Role of Thromboxane and Prostacyclin Release on Photodynamic Therapy-induced Tumor Destruction

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ABSTRACT

Thromboxane and prostacyclin levels in serum were measured following photodynamic therapy (PDT) to assess the role of these vasoactive agents on vascular damage and tumor destruction. Sprague Dawley rats were given injections i.v. of Photofrin II doses ranging from 0 to 25 mg/kg. Twenty-four h later, the right hindlimbs of animals bearing chondrosarcoma tumor or controls were exposed to 0–135 J/cm² 630 nm light. Serum concentrations of thromboxane and prostacyclin were determined by radioimmunoassay. A dose-response relationship was established between the amount of photosensitizer administered and the light dose delivered with the release of thromboxane immediately following PDT. Treatment of tumor induced higher levels of thromboxane than did the treatment of tumor-free tissue, suggesting that tumor is more sensitive to PDT-induced damage. The porphyrin and light doses found to induce the release of thromboxane into serum were the same as those required to evoke vascular stasis and tumor destruction. Prostacyclin release was not altered by PDT. The administration of indomethacin (10 mg/kg, i.p.) 3 h before light treatment was found to suppress the intravascular release of thromboxane at the highest porphyrin and light doses studied. Indomethacin treatment also inhibited PDT-induced vascular stasis and tumor destruction, suggesting that the release of thromboxane is linked to these events. Since prostacyclin levels in serum were unchanged following PDT treatment of tumor and controls, thromboxane release appears to be a specific response to PDT and may mediate the vascular stasis observed following PDT.

INTRODUCTION

Photodynamic therapy, a new modality for the treatment of solid tumors, involves the systemic administration of photosensitizing drugs and delivery of visible light to produce selective tumor destruction. Treatment of animal tumors with porphyrin- or phthalocyanine-based photosensitizers and light is characterized by rapid changes in the microvasculature. These changes include platelet aggregation and vasoconstriction, and lead to vascular stasis (1, 2). Stasis of blood flow within tumor microvasculature is known to result in progressive tissue hypoxia and deprivation of nutrients (3, 4). These processes correlate with tumor destruction in the animal models examined (5, 6).

Recent studies have examined how PDT affects eicosanoid metabolism in cells (7, 8) to further understand the process of tumor destruction. The eicosanoids are a class of biologically active molecules which are synthesized from lipid precursors in response to cellular damage. This class of molecules includes the prostaglandins, thromboxanes, and leukotrienes. Lim et al. (9) incubated murine mast cells in protoporphyrin and exposed them to visible light. Mast cells released PGD₂ and lesser amounts of other prostanooids during treatment (9). An additional series of experiments showed that light treatment to protoporphyrinic mice suppressed generation of PGI₂, PGF₂α, PGE₃, and PGD₂ upon stimuli, suggesting alteration of the eicosanoid metabolism by PDT (10). These studies were extended by Henderson and Donovan (8), who determined that mouse peritoneal macrophages and RIF tumor cells released measurable quantities of PGE following Photofrin II-mediated phototherapy. Reed et al. (11) addressed the role of eicosanoid release in PDT in an alternative manner. They found that the administration of aspirin or indomethacin to rats before Photofrin II-mediated PDT markedly inhibited alterations in blood flow normally observed during treatment. Since aspirin and indomethacin act to block the enzyme cyclooxygenase and suppress the release of certain eicosanoids, the release of these vasoactive agents may be linked to observations of vascular damage and tumor destruction.

Since tumor destruction by photodynamic therapy is intimately linked to microvascular damage, we have studied processes that could evoke vasoconstriction and platelet aggregation. One common avenue which produces both vasoconstriction and platelet aggregation is the release of thromboxane from platelets (12, 13). Thromboxane is a metabolite of arachidonic acid which was identified by Hamberg et al. (14) in 1975. We hypothesize that singlet oxygen generated during PDT causes damage to certain intravascular targets, producing lipid peroxidation and/or other membrane alterations. This membrane damage causes the mobilization of arachidonic acid and other lipids which are metabolized to thromboxane and perhaps other eicosanoids. Localized release of thromboxane from cells or platelets precipitates vasoconstriction and platelet aggregation which lead to vascular stasis. To test this hypothesis, we have examined the intravascular release of thromboxane with different sensitizer concentrations and light doses in the treatment of normal tissue and tumor. Additionally, we measured the release of prostacyclin (PGI₂), a potent vasodilating and antiaggregatory agent, which counteracts thromboxane action under physiological conditions (15).

