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Chemopreventive efficacy of all-*trans*-retinoic acid in biodegradable microspheres against epithelial cancers: Results in a 4-nitroquinoline 1-oxide-induced oral carcinogenesis model

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Abstract

Retinoids are known to suppress carcinogenesis in various epithelial tissues. Among them, all-*trans*-retinoic acid (atRA) is recognized as one such active retinoid. However, despite the known anticarcinogenic activity of atRA, it exhibits its short plasma half-life during repeated oral administration due to the "acute retinoid resistance" in the liver. This has been the major limitation in clinical applications of atRA. Therefore, in order to render atRA more suitable for clinical uses, sustained delivery of atRA using biodegradable microspheres is suggested in this study. When 50 mg atRA/kg of atRA-loaded microspheres were subcutaneously administered to rats once, the atRA concentration in plasma was maintained around 6.5 ng/ml for 7 weeks, with only minor signs of toxicity. When the chemopreventive efficacy of atRA-loaded microspheres was evaluated using a model of 4-nitroquinoline 1-oxide-induced oral carcinogenesis in F344 rats, a single injection of atRA-loaded microspheres significantly suppressed oral carcinogenesis. Additional injections of atRA-loaded microspheres, however, did not indicate further suppression of carcinogenesis.

Keywords: Chemoprevention; Oral carcinogenesis; All-*trans*-retinoic acid; Biodegradable microspheres; PDLLA

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is an aggressive epithelial malignancy that is now the sixth most prevalent neoplasm. In spite of much advancement in treatment modality for HNSCC, the long-term survival rate of HNSCC has remained less than 50% for the last 30 years. Of different problems that prevent the long-term survival rate, the frequent development of second primary cancers has posed as the most difficult (Hong et al., 1990; Vokes et al., 1993; Sturgis and Miller, 1995). In this regard, many chemotherapeutic agents were tried to reverse premalignant oral lesions as well as to prevent the second primary cancers, and studies in early 1990s showed that 13-*cis*-retinoic acid was effective in preventing second primary cancers in HNSCC (Hong et al., 1990).

All-*trans*-retinoic acid (atRA), an active isomer of 13-*cis*-retinoic acid, is known to play an essential role in the regulation of cell differentiation and proliferation in epithelial tissues (Gillis and Goa, 1995), and the effects of retinoic acid on HNSCC have been proved in vitro and in vivo (Shalinsky et al., 1995; Lotan, 1996). Presently, however, the clinical application of atRA is strictly limited due to both "acute retinoid resistance" in the liver and its systemic toxicity (Frankel et al., 1992; Muindi et al., 1992). Pharmacokinetic studies have demonstrated that when atRA was orally administered on a chronic daily basis, the area under the curve (AUC) and the half-life of atRA in plasma rapidly decreased at every repeated dose, recording a very short half-life of less than one hour (Muindi et al., 1992). Therefore, there has not been any kind of long-term treatments reporting success in preventing second primary cancers. In the

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present study, sustained drug delivery of retinoic acid was suggested to overcome such drawbacks of the drug and thereby to prevent second primary cancers. For this purpose, atRA was encapsulated in biodegradable microspheres to obtain extended plasma half-life for chemoprevention. Plasma concentration of atRA was measured up to 8 weeks after a subcutaneous injection of atRA-loaded microspheres. Then, the preventive efficacy of atRA-loaded microspheres was evaluated in a 4-nitroquinoline 1-oxide (4-NQO)-induced oral carcinogenesis model. 4-NQO was used as a carcinogen because it is known to produce a spectrum of preneoplastic and neoplastic lesions in the oral cavity (Tanaka et al., 1993, 1994). Of particular interest is that oral lesions induced in rats by oral administration of 4-NQO in drinking water have been reported to be similar to lesions in human (Steidler and Reade, 1984; Nauta et al., 1997).

2. Materials and methods

2.1. Materials

All-*trans*-retinoic acid and Lugol's solution were obtained from Sigma Chemical Co. (St. Louis, MO). Poly(D,L-lactide) (PDLLA, Res R202, M_w 17,500) was purchased from Boehringer Ingelheim Co. (Ingelheim, Germany). Monomethoxy polyethylene glycol (mPEG, M_n 5,000), L-lactide, poly(vinyl alcohol) (PVA, 98% hydrolyzed, M_w 13,000–23,000), 4-nitroquinoline 1-oxide (4-NQO), ammonium acetate, potassium hydrogen phosphate and isobutyl alcohol (HPLC grade) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Acetonitrile and glacial acetic acid were of HPLC grade, and were supplied by Mallinckrodt Baker Inc. (Phillipsburg, NJ).

