Abstract. This study was conducted to assess protective effect of an antioxidant protein, sericin, on tumor promotion in the 7,12-dimethylbenz (α) anthracene (DMBA)-initiated and 12-O-tetradecanoylphorbol 13-acetate (TPA)-promoted mouse skin tumorigenesis model. In experiment 1, sericin was applied topically to DMBA-initiated female ICR mouse skin at the doses of 2.5 and 5 mg twice per week for 16 weeks, 30 min prior to each promotion treatment with TPA. The protective effect of sericin was evident in terms of significant reduction in tumor incidence and tumor multiplicity at the doses of 2.5 and 5 mg per application, compared to the control group without receiving sericin. The expression of tumor necrosis factor (TNF-α) protein and the level of 4-hydroxynonenal (4-HNE) in normal epidermis were significantly reduced in both sericin treatment groups. In experiment 2, sericin at the dose of 5 mg was applied topically to the dorsal mouse skin 30 min before application of a TPA, and the same doses of TPA and sericin were applied twice at an interval of 24 h. Sericin treatment inhibited double TPA treatment-induced morphological changes reflecting inflammatory response, including leukocyte infiltration, hyperplasia and cell proliferation. Furthermore, sericin treatment significantly suppressed the elevation in 4-HNE level and elevated expressions of c-fos, c-myc and cyclooxygenase-2 (COX-2) in normal epidermis induced by double application of TPA. The results suggest that sericin possesses protective effect against tumor promotion in mouse skin by suppressing oxidative stress, inflammatory responses and TNF-α.

Introduction

Sericin is a silk protein that is the main constituent of silk fiber (20-30% of the total cocoon weight) (1). When cocoon is used for silk textiles, the sericin is mostly removed from the cocoon and disposed of without any use. Previously, we found that sericin has antioxidant action (2). This antioxidant effect appears to be mediated by its chelation with copper and iron. Sericin is also known to have a skin moisturizing and antiwrinkle effect due to its high content of serine (30-33% of the total amino acids) (1). Because of the characteristic effects, sericin has been widely used in Japan as an ingredient of cosmetics. We have further found that consumption of sericin suppressed 1,2-dimethylhydrazine-induced colon tumorigenesis in mice (3,4), implying that sericin could be a useful chemopreventive agent against colon cancer.

The involvement of oxidative stress in cancer induction and its subsequent development, and associated molecular mechanisms is becoming increasingly clear (5,6). The skin is directly and frequently exposed to sunlight and is always in contact with oxygen, resulting in the production of reactive oxygen species (ROS) implying that the skin is always in a state in which it is attacked by ROS (7). We postulated that topical application of sericin may suppress skin tumor promotion through its antioxidant activity. To test this possibility, we have studied the effect of sericin on the TPA-induced tumor promotion in DMBA-initiated mouse skin. Application of TPA triggers excessive ROS production by leukocytes in mouse skin ultimately leading to tumor promotion of the initiated skin, and most antioxidants afford protection against the tumor promotion (8,9). This study was undertaken to address whether sericin suppresses TPA-induced oxidative stress in mouse skin. TNF-α, one of the inflammatory cytokines, acts as an endogenous tumor promoter and a central mediator of tumor promotion, and induces similar biochemical and biological responses as known tumor promoters (10,11). Thus, we further attempted to analyze the expression of epidermal TNF-α in DMBA-initiated TPA-promoted mouse skin.
Materials and methods

Chemicals and animals. TPA and DMBA were purchased from Sigma Chemical (St. Louis, MO, USA), and sericin was prepared by the method described elsewhere (3). Six-week old female CD-1 (ICR): Crj mice were obtained from Charles River Japan Inc (Hino, Japan). The mice were housed four to a cage at 24±2°C and subjected to a 12:12-h light-dark cycle (lights on, 08:00-20:00 h). They were acclimatized for 1 week before use and fed a commercial stock diet (MF, Oriental Yeast, Tokyo, Japan) and deionized water. The animals were maintained according to the ‘Guide for the Care and Use of Laboratory Animals’ established by Hiroshima University. Prior to any study, the dorsal side of the skin was shaved using electric clippers, and the mice with hair cycle in the resting phase were used in all of the studies. Body weights were measured every week.

