Gene Transfer of Matrix Metalloproteinase-9 Induces Tumor Regression of Breast Cancer In vivo

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Abstract
Matrix metalloproteinases (MMP) are important regulators of angiogenesis and tumor progression by degradation of extracellular matrix. Clinical trials using MMP inhibitors have failed and recent studies suggest that MMPs may in contrast suppress tumor growth. It is not known, however, if MMPs or their inhibitors, tissue inhibitor of metalloproteinases (TIMP), can be used as therapy of established cancer. Here, adenovirus vectors carrying the human genes for MMP-9, TIMP-1, or empty controls were injected intratumorally in breast cancers established in mice supplemented with estradiol and treated with tamoxifen. Microdialysis was used to quantify MMP activity and sampling of endostatin and vascular endothelial growth factor (VEGF) in situ. We show that AdMMP-9 increased MMP activity and sampling of endostatin and vascular endothelial growth factor (VEGF) in vivo. We show that AdMMP-9 increased MMP activity in vivo, decreased tumor growth rate, and decreased microvesSEL area significantly. AdMMP-9 therapy resulted in significantly increased levels of endostatin in vivo, whereas VEGF levels were unaffected. As previously shown, tamoxifen exposure by itself increased MMP activity in all treatment groups. Moreover, the combined therapy with AdMMP-9 and tamoxifen further reduced tumor growth and increased the endostatin levels compared with either treatment alone. Gene transfer of TIMP-1 had no effects on tumor progression and counteracted the therapeutic effect of tamoxifen in our breast cancer model. This is the first report showing that overexpression of MMP-9 results in increased generation of antiangiogenic fragments, decreased angiogenesis, and therapeutic effects of established breast cancer.

Introduction
Degradation and proteolysis of the extracellular matrix (ECM) are critical events in cancer cell dissemination, growth, and metastasis (1). Matrix metalloproteinases (MMP) play an important role in the remodeling and proteolysis of the ECM. The functional activity of MMPs in vivo is regulated in the extracellular microenvironment where they are activated by various proteases and inhibited by naturally occurring tissue inhibitors of metalloproteinases (TIMP; ref. 2).

The essential role of the MMP/TIMP system in the metastatic process of cancers and of tumor angiogenesis has been clearly shown. Up-regulation of MMPs and paradoxically TIMPs has been correlated with tumor aggressiveness of various cancer forms, including breast cancer (3–6). Depending on the tissue distribution and the expression level of individual MMPs and TIMPs, these enzymes may either promote or inhibit tumor progression. Several recent studies have shown that up-regulation of endogenous MMPs decrease angiogenesis in tumors by the production of antiangiogenic factors such as angiotatin, tumstatin, and endostatin and thereby enhance tumor regression (7–12). Moreover, MMP inhibition in clinical trials has failed as antitumor therapy in several cancer forms (13, 14).

The in vivo activation of MMPs and the release of proangiogenic and antiangiogenic factors occur extracellularly in the tumor microenvironment. Hence, techniques that allow for in situ investigations of these events are vital. Microdialysis, a technique that mimics a blood vessel within a tissue, allows sampling of molecules such as vascular endothelial growth factor (VEGF) and endostatin directly from interstitial space and we have previously shown that this technique may also be used to directly quantify MMP activity in tumor tissue in situ (10, 15–18).

Sex steroids play an important role in breast carcinogenesis and progression and the majority of breast cancers are hormone dependent. Anti-estrogens such as the synthetic, nonsteroidal compound tamoxifen, and aromatase inhibitors are corner stones in the medical treatment of breast cancer. We recently reported that tamoxifen treatment of experimental breast cancer both in vitro and in vivo increased the expression and activity of MMP-9, whereas estradiol treatment lowered MMP-9 activity levels (10, 19). Additionally, the increased MMP-9 activity was accompanied by higher levels of interstitial endostatin and these tumors exhibited decreased angiogenesis (10, 19). This suggests that increasing the levels of MMP-9 and thereby the generation of antiangiogenic fragments are among the mechanisms that explain the antitumorigenic properties of tamoxifen.

Most studies of MMP/TIMP and tumor progression have been used in tumor cell lines overexpressing various MMPs or MMP null mice and focused on the early stages of cancer progression (9, 20). No previous studies have investigated the effects of direct treatment of established solid tumors with MMPs or TIMPs. Our previous studies demonstrating increased levels of MMP-9 by tamoxifen lead us to investigate in the present study if treatment with MMP-9 and TIMP-1 may affect tumor growth of established human breast cancer in nude mice. We show that overexpressing MMP-9 in whole tumor tissue by intratumoral injection of an adenovirus carrying the cDNA for MMP-9 induces tumor regression and the release of extracellular endostatin in vivo. The addition of tamoxifen treatment enhanced the effects of the MMP-9 gene therapy. We also show that overexpressing TIMP-1 intratumorally does not affect tumor progression but instead counteracts the beneficial effects of tamoxifen in breast cancer.