2.2. Preparation of atRA-loaded microspheres

Poly(L-lactide)-poly(ethylene glycol) diblock copolymer (PLE) was synthesized with mono-methoxy PEG (mPEG, M_n 5,000) and L-lactide by solution polymerization as described in the previous study (Choi et al., 2001). The number average molecular weight (M_n) and molecular weight distribution of the synthesized PLE were 32,500 Da and 1.46, respectively. The amphiphilic PLE polymer was introduced in microspheres during preparation procedure to control the drug release rate. PDLLA/PLE microspheres containing atRA were prepared using the solvent evaporation method in oil-in-water emulsion. PDLLA (4 g), atRA (0 wt.% for placebo microspheres and 10 wt.% for atRA-loaded microspheres) and PLE (8 wt.%) were concurrently dissolved in dichloromethane. The mixture was poured into 600 ml aqueous solution containing 2% (w/v) of PVA while mixing vigorously using a mechanical stirrer (IKA LABORTECHNIK, Selangor, Malaysia) at 1,000 rpm. After mechanical stirring for 10 min, the resulting suspension was gently stirred for 3 h at 40 °C with a magnetic stirrer to evaporate dichloromethane. The microspheres were separated by centrifugation at 12,000 rpm for 10 min. Thus obtained microspheres were washed with distilled water, centrifuged four times and freeze-dried. The size of the atRA-loaded microsphere ranged from 20 to 100 μm (Choi et al., 2002).

2.3. In vitro drug release test

In the atRA release test in vitro, 10 mg atRA-loaded microspheres were enveloped in each of the cellulose acetate membranes, whose molecular weight cut-off was 300,000 Da, and were immersed in a shaking water bath at 37 °C, which contained 40 l of PBS. The PBS medium was exchanged with a fresh solution periodically in order to maintain the sink condition. On each of the 3rd, 6th, 9th, 14th, 19th, and 24th day, the microspheres were dissolved in dichloromethane, and the amount of remaining atRA was determined by the absorbance at 365 nm. Since atRA is highly hydrophobic and has a low solubility in PBS, the amount of released atRA from microspheres was calculated from the remaining amount of atRA in the microspheres at each time. All preparation procedures were carried out in a dark room under a dim yellow light to prevent photoisomerization of atRA.

2.4. Measurement of plasma concentration of atRA after subcutaneous injection of atRA-loaded microspheres

Six F344 rats, 6 weeks old and purchased from Japan SLC Inc. (Tokyo, Japan), were used for measuring the amount of atRA released from the subcutaneously administered microspheres. After a dose of 50 mg atRA/kg of atRA-loaded microspheres had been administered subcutaneously to rats, blood was sampled at day 1, 3, 5, 7, 10, 14, 21, 35, 42, 49, and 56. They were then assayed by high performance liquid chromatography (HPLC) as described by Buggé et al. (1985) using the Hitachi HPLC system (D-7000 series Hitachi Ltd., Tokyo, Japan) with TSK-gel ODS-80T_M column (4.6 mm × 250 mm, Tosoh Co., Tokyo, Japan). Briefly, 150 μl of a 1:1 mixture of acetonitrile and isobutyl alcohol was added to 200 μl of plasma and vortexed for 1 min. After the addition of 120 μl of saturated K_2HPO_4 solution and mixing it for 30 s, the samples were centrifuged for 2 min. The organic upper layer (80 μl) was analyzed by HPLC, where a linear gradient from the initial condition of 100% phase A to 100% phase B was adopted over 15 min. The mobile phase A consisted of a mixture of water, acetonitrile and acetic acid in the ratio of 100:100:1, whereas the B phase consisted of water, acetonitrile and acetic acid in 190:10:0.08 ratio. Both mobile phases contained 10 mM of ammonium acetate and the atRA concentration was measured from the absorbance at 365 nm.

The injection dose of the microspheres for chemoprevention study was determined from previous subacute toxicity and biocompatibility studies (Choi et al., 2002, 2003a). The prepared microspheres were sterilized by gamma–irradiation before they were injected into rats (Choi et al., 2002).

