Inhibition of prostate carcinogenesis in probasin/SV40 T antigen transgenic rats by raloxifene, an antiestrogen with anti-androgen action, but not nimesulide, a selective cyclooxygenase-2 inhibitor

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The chemopreventive efficacies of raloxifene and nimesulide, an anti-estrogen but with anti-androgen action and a cyclooxygenase-2 (COX-2) selective inhibitor, respectively, were evaluated in probasin/SV40 T antigen (Tag) transgenic (TG) rats. The treatment groups were placebo, nimesulide (400 p.p.m. in basal diet p.o.), raloxifene (slow-release pellets implanted s.c., 5 mg/kg/day), raloxifene (5 mg/kg/day) plus nimesulide (400 p.p.m.), and raloxifene (10 mg/kg/day) plus nimesulide (400 p.p.m.). Animals were killed at 17 weeks of age, and prostate tissues were harvested and weighed by lobes. Tissues were evaluated by histology, immunohistochemistry, and western blot analyses and blood was collected to measure the testosterone levels. All the animals in the placebo group had tumors in each lobe compared with only 43% each in the dorsolateral (DLP) and anterior prostate (AP) of the animals treated with raloxifene (10 mg/kg/day) plus nimesulide. The total prostate weights and adenocarcinoma portions were significantly reduced in the three raloxifene-treated groups, whereas atrophic glands were increased. There were no significant differences between the nimesulide alone and placebo groups or between the raloxifene (5 mg/kg/day) alone and raloxifene (5 mg/kg/day) plus nimesulide group, suggesting a lack of cancer preventive effects of the COX-2 inhibitor in this animal model. PCNA positive rates in ventral prostate (VP) and DLP, and androgen receptor (AR) levels in VP were significantly reduced in the three raloxifene-treated groups. Furthermore, circulating testosterone was decreased after raloxifene (10 mg/kg/day) plus nimesulide treatment. These results demonstrate that raloxifene, but not nimesulide, inhibits prostate carcinogenesis in SV40 Tag TG rats associated with a decline in circulating testosterone levels and a loss of AR expression, as well as an inhibition of cell proliferation.

Abbreviations: Ap, anterior prostate; COX-2, cyclooxygenase-2; ER, estrogen receptor; IPAP, image processor for analytical pathology; NSAID, nonsteroidal anti-inflammatory drug; PCNA, proliferating cell nuclear antigen; PGE2, prostaglandin E2; SERMs, selective estrogen receptor modulators; SD, Sprague-Dawley; TRAMP, transgenic adenocarcinoma of mouse prostate; VP, ventral prostate.

Introduction

Prostate cancer is one of the most frequent malignancies among men in the Western world, with >230 110 new cases and 29 900 cases of death expected this year in the USA (1). Over 60% of these newly diagnosed cases of prostate cancer will be pathologically advanced (2). One approach to reduce the number of patients with advanced prostate cancer may be an early detection through screening programs. Another strategy is to prevent prostate cancer by effective drugs that focus on the carcinogenesis process, but not on treatment. The development of chemoprevention strategies would have an overall impact, both medically and economically against prostate cancer.

Selective estrogen receptor modulators (SERMs) are compounds that antagonize the growth promoting effects of estradiol by inhibiting the binding of the hormone to its receptor (3). Raloxifene, a prototypical SERM, has been shown to prevent osteoporosis and breast cancer (4,5). Although raloxifene binds to both estrogen receptor (ER) α and β with high affinity, the binding affinity to ERα is four times higher than that to ERβ (6,7). ERα and ERβ have been found in murine and human prostates (8,9), and there is accumulating evidence suggesting that estrogens are required for prostate carcinogenesis (10,11). In addition to its anti-estrogen activity in male guinea pigs, raloxifene is a physiological antagonist of androgen action (12). In vivo administration of raloxifene produced a regression of the ventral prostate (VP) and seminal vesicles in intact male rats, which were associated with a decline in serum testosterone levels (13). Additionally, raloxifene has been found to induce apoptosis in both androgen-dependent and androgen-independent human prostate cancer cell lines in vitro (14–16). Thus, raloxifene might be used to prevent prostate cancer through multiple mechanisms.