MATERIALS AND METHODS

Tumor System. A chondrosarcoma tumor maintained in our laboratory was used in these experiments (16). Female Sprague Dawley rats (150–200 g) were used for tumor implantation and for control studies. For the preparation of tumor cell suspensions, approximately 500 mg of nonnecrotic tumor was minced in 2 ml of Hanks' balanced salt solution containing penicillin and streptomycin. The resulting suspension was passed through a tissue sieve (Collecrator tissue sieve, 30 mesh screen), then through graded needles. For tumor implantation, 0.3 ml (1 x 10⁶ cells/ml) of tumor cell suspension was injected s.c. in the center of the thigh on the right hindlimb of animals with a 20-gauge needle.

Tumors were used for experimentation when they had reached a surface diameter of 5–8 mm and a thickness of 2–3 mm. Generally, tumors reached this size 3–4 days after implantation. Tumors were free of evident necrosis at treatment size.

Photosensitizer. Photofrin II (Quadra Logic Technologies, Vancouver, British Columbia, Canada) was used in all experiments. This compound is a purified component of hematoporphyrin derivative. Photofrin II was stored in the dark at ~20°C before use.

Indomethacin. Indomethacin (Sigma Chemical Co., St. Louis, MO)

Received 9/14/89; revised 1/19/90.

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1 This investigation was supported by the Center for Applied Microcirculatory Research, by the Department of Surgery of the University of Louisville School of Medicine, and by the J. Graham Brown Cancer Center.

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3 The abbreviations used are: PDT, photodynamic therapy; PGD₂, prostaglandin D₂; other prostaglandins are similarly designated.

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Indomethacin. Indomethacin (Sigma Chemical Co., St. Louis, MO)
was dissolved in absolute ethanol and diluted 1:20 with Hanks' balanced salt solution before use. Indomethacin was injected i.p. into rats at a dose of 10 mg/kg, 3 h before phototherapy.

Photodynamic Therapy Conditions. Two days prior to treatment, the right hindlimbs of tumor-bearing or control rats were shaved and depilated. Animals were given i.v. injections (via tail vein) of 0–25 mg/kg photosensitizer 24 h before treatment. For treatment, rats were anesthetized with an i.p. injection of sodium pentobarbital (50 mg/kg) and placed on their left sides. The right hind leg was placed in a restraint designed to preclude any leg movement without producing vascular impairment.

A 5 W argon laser (Spectra Physics Model 165, Mountain View, CA) was used to power a dye laser (Spectra Physics Model 375B containing a krypton red dye (Exililon Chemical Co., Dayton, OH). The output beam was coupled to a single 400-μm quartz fiberoptic cable (Ensign-Bickford Optics Co., Avon, CT). A microcatheter with a focal length of 3.5 mm was inserted to the end of the fiberoptic to ensure even light distribution throughout the treatment field. The wavelength, measured by a monochromator (Oriel Optical Corp., Stamford, CT), was tuned to 630 nm by a birefringent filter. Power density of delivered light was adjusted to 75 mW/cm² for a spot size of 2-cm diameter as measured by a thermopile (Coherent Model 210, Auburn, CA). For treatment, the dorsal surface of legs containing tumor, or tumor-free controls, were illuminated with 0–135 J/cm² light (0–30-min treatment).

Assay of Thromboxane and Prostacyclin. Animals were anesthetized with sodium pentobarbital (50 mg/kg), at least 1 h before treatment. Three to 5 animals were studied for each different treatment condition. A 1- to 1.5-cm midline incision was made in the cervical region to expose the left internal carotid artery. A heparinized polyethylene catheter (PE-50; Clay Adams, Parsippany, NJ) was inserted into the carotid in a retrograde manner to permit blood sampling. To reduce blood clotting, animals received 100 units of sodium heparin after the placement of the catheter. The surgical incisions were then closed, and the animals were monitored for changes in blood pressure and heart rate before phototherapy.

