Chemoprevention of 4-NQO-induced oral carcinogenesis by co-administration of all-trans retinoic acid loaded microspheres and celecoxib

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Abstract

All-trans retinoic acid (atRA) is one of the most potential chemopreventive agents for head and neck squamous cell carcinoma (HNSCC). However, the induced metabolism of atRA by cytochrome P450s in the liver limits its clinical applications. To overcome such limitation, we had developed atRA-loaded microspheres designed to release atRA for a long period. Unfortunately, the atRA-loaded microspheres severely induced inflammatory responses: that is, atRA released from the microspheres significantly induced the proliferation of fibroblasts and collagen deposition, thereby causing a permeability barrier for drugs from entering the blood stream. In the present study, the effects of celecoxib as an anti-inflammatory drug are investigated when it is concurrently used with atRA-loaded microspheres to treat 4-NQO-induced oral carcinogenesis. We investigated if it might influence the plasma concentration of atRA and its metabolism by preventing the fibroblast proliferation and collagen deposition, reduce the toxicity level of atRA, and improve the chemopreventive efficacy of atRA-loaded microspheres. The concurrently administered celecoxib prevented inflammatory responses and suppressed the number of fibroblasts and collagen deposition in the fibrous capsules for 14 days. The atRA concentration in plasma was also increased and the metabolism of atRA was significantly decreased within 2 weeks. In the 4-NQO-induced oral carcinogenesis study, the incidence of invasive SCC was above 44% when F344 rats were treated with atRA-loaded microspheres. However, the treatment using atRA-loaded microspheres and celecoxib concurrently could reduce the incidence of invasive SCC up to 28%, and three of 25 rats were found to have no tongue lesions. In conclusion, the concurrent use of celecoxib could maintain the atRA concentration in plasma at a higher level while reducing its

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metabolism by preventing inflammatory responses, thereby improving their chemopreventive effects against 4-NQO-induced oral carcinogenesis.

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Keywords: All-trans retinoic acid; Celecoxib; Biodegradable microspheres; Inflammation; Carcinogenesis

1. Introduction

All-trans retinoic acid (atRA), an active isomer of 13-cis-retinoic acid, plays an essential role in the regulation of cell differentiation and proliferation in epithelial tissues [1–3], and the effects of retinoic acid on HNSCC have been proved in vitro and in vivo [4,5]. Presently, however, the clinical application of atRA is compromised due to both “acute retinoid resistance” and atRA toxicity [6,7]. 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 1 h [6]. This observation has been attributed to the accelerated metabolism brought on by a specific cytochrome P450, which is induced by atRA in the liver [8,9]. Consequently, the atRA concentration in plasma cannot be maintained within the therapeutic range in long-term treatments.

In our previous study, atRA-loaded biodegradable microspheres have been found to maintain the plasma concentration of atRA in the therapeutic range for a long period. When atRA was released from the subcutaneously injected microspheres, the first-pass metabolism was avoided, and the continuously released atRA could maintain its plasma concentration in the therapeutic range for a long period [10]. However, atRA-loaded microspheres, which were subcutaneously administered, stimulated severe inflammation at the implant sites, and these inflammatory responses seemed to be associated with the production of prostaglandins following the action of COX-2. The newly developed fibrous capsules by atRA-loaded microspheres had many inflammatory cells as well as fibroblasts [11]. The thick bands of fibrous capsules that developed around the implanted atRA-loaded microspheres contained inflammatory cells, fibroblasts, and collagen, and could affect drug permeation to the capillaries as well as its function [12]. Randolph and Simon reported that fibroblasts could limit the delivery of retinoic acid due to its metabolic capacity and abundance in the dermis [13]. In addition, the newly developed fibrous capsules surrounding drug eluting polymeric devices might influence the release behavior of a drug [14].

