

Association of Preoperative Radiation Effect with Tumor Angiogenesis and Vascular Endothelial Growth Factor in Oral Squamous Cell Carcinoma

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This study examined the relationship between tumor angiogenesis and the radiation-induced response, evaluated based on pathological changes, in oral squamous cell carcinoma patients treated with preoperative radiation therapy. Forty-one cases of squamous cell carcinoma treated with preoperative radiation therapy were investigated. Tumor angiogenesis was assessed by scoring the intratumor microvessel density (IMVD). Expression of vascular endothelial growth factor (VEGF) was also evaluated before and after preoperative radiotherapy. There was no correlation between IMVD in the specimens before therapy and the pathological response to radiation therapy. However, radiation therapy decreased IMVD in the specimens after therapy. A significant association was observed between VEGF expression and resistance to radiation therapy: only 4 of the 21 patients whose tumors exhibited a high level (2+ or 3+) of VEGF staining experienced a major (3+ or 4+) pathological response to radiation therapy. Furthermore, an increasing level of VEGF expression after radiation therapy was observed in non-effective (0 to 2+) response cases. These results suggest that VEGF expression and the induction of this protein are related to radiosensitivity and could be used to predict the effects of preoperative radiation therapy on oral squamous cell carcinoma.

Key words: Radiosensitivity — Intratumoral microvessel density — Oral cancer

Preoperative radiation therapy is often effective to reduce the area of tumor infiltration. It allows the overall survival rates to be improved and maintains oral morphology and function. However, the reaction of the carcinoma to radiation varies from patient to patient. To choose the proper therapy as well as to avoid untoward side effects, a useful method of predicting radiotherapeutic effectiveness must be established.

Tumors induce angiogenesis to supply their oxygen and nutrient needs, and are dependent on an adequate blood supply for maintenance and growth. The clinical importance of tumoral angiogenesis as a negative prognostic factor has been demonstrated in a number of tumors.^{1–3)} On the other hand, hypoperfusion of tumors with resulting hypoxia is considered to be one of the major causes of radiotherapy failure.^{4,5)} In this sense, a high grade of tumor vascularization facilitates the effects of radiation and, at the same time, paradoxically implies a poor prognosis. We have previously reported a correlation between increased tumor vascularity and tumor progression in oral squamous cell carcinoma.³⁾ In the current study, changes in the intratumour microvessel density (IMVD) and vascular endothelial growth factor (VEGF) expression in cases of oral squamous cell carcinoma were examined in relation to the pathological effects of radiation therapy, and the use-

fulness of the VEGF expression for predicting the therapeutic sensitivity of these preoperative procedures was assessed.

MATERIALS AND METHODS

Materials Forty-one oral squamous cell carcinomas were analyzed from patients who were given preoperative external radiation therapy before surgery at the Department of Oral and Maxillofacial Surgery II, Okayama University Dental School. Of the 41 patients, 28 were male and 13 female. Their ages ranged from 44 to 81 years and, the average age was 61.2 years. Tumor extent was classified according to the TNM system by UICC.⁶⁾ There were 5 cases of T1, 16 cases of T2, 10 cases of T3 and 10 cases of T4, 29 cases of N0 and 12 cases of N1. All cases were M0. Accordingly, they were classified into 5 cases of stage I, 14 cases of stage II, 12 cases of stage III, and 10 cases of stage IV. Radiation was performed five times a week with 2 Gy at each session. The mean dose was 30.2 Gy. All cases were operated on 2 to 3 weeks after the radiation therapy. Cases were selected for this study only when tumor specimens were available both before and after radiation therapy.

Tumor response assessment Standard criteria were used to classify the tumor response. A response was termed complete (CR) if there was no evidence of visible or palpable tumor on gross inspection. A response was partial

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(PR) if there was a greater than 50% decrease in the product of the longest tumor dimension and the perpendicular diameter and no increase in the extent of any other known disease. Tumor regression less than 50% of the initial tumor size was termed stable disease unless the product of the two diameters showed a greater than 25% increase over the initial product, in which case the response was categorized as progression.