Experiment 1 (long-term study)

Two-stage carcinogenesis in mouse skin. The animals were randomly divided into three groups of 16 animals each (control group, 2.5-mg sericin group, 5-mg sericin group). Mice were initiated with a single application of 190 nmol of DMBA in 0.2 ml of acetone painted to the back of each mouse. After one week, the animals in control group were treated topically with 0.2 ml of acetone, and the animals in the treatment groups were treated with either 2.5- or 5-mg doses of sericin per mouse, in 0.2 ml of acetone. Thirty min after these treatments, a 3.2 nmol dose of TPA per mouse was applied topically to animals in all three groups. The TPA alone or two doses of sericin plus TPA treatments were repeated two times per week up to the termination of the experiment at 16 weeks from the start of TPA treatment. Skin tumor formation was recorded weekly, and tumors of >1 mm in diameter were included in the cumulative total if they persisted for 2 weeks or more. Latent periods for the onset of tumor in various groups were computed, and the tumors were diagnosed histologically at the termination of the experiment. At this point, the average tumor volume per mouse was also recorded.

Histological observation and immunohistochemical staining. Chemically exposed skin tissues from all groups of animals were removed and immediately fixed in 10% neutral-buffered formalin at 4°C overnight and embedded in paraffin. Biopsied tumors were sectioned to 4-μm thickness, and stained with hematoxylin and eosin (H&E) and diagnosed histologically.

Immunohistochemical staining, paraffin-embedded tissues were used. Immunohistochemical analysis of apoptosis labeling was examined by the TUNEL method. This method is based on TdT-mediated dUTP-biotin nick end labeling of fragmented DNA (12). After deparaffinization, the specimens were stained by an ‘Apoptosis in situ Detection Kit’ (Wako Pure Chemicals, Osaka, Japan). The quantitation was made by counting the apoptosis-positive cells as well as the total cells at 10 arbitrarily selected fields at x40 magnification within the normal epithelial regions in a blinded manner. The percentage of the apoptosis-positive cells for different treatment samples was determined as: number of the positive cells x 100/total number of cells (labeling index cells).

Experiment 2 (short-term study)

Double TPA treatment of mouse skin. The double-treatment protocol was based on the method of Nakamura et al (9). Briefly, the back of each mouse was shaved using electric clippers 2 days before each experiment; each experimental group consisted of 5 mice. Sericin (5-mg in 0.2 ml of acetone) was applied topically to the shaved area of the dorsal skin 30 min before application of a TPA solution (8.1 nmol in 0.1 ml of acetone). In the double-treatment protocol, the same doses of TPA and the test compounds or acetone were applied twice at an interval of 24 h. Mice were divided into three groups as follows: Acetone (acetone-acetone/acetone-acetone), TPA (acetone-TPA/acetone-TPA), Sericin + TPA (sericin-TPA/sericin-TPA).

Epidermal hyperplasia and leukocyte infiltration. Mice treated by the double-treatment protocol were sacrificed 1 h after the last TPA treatment. For the epidermal hyperplasia study, skin samples from different treatment groups were fixed in 10% buffered formation and embedded in paraffin. Vertical sections (4-μm thick) were cut, and stained with H&E. Epidermal hyperplasia was determined as mean vertical epidermal thickness and mean number of vertical epidermal cell layers by microscopic examination of different treated skin tissue sections. For each section of skin, the thickness of the epidermis from the basal layer to the stratum corneum was measured at 10 equal distance interfollicular sites using light microscope equipped with an eyepiece micrometer. A total of 50 sites (10 sites per skin section per sample, a total of five skin samples were determined) were examined per group. The number of nucleated cell layers was counted in the same areas. The number of infiltrating leukocytes was counted at five different areas of each section using a digital image analysis with micro analyzer (Poladigital Co. Ltd., Tokyo, Japan).

Immunohistochemical staining. In order to document TPA-induced increases in epidermal proliferation, the present study used immunohistochemical detection methods combined with antibody directed against proliferating cell nuclear antigen (PCNA). The staining method was as follows. After deparaffinization, the skin sections were treated with 1.2% H2O2 in absolute methanol 20 min. Then Dako Epos anti-PCNA/HRP antibody (PC10; Dako A/S, Denmark) was put
The quantitation of proliferating cells was made by counting the PCNA-positive cells as well as the total cells at 10 arbitrarily selected fields at x40 magnification within the normal epithelial regions in a blinded manner. The percentage of PCNA-positive cells per 10x40 fields for different treatment samples was determined as: number of the PCNA-positive cells x 100/total number of cells (labeling index of PCNA).