Materials and Methods

Adenoviral constructs. The human cDNA for TIMP1 was a gift from C. Richards (Department of Pathology and Molecular Medicine, Centre for
Gene Therapeutics, McMaster University, Hamilton, Ontario, Canada). The human cDNA for MMP-9 (hMMP-9) was purchased from American Type Culture Collection (ATCC). Both cDNAs were cloned into theloxP-containing shuttle vector, pDCl36, for viral assembly. Cotransfection of recombinant pDCl36 and adenovirus genomic plasmid, pBHGlOxΔE1,3Cre, subsequent purification and amplification were carried out as previously described (21).

Cell culture. The cell line 293 was used to confirm expression of hMMP9. Cells were grown on 10-mm dishes (BD Falcon) to 70% to 80% confluence in α-MEM (Sigma) supplemented with 10% fetal bovine serum (FBS; Sigma), 2 mmol/L L-glutamine (Sigma), 50 IU/mL penicillin G, 50 µg/mL streptomycin (Pen-strep, Sigma), and 5 µg/mL fungizone at 37°C in a humidified atmosphere of 5% CO2. Cells were serum starved overnight, after which cells were infected with 10 µL of adenovirus solution and allowed to incubate for a further 24 h. An empty control vector, Add707-3, was used in parallel.

MCF-7 (HTB-22; human breast adenocarcinoma, estrogen receptor and progesterone receptor positive) cells were obtained from ATCC. Cells were cultured in DMEM without phenol red, supplemented with 2 mmol/L L-glutamine, 50 IU/mL penicillin G, 50 µg/mL streptomycin, and 10% FBS, at 37°C in a humidified atmosphere of 5% CO2. Cell culture medium and additives were obtained from Invitrogen unless otherwise stated.

Zymography. To quantitate transgene activity of TIMP-1, reverse zymography was used. Twenty micrograms of total protein from bronchoalveolar lavage fluid prepared from AdTIMP1-infected rats, as previously described (22), were loaded onto a 12% SDS-PAGE gel containing 0.1% gelatin purified gelatinases. To obtain gelatinases, A549 cells were grown to confluence in 100-mm plates in DMEM supplemented with 2 mmol/L L-glutamine, 50 IU/mL penicillin G, 50 µg/mL streptomycin (Pen-strep, Sigma), 5 µg/mL fungizone, and 10% fetal bovine serum (FBS). The cells were then trypsinized and resuspended in serum-free medium containing 50 ng/mL 4-aminophenylmercuric acetate (Sigma) and allowed to incubate for 48 h. The supernatants were collected, mixed with 500 µL of gelatin-agarose beads, and incubated at 4°C for 16 h. Beads were spun down and washed with buffer containing 50 mmol/L Tris (pH 7.6), 0.15 mol/L NaCl, and 0.1% Tween 20. Gelatinases were eluted from the beads with buffer containing 50 mmol/L Tris (pH 7.6), 1 mol/L NaCl, 7.5% DMDSO, and 0.1% Tween 20. A total of 100 µg of gelatinase protein was copolymerized with the gelatin. Gelatin zymography for hMMP9 activity was carried out in the same manner without the addition of gelatinase beads to the gels. Gels were run at a constant voltage of 100 V for 16 h. Beads were then stained and subjected to von Willebrand immunohistochemistry (rabbit anti-human von Willebrand; dilution 1:500; DakoCytomation) and counterstained with Mayer's hematoxylin. Negative controls did not show staining. In a blinded manner, 10 high-power fields (>200) were examined per section of three different tumors in each group. Vessel quantification of tumor sections was conducted as described previously using a Nikon microscope equipped with a digital camera (24). Percentage of area stained positively for von Willebrand factor was assessed using Easy Image Measurement software (Bergstrom Instruments).

Statistical analysis. Data are expressed as means ± SE. ANOVA and two-tailed Student’s t test was used for simple comparison of values where appropriate. All statistical tests were two-sided. P < 0.05 was considered statistically significant.

Results

Vector function and gene transduction in tumor tissue. Bronchoalveolar lavage fluids prepared from virus-injected (AdMMP-9 and AdTIMP-1) rats were loaded on zymogram gels. Figure 1A shows MMP-9 activity on a zymogram and Fig. 1C depicts a reverse zymogram of TIMP-1 function.
One week after intratumoral injections of the vectors, tumors were excised, snap frozen in liquid nitrogen, and homogenized. Protein levels of MMP-9 and TIMP-1 were analyzed in all treatment groups. There was a significant increase of MMP-9 protein content after intratumoral injections of AdMMP-9 compared with control, E+Addl70-3 versus E+AddMMP-9 (4.67 ± 0.56 versus 350 ± 51.5 ng/mg protein; P = 0.0005; n = 4–5 in each group; Fig 1B). In the tamoxifen-treated tumors, the increase after AdMMP-9 was more pronounced, E+TAM+Addl70-3 versus E+TAM+AdMMP-9 (18.5 ± 4.4 versus 858 ± 125 ng/mg protein; n = 4–5 in each group, P = 0.0006; Fig 1B). In line with our previous data (10, 19), tamoxifen treatment by itself significantly increased the MMP-9 levels in all adenovirus treatment groups, E+Addl70-3 versus E+TAM+Addl70-3 (4.67 ± 0.56 versus 18.5 ± 4.4 ng/mg protein; n = 4–5 in each group, P = 0.02; Fig 1B).