Celecoxib, a kind of nonsteroidal anti-inflammatory drug (NSAIDs) that selectively inhibits COX-2 and exhibits anti-inflammatory and anti-tumor effects, was used in this study to prevent severe inflammation caused by atRA-loaded microspheres. COX-1 and COX-2 are two isoforms of cyclooxygenases—key enzymes in the production of prostaglandins. COX-1 is constitutively expressed in most normal tissues and appears to be responsible for the production of prostaglandins that mediate normal physiological functions, such as the maintenance of the gastrointestinal mucosa, kidney, and platelet functions [15]. On the other hand, COX-2 is induced by inflammatory stimuli, including cytokines, growth factors, and tumor promoters, and is related to inflammatory diseases [16]. The stimulation of prostaglandin production at sites of inflammation responses is largely done through the induction of COX-2. Conventional non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ketoprofen, indomethacin, and corticosteroids, including prednisolone and dexamethasone, inhibit both COX-1 and COX-2 without much selectivity, thereby causing serious side effects. However, selective COX-2 inhibitors including celecoxib and rofecoxib induce substantially less gastrointestinal injury than other NSAIDs [17–19] because these COX-2 inhibitors have high selectivity for COX-2. Celecoxib could significantly suppress the gastrointestinal lesions by approximately 50–60% compared to the traditional NSAIDs containing aspirin and indomethasine [20]. Since celecoxib is known to selectively inhibit COX-2, the mechanism of their anti-inflammatory responses is conceivably
the altered metabolism of arachidonic acid and, subsequently, prostaglandins. According to the report by Igarashi et al., the selective COX-2 inhibitor celecoxib could effectively prevent prostaglandin synthesis caused by inflammatory cytokines [21]. Therefore, it is considered that celecoxib can be especially suitable to suppress the inflammation on the dermis by atRA-loaded microspheres.

In this study, celecoxib was orally administered to rats, to which atRA-loaded PLGA/PLGA–PEG microspheres had been injected subcutaneously, in order to reduce the inflammatory responses induced by atRA released from the PLGA/PLGA–PEG microspheres. Herein, we studied whether the prevention of atRA-induced inflammation by celecoxib affected the atRA metabolism and atRA concentration in plasma. Furthermore, we investigated the effects of concurrently using celecoxib on the cancer preventive efficacy of atRA-loaded PLGA/PLGA–PEG microspheres in a 4-nitroquinoline 1-oxide-induced oral carcinogenesis model.

2. Materials and methods

2.1. Materials

All-trans retinoic acid (atRA) was obtained from Sigma Chemical Co. (St. Louis, MO). Poly(D.L-lactide-co-glycolide) (PLGA; Resomer R503H, $M_w$ 33,000) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Poly(vinyl alcohol) (PVA; 98% hydrolyzed, $M_w$ 13,000–23,000), N-hydroxysuccinimide (HOSu), and N,N-dicyclohexyl carbodiimide (DCC), 4-nitroquinoline 1-oxide (4-NQO), ammonium acetate, potassium hydrogen phosphate, and isobutyl alcohol were of HPLC grade and were supplied by Mallinckrodt Baker (Phillipsburg, NJ).

2.2. Preparation of microspheres

Poly(D,L-lactide-co-glycolide)–poly(ethylene glycol) diblock copolymer (PLGA–PEG) was synthesized by coupling PLGA containing a mono-carboxyl group with mono-methoxy PEG-NH$_2$ (mPEG-NH$_2$, $M_n$ 2000). The number average molecular weight ($M_n$) and the weight average molecular weight ($M_w$) of synthesized PLGA–PEG were 18,090 and 25,760 Da, respectively. Microspheres composed of PLGA and PLGA–PEG (PLGA/PLGA–PEG microspheres) containing atRA were prepared using a solvent evaporation in oil-in-water emulsion as described in a previous report [22]. Briefly, PLGA (3.6 g), PLGA–PEG (0.4 g), and atRA (0 wt.% for control microspheres, and 3 wt.% for atRA-loaded microspheres) were concurrently dissolved in 50 ml of dichloromethane. This mixture was poured into 600 ml of aqueous solution containing 2 wt/vol.% of PVA while mixing vigorously using a mechanical stirrer (Ikala-bortechnik, Selangor, Malaysia) at 1000 rpm for 10 min. The suspension was then gently stirred at 35 °C for 2 h with a magnetic stirrer to evaporate dichloromethane. The microspheres were collected by centrifugation at 7000 rpm for 10 min. The collected microspheres were washed with distilled water four times and then lyophilized. The loading efficacy of atRA was about 97% in PLGA/PLGA–PEG microspheres, with the size of microspheres ranging from 20 to 80 μm.

