-inbred male adult (10–12-wk-old) Syrian golden hamsters weighing 120–150 g, were divided into one control and seven experimental groups, each containing six animals. They were fed a commercial diet and given tap water. 0.5% 7,12-dimethylbenz[a]anthracene (DMBA) in heavy mineral oil, was applied thrice weekly with a No. 4 camel's hair brush to the right cheek pouches of all the animals of the seven experimental groups. The cheek pouches of the control group and the left pouch of each animal of the experimental groups was left untreated and served as a built in control. At the end of each experimental week (3 days following DMBA painting), one group of six animals was omitted from the subsequent application. Then, under ether anesthesia, 0.5 to 1.0 ml of blood was drawn intracardially with a No. 19 gauze needle. The blood was then centrifuged at 3000 rpm for 10 min. The supernatant (serum) was transferred to eppendorf tubes in aliquots of 25  $\mu$ l and quick-frozen in liquid nitrogen. The animal was then killed by a lethal dose of diethyl ether. The buccal pouches were everted, examined grossly and the mucosa was excised. The samples comprised areas where lesions were grossly visible; otherwise, they were taken longitudinally, at random, from the pouches. All the tissues were quick-frozen in liquid nitrogen for CK isoenzyme study (30, 31). The remaining pouch tissue was immersed in 10% buffered formalin, dehydrated in ascending alcohols, cleared in xylene and embedded in paraffin. Sections were cut to 4  $\mu$ m, stained with hematoxylin-eosin and examined by light microscopy. The same procedures were repeated at a bi-weekly interval until all the animals (including the control group) had been killed.

### CK isoenzyme study

The tissue homogenates of pouch tissues were prepared as described by

KANEMITSU *et al.* (30). Before extracting the CK isoenzymes, the tissue was thawed on ice and measured for its wet weight. Then, the tissue was cut into small strips and homogenized on ice with 10 vol (v/w) of 10 mmol/l tris-HCl (pH 7.4) buffer with the Kinematica AG (polytron PT3000) homogenizer at 600 rpm for 20 strokes. Cellular debris was removed by centrifuge at 12,000 g for 10 min at 4°C. The pellets were then thoroughly washed five times with the tris-HCl buffer and suspended in 50 mmol/l  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  buffer (pH 7.4, 4°C) containing 50 g/l of human albumin and 5 mmol/l glutathione. After incubation at 4°C for 1 h, the suspension was centrifuged at 105,000 g for 30 min at 4°C and the supernatants possibly containing mitochondrial CK (CK-Mt) were obtained. The total CK activity of serum and supernatant fluid of tissue homogenates were estimated at room temperature with a Shimadzu UV-visible Recording Spectrophotometer, based on the kinetic method of ROSALKI (31), with a reagent from Merck company (No. 14328 Merck-1-Test). This reagent contains AMP and diadenosine pentaphosphate to inhibit adenylate kinase (E.C.2.7.4.3.) activity (32).

The serum and supernatant fluid of tissue homogenates was electrophoresed at 225 V for 20 min at pH 8.8 on cellulose acetate film with reagents and equipment from Gelman Science Inc. This was done according to the manufacturer's instructions (Gelman Sci No. 51914). The samples were run simultaneously with the CK isoenzyme marker, CK/LD isoenzyme control (Cat. No. ECA 051317, ECA Inc.), which contains monkey CK-MM, CK-MB and CK-BB respectively. To develop the CK activity, the CK/UV reagent from ECA company (Cat. No. ECA 05131), was prepared 5 min before the end of electrophoresis, according to the manufacturer's instructions. Then, 1 ml of the prepared CK/UV reagent was spread evenly over the film and was incubated in a moist chamber for 30 min at 37°C and dried in a 45°C forced air oven. The film was illuminated from below and the isoenzyme bands were observed in a view box with two long wave ultraviolet (UV) lamps (365 nm) (33). The migration distance of each CK isoenzyme band of the samples was compared with that of CK control bands. Then, the activity, expressed as a per-

centage of the total intensity of each CK isoenzyme, was determined and recorded under the UV light with a wavelength of 365 nm, by a scanning densitometer (DC 2020 Gelman Science Inc.).