Levels of cyclooxygenase-2 (COX-2) have been found to be closely associated with the growth and progression of many human cancers, including prostate cancers (17,18). It is now clear that COX-2 is an inducible immediate early gene, involved not only in inflammation and cell proliferation (19) but also in differentiation (20), apoptosis (21), metastasis (22) and angiogenesis (23). Nimesulide, a preferential COX-2 inhibitor of the nonsteroidal anti-inflammatory drug (NSAID) class, inhibits the chemically induced colon (24), mammary (25) and urinary bladder (26) carcinogenesis in rats. As COX-2 catalyzes the synthesis of prostaglandin E2 (PGE2), while PGE2 increases aromatase cytochrome P450 activity that is involved in estrogen biosynthesis, COX-2 inhibitors are expected to prevent hormone-dependent cancers by deprivation of estrogen (27).

Probasin/SV40 T antigen (Tag) transgenic (TG) rats were originally generated by the microinjection of recombinant DNA of the probasin gene promoter, fused to the SV40 Tag, into the pronuclei of Sprague-Dawley (SD) rat embryos (28). All TG rats develop adenocarcinomas in the prostate by
15 weeks of age. This model provides a good tool to evaluate strategies for the prevention and treatment of prostate cancer in the relative short term. In the present study, the effects of raloxifene, nimesulide, and these compounds in combination on prostate carcinogenesis were examined in SV40 Tag TG rats. We show that raloxifene, but not nimesulide, inhibits prostate carcinogenesis in the present rat model.

**Materials and methods**

**Animals and chemicals**

Original homozygous TG rats (SPF) were kindly provided by the Department of Experimental Pathology and Tumor Biology, Graduate School of Medical Sciences, Nagoya City University, F1 female homozygous SV40 Tag TG rats were cross-bred with SD wild-type strain males (SPF) (CLEA, Japan Inc., Tokyo, Japan), and the hybrid litters were screened by PCR, as described previously (28), for the presence of the SV40 Tag transgene at 4 weeks of age. Only male rats were used in the experiment. All animals were maintained in the Kagawa Medical University Animal Facility according to the institutional animal care guidelines. They were housed 2/cage on wood-chip bedding in an air-conditioned room at 23 ± 2°C and 60 ± 10% humidity. Food (CE-2, CLEA, Japan Inc.) and tap water were available *ad libitum*. Raloxifene (Eli-Lilly, Indianapolis, IN) was incorporated into slow-release pellets (Innovative Research of America, Sarasota, FL), and the drug dose was adjusted for growth-related changes in weight. Nimesulide was purchased from Cayman Chemical Co. (Ann Arbor, MI).

**Treatment of animals**

The raloxifene pellets were implanted s.c. through a 1 cm incision in the flank of SV40 Tag TG rats anesthetized with ether as described previously (2). Beginning at the age of 5 weeks, one group of 11 TG rats were fed basal diet and received a 90-day-release drug pellet of raloxifene (5 mg/kg/day); three groups of 7–11 TG rats each were fed diet supplemented with 400 p.p.m. nimesulide and received a 90-day-release drug pellet of either a dose of 5 mg/kg/day raloxifene or a dose of 10 mg/kg/day raloxifene or a placebo; one group of 12 TG rats were continuously fed basal diet and received a placebo pellet as controls. Additionally, 10 male hybrids that were negative with SV40 Tag were used as non-TG normal controls. Body weight and food consumption were recorded biweekly. At the end of week 17 of age, all surviving rats were killed under ether anesthesia.

**Tissue processing**

At autopsy, prostates were removed and, after weighing, half of each VP was immediately frozen in liquid nitrogen for storage until western blot analysis. The remainder of the prostate was fixed in 10% neutral buffered formalin. Livers, kidneys and levator ani muscle were also excised, weighed and fixed. Weights of the levator ani muscle provide a good index of anabolic activity for the hormone-dependent organ toxicological studies. After fixation for 48 h, the dorsolateral prostate (DLP) and anterior prostate (AP), as well as the remaining half of VP were carefully dissected into individual lobes whenever possible, and then each was weighed. The tissues were routinely embedded in paraffin for histopathological evaluation and immunohistochemistry. Blood was collected, and serum was stored at −20°C for the assay of testosterone levels.