Before injecting microspheres into rats, the microspheres were sterilized by a total dose of 25 kGy gamma irradiation from a $^{60}$Co source in open air at room temperature.

2.3. Evaluation of inflammation

To evaluate inflammatory response induced by subcutaneously injected atRA-loaded microspheres, 40 SD rats (140–150 g, male) were divided into four groups (10 rats in each group) as follows: control microspheres treated group, atRA-loaded microspheres treated group, atRA-loaded microspheres and celecoxib (1.0 mg/kg) treated group and atRA-loaded microspheres and celecoxib (3.0 mg/kg) treated group. The atRA-loaded microspheres containing 50 mg/kg atRA were dispersed in normal saline and subcutaneously administered at the back of the neck. Celecoxib was orally administered through an oral gavage once a day for 2 weeks. SD rats, bred under specific pathogen-free (SPF) conditions, were obtained from Samtako (Osan, Korea). The rats were
transferred to a holding room and kept under controlled condition of 23±3 °C temperature, 50±10% humidity, and a 12 h light/dark cycle.

To compare the inflammation caused by atRA-loaded microspheres in each group, edema size was measured with a caliper every week and calculated by the equation: width×length×height×1/2 [23]. Furthermore, microscopic analyses of all implantation sites were carried out at the 14th and 28th days as described by Yamaguchi and Anderson [24]. The implantation sites were cut with a razor blade and were fixed in 10% buffered formaldehyde solution, followed by embedding in paraffin and sectioning with a microtome. The samples were stained with hematoxylin and eosin for cellularity and counting of the cells, and Masson’s Trichrome for fibrous capsule formation and collagen deposition.

2.4. Measurement of atRA concentration in plasma

Five SD rats in each group were used for the measurement of atRA concentration in plasma. After subcutaneous injection of atRA-loaded microspheres at the dose of 50 mg/kg atRA, blood was sampled at 1, 7, 14, 21, and 28 days, and the drug concentrations were measured using HPLC as described by Bugge et al. [25]. A Hitachi HPLC system (D-7000 series; Hitachi, Tokyo, Japan) and TSK gel ODS-80Ts column (4.6×250 mm; Tosoh, Tokyo, Japan) were used. Briefly, 150 μl of the 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 a saturated K₂HPO₄ solution and mixing it for 30 s, the samples were centrifuged for 2 min. The organic upper layer (80 μl) was analyzed. The concentration of atRA was detected by measuring the UV absorption at 365 nm.

2.5. Degradation of microspheres in vivo

To investigate the degradation of microspheres in vivo, three rats in each group were sacrificed at the 14th and 28th days, respectively, after implantation. The isolated edema tissue was frozen in cryomatrix (frozen specimen embedding medium) at −20 °C and sectioned with a microtome. The morphology of microspheres in the edema tissue in each group was observed with SEM.

2.6. Subacute toxicities of atRA-loaded microspheres and celecoxib

One hundred and twenty SD rats (6 weeks old) were randomized into six groups as follows: a non-treated group as a control, an atRA-loaded microsphere treated group, a celecoxib (9 mg/kg) treated group, three atRA-loaded microspheres, and celecoxib treated group, where the doses of celecoxib were 1.0 (group T₁), 3.0 (group T₂), and 9.0 mg/kg (group T₃), respectively. Each dosage group consisted of 20 rats (10 males and 10 females), which were used to evaluate the subacute toxicity of atRA-loaded microspheres. The sterilized microspheres were dispersed in a normal saline solution, and injected subcutaneously into the back of the rat’s neck. Celecoxib was orally administered once a day for 28 days through an oral gavage. For hematological evaluation, white blood cells (WBC), red blood cells (RBC), platelet counts (PLT), hemoglobin concentrations, activated partial thromboplastin time (aPTT), and prothrombin time (PT) were measured at 28 days. In addition, plasma concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholesterol, blood urea nitrogen (BUN), and creatinine were measured.

2.7. Experimental protocol of carcinogenesis

Fischer 344 male rats (Japan SLC, Hamamatsu, Japan), 6 weeks old at the commencement of the experiments, were housed, three to a plastic cage with hardwood chips for bedding, under controlled condition of 23±3 °C temperature, 50±10% humidity, and a 12 h light/dark cycle. Drinking water containing 20 ppm of 4-NQO was prepared twice a week by dissolving the carcinogen in distilled water and was given in light-opaque bottles. Diets containing 50 ppm of celecoxib were prepared once a week by mixing the compound with a powdered basal diet and were given to the animals in stainless steel containers. The diet and water were available ad libitum.