## Results

Gross observation of the DMBA-treated pouches revealed that the pouch tissues initially exhibited erythema and erosion followed by repair. Later, there was a rough, thickened mucosa with a whitish granular appearance (5–7 wk). Then, various papillomatous growths and, subsequently, squamous cell carcinomas developed in the last period of the experiment (9–13 wk). The average number and dimension of the induced tumors in the later stages (9–13 wk), are shown in Table 1.

Microscopically, the changes in the pouches began with acute inflammation and severe mucositis (3 wk), followed by hyperkeratosis, acanthosis, mild dyskeratosis and lymphocytic infiltration and a subsequent period of repair (5–7 wk). Between 9–11 wk, exophytic buccal pouch tumors, featuring hyperchromatism, cellular pleomorphism, disarray and an increase in the numbers of abnormal mitotic figures were seen. During the final stages (11–13 wk), poorly differentiated invasive tumor cells were found within the underlying fibrous connective tissue and muscle layers (Table 1).

### Total creatine kinase activity

**Serum** – The results of the evaluation of total serum CK activity during DMBA-induced chemical carcinogenesis of hamster buccal pouch tissue is shown in Fig. 1. The mean total serum CK activity in the control group was  $103.05 \pm 8.257$  U/l and increased gradually to reach a maximum value in the 7-wk DMBA-treated group ( $218.04 \pm 10.96$  U/l) (Fig. 1). As from the 9-wk DMBA-treated group ( $152.84 \pm 4.481$  U/l), the mean serum total CK activity decreased gradually until the end of the experiment ( $52.92 \pm 10.05$  U/l) (Fig. 1). Statistically (Student's two-sample t test, unpaired), with the exception of the 1-wk DMBA treated group, all the total CK values of the 3-wk, 5-wk and 7-wk DMBA treated groups were significantly higher than that of the control group ( $P < 0.01$ ). Moreover, all the total CK values of the 9-wk, 11-wk and 13-wk DMBA treated groups were significant-



Table 1. Average number and dimension of tumors induced per hamster and degree of malignancy during DMBA-induced chemical carcinogenesis of hamster buccal pouch tissue (HBPT)

	Average number of tumors/hamster	Average dimension (mm) of tumors/hamster	Degree of malignancy
Wk 9	1.33	2.00	+
Wk 11	1.50	2.57	±
Wk 13	2.33	2.89	—

+: well-differentiation; ±: well or poor-differentiation; —: poor-differentiation.

ly lower than the value of the control group ( $P < 0.01$ ).

**Hamster buccal pouch tissue** – The result of the evaluation of total HBPT CK activity during DMBA-induced chemical carcinogenesis of hamster buccal pouch tissue is shown in Fig. 1. We found that the pattern of changes in total HBPT CK activity was consistent with that of serum activity. However, the values of total CK activity in HBPT for each painting period were much higher than those of serum. The mean total HBPT CK activity of the non-painted group was  $350.29 \pm 13.99$  U/l, and gradually increased to reach the maximum value in the 7-wk DMBA treated group ( $636.10 \pm 49.31$  U/l) (Fig. 1). Conversely, the value was declined gradually as from the 9-wk DMBA treated group ( $309.96 \pm 23.55$  U/l) until the end of the experiment ( $234.91 \pm 28.17$  U/l) (Fig. 1). Statistically (student's two-sample *t* test, unpaired), the value of the total HBPT CK activity, for the 1-wk DMBA painting period, was marginally to significantly higher than that of the control group ( $0.01 < P < 0.05$ ). The values of the 3-wk, 5-wk and 7-wk groups were all significantly higher than those of the control group ( $P < 0.01$ ), while those of the 9-wk, 11-wk and 13-wk groups were significantly lower than that of non-painted group ( $P < 0.01$ ). All the values of the pouches left unpainted were lower

than those of the painted right counterparts and, with the exception of the 7-wk group, which were only slightly higher than those of the control group.