**Histopathological analysis and serum testosterone levels**

Paraffin-embedded prostate tissue sections (4 µm) were stained with hematoxylin and eosin. Neoplastic lesions of the prostate were classified as PIN and adenocarcinomas. Atrophic glands were also assessed. Each of the prostate lobes was scored and the area percentage of different component was recorded quantitatively with an image processor for analytical pathology (CLEA, Japan Inc.) and tap water were available *ad libitum*. Raloxifene (Eli-Lilly, Indianapolis, IN) was incorporated into slow-release pellets (Innovative Research of America, Sarasota, FL), and the drug dose was adjusted for growth-related changes in weight. Nimesulide was purchased from Cayman Chemical Co. (Ann Arbor, MI).

**Immunohistochemistry**

Immunohistochemical staining was performed using a VENTANA HX system. Prostate tissue sections (4 µm) were treated with primary polyclonal antibodies to SV40 large Tag (1:400), AR (1:200), proliferating cell nuclear

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<table>
<thead>
<tr>
<th>Table 1. Final body, prostate and levator ani muscle weights (g), and serum testosterone levels in SV40 Tag TG rats.</th>
<th></th>
<th></th>
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<tr>
<td>Treatment</td>
<td>No. of rats</td>
<td>Body wts (g)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VP wts (g)</td>
</tr>
<tr>
<td>Placebo</td>
<td>12</td>
<td>454.7</td>
</tr>
<tr>
<td>Nimesulide (5 mg/kg/day)</td>
<td>10</td>
<td>485.6</td>
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<tr>
<td>Raloxifene (5 mg/kg/day)</td>
<td>11</td>
<td>421.6</td>
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<td>Raloxifene (10 mg/kg/day)</td>
<td>7</td>
<td>398.9</td>
</tr>
<tr>
<td>Non-TG</td>
<td>10</td>
<td>527.1</td>
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</table>

*P < 0.05 vs group of non-TG rats.*
antigen (PCNA) (1:200) (all above antibodies from Santa Cruz Biotechnology, CA), and then sequentially with secondary antibody and avidin–biotin complex (ABC). To quantitatively evaluate cell proliferation activity, morphometric analysis was performed using an IPAP image analyzing system. Labeling indices for PCNA were analyzed in terms of the positive nuclear area/total nuclear area in at least 20 cross sections (μm²).

Western blot analysis
Western blot analysis was performed as described previously (30). Briefly, frozen tissues were homogenized in lysis buffer and centrifuged at 12,000 r.p.m. for 10 min at 4°C. After determination of the concentration, cytosolic proteins (50 μg) were separated by electrophoresis on 8% sodium dodecyl sulfate–polyacrylamide gels. After transfer to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) and incubation for 1 h at room temperature with blocking buffer, blots were incubated with primary polyclonal antibodies to AR (1:500), ERα (1:200), ERβ (1:200), or β-tubulin (1:500) (all the above antibodies from Santa Cruz Biotechnology) overnight at 4°C, and then with goat anti-rabbit IgG, HRP-linked secondary antibody for 1 h at room temperature. The immunoreactive bands were detected by the ECL plus Western Blotting Detection System (Amersham Biosciences, UK).

Statistical analysis
The incidences of lesions were analyzed by the Fisher’s exact probability test. The mean percentage of each pathological stage was expressed as mean ± SD. Differences among means were assessed using analysis of variance (ANOVA).