Pathological changes in the resected tumors after radiation therapy varied from case to case and between different regions of individual tumors. Changes regarded as reflecting a response to therapy included evidence of necrosis and fibrosis. Foci of necrotic tumor were noted less frequently and occasionally were difficult to distinguish from tumor necrosis, which is often encountered in untreated tumors. The areas of fibrotic tumor were distinct. Paucicellular regions of hyalinized tissue with abundant small vessels and sparse inflammatory cells were found. Aggregates of foamy histocytes, hemosiderin-laden macrophages, and inflammatory cells were present within the more densely fibrotic areas, as were nests of viable carcinoma in the tumors showing a partial response. Residual tumor cells often showed cytomegaly and nuclear pleomorphism exceeding that found in the pretherapy biopsies. The pathological response in the primary tumor was scored as follows: none, no evidence of treatment effect; +, treatment effect involving up to one-third of the gross tumor mass; 2+, effect involving one-third to two-thirds of the gross tumor mass; 3+, treatment effect in more than two-thirds of the gross tumor mass; 4+, treatment effect on the entire tumor with no viable carcinoma apparent. The pathological response was scored by two authors (S. S., A. K.), who were unaware of the patients' clinical response to radiation therapy.

Immunohistochemistry Specimens were fixed in 10% formalin and embedded in paraffin. Serial sections with 4- μ m thickness from each specimen were made for immunohistochemistry. Immunohistochemical detection of endothelial cells (CD31) and VEGF was performed using anti-CD31 (Dako, Kyoto) and anti-VEGF polyclonal antibodies (A-20, Santa Cruz Biochemistry, Inc., Santa Cruz, CA). This anti-VEGF antibody recognizes the three isoforms, VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉. Tissue sections were deparaffinized and dehydrated in graded alcohols. The sections were treated for 30 min with absolute methanol, including 0.3% H₂O₂, to inhibit endogenous peroxidase activity, then incubated with trypsin (DIFCO Lab., Detroit, MI) and 1.5% normal horse serum diluted 1:75 in Tris-buffered saline (145 mmol/NaCl, 20 mmol/liter Tris, pH=7.6) for 10 min. Both anti-CD31 and VEGF antibodies were diluted 1:100 and the specimens were incubated in primary antibodies at 4°C for 16 h. Bound antibody was detected using the Envision system (Dako). Diaminobenzidine (1 mg/ml) in the presence of 0.03% hydrogen peroxi-

dase was used to visualize any bound peroxidase and sections were counterstained with methyl green. Negative controls were obtained by omission of the primary antibody.

Assessment of IMVD and VEGF expression Immunohistochemical staining for CD31 to highlight the endothelial cells was performed. After the immunostaining, the sections frequently showed a heterogeneous staining pattern for anti-CD31 antibody. For the determination of IMVD, the five most vascular areas within a section were selected and counted under a light microscope with a 200-fold magnification (i.e., $\times 20$ objective lens and $\times 10$ ocular lens; 0.7386 mm²/field) as described by Weidner *et al.*¹⁾ Each of five areas was counted twice and the arithmetical mean in each area was used to calculate the mean IMVD for each tumor section.

The immunoreactivity for VEGF was semiquantitatively evaluated by two of the authors on blinded sections. All cases were assigned to one of four subgroups: (a) 0, positive cells were <5% and/or staining was missing; (b) 1+, positive cells were 5–25% and the staining was weak; (c) 2+, positive cells were 25–50% and the staining was moderate; (d) 3+, positive cells were >50% and the staining was pronounced.

Western blot analysis The assessment of VEGF expression level by western blotting was performed on 8 cases that did not respond to radiation therapy (none, 6 cases; and +, 2 cases). Frozen tissue was pulverized on dry ice and immediately homogenized in a buffer [2% sodium dodecyl sulfate (SDS), 50 mM Tris pH=7.6] containing a cocktail of protease inhibitors. The tissue homogenate was centrifuged at 1500g to remove unbroken cells and large particles. An aliquot of the supernatant was used for protein determination and the remainder was stored at -70°C until used for western blot analysis. A portion of the supernatant was diluted in gel loading buffer to the final concentration of 1 mg/ml, and 50 μ g of protein was used for western blot analysis. Proteins were resolved on SDS-polyacrylamide (SDS-PAGE) gels under denaturing conditions. A polyacrylamide concentration of 12% was used. Following SDS-PAGE, the separated proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA) overnight at 4°C. The membranes were blocked for a minimum of 30 min in a 3% w/v solution of nonfat milk in TBS-T [10 mM Tris (pH 7.6), 150 mM NaCl, and 0.01% Tween 20] at room temperature. A rabbit polyclonal antibody raised against a peptide corresponding to VEGF (A-20, Santa Cruz Biochemistry, Inc.) was used. The antibody concentration used for immunodetection was 1:1000. Blots were incubated overnight at 4°C with the primary antibody. The blots were washed for 1 h, then incubated for 1 h with an anti-rabbit IgG goat secondary antibody complexed to horseradish peroxidase (Santa Cruz Biochemistry, Inc.). Following incubation with the secondary antibody, the