Immediately after the specified light treatment, 1–2 ml blood were collected from the carotid artery, into prechilled polypropylene centrifuge tubes coated with a solution of 4.5 mM EDTA and 10 μg/ml indomethacin. Samples were centrifuged at 1000 × g for 10 min at 2–4°C. The plasma fraction was removed and acidified to pH 3.0 with 100 μl/ml of 2 M citric acid to inhibit all enzyme activity and was centrifuged again to remove proteins which may have denatured and precipitated. The plasma was removed and frozen at −80°C until assayed.

Eicosanoids were extracted by using successive chromatography on C18 and silica columns (Analytichem International, Harbor City, CA), using a modification of the procedure outlined by Skrinska and Lucas (17). C18 columns were prepared by washing with 2 ml methanol and 2 ml distilled water (pH 3.5). Plasma samples (≤1 ml) were loaded onto the C18 columns and passed through under vacuum at a flow rate of no more than 1 ml/min. Columns were then washed with 2 ml distilled water (pH 3.5) followed by 2 ml of 10% methanol and 2 ml benzene.

Eicosanoids were eluted from the C18 columns with 1 ml ethyl acetate directly onto silica columns. The silica columns were then washed with solvent mixtures of increasing polarity (additions of methanol to a 60:40 mixture of benzene:ethyl acetate) to separate the eicosanoids from other contaminants. Eicosanoids were finally extracted from the silica columns with a solution of benzene:ethyl acetate:methanol (60:40:30) into polypropylene tubes.

Extracts were dried under nitrogen and reconstituted to 0.5 ml by using the assay buffer supplied in the radioimmunoassay kits. Method blanks (aliquots of distilled water, or known concentrations of eicosanoids) were run with each batch of samples. Recoveries of thromboxane or prostacyclin were greater than 90% with this method.

Levels of thromboxane A2 were determined by radioimmunoassay of its stable hydrolysis product, thromboxane B2 (18). Prostacyclin levels in serum were assessed by radioimmunoassay for one stable conversion product, 6-keto prostaglandin F1α (19, 20). Radioimmunoassay kits contained antibodies specific for thromboxane B2 or 6-keto prostaglandin F1α, and 125I-labeled ligand (New England Nuclear Kits NEK-024, NEK-025; NEN Products, Boston, MA).

Assessment of Tumor Response. Rats were examined for tumor regrowth daily for the first 14 days after treatment, and weekly thereafter for a total of 91 days. Ten to 20 animals were used in each treatment group.

Assessment of Vascular Damage. The extent of vascular stasis in animals given PDT was determined immediately after treatment. Animals were given injections i.v. of 5 mg sodium fluorescein (Sigma Chemical Co., St. Louis, MO) diluted in 0.5 ml normal saline. The right hindlimbs of treated and control rats were then exposed to UV light (Spectroline Model MB-100 365 nm UV lamp, Spectronics Co., Westbury, NY) to assess fluorescein dye exclusion in the treatment area. Three to five animals were examined in each treatment group. Fluorescence patterns were photographed and recorded.

Tissue Extraction of Porphyrins. Animals were given injections i.p. of 25 mg/kg Photofrin II. Tissue samples of skin, muscle, and tumor were obtained from the right hindlimb of animals 24 h after. Porphyrin extraction and quantitation were done by using the method described by Mang et al. (21). Porphyrin levels were calculated on a per g wet weight basis. Three to five samples of each tissue were assayed.

Statistical Analysis of Data. Data from experiments were grouped and the means were calculated. Data are presented as the mean ± 2 SEM. This value estimates the 95% confidence interval for each set of data. Groups of data were compared by using Student’s t test, and verified with the Wilcoxon two-sample rank test (22). Differences between groups were considered statistically significant if P < 0.05. In cases where linear regression was done, the coefficient of determination (r²) and probability (significance) was determined (22).

RESULTS

Treatment of tumor-free hind legs with photodynamic therapy (25 mg/kg Photofrin II, 24 h, 135 J/cm² 630 nm) induced the release of significant levels of thromboxane into serum compared to controls. These data are described in Fig. 1. Animals given neither photosensitizer nor light treatment had thromboxane levels of 858 pg/ml serum (95% confidence interval, 762–954). Animals exposed to drug alone or light alone had thromboxane levels of 795 pg/ml (749–841 pg/ml) and 1000 pg/ml (943–1057 pg/ml), respectively. Treatment of the legs with photosensitizer and light produced levels of 3300 pg/ml thromboxane (2940–3660 pg/ml). This is more than a 3-fold increase in systemic thromboxane in serum compared to controls (P < 0.001).