2.5. Prevention of oral carcinogenesis

Male F344 rats were housed in a holding room kept under the following controlled conditions: 23 ± 3 °C temperature, 50 ± 10% humidity, and 12 h light/dark cycle. After 2 weeks of quarantine, healthy rats were randomized into four groups. A 4-NQO solution was prepared at the concentration of 20 ppm in tap water and stored in dark at 4 °C until used (Tanaka et al.,

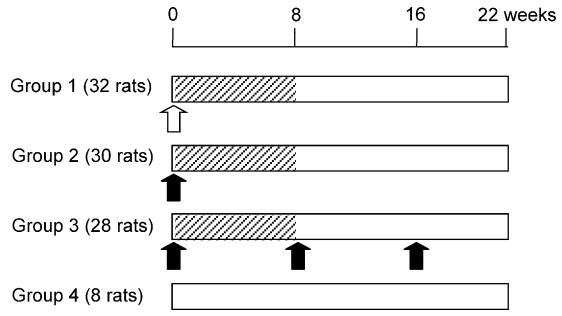

Fig. 1. Experimental protocol. (▨) 4-NQO, 20 ppm in drinking water; (□) basal diet and tap water; (⇧) injection of placebo microspheres; (⬆) injection of atRA-loaded microspheres, 50 mg atRA/kg.

1994). Dark amber bottles were used for the 4-NQO solution to protect it from decomposition by light.

Ninety-eight rats were divided into four groups as shown in Fig. 1. Rats from the groups 1–3 were freely given 20 ppm 4-NQO in drinking water for 8 weeks to induce oral carcinogenesis. Placebo microspheres without any atRA were administered subcutaneously to rats of the group 1. For the rats of groups 2 and 3, a dose of 50 mg atRA/kg of atRA-loaded microspheres was injected subcutaneously when the feeding of 4-NQO was started. The sterilized atRA-loaded microspheres were dispersed in a sterile, non-pyrogenic normal saline solution at the concentration of 0.5 g/ml without the addition of any surfactants, and were subcutaneously injected by a sterilized disposable syringe with a 20-gauge needle. For the group 3, the same dosage of atRA-loaded microspheres was subcutaneously administered at an interval of 8 weeks. The group 4 was given the basal diet and tap water without 4-NQO, and it served as an untreated control. Five rats from the group 4 were used to measure atRA concentration under the fed condition. The dosage of atRA-loaded microspheres was determined based on the results of our previous study on subacute toxicity, in which we evaluated the potential toxicities on body weights, vital organs and blood components of rats (Choi et al., 2003a).

All rats were carefully observed daily and their body weights were measured weekly during the experiment period of 22 weeks. At necropsy, hard palates and tongues of each rat were collected for histological analysis. During the collection of the tissues, the size of the projected tumors on tongue mucosa was measured using the equation of width × length × height × $\pi/6$ (Tomayko and Reynolds, 1989). The mucosa of palate and tongue were brushed over with a 2% Lugol's dye solution to detect dysplasia and carcinoma in the mucosal layer (Tincani et al., 2000). Suspicious areas of dysplasia and carcinoma appeared

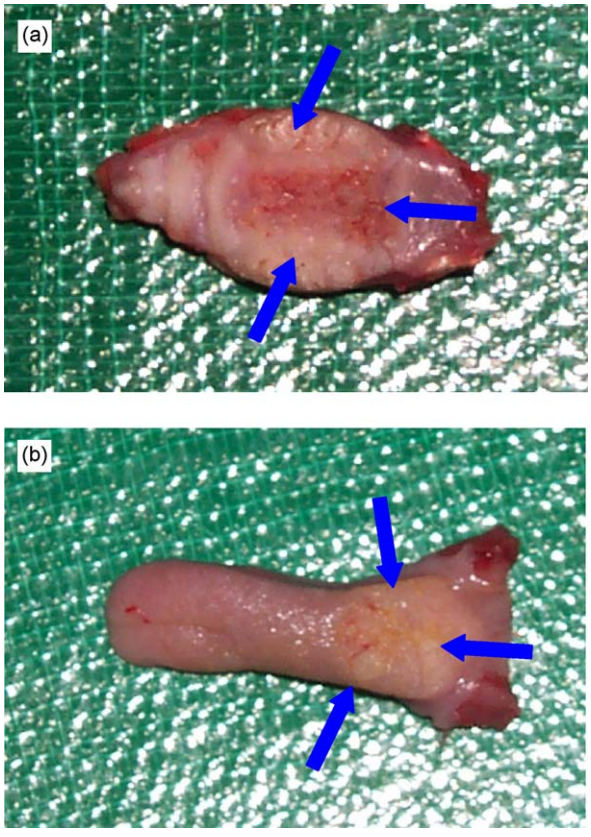

Fig. 2. (a) Hard palate and (b) tongue of 4-NQO treated rats after staining with Lugol's dye solution (yellowish areas were considered as suspicious regions of dysplasia and carcinoma, and transverse cutting of these tissues were performed through the regions indicated by arrows).