#### Activity of creatine kinase isoenzymes

**Serum** – On electrophoresis, the serum of the control and 1-wk DMBA treated groups yielded three bands, namely, CK-MM, CK-MB and CK-BB (Fig. 2A). An additional band of MCK-2 was seen cathodically with the CK-MM band in the 3-wk DMBA treated group and continuing throughout until the end of the experiment (Figs. 2B, C). Fig. 3 summarizes the activity, expressed as the percentage of the total intensity, of each CK isoenzyme. The activities of CK-MM, CK-MB and CK-BB fluctuated throughout the experimental period (Fig. 3A). Note that the activity of MCK-2 increased gradually from the 3-wk DMBA group until the end of the experiment (Fig. 4). No MCK-2 activity could be detected in any of the sera of the control, or the 1-wk DMBA treated group (Fig. 2A).

**Hamster buccal pouch tissue** – The change in the HBPT CK-isoenzymes pattern during DMBA-induced chemical carcinogenesis was similar to that in the serum. The results are summarized in Figs. 2D-F, 3B, and 4 respectively. Note that MCK-2 activity gradually increased from 3-wk DMBA painted group to the end of the experiment (Fig. 4) and that no MCK-2 activity could be detected in any of the control, or 1-wk DMBA treated hamster pouch tissues or pouches left untreated (Fig. 2D).

In addition, by comparing the MCK-2 level to the average number and dimension of tumors formed during the DMBA-induced hamster buccal pouch carcinogenesis (9–13 wk), we found a positive association between the increasing size and number of induced tumors and the increase in serum (Figs. 5A, B) and HBPT (Figs. 6A, B) MCK-2 levels respectively. This association is especially obvious in the MCK-2 level of HBPT (Figs. 6A, B).

## Discussion

The results of the present study have demonstrated that the total CK level in both serum and HBPT increases gradually during the premalignant period (3–7 wk) (Fig. 1), during DMBA-induced chemical carcinogenesis. The increasing CK activity may be a result of the progressive tissue necrosis and cellular degeneration of the HBPT, caused by the carcinogen-DMBA. Then, CK isoenzymes are released into the circulatory system (34).

We also found that the total CK activity declined during the malignant stage (9–13 wk) of DMBA-induced carcinogenesis (Fig. 1). This result is consistent with a few human studies which demonstrated the decrease of total CK activity in most malignancies (7) and neoplastic tissue of the gastrointestinal tract (19). However, it is difficult to elucidate the exact reason for the decrease in CK levels as all cancer studies on enzymes report increased activity, with the exception of alkaline phosphatase (37). There are reports indicating that the mis-programming of genetic expression in cancers produces notable changes in isoenzyme composition during malignant transformation (38–41). These reports may imply that there may be some unknown mechanisms controlling the production of CK in tissue and in release of CK into the serum or its removal from tissue and serum.

The activity of CK-MM, CK-MB and CK-BB fluctuated throughout the experimental period (Figs. 3A, B). No obvious relationship could be established between the activity and the progression of the DMBA-induced carcinogenesis. However, beginning from the 3-wk DMBA painting group, MCK-2 was detected in both the sera and tissues (Figs. 2B, C & E, F). The possibility that the cathodic band is adenylate kinase is extremely small, as its activity had already been inhibited by the AMP and diadenosine pentaphosphate present in the CK reagent (32).

There is a direct relationship between MCK-2 activity and total CK activity, during the premalignant period of DMBA-induced carcinogenesis. On the other hand, we observed that there were discrepancies between the amount of MCK-2 seen on the electrophoretogram and total CK activity, during the malignant period. A strong MCK-2 fluorescent band could be seen when the total CK level was low. An inverse relationship between the activity of MCK-2 and

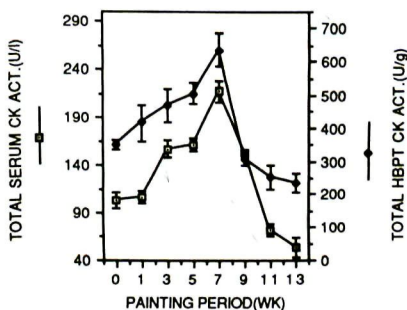
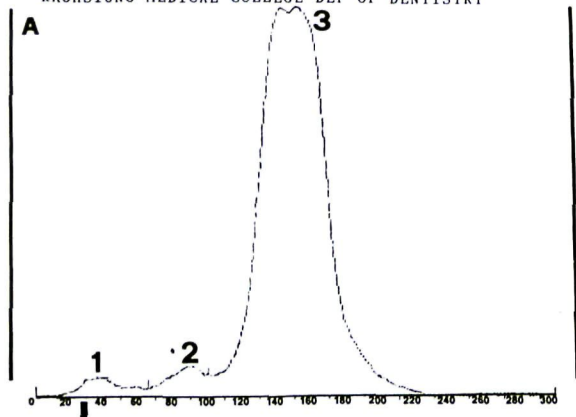


Fig. 1. Total HBPT and CK activity during DMBA-induced chemical carcinogenesis of hamster buccal pouch tissue (HBPT).