blots were washed and immunoreactivity was detected by chemiluminescence using a commercial kit (ECL Amersham Corp., Arlington Heights, IL) in accordance with the manufacturer's instructions. To reduce methodological variation in the results due to differences of gel loading and electrotransfer, following immunodetection for VEGF, the blots were reprobated for actin (sc1616, Santa Cruz Biochemistry, Inc.). Immunoreactivity corresponding to VEGF was quantified by densitometry relative to a standard reference preparation contained on each blot. Results are expressed as relative densitometry units normalized to the expression level of actin.

Statistical analysis Statistical analysis of the correlation between IMVD, VEGF expression and response to radiation therapy was performed by means of the two-sided Fisher's exact test. Mean values were compared using the Mann-Whitney test. The significance level was set at $P < 0.05$ for each analysis.

RESULTS

The correlation between the clinical and pathological response to radiation therapy is shown in Table I. A total of 26 patients had objective response, of which 15 were partial. There was a good correlation between the clinical and pathological responses, and in the present study, we selected the pathological response to evaluate the effect of radiation therapy. The correlation between IMVD in the specimens before radiation therapy and the pathological response is shown in Table II. A total of 34 patients had a pathological response. This was scored as 1+ to 2+ in 13 patients and 3+ to 4+ in 21 patients. Because of the small numbers of patients, the levels were collapsed before a Fisher's exact test was used to test for association. All patients were divided into four groups by IMVD level as follows: <50, 50–100, 100–200, >200 per one field. No association between IMVD and the pathological response was found (Table II). A comparison of IMVD before and after radiation therapy is shown in Fig. 1, a and b. IMVD was decreased in all cases (Fig. 1, a and b, Fig. 2). However, there was no relation between the change of IMVD and the pathological response to radiation therapy (data not shown).

VEGF immunoreactivity was observed in the cytoplasm of cancer cells (Fig. 3, a and b). There was no correlation between VEGF immunoreactivity and IMVD (data not shown). The correlation between VEGF expression and the pathological response is shown in Table III. A statistically significant association was found when VEGF expression and pathological response were grouped into the low and high categories. Only 4 of the 21 patients who had tumors exhibiting a high level (2+ to 3+) of VEGF tumor staining before radiation therapy had a 3+ to 4+ pathological response ($P < 0.01$).

Table I. Correlation between Clinical Response and Histological Evaluation of the Effect of Radiation Therapy

Pathological evaluation of radiosensitivity	Patients' clinical response				
	Complete	Partial	Stable	Progression	Total
0	0	0	2	5	7
1+	0	3	4	1	8
2+	0	2	3	0	5
3+	2	7	0	0	9
4+	9	3	0	0	12
Total	11	15	9	6	41

Table II. Correlation between Pathological Response and Intratumor Microvessel Density (IMVD)

Intratumor microvessel density (IMVD)	Pathological response ^{a)}				
	0	1+	2+	3+	4+
<50	0	1	2	3	2
50–100	3	5	1	2	3
100–200	2	1	1	3	4
200<	2	1	1	1	3
Total	7	8	5	9	12

	Pathological response ^{a)}	
	0–2+	3+–4+
<100	12	10
100<	8	11

a) When tumor specimens are grouped into high and low categories there is no significant relationship between pathological response and IMVD.

NS: not significant.