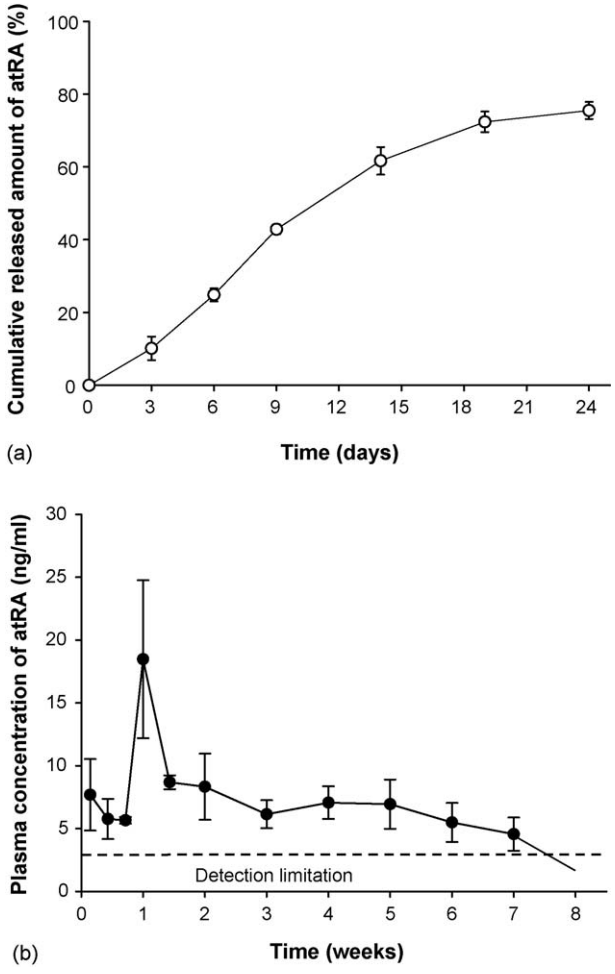

Fig. 3. (a) The in vitro release profiles of atRA from PDLLA/PLE microspheres, (b) The concentration profile of atRA in plasma after injecting atRA-loaded microspheres subcutaneously at 50 mg atRA/kg.

either white (i.e., unstained) or yellow. Each hard palate and tongue was sliced across the suspicious areas into three pieces, as shown in Fig. 2. The sliced tissues were fixed in a 10% buffered formaldehyde solution, embedded in paraffin, sectioned with a microtome, and stained with hematoxyline and eosin for histological examination. The most advanced stage of carcinogenesis as viewed from various histological pictures of the hard palate and tongue in a rat was taken as the stage of carcinogenesis for that rat. Epithelial lesions (hyperplasia, dysplasia, and neoplasm) in the oral cavity were diagnosed according to the criteria described by Bánóczy and Csiba (1976).

Procedures of animal experimentation were reviewed and approved by the Institute's Animal Research Committee. Care of all animals was carried out in accordance with the guidelines of the National Animal Care Committee.

2.6. Statistical analysis

Preventive effects of the microspheres against the development of lesions were statistically evaluated using a χ^2-test. Other data were expressed as mean ± S.D. values, and statistical analyses of the data were performed with the use of the Student's *t*-test.

3. Results

From the in vitro release test, atRA encapsulated in the microspheres was almost constantly released from the microspheres up to 9 days, and release rate of atRA gradually decreased thereafter (Fig. 3a). About 75.5 ± 2.3% of atRA was released from the microspheres at day 24 in vitro, indicating that the release of atRA from the microspheres is still going on at this time.

When atRA-loaded microspheres were subcutaneously administered to rats at the dose of 50 mg atRA/kg, the atRA concentration in the plasma reached 18.5 ± 6.3 ng/ml at one week, as shown in Fig. 3b. Afterwards, the atRA concentration in the plasma was reduced gradually but maintained in the range of 3.2–9.3 ng/ml up to the 7th week with the mean value of around 6.5 ng/ml. After 7 weeks, the concentration of atRA in the plasma decreased below the detection limit (3 ng/ml). Although the method used for HPLC analysis is one of the well established methods for atRA analysis and has been validated for use in the rat plasma (Buggé et al., 1985), atRA could not be extracted well from the plasma samples into an organic solvent when the atRA concentration in the plasma samples was below 3 ng/ml. On the other hand, the extraction of atRA was reproducible when the atRA plasma concentration was greater than 3 ng/ml.