Results
Effects of raloxifene and nimesulide on prostate weights
Data for final body, prostate and levator ani weights, and serum testosterone levels, are summarized in Table I. Significant increases of the absolute prostate weights and the relative values to body weight were observed in the placebo-treated TG rats compared with the non-TG rats. The DLP and the AP weights as well as total prostate weights were decreased in the group that received a dose of 5 mg raloxifene alone, 5 mg dose of raloxifene plus nimesulide, and 10 mg dose of raloxifene plus nimesulide groups, but the VP weights were decreased only in the last group compared with placebo treatment. Ten milligram dose of raloxifene plus nimesulide treatment reduced both the absolute and the relative prostate weights, which was close to non-TG rats, although the body weights themselves were significantly decreased in this group. The VP weights in the 10 mg dose raloxifene plus nimesulide-treated TG rats were even lower than in the 5 mg dose raloxifene plus nimesulide treatment group. This result suggests that the 10 mg dose raloxifene treatment has a marked effect on prostate weight, particularly for VP. A reduction in serum testosterone levels and levator ani muscle weights were also observed with the 10 mg dose raloxifene plus nimesulide treatment. They also showed a similar tendency for decrease in the 5 mg dose raloxifene treatment alone and 5 mg dose raloxifene plus nimesulide treatment groups, but without significance when compared with the placebo group.

Raloxifene but not nimesulide prevents the development of prostate cancer
The effects of raloxifene and nimesulide on prostate morphology and cancer progression were investigated by histological evaluation. The VP and DLP of non-TG rats had delicate epithelial ducts with sparse intervening stroma (Figure 1a and g).
In contrast, VP or DLP of placebo-treated SV40 Tag TG rats were completely occupied by PIN and adenocarcinoma lesions (Figure 1b and h). PIN developed at a 100% incidence in all the lobes and all the groups. Adenocarcinoma was found at high incidence in each prostate lobe of all groups except for the 10 mg dose raloxifene plus nimesulide treatment group, in which adenocarcinoma was significantly decreased in DLP and AP (Table II). Small cell carcinomas were only found in DLP of two rats from the placebo-treated and 10 mg dose raloxifene plus nimesulide treatment groups, respectively.

Table II. Incidences of PIN and adenocarcinomas in prostates of SV40 Tag TG rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>VP PIN</th>
<th>Adenoarcinoma</th>
<th>DLP PIN</th>
<th>Adenoarcinoma</th>
<th>AP PIN</th>
<th>Adenoarcinoma</th>
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<td>Placebo</td>
<td>12</td>
<td>12 (100)a</td>
<td>12 (100)</td>
<td>12 (100)a</td>
<td>12 (100)</td>
<td>11 (92)</td>
<td></td>
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<tr>
<td>Nimesulide</td>
<td>10</td>
<td>10 (100)</td>
<td>10 (100)</td>
<td>10 (100)</td>
<td>10 (100)</td>
<td>10 (100)</td>
<td>7 (70)</td>
</tr>
<tr>
<td>Raloxifene (5)b</td>
<td>11</td>
<td>11 (100)</td>
<td>11 (100)</td>
<td>11 (100)</td>
<td>10 (91)</td>
<td>11 (100)</td>
<td>10 (91)</td>
</tr>
<tr>
<td>Raloxifene (5)b + nimesulide</td>
<td>11</td>
<td>11 (100)</td>
<td>11 (100)</td>
<td>11 (100)</td>
<td>9 (82)</td>
<td>11 (100)</td>
<td>9 (82)</td>
</tr>
<tr>
<td>Raloxifene (10)c + nimesulide</td>
<td>7</td>
<td>7 (100)</td>
<td>7 (100)</td>
<td>7 (100)</td>
<td>3 (43)</td>
<td>7 (100)</td>
<td>3 (43)</td>
</tr>
</tbody>
</table>

*aPercentages of lesions in parentheses.
+bRaloxifene was implanted as slow-release pellets, s.c. at 5 mg/kg/day.
+cRaloxifene was implanted as slow-release pellets, s.c. at 10 mg/kg/day.
+P < 0.05 vs placebo.
+P < 0.05 vs group of raloxifene (5) + nimesulide.

Effects of raloxifene and nimesulide on apoptosis

To further explore the chemopreventive effects of raloxifene, apoptosis was analyzed in VP and DLP using the TUNEL assay. Interestingly, although prostate weights were decreased, apoptosis rates were decreased rather than increased in the raloxifene-treated groups, particularly with the 10 mg dose raloxifene plus nimesulide treatment (Figure 3). Nimesulide alone did not affect apoptosis. There was no obvious increase of apoptosis in atrophic glands associated with raloxifene treatment. PIN showed a reduction in apoptosis compared with adenocarcinomas (Figure 4).