4-HNE, c-fos, c-myc and COX-2 staining methods were as follows: After deparaffinization, monoclonal anti-4-HNE antibody (Nippon Yushi, Tokyo, Japan), rabbit polyclonal anti-c-fos antibody (Oncogene Research Product, Cambridge, MA, USA), rabbit polyclonal anti-c-myc antibody (Santa Cruz Biochemistry, Santa Cruz, CA, USA) and affinity-purified goat polyclonal COX-2 antibody (Santa Cruz Biochemistry, Santa Cruz, CA, USA) were put on the specimens. Then, the 4-HNE and COX-2 staining specimens were stained by a Vectastain Universal Quick Kit and the c-fos and c-myc staining specimens were stained by Vectastain Elite ABC Kit. The quantitation was made by counting the various positive cells as well as the total cells at 10 arbitrarily selected fields at x40 magnification within the normal epithelial regions in a blinded manner. The percentage of the various positive cells for different treatment samples was determined as: number of the positive cells x 100/total number of cells (labeling index of PCNA).

**Statistical analysis.** Values were presented as means ± SE. The tumor incidence and tumor multiplicity were analyzed by χ² test and Wilcoxon rank sum test, respectively. Other data were analyzed by Student’s t-test. Differences with p<0.05 were considered significant. Some data were analyzed by regression analysis.

**Results**

**Experiment 1**

Preventive effect of sericin against tumor promotion. Topical application of sericin prior to each TPA application resulted in highly significant preventive effect against TPA-induced tumor promotion in DMBA-initiated ICR mouse skin (Fig. 1).

In terms of anti-tumor promotion results, when the data were analyzed for tumor incidence (the percentage of mice with tumors), as shown in Fig. 1A, topical application of sericin prior to that of TPA in DMBA-initiated ICR mouse skin resulted in significant protection. Compared to the non-sericin-treated control group of mice, the time of appearance of first tumor was delayed by 1 week in 2.5-mg sericin-treated animals. However, application of 5-mg sericin-treated animals did not produce any tumors on the skin by week 15, and only one small tumor in one mouse was seen at week 16 of tumor promotion. When these data were assessed at week 10 of tumor promotion, compared to 100% mice with skin tumors in non-sericin-treated group, only 61% of animals in the 2.5-mg sericin-treated group (p<0.01). At the termination of the experiment at 16 weeks, compared to 100% animals with skin tumors in the non-sericin-treated group, only 88% and 6% of the animals in the 2.5- and 5-mg sericin-treated groups respectively, exhibited skin tumors accounting for 13 and 94% (p<0.001) inhibition in tumor incidence, respectively (Fig. 1A).

Similarly, when the tumor data were evaluated for tumor multiplicity (the number of tumors per mouse), beginning with the first tumor appearance up to the termination of the experiment, all of the two doses of sericin used produced highly significant protection against TPA-induced complete tumor promotion in mouse skin (Fig. 1B). At the termination of the experiment at 16 weeks, compared to 17.6±1.2 tumors per mouse in non-sericin-treated group, only 5.2±0.7 and 0.1±0.1 tumors per mouse were observed in the 2.5- and 5-mg...
sericin-treated groups, respectively, accounting for 69 and 99% inhibition (p<0.05), respectively.

When the tumor promotion data were analyzed in terms of tumor volume, compared to non-sericin-treated control group, the tumor volume per mouse and tumor volume per tumor were found to be significantly lower (p<0.001) in different doses of sericin-treated groups (Table I). No difference was observed in the average body weight between two doses of sericin-treated and non-sericin-treated groups of animals throughout the experiment (data not shown).

Histological observation and immunohistochemical staining. The tumors in each group of mice were histologically identified as papillomas. The labeling index of apoptosis cells was unaffected by topical application of sericin (data not shown). A strong cytoplasmic and membrane TNF-α expression was observed in the epidermal cells from the non-sericin-treated control group (Fig. 2A), whereas only very rare staining for TNF-α was observed among the epidermal cells in samples from both sericin-treatment groups (Fig. 2B and C). A weak 4-HNE immunoreactivity was seen in cytoplasm of epidermal cells from both sericin-treatment groups (Fig. 3B and C), while it was markedly increased in the non-sericin-treated control group (Fig. 3A).

Experiment 2
Inhibitory effect of sericin on TPA-caused induction of epidermal hyperplasia, leukocyte infiltration and epidermal proliferation. As shown by data in Table II, compared to double application of acetone as vehicle, application of double dose of acetone and TPA to the shaved mouse skin resulted in a significant induction of epidermal hyperplasia, namely vertical epidermal thickness and vertical epidermal cell layers. However, pre-application of sericin at 5 mg dose prior to each TPA application in double-TPA-application protocol resulted in marked inhibition (p<0.01) of TPA-induced...
epidermal hyperplasia. Compared to acetone control, pre-application of sericin to that of each TPA treatment did not result in an increase in either mean epidermal thickness or mean vertical epidermal cell layers.