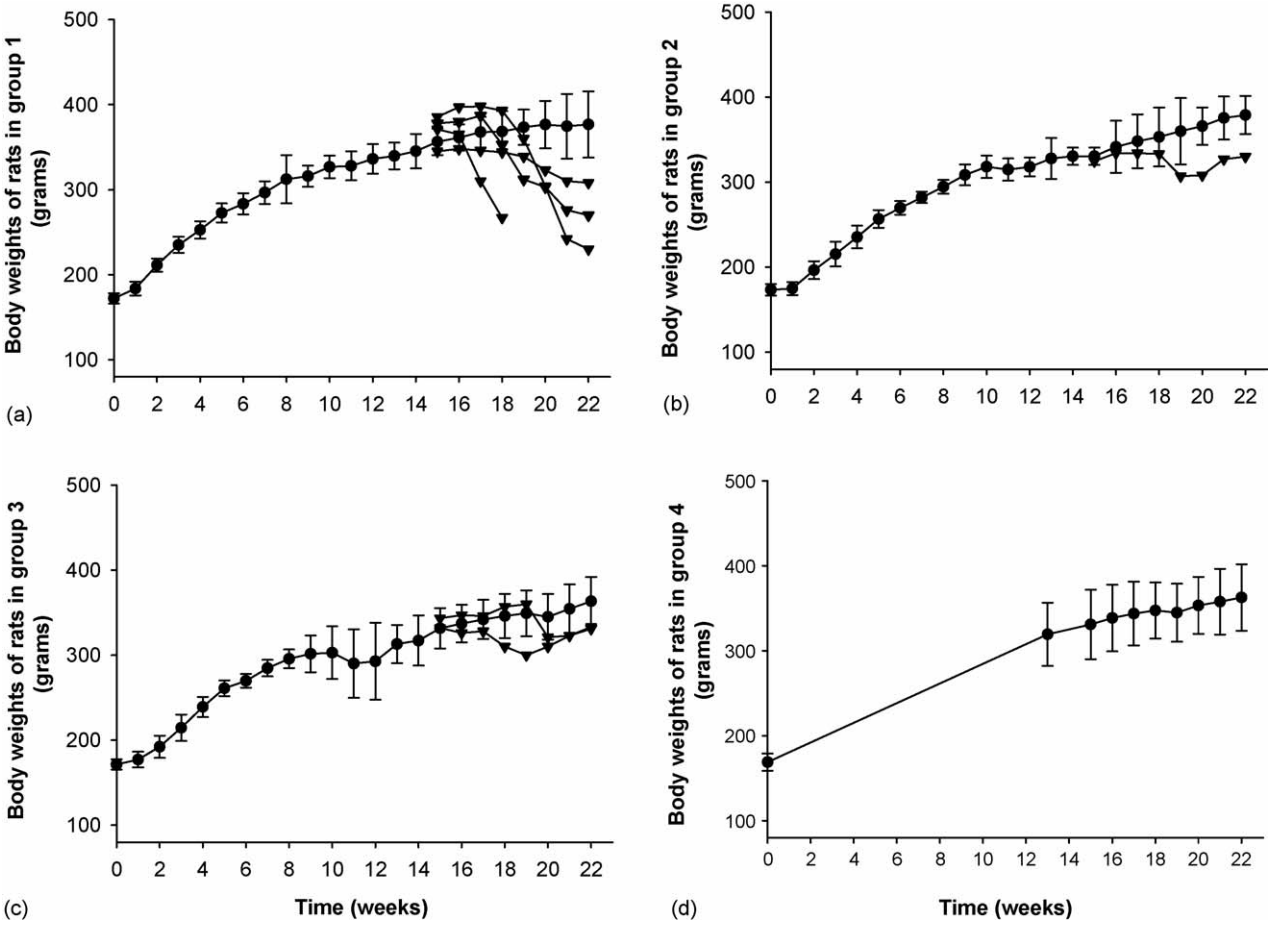

Fig. 4. Body weight changes: (a) the group 1, (b) the group 2, (c) the group 3, and (d) the group 4. (●) represents the mean body weight of rats and (▼) represents the body weight changes of rats with projected tumor.

The C_{max}, $T_{max,}$ and AUC of atRA in microsphere formulation were calculated to be 18.6 ± 6.1 ng/ml, 230.4 ± 177.6 h, and 8424 ± 1200 ng/ml h, respectively. When the same dose of placebo microspheres was administered, atRA was not detected by HPLC, indicating that the atRA concentration in the plasma was maintained below 3 ng/ml under the fed condition.