Fig. 2. Quantitative analysis of the proportions of different histological components in VP (a) and DLP (b) of SV40 Tag TG rats. Norm, normal glands; PIN, prostatic intraepithelial neoplasia; Atro, atrophic glands; Ca, adenocarcinoma; Nime, nimesulide; Ral, raloxifene. *, without nimesulide treatment or with placebo treatment; 5, raloxifene at 5 mg/kg/day; 10, raloxifene at 10 mg/kg/day. **P < 0.01 versus placebo-treated control group; #P < 0.05 versus raloxifene (5 mg/kg/day) plus nimesulide.

Raloxifene but not nimesulide decreases cell proliferation

We next investigated PCNA expression in the VP and DLP to check cell proliferation. Notably, atrophic glands nearly lost the expression of PCNA. On the other hand, adenocarcinoma expressed PCNA more strongly than PIN (Figure 4). PCNA expression rates were significantly decreased by raloxifene treatment compared with the placebo treatment (Figure 5).
weights of SV40 Tag TG rats and reduced the incidence and the proportion of adenocarcinoma. In the other cases, the reduction in tumor area was independent of any additional treatment with nimesulide. These results suggest that the raloxifene-based treatment may be an effective strategy for the prevention of prostate cancer.

We show here that the prostate cancer regressive responses to raloxifene in SV40 Tag TG rats are associated with a decline in serum testosterone levels and a loss of AR expression in VP. In line with the present results, raloxifene has also been found to produce a regression of the male accessory sex organs in intact SD rats, which correlated with decreases in circulating androgens (13). In contrast, another SERM toremifene was recently found to prevent prostate cancer in the transgenic adenocarcinoma of mouse prostate (TRAMP) model with elevation of serum testosterone levels but without altering AR expression in the VP (31). Being a different subclass of SERM with tamoxifen, toremifene and idoxifene, raloxifene shows some special characters. Raloxifene is unique in that it is an estrogen antagonist in the uterus (32), and only raloxifene has been found to have an anti-androgen action in vivo. However, raloxifene decreases the levels of serum testosterone not through affecting the hypothalamic–pituitary–gonadal axis, because it did not inhibit prostatic 5α-reductase or testicular 17α-hydroxy/C17,20α-lyase activities (13). Whether raloxifene, like estrogens, reduces circulating testosterone by enhancing metabolic clearance (33) or altering hepatic function to change the secretion of circulating proteins capable of binding steroids (34) still remains to be elucidated. Based on the fact that most prostate cancer is androgen-dependent when it is primarily developed, raloxifene may be superior to other SERMs in chemopreventive efficacy for this tumor.

In the present study, raloxifene treatment not only induced adenocarcinoma regression in VP but also inhibited prostate carcinogenesis in DLP, in which atrophic glands were not evident. Unlike VP, there was no evidence of lost expression of AR and SV40 Tag in DLP. Raloxifene appears to prevent cancer progression by some androgen-independent mechanism(s), which may be related to a decrease in the cell proliferation rate. Human stromal cells have been found responding to anti-estrogens by increasing the synthesis of the growth regulatory factor, transforming growth factor β (TGFβ). This TGFβ can alter the growth of mammary adenocarcinoma cells (35). The involvement of growth regulatory factors as mediator of raloxifene tissue reductive responses needs to be further studied. It is interesting that apoptosis cells were found to be reduced in the prostates of raloxifene-treated rats in the present study. This may be owing to the decrease in the cell proliferation rates in these rats. It has been found that apoptosis and PCNA expression levels positively correlate with the progression of prostate lesion in SV40 Tag TG mice (36). Although the currently approved raloxifene used to treat osteoporosis does not appear to exert any serious side-effects, we observed a body weight reduction in the 10 mg dose raloxifene-treated SV40 Tag TG rats. However, the liver weights were not different between the 10 mg dose raloxifene group and the others. Furthermore, there were no histopathological findings suggestive of toxicity in the livers of any raloxifene-treated rats (data not shown). The loss of body weights may be attributed to the effect of raloxifene on eating behavior, but not on toxicity (13). A similar influence has also been reported in estrogens and their antagonists (37,38).