A comparison of VEGF expression before and after radiation therapy is shown in Table IV. This comparison could not be made in cases in whom a major pathological response to radiation therapy resulted in little or no viable tumor on which to perform staining for VEGF. In 29 of the 41 evaluable specimens, 14 of 29 cases showed no change in the level of VEGF staining. In 8 cases, the level of VEGF staining decreased, and in 7 cases it increased. However, there were only 7 cases with an increase in the level of VEGF in the specimens after radiation therapy compared to that before therapy, and in whom there was a non-effective (0–1+) pathological response (Table V, Fig. 3, a and b).

The assessment of VEGF expression level by western blotting was possible in 8 cases in whom radiation therapy was non-effective. The band of VEGF at 21 kDa (corresponding to the VEGF₁₆₅ protein) was dominantly observed. An increase in VEGF levels after therapy was observed in the specimens from cases who did not respond to radiation therapy (Fig. 4).

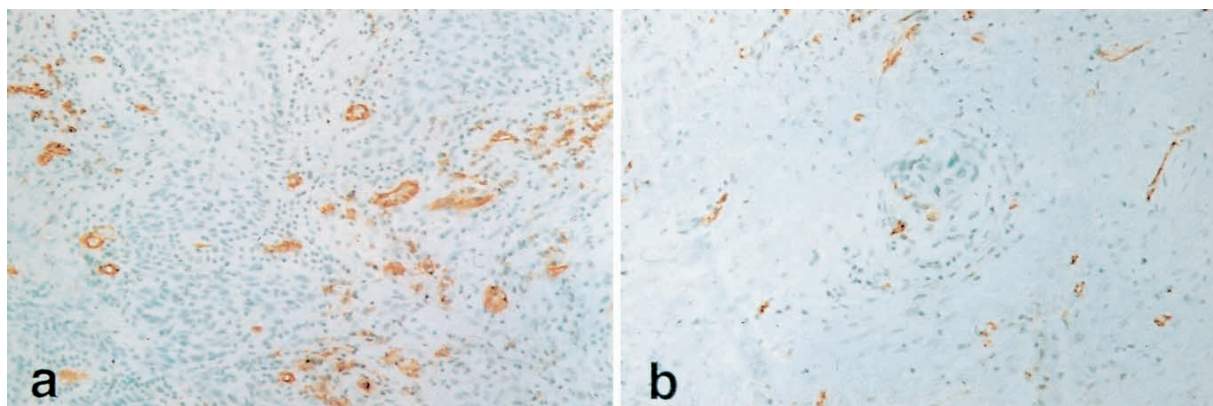


Fig. 1. Intratumoral microvessels in the specimens before preoperative radiation therapy (a), and after radiation therapy (b) of oral squamous cell carcinomas ($\times 200$). IMVD decreased after radiation therapy compared to before therapy.

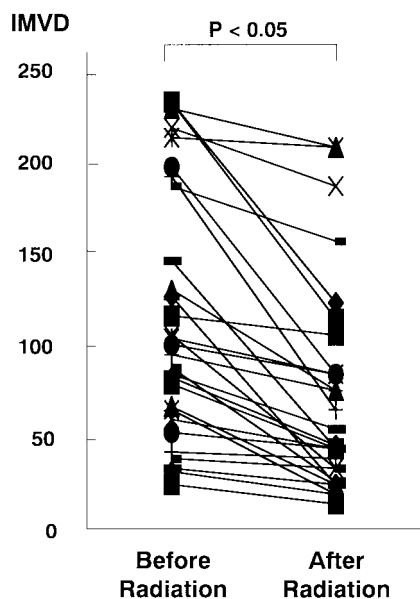


Fig. 2. Changes in IMVD after radiation therapy. There was a significant decrease in IMVD after radiation therapy ($P < 0.05$). Number of intratumoral microvessels within five fields at $\times 200$.