For the carcinogenesis experiments, the injection interval of the microspheres was decided as 8 weeks since the plasma concentration of atRA was maintained above 3 ng/ml for 7 weeks. Repeated injections of atRA-loaded microspheres were highly tolerable, and minor signs of toxicity such as decrease in activity and body weight gain of the rats in the atRA-treated groups had been observed transiently after 2 weeks of microsphere injection. The state of the rats was gradually improved with time and no adverse tissue reactions were observed at the injection sites of atRA-loaded microspheres at this dose for about 25 weeks as observed before (Choi et al., 2002). On the other hand, placebo microspheres, which had been confirmed as inert materials in our previous studies (Choi et al., 2002, 2003a), did not induce any toxicity. According to our previous observation, placebo microspheres had no effect on oral carcinogenesis.

Rats from the groups 2 and 3 showed a temporary decrease both in their activity and body weight gain; gradually, there was improvement in their activity and body weight gain after 2 weeks. Ultimately, body weights of rats were not different statistically between each group at 22 weeks ($P > 0.15$, Fig. 4). However, the rats, which developed large tumors, showed a significant decrease in their body weights while big tumor masses had projected on the tongue. The projected tumors were observed on the tongues and not on the hard palates (Fig. 5a). In the group 1, a projected tumor lager than 10 mm^3 was observed in the tongue of 7 rats (21.9%); in 4 of these 7 rats, tumor was larger than 100 mm^3. For the group 2 and 3, the size of the projected tumors was greater than 10 mm^3 and the incidence rates were 13.3% and 7.1%, respectively (Fig. 5b).

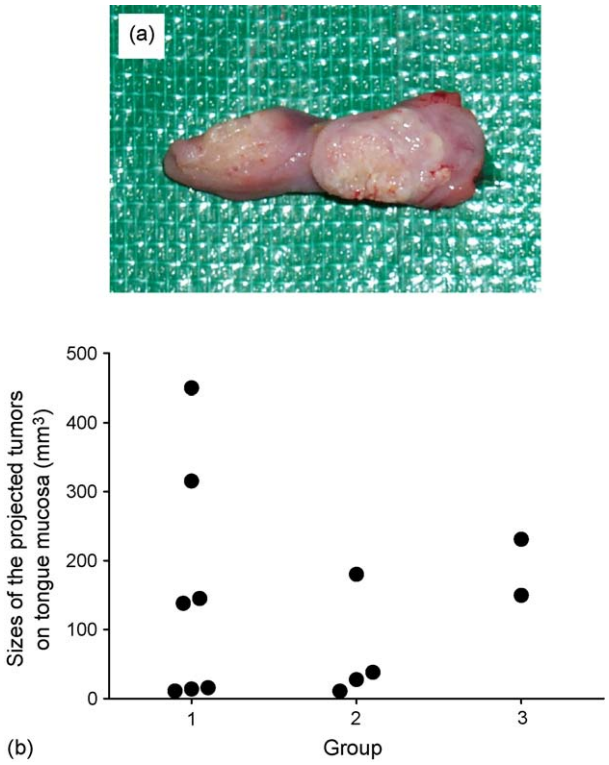

Fig. 5. (a) Projected tumors on tongue mucosa and (b) sizes of the projected tumors of each tongue.

Histological evaluations for carcinogenesis were performed for hard palates and tongues (Fig. 6). The basal layer was thickened, and the loss of polarity was initiated in rats with hyperplasia. Moreover, the loss of polarity of epithelium was found throughout the whole thickness in rats with dysplasia. Invasion into dermis, well-differentiated SCC in dermis, and keratin pearls were observed in rats with invasive SCC. The develop-