The experimental protocol of carcinogenesis is shown in Fig. 1. One hundred and four F344 rats were divided into five groups. Rats in group 1 (five rats) served as controls and were given tap water and a basal diet for 22 weeks. Rats in groups 2 (24 rats) and 3 (25 rats) were given 20 ppm 4-NQO in their
drinking water for 8 weeks, followed by tap water and a basal diet. Rats in groups 4 (25 rats) and 5 (25 rats) were given 20 ppm 4-NQO in their drinking water for 8 weeks, followed by tap water and were put on a diet containing 50 ppm celecoxib for 22 weeks. In the cases of groups 3 and 5, atRA-loaded microspheres at a dose of 50 mg/kg atRA were subcutaneously injected every 4 weeks. All of the animals were sacrificed under CO2 anesthesia at 22 weeks. Their tongues were excised, fixed in a 10% buffered formaldehyde solution, embedded in paraffin, sectioned with a microtome, and stained with hematoxylin and eosin for histological evaluation. Epithelial lesions (normal, hyperplasia, and neoplasm) in the oral cavity were diagnosed according to the criteria described by Báno´czy and Csiba [26].

2.8. Statistical analysis

All results were given as mean±S.E. The significant differences between group values were statistically analyzed using Mann–Whitney U test and \( \chi^2 \) test.

3. Results

3.1. Evaluation of inflammation

When atRA-loaded microspheres were administered via parenteral route, a severe inflammatory response was induced by atRA-loaded microspheres as shown in Fig. 2. The edema size observed in the group treated with control microspheres was very small and slightly increased at 2 weeks and then decreased. However, the edema size in the group that received the treated atRA-loaded microspheres was significantly increased to 4350.1±325.8 mm\(^3\), by about 8.7 times compared to that of the control group at 2 weeks. The edema size was gradually decreased over time after 2 weeks. On the other hand, when celecoxib (1.0 and 3.0 mg/kg) was co-administered orally into rats with the injection of atRA-loaded microspheres, the edema size was significantly decreased in a dose-dependent manner, compared to the treatment of atRA-loaded microspheres alone. In particular, the edema size was effectively decreased by about 61.2% when 3.0 mg/kg celecoxib was administered.

Histological evaluations of edema tissues were performed with hematoxylin and eosin and Masson’s Trichrome for the cellularity and collagen deposition, respectively (Fig. 3). In the group treated with atRA-loaded microsphere alone, interstices of the trapped microspheres in the newly formed fibrous tissue were filled with many macrophages and fibroblasts. The inner fibrous capsule was filled with bloody exudates containing many red blood cells. In addition, severe collagen deposition in the fibrous tissue was detected.

![Fig. 2. The edema sizes of each group induced by atRA-loaded microspheres with or without celecoxib treatment; (□) control microsphere treated group; (■) atRA-loaded PLGA/PLGA–PEG microsphere treated group; (▲) atRA-loaded PLGA/PLGA–PEG microsphere and 1.0 mg/kg celecoxib treated group; (▲) atRA-loaded PLGA/PLGA–PEG microsphere and 3.0 mg/kg celecoxib treated group. *P<0.05 vs. atRA loaded PLGA/PLGA–PEG microspheres alone. **P<0.005 vs. atRA loaded PLGA/PLGA–PEG microspheres alone.](image-url)
However, in the group that received the concurrent treatment of atRA-loaded microspheres and celecoxib, the number of fibroblasts and collagen deposition in the fibrous tissue were significantly decreased.