DISCUSSION

The growth of tumors is dependent upon angiogenesis.⁷⁾ Initial tumor growth is associated with the passive diffusion of nutrients and waste products, but subsequent growth must be accompanied by the development of blood vessels. This had led to interest in studying the relationship between tumor angiogenesis and prognosis in cancer patients.⁸⁾ Several groups have reported a correlation

Table III. Correlation between Pathological Response and VEGF Immunohistochemical Staining

Immunohistochemical staining of VEGF before radiation therapy	Pathological response ^{a)}				
	0	1+	2+	3+	4+
0	0	0	0	3	5
1+	1	3	3	3	6
2+	3	5	1	3	1
3+	3	0	1	0	0
Total	7	8	5	9	12

	Pathological response ^{a)}	
	0-2+	3+-4+
0-1+	7	17
2+-3+	13	4

$P < 0.01$

a) When tumor specimens are grouped into high and low categories only 4 of the 21 patients with a +++ or ++++ pathological response had a high level (++ to +++) of VEGF staining before radiation.

between increased tumor vascularity and poor prognosis.^{1,9,10)} Since tumor vascularization is of the utmost importance for tumor progression, it is suggested that the anti-vascular effect of irradiation may be partly involved in the anti-tumoral effect of radiotherapy. On the other hand, an impaired tumoral vascular network may reduce oxygenation and thus reduce tumor cell vulnerability to radiation, since hypoxia is considered to be one of the major causes of radiotherapy failure.^{4,5)}

This study demonstrated that the tumoral vascular network was significantly affected by radiation, with a decrease in microvessel density. Furthermore, the VEGF level is related to radiosensitivity, and the induction of VEGF is observed.

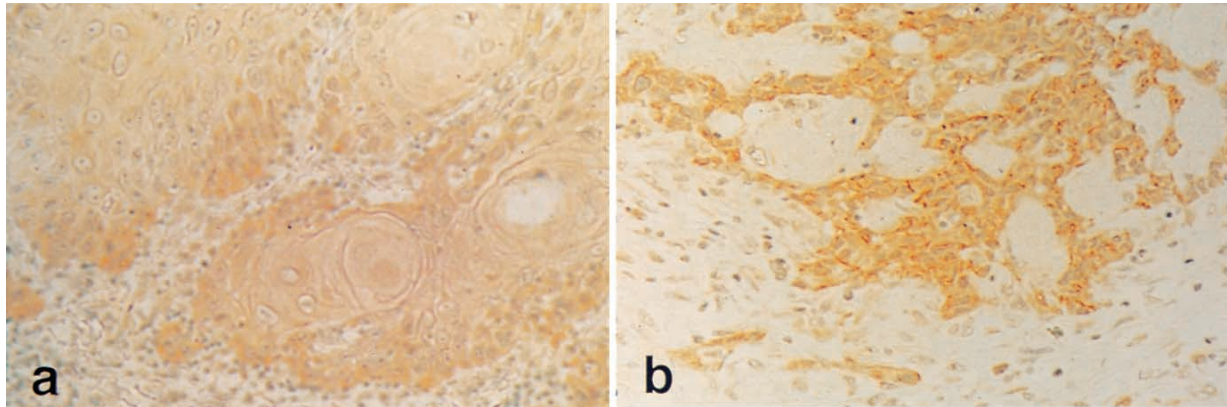


Fig. 3. Immunohistochemical staining of VEGF in oral squamous cell carcinomas. Positive staining is seen dominantly in the cytoplasm of the cancer cells. The intensity of VEGF staining was stronger in the tumors that survived radiation therapy (b) than before preoperative radiation therapy (a) ($\times 200$).

Table IV. Comparison of VEGF Immunohistochemical Staining before and after Radiation Therapy^{a)}

Immunohistochemical staining of VEGF before radiation therapy	After radiation therapy			
	0	1+	2+	3+
0	3	0	0	0
1+	4	4	2	0
2+	2	1	4	5
3+	0	1	0	3
Total	9	6	6	8

a) Cases in which there was complete pathological response after radiation therapy could not be assessed because there was no residual viable tumor on which to perform immunohistochemical staining.

Table V. Correlation between VEGF Immunohistochemical Staining Levels before and after Radiation Therapy^{a)}

Changes of VEGF immunohistochemical staining after radiation therapy	Pathological response ^{a)}			
	0	1+	2+	3+
Increase	3	4	0	0
No change	4	4	1	5
Decrease	0	0	4	4

a) The patients achieving complete response after radiotherapy are not included because immunohistochemistry was not possible due to the absence of viable tumor tissue.