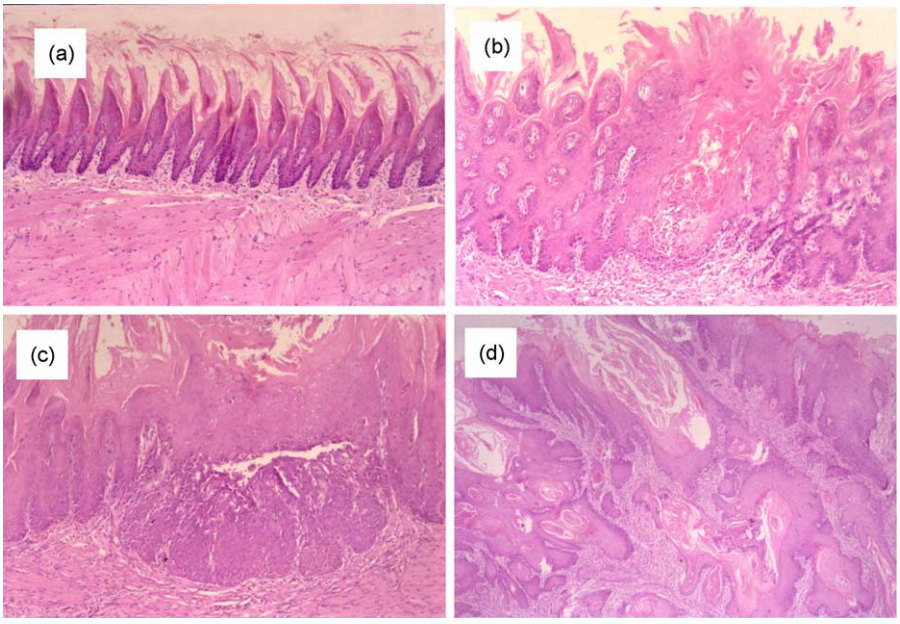

Fig. 6. Representative histology appearance of hard palate epithelium of rat (H&E staining): (a) normal, (b) hyperplasia, (c) dysplasia and (d) invasive SCC.

Table 1
Incidence of neoplasm on hard palate in F344 rats

Group	No. of rats examined	No. of rats (%)			
		Normal	Hyperplasia	Dysplasia	Invasive SCC
1	32	0/32 (0.0)	5/32 (15.6)	13/32 (40.6)	14/32 (43.8)
2	30	0/30 (0.0)	6/30 (20.0)	22/30 (73.3)	2/30 (6.7)*
3	28	1/28 (3.6)	7/28 (25.0)	18/28 (64.3)	2/28 (7.1)*
4	8	8/8 (100.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)

* Significantly different from group 1 ($P < 0.01$).

Table 2
Incidence of neoplasm on tongue in F344 rats

Group	No. of rats examined	No. of rats (%)			
		Normal	Hyperplasia	Dysplasia	Invasive SCC
1	32	4/32 (12.5)	5/32 (15.6)	9/32 (28.1)	14/32 (43.8)
2	30	1/30 (3.3)	12/30 (40.0)	8/30 (26.7)	9/30 (30.0)
3	28	2/28 (7.1)	7/28 (25.0)	12/28 (42.9)	7/28 (25.0)
4	8	8/8 (100.0)	0/8 (0.0)	0/8 (0.0)	0/8 (0.0)

ment of preneoplasm and neoplasm in both sites are shown in Tables 1 and 2. In hard palates of most rats from the group 1, carcinogenesis proceeded up to dysplasia (40.6%) or invasive SCC (43.8%). On the other hand, of the group 2 rats that received atRA-loaded microspheres once, 73.3% showed dysplasia and only 6.7% showed incidences of invasive SCC. In the group 3 that received atRA-loaded microspheres repeatedly, carcinogenesis was observed to be further suppressed as 64.3% showed dysplasia, 25% hyperplasia, and one no incidence of carcinogenesis. Therefore, carcinogenesis was significantly inhibited in the group 2 ($P < 0.01$) and group 3 ($P < 0.01$) as compared to group 1. However, repeated administration of atRA-loaded microspheres did not induce further significant reduction in the incidence of invasive SCC. The chemopreventive effect of atRA-loaded microspheres was also observed in the tongue (Table 2). As in the case of hard palate, there was no statistically significant difference in the chemopreventive effect between the groups 2 and 3 with regard to chemopreventive effects of atRA-loaded microspheres in the tongue.

4. Discussion

In this study, in vitro atRA release test revealed that atRA was continuously released from the biodegradable microspheres; it was still being released at 24th day. It is well explained by the data from *in vivo* release test. The atRA in plasma, which ranged from 3.2 to 9.3 ng/ml, was measured up to 7 weeks in vivo. The gradual decrease in the plasma concentration of atRA after 1 week seems to be due to the decreased release rate of atRA from the microspheres. Interestingly, the atRA concentration measured in plasma for 7 weeks was around 6.5 ng/ml, which was three times greater than the physiological level. The endogenous level of atRA in the plasma is reported to be very low (mean value: around 1.5 ng/ml), being in the range of 0.5–2.7 ng/ml in rats (Cullum and Zile, 1985; Kurlandsky et al., 1995), and 0.92–3.54 ng/ml in human (Lehman and Franz, 1996). Although the minimum effective plasma concentration of atRA needed to prevent carcinogenesis has not been established, several in vitro cell studies have indicated that there is a dose- and time-dependent transcriptional activation of nuclear retinoic acid receptors (RARs: RAR-α, -β, and -γ) above the atRA concentration of 3 ng/ml (Lehmann et al., 1991; Yang et al., 1997). Our previous study using human head and neck cancer xenograft model in athymic nude mice also showed decreased tumor growth by 50% when the plasma concentration of atRA was maintained in the range of 3.6–11.1 ng/ml for 4 weeks (Choi et al., 2003b).