3.2. atRA concentration in plasma

After implantation of atRA-loaded microspheres in rats, the atRA concentration in plasma was measured by HPLC for 4 weeks, as shown in Fig. 4. When 50 mg/kg atRA of atRA-loaded microspheres was subcutaneously administered to rats, the atRA concentration in plasma reached 7.09 ± 1.06 ng/ml at 7 days. On the other hand, the atRA concentration in plasma at 7 days was increased up to 11.56 ± 0.77 and 13.41 ± 0.34 ng/ml when 1.0 mg/kg and 3.0 mg/kg celecoxib were orally administered, respectively. The atRA concentration was more highly maintained in the group treated with celecoxib than in the group

![Fig. 3. Histological evaluation of fibrous capsules developed by the atRA-loaded PLGA/PLGA–PEG microsphere without (a and c) or with (b and d) 3.0 mg/kg celecoxib at 14 days: without (a) and with (b) 3.0 mg/kg celecoxib at 14 days; H and E stain (×400); M indicates microsphere and arrowheads represent the fibroblasts. Without (c) and (d) with 3.0 mg/kg celecoxib at 14 days, Masson’s Trichrome stain (×400). Blue color represents newly formed collagen deposition.](image)

![Fig. 4. The plasma concentration of atRA after treatment of atRA-loaded PLGA/PLGA–PEG microspheres with (●) 0 mg/kg, (■) 1.0 mg/kg, and (▲) 3.0 mg/kg celecoxib. *P<0.05 vs. atRA loaded PLGA/PLGA–PEG microspheres alone. **P<0.01 vs. atRA loaded PLGA/PLGA–PEG microspheres alone.](image)
treated with the atRA-loaded microspheres alone for 28 days.

3.3. Metabolism of atRA

To evaluate the relationship between the extent of inflammatory responses and the metabolism of atRA, the percentage metabolism of atRA was calculated from the peak areas by using the following equation:

\[
\text{Metabolism} \% = \left( \frac{\text{area of metabolite peaks}}{\text{area of metabolite and atRA peaks}} \right) \times 100.
\]

As shown in Fig. 5, the percentage metabolism of retinoic acid in the group treated with atRA-loaded microspheres alone showed very high values of 82% at 1 day. However, with 1.0 mg/kg and 3.0 mg/kg celecoxib treatments, the percentage metabolism of retinoic acid was significantly decreased about 54% and 48%, respectively, at 1 day.

3.4. Degradation of microspheres in vivo

The degradation of microspheres at inflammatory sites was performed to compare the degradation rates of microspheres with and without the treatment of celecoxib at 1.0 and 3.0 mg/kg. As shown in Fig. 6, very small pores were detected in the inner part and on the surface of microspheres at 1 week. At 2 weeks, many large pores were observed in the inner part of microspheres. The microspheres were almost degraded in fibrous tissues at 4 weeks. The degradation pattern and rates of microspheres containing retinoic acid in all groups were very similar regardless of the presence or absence of celecoxib treatment.

3.5. Subacute toxicity of atRA-loaded microspheres and celecoxib

Blood chemistry and hematological parameters were tested to evaluate subacute toxicities of the treatment of atRA-loaded microspheres and celecoxib in male and female SD rats (Table 1). In male rats that received the treatment of atRA-loaded microspheres alone, the red blood cell count was not changed compared with non-treated group. But, the concentration of hemoglobin and the aminotransferases (ALT and AST) showed statistical differences compared with control group. Also, the white blood cell count was slightly increased when compared with the control, T2, and T3 groups, respectively, indicating that the systemic inflammation was more preferentially induced by treating atRA-loaded microspheres. The levels of creatinine, BUN, and cholesterol were normal in all groups, indicating that the renal function was normal at 28 days. However, the increase in the ALT and AST levels in the serum was not considered as a toxic effect because other parameters were normally maintained and no changes of body weight were observed. On the other hand, when celecoxib was concurrently administered with atRA-loaded microspheres in all dosage groups, all of the parameters were normally maintained; also, when compared to the control, there were no statistically significant differences between male and female rats with respect to hematological and clinical chemistry parameters at 28 days.