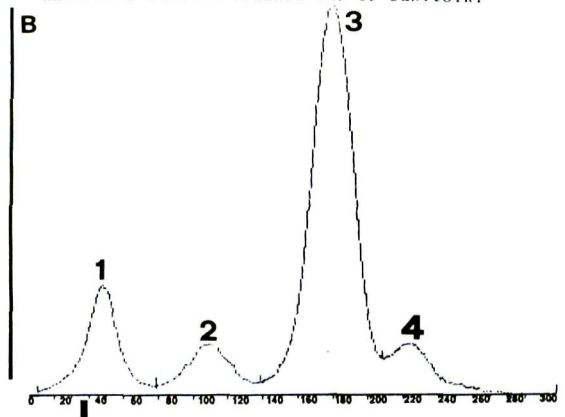
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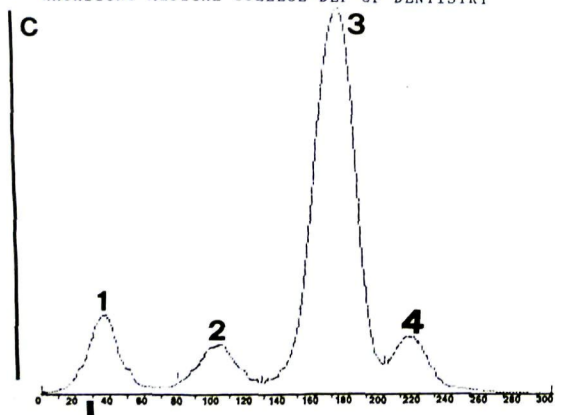
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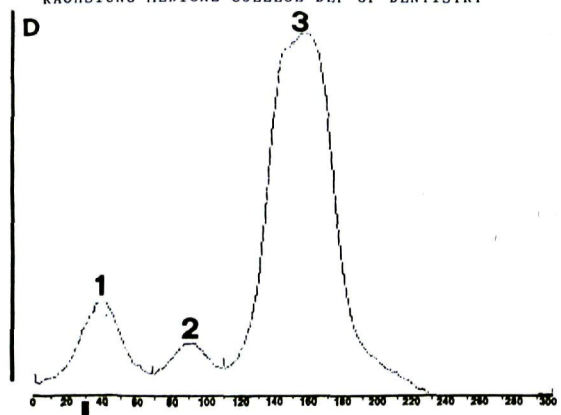
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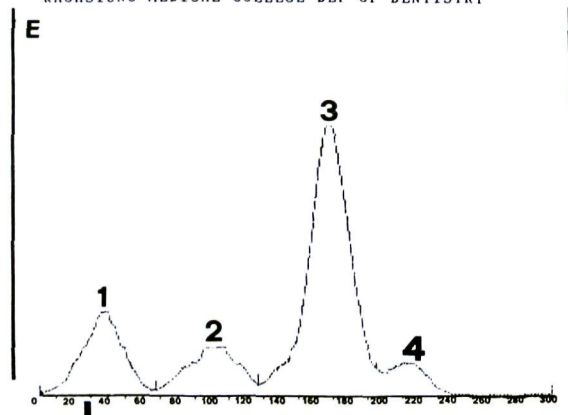
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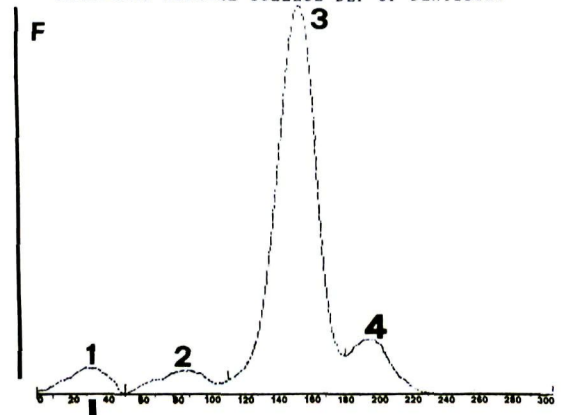