A second series of studies established a dose-response relationship between the amount of photosensitizer administered, and thromboxane levels in tumor-free animals treated with PDT, or controls. Animals in the appropriate groups were given injections of 25 mg/kg Photofrin II i.v., 24 h prior to light treatment, and/or given 135 J/cm² 630 nm light treatment to the right hindlimb (30-min treatment). Columns, mean of 3–5 experiments; bars, ±2 SEM.

Fig. 1. Thromboxane B2 levels in serum of tumor-free animals treated with PDT, or controls. Animals in the appropriate groups were given injections of 25 mg/kg Photofrin II i.v., 24 h prior to light treatment, and/or given 135 J/cm² 630 nm light treatment to the right hindlimb (30-min treatment). Columns, mean of 3–5 experiments; bars, ±2 SEM.
the light dose delivered, and systemic concentrations of thromboxane immediately following PDT. A linear relationship was found to exist between the injected photosensitizer dosage and thromboxane released into serum (Fig. 2) \( r^2 = 0.99, P < 0.01 \).

Fig. 3a describes the light dose response for thromboxane release following the treatment for 0 to 30 min (0 to 135 J/cm\(^2\)) at Photofrin II doses of 5, 10, and 25 mg/kg. Progressive amounts of thromboxane were released through the treatment period when drug doses of 25 mg/kg were used \( (P < 0.05) \). Short durations of light (5 min or less) were sufficient to induce measurable levels of thromboxane. At 10 mg/kg injected Photofrin II doses, increases in systemic thromboxane concentrations were noted. Treatment times of more than 15 min (67.5 J/cm\(^2\)) produced no further elevation in thromboxane levels. Photofrin II doses of 5 mg/kg did not induce any significant increases in systemic thromboxane levels at any light dose \( (P > 0.05) \).

These results were compared to the thromboxane release observed when tumor is treated instead of normal tissues. Fig. 3b illustrates the porphyrin and light dose response for thromboxane release after tumor treatment. Significant increases in systemic thromboxane concentration were observed following PDT by using Photofrin II doses of 10 and 25 mg/kg, and light doses of 22.5 J/cm\(^2\) and above \( (P < 0.05) \). No increases in thromboxane in serum were noted when Photofrin II doses of 5 mg/kg were used \( (P < 0.05) \). Treatment of tumor precipitated the release of 3300 pg/ml (2275–4325 pg/ml) thromboxane at a drug dose of 10 mg/kg and 4300 pg/ml (2910–5690 pg/ml) with drug doses of 25 mg/kg.

These samples were also assayed for PDT-induced release of prostacyclin from endothelial cells. No changes were observed in the release of prostacyclin into serum following photodynamic treatment of the surface of the right hind leg of rats when porphyrin doses of up to 25 mg/kg Photofrin II, and light doses from 0 to 135 J/cm\(^2\) were administered. Control values were 186 pg 6-keto-PGF\(_{1\alpha}\)/ml serum collected (95% confidence interval, 151–221 pg/ml) for animals given no photosensitizer, 151 pg/ml (122–180 pg/ml) for animals given photosensitizer alone (25 mg/kg Photofrin II, 24 h), and 160 pg/ml (130–190 pg/ml) for animals given 135 J/cm\(^2\) light treatment alone. Animals exposed to 5, 10, and 25 mg/kg photosensitizer, and 135 J/cm\(^2\) light had 6-keto-PGF\(_{1\alpha}\) levels of 160 pg/ml (130–190 pg/ml), 172 pg/ml (152–192 pg/ml), and 163 pg/ml (117–209 pg/ml), respectively. These values were not statistically different from controls \( (P > 0.05) \).