The potential role of COX-2 in prostate cancer has received considerable attention in recent years, because human prostate

Discussion

In this study, we have demonstrated an inhibitory effect of raloxifene, but not nimesulide, on prostate carcinogenesis in the SV40 Tag TG rat on the basis of the following observations: raloxifene treatment significantly decreased the prostate weights of SV40 Tag TG rats and reduced the incidence and
cancer overexpresses COX-2 in a consistent manner (39,40). COX-2 expression was also reported in high-grade PIN lesions in LPB-tag mice (41). More recently, a selective COX-2 inhibitor celecoxib was demonstrated to suppress prostate carcinogenesis in the TRAMP model (42). However, we did not observe any significant inhibitory effects of nimesulide on prostate carcinogenesis in the present SV40 Tag TG rats. In our previous study and others, two plant lignans, arcttin and flaxseed, have also been shown to have a distinct inhibitory effect on prostate carcinogenesis in SV40 TG rats and the TRAMP model, respectively (43,44). Although the biochemical characteristics of closely constructed compounds, like celecoxib and nimesulide or arcttin and flaxseed, might be varied (45,46), the influence shown from different animal models should be primarily discussed. TRAMP model, which is generated using the same gene construct to SV40 Tag TG rats, is one that is widely used, and has shown promise as a good prevention experimental animal model (47). Similar to TRAMP, all SV40 Tag TG rats express the transgene in an androgen-dependent manner and spontaneously produce prostate cancer that mirrors human prostate cancer in a short period (28,48). However, there are still distinct characters in two models. First, SV40 Tag TG rats would not develop androgen-independent tumors after castration, but the TRAMP model do develop this tumour (28,49). Second, the prostate adenocarcinoma in SV40 Tag TG rats rarely metastasize even with long time bredrats, while it frequently happens in the TRAMP model (28,48). Third, small cell carcinoma developed in SV40 Tag TG rats has been proved to have neuroendocrine tumor characteristics (50), such as synapophysin (51) and protein gene product 9.5 expression (52), which were lacking in the TRAMP mice (49). Finally and importantly, the carcinogenesis appears to be stronger in the SV40 Tag TG rats than in TRAMP model because PIN is found as early as 4 weeks and the adenocarcinoma is developed within 15 weeks of age in the SV40 Tag TG rats, whereas they are formed at 10 weeks and 18 weeks of age, respectively, in the TRAMP model (28,49,50). These facts argue that although the prostatic carcinogenesis in the two models is genetically initiated in a same manner, the endogenous mechanisms for promotion may be different. By analogy, genetic background has been suggested to be involved in the risk of prostate cancer in human as evidenced by the greater incidence of prostate carcinomas in African-American men than in white men (53). Additionally, when male SV40 Tag TG rats with SD genetic background were mated with wild-type females of F344, Wistar and ACI strains, the F1 male TG hybrids with Wistar and ACI background had significantly lowered incidences of prostate carcinomas (54). Therefore, the different preventive effect of COX-2 inhibitor hints at a probably divergent involvement of COX-2 in the progression of prostate cancer in the two rodent models. COX-2 may not impact prostatic carcinogenesis in SV40 Tag TG rats so much as in the TRAMP model. The present results suggest that SV40 Tag TG rats are not susceptible to COX-2 inhibitors in preventing prostate cancer.

**Fig. 4.** Representative immunohistochemical staining of AR, SV40 Tag, PCNA, and in situ end labeling of fragmented DNA in raloxifene (10 mg/kg/day) plus nimesulide-treated prostate of SV40 Tag TG rats. AR and SV40 Tag are readily expressed in adenocarcinoma (Ca) and PIN, but not in atrophic glands (Atro) (asterisks); PCNA and apoptotic bodies (arrows) are found decreased in PIN as compared with adenocarcinoma, and lost in atrophic glands (asterisks). Scale bars in all panels are 200 μm.
expression, as well as an inhibition of cell proliferation. These findings suggest raloxifene as a potential chemopreventive agent for prostate cancer.

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