As shown in Table II, a greater number of leukocytes were found to have infiltrated the dermis by double-TPA application as compared with the acetone-treated control, whereas double pre-treatment with sericin significantly inhibited the leukocyte infiltration (82% reduction, p<0.01) compared to the TPA-treated group without receiving sericin. There was no significant difference between acetone control and sericin-treatment group (p>0.05).

The PCNA-labeling index, a marker for cell proliferation, in the epidermis of the double application of TPA mice increased by 3.2-fold over that of acetone control group (Table II, p<0.01). However, pre-treatment with sericin at 5 mg dose prior to each TPA application significantly reduced the PCNA-labeling index (Table II, 89% inhibition, p<0.01) compared to the TPA-treated group without receiving sericin. Compared to acetone control, pre-application of sericin to that of each TPA treatment did not result in an increase in the PCNA-labeling index.

Inhibitory effect of sericin on TPA-induced elevations of 4-HNE, and of the expressions of c-fos, c-myc and COX-2 proteins in ICR mice skin. As shown in Fig. 4, compared with the acetone-treated control, the double application of TPA resulted in significant induction of epidermal 4-HNE labeling index and the expressions of c-fos, c-myc and COX-2. However, double pre-treatment with sericin at a 5-mg dose prior to that of each TPA application significantly reduced the labeling index of 4-HNE and the expressions of c-fos, c-myc and COX-2 (83, 91, 88 and 76% reduction respectively, p<0.01), compared to the TPA-treated group without receiving sericin. In addition, the labeling index of 4-HNE observed in all epidermis was significantly correlated with those of c-fos, c-myc and COX-2 proteins in all epidermis (r=0.782, 0.691 and 0.513 respectively, p<0.01).

Table II. Effect of sericin on double-TPA-induced morphological changes in mice skin.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Epidermal thickness (µm)</th>
<th>No. of nucleated epidermal cell layers</th>
<th>No. of leukocytes in the cutis (No./mm²)</th>
<th>PCNA-labeling index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone (Acetone/Acetone -Acetone/Acetone)</td>
<td>16.4±4.4</td>
<td>1.4±0.1</td>
<td>86.3±4.1</td>
<td>18.6±0.7</td>
</tr>
<tr>
<td>TPA (Acetone/TPA -Acetone/TPA)</td>
<td>28.2±1.4*a</td>
<td>3.1±0.1*a</td>
<td>253.3±23.9*a</td>
<td>60.9±1.6*a</td>
</tr>
<tr>
<td>Sericin + TPA (Sericin/TPA -Sericin/TPA)</td>
<td>16.5±0.7</td>
<td>1.7±0.2</td>
<td>116.7±11.4</td>
<td>23.2±1.1</td>
</tr>
</tbody>
</table>

Values are mean ± SE (N=5). *Significantly different from other groups by Student’s t-test (p<0.01).
Discussion

The central finding in this study was that an antioxidant protein, sericin, showed strong anti-tumor-promoting effect in the mouse skin two-stage tumorigenesis model, suggesting that sericin could be a useful cancer chemopreventive agent against skin cancer.

Double applications of TPA to mouse skin lead to excessive ROS production (9). The available data suggest that each application induces two distinguishable biochemical events, namely priming, which is characterized by infiltration of inflammatory leukocytes and activation, which is characterized by ROS production from accumulated leukocytes (13). Thus, induction of an inflammatory response, as seen by dermal recruitment of inflammatory cells, is an integral part of the response of mouse skin to TPA (14). It has been also revealed that the second TPA application significantly increases leukocyte infiltration in mouse skin (9). Consistent with this finding, the results of the present study clearly demonstrated that application of double dose of acetone and TPA on the mouse skin resulted in a significant induction of leukocyte infiltration. Our study further demonstrated that double application of sericin significantly reduced TPA-induced leukocyte infiltration in the cutis, implying that sericin suppresses inflammatory responses.

One of the most common events after topical application of TPA on the mouse skin is a hyperproliferative response, namely epidermal hyperplasia and proliferating cell nuclear antigen-positive cells in epidermis mediated by inflammatory response (9,15). The measurement of proliferating activity has been used to determine the grade of precancerous lesions during tumorigenesis, as well as in predicting the prognosis of malignant tumors (16,17). In the present study, treatment of mice with sericin prior to TPA resulted in a highly significant inhibition of TPA-induced morphological changes. The results suggest that the reduction in epidermal hyperproliferation is, at least in part, responsible for the protective effect of sericin against skin tumorigenesis.