3.6. Incidence of oral carcinogenesis in tongue

To investigate the preventive effects of the concurrent treatment of the atRA-loaded microspheres with celecoxib, oral carcinogenesis was induced by 4-NQO and histological evaluation was performed for
carcinogenesis of tongues. The incidences of dysplasia and invasive squamous cell carcinoma (SCC) in tongues are shown in Fig. 7. In tongues of groups 2, 3, and 4, carcinogenesis proceeding up to dysplasia (54.2%) was observed in most of rats. More specifically, invasive SCC (45.8%) was found in group 2, dysplasia (44.0%) and invasive SCC (56.0%) in group 3, and dysplasia (56.0%) and invasive SCC (44.0%) in group 4. These results were not statistically different from each other since $p$ values in the $\chi^2$ test were higher than 0.05. However, in group 5 under 4-NQO that has received celecoxib and atRA-loaded microspheres, 3 of 25 rats (12.0%) showed no incidence of carcinogenesis (i.e., normal state) and
the incidence of invasiveness SCC (28.0%) was further reduced when compared to groups 2–4, whose \( p \) value in the \( \chi^2 \) test was lower than 0.05. When either atRA-loaded microspheres or celecoxib was used alone in treatment, it did not prevent the incidences of oral carcinogenesis induced by 4-NQO, but when concurrently used, the treatment of atRA-loaded microspheres and celecoxib could reduce the incidence of invasive SCC. We found that 12% of the rats in group 5 were in the normal state.

### 4. Discussion

Although one of the most potent chemopreventive agents, the application of all-trans retinoic acid is compromised because of its metabolism by the induced P450s in the liver (acute retinoid resistance). In order to prevent acute retinoid resistance in the liver while releasing atRA for a long period, atRA-loaded microspheres were developed for parenteral administration [10,22]. In general, implantable devices are associated with critical problems of inflammatory responses that maybe caused by various factors such as surface area, shape, degradation products, and degradation rates of the polymer matrices and released drugs. Expectedly, topical application of atRA has been found to induce an intensive local edema and erythema without necrosis [27], and we found that atRA-loaded microspheres stimulated severe inflammation at the implant site.

In our research, however, PLGA/PLGA–PEG microspheres caused only moderate inflammation. This result indicates that carboxylic groups of degraded fragments and atRA released from PLGA/PLGA–PEG microspheres induce severe inflammation. The degradation patterns of atRA-loaded microspheres with or without the presence of celecoxib were very similar in fibrous capsules. During the degradation of microspheres, most of atRA were released from the microspheres within 14 days, and the microspheres were degraded fast and almost disappeared from the fibrous capsules at 28 days.

Topically applied atRA has also been found to significantly proliferate fibroblasts and cause extensive collagen deposition [28,29]. The released atRA from microspheres could activate macrophages to produce IL-1, which stimulates fibroblast proliferation [30] and induces COX-2 expression that triggers the prostaglandin synthesis. Also, it has been reported that

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### Table 1

Hematology and blood biochemistry in male and female rats

<table>
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<tr>
<th></th>
<th>Control</th>
<th>RA MS</th>
<th>Celecoxib</th>
<th>T1</th>
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<td>WBC (10³ mm⁻³)</td>
<td>3.9±0.3</td>
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<td>Hb (g/dl)</td>
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<td>15.5±0.7**</td>
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<tr>
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* Significantly different from control group (\( P<0.05 \)).

** Significantly different from control group (\( P<0.005 \)).

*** Significantly different from control group (\( P<0.001 \)).
prostaglandin D$_2$, one of the various prostaglandins, could enhance fibroblast proliferation [31]. Therefore, compared to the control microspheres, the implant sites of atRA-loaded microspheres contain not only many more inflammatory cells but also many fibroblasts. The proliferated fibroblasts migrate around the microspheres, and make thick fibrous capsules.

In the present study, we found that when celecoxib was used concurrently with atRA-loaded microspheres, it could efficiently suppress the fibroblast proliferation and collagen deposition in fibrous capsules resulting from the atRA-loaded microspheres. The prevention of fibroblast proliferation and collagen deposition in fibrous capsules could be due to the suppressed production of prostaglandins following the inhibition of COX-2 activity by celecoxib. Orally administered celecoxib of 3.0 mg/kg efficiently suppressed the inflammatory responses and also decreased the edema size. Furthermore, the selective COX-2 inhibitor celecoxib could decrease fibroblast proliferation and collagen deposition in fibrous capsules.