No changes in prostacyclin release into serum were observed following photodynamic treatment of chondrosarcoma tumor implanted in the right hind leg of animals. Animals exposed to 5, 10, and 25 mg/kg Photofrin II, and 135 J/cm\(^2\) light had 6-keto-PGF\(_{1\alpha}\) levels of 155 pg/ml (143–167 pg/ml), 190 pg/ml (131–249 pg/ml), and 185 pg/ml (175–195 pg/ml), respectively. These values were also not statistically different from controls \( (P > 0.05) \).

The consequence of indomethacin pretreatment of animals on thromboxane release was next examined. The administration of indomethacin 3 h before PDT was found to inhibit the release of thromboxane during light treatment \( (P < 0.001) \). Animals exposed to 25 mg/kg Photofrin II and light doses of 135 J/cm\(^2\) following indomethacin injection had thromboxane levels of 250 pg/ml (95% confidence interval, 0–500 pg/ml) compared to untreated control values of 858 pg/ml serum (762–954 pg/ml) and PDT-treated values of 3300 pg/ml thromboxane (2940–3660 pg/ml).

Fluorescein dye exclusion immediately after PDT treatment gave an indication of vascular stasis produced in the tumor and surrounding skin. Porphyrin doses of 5 mg/kg and 135 J/cm\(^2\) light were ineffective in producing vascular stasis in either tumor or skin. Porphyrin doses of 10 mg/kg or 25 mg/kg and 135 J/cm\(^2\) light caused exclusion of fluorescein dye to tumor. Vascular stasis to skin surrounding tumor was only apparent
at porphyrin doses of 25 mg/kg and 135 J/cm² light. The administration of indomethacin prior to PDT inhibited vascular stasis at all porphyrin and light doses examined in this study.

Tumor response studies were performed to assess if the inhibition of thromboxane release by indomethacin would modify tumor destruction (Fig. 4). Treatment of the chondrosarcoma tumor on the hindlimb of rats with 25 mg/kg Photofrin II i.v. 24 h, and 135 J/cm² 630 nm light produces apparent tumor destruction in 90% of animals. However, by 14 days post treatment, many of these tumors had regrown, leaving only 10% of animals tumor free at 42 days posttreatment. Injection of indomethacin 3 h before phototherapy completely inhibited tumor destruction in all animals treated. The only reaction observed was some slight swelling within the treatment field.

Representative samples of tumor, muscle, and skin from the right hindlimbs of animals were assayed for relative porphyrin accumulation 24 h after injection of Photofrin II. Tumor and skin had nearly identical porphyrin levels of 20.1 ± 1.1 µg/g and 19.8 ± 2.2 µg/g tissue, respectively. Muscle underlying skin contained 14.4 ± 2.0 µg porphyrin/g tissue.

DISCUSSION

PDT treatment of the hind leg of rats, whether free of, or implanted with chondrosarcoma, was found to induce the release of thromboxane into the serum. Thromboxane release followed a light and photosensitizer dose-dependent relationship, consistent with the in vitro studies by Henderson and Donovan (8). At the highest photosensitizer (25 mg/kg) and light dose (135 J/cm²) delivered, treatment of tumor produced a 4-fold increase in thromboxane in serum as compared to controls (P < 0.001). Porphyrin doses of 5 mg/kg, however, were ineffective in inducing thromboxane release regardless of the light dose used. These results correlate well with the observed changes in blood flow by fluorescein dye exclusion and with tumor response in this model. No difference in tumor blood flow was found in animals treated with 5 mg/kg Photofrin II, and 135 J/cm² light compared to controls. This coincides with a lack of tumor regression under these conditions. At porphyrin doses of 10 or 25 mg/kg and a light dose of 135 J/cm², we observed dye exclusion, indicating vascular occlusion, and short-term tumor regression following PDT.

The difference between the amount of thromboxane released into serum after the treatment of the tumor-free leg and the amount released after treatment of chondrosarcoma at the same site can be calculated as a ratio and may be compared to a therapeutic difference for damage to these tissues. At injected doses of 10 mg/kg Photofrin II, 1.65 times more thromboxane was released from tissue containing tumor than controls (P < 0.05). This value was less at 25 mg/kg injected doses, with a ratio of 1.30 (P < 0.1). These data indicate that tissues containing tumor may be more responsive to the effects of PDT than tumor-free tissue per unit basis, thereby producing more thromboxane. Several theories can be suggested to explain these results.