Fig. 2. Densitometer tracings of electrophoretic CK-isoenzyme patterns in serum (A-C) and pouch tissue (D-F) of hamster where (A) serum of non-painted hamster, (B) serum in 3-wk, (C) serum in 13-wk DMBA painted hamster, (D) unpainted pouch tissue, (E) 3-wk and (F) 13-wk DMBA painted pouch tissue. Note that serum (A) and pouch tissue (D) of non-painted hamsters show bands (1) CK-BB, (2) CK-MB and (3) CK-MM; serum (B) and pouch tissue (E) of 3-wk DMBA painted hamsters show bands similar to those of serum (A) and unpainted pouch tissue (D) respectively, but exhibited an additional band (4) MCK-2; the serum (C) and pouch tissue (F) of 13-wk DMBA painted hamsters show bands similar to those of (B) and (E), respectively.

total CK activity seems to exist in the malignant stage. A similar phenomenon of discrepancy between fluorescence intensity of MCK-2 and total CK activity,

in serum and colonic tissue, was reported by ROGALSKY *et al.* (42), who also suggested that there were still other unknown mechanisms affecting the re-

lease of MCK-2 into the circulatory system.

On comparing the MCK-2 level to the average number and dimension of



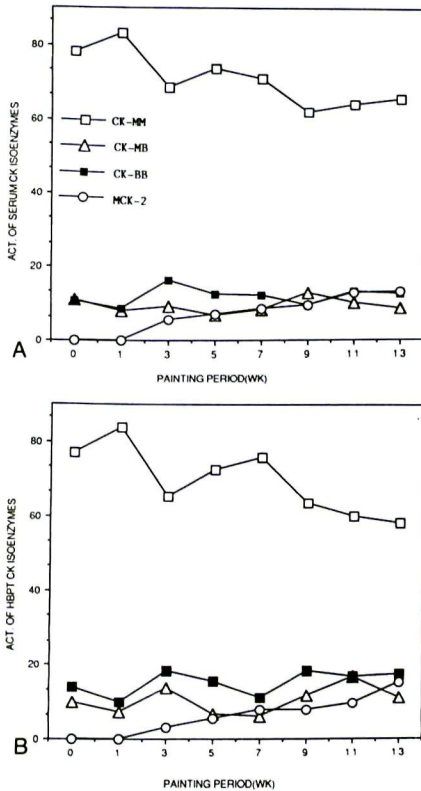


Fig. 3. Activity of serum (A) and HBPT (B) CK-isoenzymes (CK-MM, CK-MB, CK-BB and MCK-2), during DMBA-induced chemical carcinogenesis of hamster buccal pouch tissue (HBPT).

tumors induced during the DMBA-induced hamster buccal pouch carcinogenesis (9–13 wk), we found that the increasing size and number of tumors induced in HBPT (Table 1) were associated with a rise in MCK-2 levels in serum and HBPT (Figs. 5A, B & 6A, B). This is consistent with the fact that a bulky tumor in the advanced stage of malignancy is a requisite for the release of detectable MCK-2 (7, 43). Moreover, we can correlate the MCK-2 level with the degree of differentiation of hamster

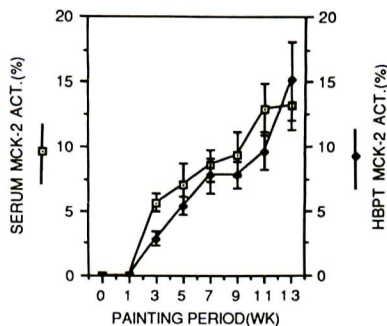


Fig. 4. Activity of HBPT and serum MCK-2 during DMBA-induced chemical carcinogenesis of hamster buccal pouch tissue (HBPT).