Oral administration of atRA in the clinics is not recommended due to the induced metabolism by specific cytochrome P450s in the liver. In humans, CYP26 is primarily related to the atRA metabolism and other cytochrome P450s (i.e., CYP2C8, CYP3A7, 3A5, 2C18, 3A4, 2C9, and 1A1) have been identified in the metabolism of atRA [32–35]. In addition, administration of atRA leads to a substantial increase in the levels of CYP26 in the liver [34]. Therefore, after continuous oral administration of atRA, atRA concentration in plasma rapidly decreased due to the catabolism of CYP26, and atRA-inducible cytochrome P450. On the other hand, celecoxib is primarily metabolized by human liver CYP2C9, although CYP3A4 also plays a role in its metabolism [36]. But, the repeated administration of celecoxib

Fig. 7. (a) Histological evaluation of normal epithelium (left), dysplasia (middle), and invasive SCC (squamous cell carcinoma, right) on tongue (H&E stain) induced in rats by giving 20 ppm of 4-NQO in the drinking water for 8 weeks. (b) Incidence of oral carcinogenesis in tongue: (■) normal epithelium, (□) dysplasia, and (□) invasive SCC. Group 1: control group; group 2: 4-NQO; group 3: 4-NQO+atRA-loaded microspheres; group 4: 4-NQO+celecoxib; and group 5: 4-NQO+atRA-loaded microspheres+celecoxib.
does not affect the levels of cytochrome P450s, which metabolize atRA in the liver.

In order to avoid atRA metabolism in the liver in oral administration, atRA-loaded microspheres were locally applied via subcutaneous injection in this study. Orally administered celecoxib of 3.0 mg/kg efficiently suppressed the inflammatory responses and decreased edema size. Furthermore, the selective COX-2 inhibitor celecoxib could decrease fibroblast proliferation and collagen deposition in fibrous capsules. According to reports, dermal fibroblasts actively metabolize all-trans retinoic acid to polar metabolites [13] and thick fibrous capsules composed of intensively deposited collagen act as a permeability barrier that might block the diffusion of drugs into the blood [12,14]. In our research, the atRA concentration in plasma was more highly maintained when celecoxib was concurrently used, compared to when atRA-loaded microspheres were used alone. The highly maintained atRA concentration in plasma by concomitant use of celecoxib seemed to be related to the inhibition of COX-2 activity and prostaglandin synthesis, both of which acted together to prevent fibroblast proliferation and collagen deposition, which in turn is closely related with atRA metabolism and diffusion barrier of drugs. Therefore, we suggest that celecoxib facilitates atRA uptake in plasma by reducing local inflammation induced by the atRA-loaded microspheres.

We employed a 4-NQO model in F344 rats [37,38] to evaluate the chemopreventive efficacy of atRA-loaded microspheres that were concurrently used with celecoxib. The investigation finds that the concurrent treatment using celecoxib with atRA microspheres produces a synergistic effect on the chemoprevention of 4-NQO-induced oral carcinogenesis, where celecoxib prevents the inflammation caused by the released atRA and helps to maintain the plasma level of atRA high for a long period, thereby improving the efficacy of atRA.

Furthermore, this scheme might help to reduce the toxicity of atRA which was characterized by dyspnea, fever, dryness of nasal mucosa with nosebleed, hepatic toxicity, etc. [7,39,40]. In this study, a more moderate level of toxicity was observed when atRA-loaded microspheres containing 50 mg/kg atRA were used concurrently with celecoxib, compared to the observation found in our earlier study using atRA-loaded microspheres alone [41]. The analyses of both hematology and clinical chemistry showed that there were no significant differences among parameters for females in all groups at 28 days.

Statistics on cancer in the United States for 2004 showed that there were 48,520 cases of head and neck cancer including oral cavity, pharynx, and larynx cancers [42]. It indicates that despite recent improvements in treatment and successful treatment of a primary head and neck cancer, there have been no changes in the 5-year survival rates over the last few decades and there is a high occurrence of second primary cancers [43]. The second primary cancers are a significant problem in treating head and neck cancer as they have a negative impact on survival. Therefore, the new chemoprevention system using atRA proposed in this study could serve as an effective method for the prevention of second primary cancer occurrence.

5. Conclusions

In this study, the concurrent treatment of celecoxib with atRA-loaded microspheres could effectively prevent the severe inflammation induced by atRA released from the PLGA/PLGA–PEG microspheres, thereby maintaining the effective concentration of atRA in plasma for a long period and reducing its metabolism in fibrous capsules. In addition, celecoxib could reduce the toxicity of atRA. Therefore, the concurrent use of atRA-loaded microspheres with celecoxib could greatly improve the atRA efficacy and show chemopreventive effects against 4-NQO-induced oral carcinogenesis.

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