ROS production by double or multiple TPA treatment is closely associated with the metabolic activation of proximate carcinogens and the increased levels of lipid peroxidation and oxidized DNA bases (9,18). The present study indicated that topical application of sericin suppressed one major TPA-induced epidermal oxidative stress marker, 4-HNE. It is one of the major products of membrane peroxidation and reacts with proteins to form stable adducts (19). It has been demonstrated that the level of 4-HNE was elevated in DMBA/TPA-induced mouse skin (20). As expected, the elevation of 4-HNE in DMBA/TPA-induced mouse skin, as well as in double-TPA-treated skin was significantly suppressed by topical application of sericin. We further found a suppression in the protein expression of proliferation-related genes, c-fos and c-myc, by topical application sericin. c-fos and c-myc have been associated with a variety of carcinogenesis (21). In vitro studies have shown that ROS stimulate expression of proto-oncogenes c-fos, c-myc and others in various cell systems (22,23), and that TPA could stimulate the generation of such active oxygen species in vivo (24,25). It has been shown that the expression of c-fos was elevated in DMBA/TPA-induced mouse skin (20,26). This study revealed that the topical application of sericin significantly reduced the expression of epidermal c-fos and c-myc and that labeling index of 4-HNE was correlated with those of c-fos and c-myc. These results imply that sericin suppresses epidermal hypercell proliferation by reducing oxidative stress.

This study indicated that topical application of sericin suppressed the elevation in the expression of epidermal COX-2 protein induced by TPA. COX-2, an enzyme responsible for catalyzing the committed step in prostanoid biosynthesis, is the product of an immediate early gene capable of being upregulated by diverse stimuli. It is known that oxidative stress is associated with the upregulation of COX-2 (27). In vitro studies have shown that 4-HNE, the end product of lipid peroxidation is a specific inducer of COX-2 gene expression (28). Some studies have indicated that COX-2 is constitutively overexpressed in epidermal tumors obtained from the initiation-promotion protocol in mouse skin (29,30). As expected, topical application of sericin significantly inhibited the expression of epidermal COX-2. There was a significant correlation between the labeling index of 4-HNE and the expression of COX-2 in all epidermis. From these facts, the reduction of epidermal COX-2 expression by sericin might be mediated by suppressing oxidative stress. In addition, COX-2 is an important pro-inflammatory mediator (such as release of pro-inflammatory cytokine) and plays an important role in skin inflammation, cell proliferation and skin tumor promotion (18). Thus, the inhibition of inflammatory responses by sericin may be partly attributable to the suppression of COX-2 expression.

The present study further demonstrated that topical application of sericin inhibited expression of epidermal TNF-α protein in the mouse skin. TNF-α, one of the pro-inflammatory cytokines that is produced by a number of different cell types including keratinocytes under a variety of inflammatory conditions and is known to prime inflammatory cells to produce enhanced levels of reactive oxygen (31,32). Importantly, topical application of sericin inhibited TPA-caused induction of inflammatory leukocytes, implying that the sericin suppressed expression of epidermal TNF-α protein possibly by inhibiting inflammatory responses and further reduced epidermal oxidative stress. It has recently been revealed that TNF-α acts as an endogenous tumor promoter and a central mediator of tumor promotion via a PKCα (one major receptor for TPA-induced signalling in basal keratinocyte)- and AP-1-dependent pathway (10,33,34). Moore et al. (35) reported that mice deficient in TNF-α are resistant to DMBA/TPA-induced skin tumorigenesis, implying the tumor promotion by TPA is critically dependent on TNF-α. The present study demonstrated that topical application of sericin inhibited expression of epidermal TNF-α in the mouse skin two-stage tumorigenesis model. This implies that sericin exerts chemopreventive effects against TPA-induced tumor promotion by inhibiting endogenous tumor promoter TNF-α.

This study provided evidence that sericin has a suppressing activity against TPA-induced tumor promotion in mouse skin and that the underlying mechanism may involve inhibition of promoter-induced leukocyte infiltration, epidermal hyperproliferation, oxidative stress and endogenous tumor promoter TNF-α. Our recent study has further demonstrated a strong inhibitory effect of application of sericin against UVB-promoted skin tumorigenesis and oxidative stress in hairless
mice (Zhaorigetu et al, unpublished data). In conclusion, these findings suggest that sericin has suppressive activity against both chemical- and UV-radiation-induced skin tumorigenesis by reducing oxidative stress.

References