According to Tembe et al. (1996), the pharmacokinetic data (C_{max} and T_{max}) for atRA itself for rats which were given single oral doses of 50 mg/kg were 1880 ± 420 ng/ml, and 2.1 ± 0.3 h, respectively, and its AUC value was 5040 ± 1350 ng/ml h. The atRA given via oral route was detected in 8 h. Unlike the above data from the oral route, the T_{max} value of 230.4 ± 177.6 h and the AUC value of 8424 ± 1200 ng/ml h in this study were obtained from the subcutaneous injection of atRA-loaded microspheres administered at the same dose concentration. This confirms that atRA in the microsphere formulation has both the extended half-life and enhanced bioavailability. When atRA is administered orally, the route of passage is first the portal vein, then the liver, systemic circulation, and finally the target organs. So, the absorbed atRA is metabolized in the liver before it reaches the systemic blood circulation and the target site (first-pass metabolism). In contrast, atRA released from subcutaneously implanted microspheres would go to the systemic blood stream first and then to the target organs. So this would be the reason for the improved bioavailability of atRA when the route of administration is changed. In addition, reduced acute retinoid resistance may be another possibility for the increased bioavailability of atRA-loaded microsphere formulation. Recent studies have shown that induction of CYP26, the most important specific P450 enzyme responsible for atRA metabolism, is dependant on the dose of the concentration of atRA treated

(Ozpolat et al., 2005). Since the mean plasma concentration of atRA for the microsphere treated rats was maintained at much lower levels than the case of oral administration, the reduced induction of CYP26 expression is expected. The resistance, however, may not be completely inhibited by using subcutaneously implanted microsphere formulation because the plasma concentration of atRA was still maintained above the normal level for a long time.

According to Tzimas et al. (1997), the improved bioavailibility of atRA and the reduced formation of atRA metabolites were observed after subcutaneous injection of atRA into rats than oral administration at the same dose. Since the plasma level after the subcutaneous injection of atRA without microencapsulation was maintained only for about 24 h (Tzimas et al., 1997), frequent injection of drug solutions is required. Therefore, atRA in microsphere formulation would be a more convenient and practical design for chemoprevention than atRA without microencapsulation.

In the previous biocompatibility and toxicity study (Choi et al., 2003a, 2002), we had already investigated the relationship between the doses of atRA-loaded micropsheres and toxic effects on the body weight, bleeding, bone fracture, red blood cell, reticulocyte, hemoglobin, hematocrit, plasma alkaline phosphatase, aminotransferases, cholesterol, and etc. The toxicity study revealed only minor signs of toxicity for the rats receiving atRA-loaded microspheres at 50 mg atRA/kg. In the current study, therefore, side effects appearing at this dosage due to repeated injections were briefly checked against the body weight changes and gross observation of rats. No statistical difference in the body weights of rats between groups and no apparent abnormality upon gross observation at week 22 indicate that this dosage and administration schedule of the drug is "highly tolerable" despite the transient signs of minor toxicity during the treatment. Since only one or two rats in the groups 2–3 showed decreased body weights with projected tumors, their reduced body weights did not significantly affect the mean body weights of the rats in the drug-treated groups at week 22.

As shown in the tables, the higher frequency of dysplasia was observed in the atRA-treated groups with more frequent reduction of invasive cancer, suggesting that the progression of carcinogenesis was effectively arrested just by one time administration of atRA-loaded microspheres. The proliferation of tumors in the tongues also seems to be inhibited as observed previously in the tumor xenograft model (Choi et al., 2003b). The chemoprevention effect, however, was not significantly improved by additional injections of the atRA-loaded microspheres, although the formation of large tumors had somewhat decreased compared to the administration of single injections. For further study, we need to investigate the reasons for the reduced effectiveness of the microsphere formulation during repeated administration. In addition, factors including injection timing and dose should be considered in the further studies to optimize the efficacy of atRA-loaded microspheres.

In conclusion, the encapsulation of atRA in the microspheres provided maintenance of the plasma concentration of atRA in the therapeutic range for a long period. Furthermore, atRA released from the microspheres effectively suppressed oral carcinogenesis with minor signs of toxicity that was transient. Therefore, this new drug delivery system may serve as an effective method by which atRA can be used as a chemopreventive agent.

Acknowledgements

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