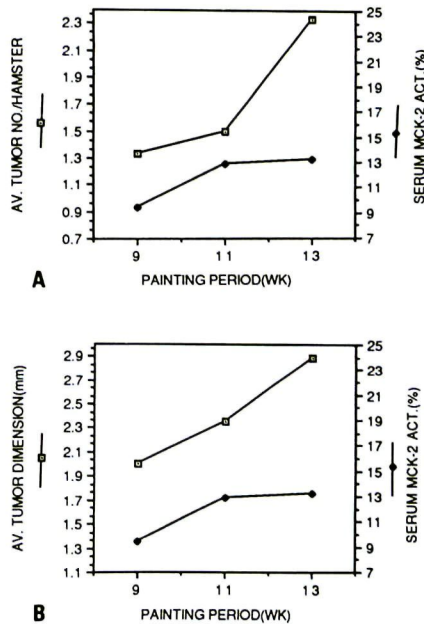


Fig. 5. Relationship between average tumor number (A) and dimension (B) and serum MCK-2 activity during 9th, 11th and 13th wk DMBA-induced chemical carcinogenesis of hamster buccal pouch tissue (HBPT).

buccal pouch carcinoma. We found that the well-differentiated exophytic carcinoma induced in HBPT (9 & 11 wk) was associated with a lower level of MCK-2. The opposite was true for the poorly differentiated invasive carcinoma (13 wk). This may be due to an induction

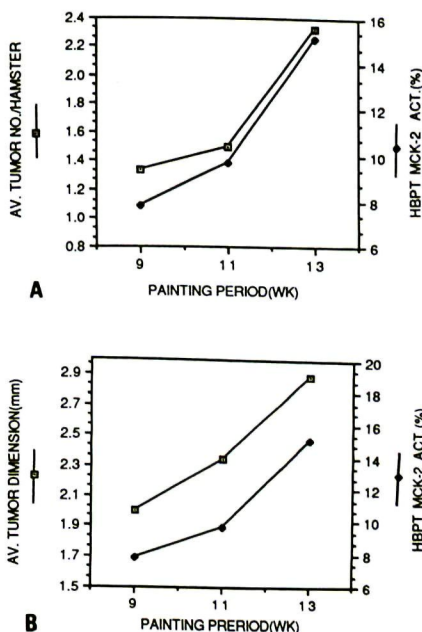


Fig. 6. Relationship between average tumor number (A) and dimension (B) and HBPT MCK-2 activity during 9th, 11th and 13th wk DMBA-induced chemical carcinogenesis of hamster buccal pouch tissue (HBPT).

process by the invasive tumor cells in the underlying fibrous connective tissue and muscle layers (44).

KANEMITSU *et al.* (26) suggested that MCK-2 originates from liver mitochondria. However, in a previous study (unpublished data of LM LIN), we found autopsy hamster specimens with fatty degeneration and angioblastic changes in their liver tissue, during DMBA-induced carcinogenesis. In addition, the possibility that DMBA-induced buccal pouch carcinoma should metastasize to the liver is extremely low and only a few reports of lymph node metastasis (45, 46) have been presented. A spectrum of abnormalities involving mitochondrial membrane potential, pH gradient and total electrochemical gradient in normal and cancer cells was reported (47). All these abnormalities may facilitate the release of the mitochondria CK-isoenzymes into the circulatory system as MCK-2 is located in the inner mitochondrial membrane (48). Therefore, it is suggested that MCK-2 is released from the mitochondria of transformed or tumor cells of HBPT.

In this study, MCK-2 was initially detected in the 3-wk DMBA-painted groups and its activity increased through the premalignant and malignant period, during DMBA-induced carcinogenesis. In addition, we observed that the serum MCK-2 level was consistent with that of HBPT. MCK-2 activity was detected neither in serum nor in HBPT of the normal or 1-wk DMBA-treated groups. Therefore, the sequential changes in MCK-2 activity may provide valuable information on the state and progression of the oral malignancy. We propose that the serum MCK-2 level may be a promising tumor marker in the oral cavity.

Though a tumor marker such as squamous cell carcinoma-antigen (SCC-Ag) (49, 50) has been proposed for use in the screening and monitoring of oral malignant tumors, no definite reliable tumor markers of oral cancer, to our knowledge, have been reported yet. In the future, more extensive studies to estimate actual MCK-2 activity using chromatography (22), and identify MCK-2 histochemically, using monoclonal antibody against MCK-2 (27) are needed before a definite statement concerning the clinical application of the appearance of and changes in MCK-2 levels can be made.

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