Tumor microvasculature may be more susceptible to PDT damage than normal tissue vessels. Many tumors lack a mature vascular system, exhibiting areas with leaky vessels and regions with incomplete endothelial linings (23). These vessels may be irreversibly damaged by much less trauma than fully developed normal vessels. This hypothesis may explain why several authors have observed PDT-induced vasoconstriction and blood flow stasis earlier in tumor and peritumoral microvasculature than in normal vessels (24, 25).

Increased levels of porphyrin in regions containing tumor may also account for the observed phenomenon. Areas with elevated porphyrin concentration would be expected to show greater response to PDT. However, porphyrin uptake in our chondrosarcoma is similar to uptake in surrounding skin and thus cannot explain the differences in thromboxane release.

Tissue containing tumor may have a greater blood volume/tissue area ratio. Treatment of an area with greater blood supply will result in a higher probability of thromboxane release since there would be a greater number of platelets that are present in the treatment field. Additionally, a larger vessel surface area would be available for damage to vascular structures and indirectly induce platelet activation.

Reduced blood flow velocity through tumor as compared to normal tissue may account for the observed differences in thromboxane release. Low flow velocity would effectively increase the transit time for any given platelet to cross the treatment field and increase the probability of platelet activation.

It is of interest that tumor cure occurred only when very high porphyrin doses were used. This differs from PDT treatment of several murine tumors, where injected porphyrin doses as low as 5 mg/kg Photofrin II are sufficient to produce significant tumor cure (4, 6). We only observed cure in the chondrosarcoma model at injected drug doses of 25 mg/kg. This decreased sensitivity is consistent with reports by Hilf et al. (26, 27, 28), and others (2, 28), who also required high injected porphyrin and light doses to damage rat tumor models. The difference in response for murine and rat tumor models may be related to the decreased reactivity of platelets to thromboxane (29). This phenomenon appears to be species specific for rats. Since larger amounts of thromboxane and other vasoactive agents are required to produce thrombus formation and vascular stasis in rats compared to other animals (mice), it follows that higher porphyrin and/or light doses would be required to produce stasis.

In contrast to changes in thromboxane levels following PDT, no release of prostacyclin was observed at any of the light and drug dose combinations studied. This indicates that PDT does not act as a general stimulus for the release of eicosanoids, and that thromboxane release is a specific response to PDT.
How is thromboxane release from platelets related to the mechanism of PDT-induced tumor destruction? It is generally accepted that vascular stasis plays a predominant role in tumor destruction (4, 30). Blood flow stasis has been shown to be a result of vessel constriction, platelet aggregation, and thrombus formation (1, 31). These observations are consistent with the intravascular release of thromboxane from platelets. The action of cyclooxygenase inhibitors such as indomethacin further strengthens the concept that thromboxane release is important in PDT-induced vascular stasis. Administration of indomethacin suppressed thromboxane release during PDT, inhibited vascular stasis and the destruction of tumors. These data establish a cause and effect relationship between the release of proaggregatory-proconstrictor agents such as thromboxane and tumor response following PDT. The events which elicit thromboxane release from platelets remain unknown. Two hypotheses offer possible mechanisms. Direct membrane damage of platelets may trigger biochemical pathways leading to thromboxane release. Peroxidation of the lipid components of platelet membranes by singlet oxygen could cause platelet activation and induce the release of thromboxane. Damage to platelets following PDT has been examined by Zieve et al. (32) and Solomon et al. (33). Platelets were shown to accumulate porphyrins ex vivo, and when irradiated, exhibited a shape change and loss of serotonin and ATP proportional to the porphyrin dose used. Thromboxane was not examined.

Damage to endothelial cells during PDT may expose collagen at the basement membrane (34) and indirectly induce platelet aggregation. Direct damage to endothelial cell membranes by singlet oxygen may cause cell contraction, and/or detachment of cells from the basement membrane. Endothelial cell changes of this magnitude have been reported by several investigators (35, 36). Platelets rapidly attach to the site of injury, primarily to the collagen of the subendothelial layer. This induces thromboxane release and platelet aggregation (12, 13). It is most likely that a combination of direct damage to platelets and endothelial cells is responsible for the release of thromboxane observed in this study. Studies are in progress to further define the target and mechanism which induce the release of thromboxane and produce vascular stasis.

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