The effect of radiotherapy on normal tissue vasculature is well known and is believed to be the main cause of late radiation damage.¹¹⁾ The slow turnover rate of non-proliferating endothelial cells leads to delayed damage to the

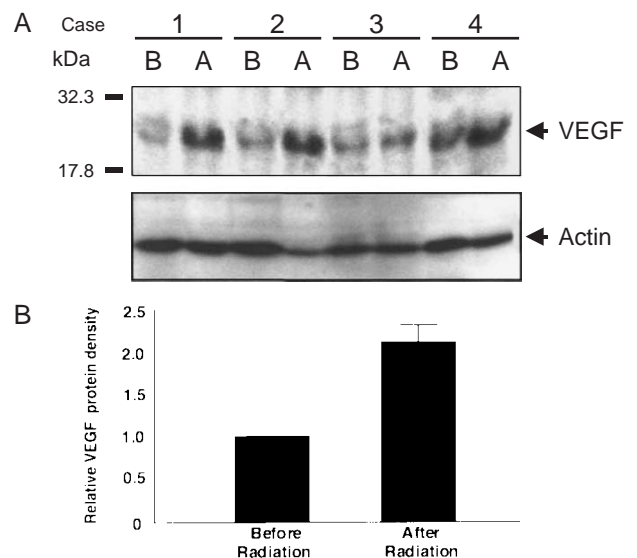


Fig. 4. Western blot analysis of VEGF expression in cytosolic extracts of oral squamous cell carcinoma (lanes 1–4). A: Western blots before (B) and after (A) preoperative radiation therapy are shown. B: Protein expression of VEGF; average of 8 cases of non-effective (with a 0 or + pathological response to radiation therapy) tumors. Bars, SE.

vasculature. However, the effect on the tumor vasculature may be different. The rapid turnover rate of proliferating endothelial cells in the tumor stroma makes them potentially more vulnerable to radiation than normal tissue.¹²⁾ Our study demonstrated that the IMVD was decreased in the specimens after radiation. These findings suggest that

the anti-vascular effect may well be mediated by a direct anti-proliferative effect on the endothelial cells. The anti-tumor effects of preoperative radiation therapy may be associated not only with the radiosensitivity of the individual tumor cells themselves, but also with the extent of tumor angiogenesis inhibition.

VEGF is a heparin-binding polypeptide growth factor and that is one of the most important angiogenic proteins.^{13,14} Four VEGF protein isoforms of 121, 165, 189 and 206 amino acids have been characterized.¹³⁻¹⁵ However, the mode of VEGF subtype expression might depend upon tissue specificity, and the populations of VEGF₁₆₅ and VEGF₁₂₁ are dominant in some carcinomas.¹⁶⁻¹⁹ Some reports suggested that the levels of VEGF₁₆₅ and VEGF₁₂₁ might be critical for the total angiogenic activity supplied by VEGFs.^{18,19} In the current study, VEGF₁₆₅ was detected mainly by western blotting, and its expression level correlated with the radiosensitivity. Up-regulation of VEGF was observed in the tumors surviving after radiation. These findings indicate that VEGF expression may represent a tumor response to radiation stress. In hematopoietic cells, VEGF inhibits apoptotic death induced by various stresses, such as ionizing radiation.²⁰ Hypoxia is known to be the most important physiological stimulus for VEGF up-regulation *in vitro*²¹ as well as *in vivo*.^{22,23} There is a possibility that radiore-

sistant cells may have the potential to up-regulate VEGF when they undergo DNA damage. Our present study of human oral cancer indicated that VEGF immunoreactivity in tumor cells was not well correlated with IMVD. Various other peptide growth factors, such as basic fibroblast growth factor,²⁴ and transforming growth factor- α ,²⁵ have been found to stimulate the proliferation and motility of endothelial cells, thus inducing new blood vessel formation. Since tumor angiogenesis is a complex multistep process controlled by various growth factors, it is unlikely that the inhibitory effects of preoperative radiation on the angiogenic phenotype of oral cancer can be explained by a change in the expression of VEGF alone. Additional studies are needed to clarify the molecular events leading to the tumor angiogenesis inhibition induced by radiation therapy.

This study demonstrated that the tumoral vascular network is significantly affected by radiation, and VEGF is related to radiosensitivity. Our results suggest that combining radiation with an angiogenesis inhibitor may provide additive antitumor effects, because the tumor cells, tumor stroma, and their interactions would be targeted by this combined therapy.

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