AdTIMP-1 injections into tumors resulted in a significant increase of TIMP-1 in tumor tissue, E+Addl70-3 versus E+AdTIMP-1 (2.3 ± 0.48 versus 68.4 ± 10.3 ng/mg protein; n = 4–5 in each group, P = 0.0008; Fig 1D). A similar significant increase was found in the tamoxifen-treated animals, E+TAM+Addl70-3 versus E+TAM+AdTIMP-1 (3 ± 0.47 versus 95 ± 9.6 ng/mg protein; n = 4–5 in each group, P < 0.0001; Fig 1D).

AdMMP-9 injection resulted in increased MMP-9 activity in tumors in vivo. Although gene transduction successfully induced elevated levels of total protein, we investigated if the high protein levels of MMP-9 also resulted in increased MMP-9 activity in tumor tissue in vivo. One week after adenovirus injections, microdialysis catheters were inserted into tumor tissue and perfused with a quenched fluorescent MMP-9 substrate. During perfusion, the substrate will passively diffuse from the inner side of the microdialysis membrane into the tissue. When active MMP-9 is present in the tissue, the substrate will be enzymatically cleaved and fluorescence will develop. The cleaved substrate diffuses from the tissue across the microdialysis membrane into the outgoing perfusion fluid, which can be directly analyzed in a fluorescence spectrophotometer. The developed fluorescence will thereby directly correlate with the MMP-9 activity in vivo. During the experiment, a steady state was reached after 30 minutes in all groups except the control E+Addl70-3. As previously shown, tamoxifen by itself induced an increase in the MMP activity, as measured by relative fluorescence units (RFU) at 120 minutes, E+Addl70-3 versus E+TAM+Addl70-3 (23,760 ± 2,769 versus 32,060 ± 125 RFU; n = 3–4 in each group, P = 0.048, Fig 2D). AdMMP-9 injection resulted in a significant increase in MMP activity both in estradiol and E+TAM–treated tumors, E+Addl70-3 versus E+AdMMP-9 (23,760 ± 2,769 versus 39,003 ± 1,955 RFU; n = 3–4 in each group, P = 0.01, Fig 2D) and E+TAM+Addl70-3 versus E+TAM+AdMMP-9 (32,060 ± 1,044 versus 45,957 ± 1,490; n = 3–4 in each group, P = 0.001, Fig 2D). AdTIMP-1 injection resulted in a nonsignificant increase compared with control, E+Addl70-3 versus E+AdTIMP-1 (2,769 ± 32,060 versus 30,849 ± 1,704 RFU; n = 3–4 in each group, P = 0.07; Fig 2).

Intratumoral gene transfer of AdMMP-9 induced tumor regression whereas AdTIMP-1 induced tumor progression in MCF-7 tumors in nude mice. Tumor growth was followed during
24 days after virus injections. MCF-7 tumors require estrogen for growth in nude mice and therefore the estrogen treatment was not discontinued during the experiment. As expected and as previously shown, tamoxifen induced reduced tumor volume in the control groups at the end of treatment, E+Addl70-3 versus E+TAM+Addl70-3 ($624 \pm 84$ versus $89 \pm 5.2$ mm$^3$; $n = 4–8$ in each group, $P = 0.0031$; Fig. 3D). In the estrogen-exposed group, AdMMP-9 injections significantly reduced tumor growth over time and at sacrifice the tumor volume was significantly lower in AdMMP-9–treated tumors compared with control tumors, E+Addl70-3 versus E+AdMMP-9 ($624 \pm 84$ versus $128 \pm 27$ mm$^3$; $n = 4–8$ in each group, $P = 0.0002$; Fig. 3D). Tumors injected with AdTIMP-1 exhibited a similar growth curve compared with Addl70-3–treated tumors and there was no significant difference in tumor volume at sacrifice (Fig. 3D).

In the group of mice treated with a combination of E+TAM, the addition of AdMMP-9 significantly reduced the final tumor volume compared with control vector, E+TAM+Addl70-3 versus E+TAM+AdMMP-9 ($89 \pm 5.2$ versus $41 \pm 6$ mm$^3$; $n = 4–8$ in each group, $P = 0.0031$; Fig. 3D). Treatment with E+TAM+AdTIMP-1 increased tumor volume significantly compared with the control E+TAM+Addl70-3 ($160 \pm 10$ versus $89 \pm 5.2$ mm$^3$; $n = 4–8$ in each group, $P = 0.0033$; Fig. 3D).

Release of soluble extracellular VEGF in vivo after AdMMP-9 and AdTIMP-1 therapy. Previous studies have implicated that increased MMP-9 levels lead to increased release of soluble bioactive VEGF from the ECM (25). However, no previous study has sampled VEGF directly from its bioactive site, the extracellular space in vivo after MMP treatment. We have previously used microdialysis as a technique for sampling molecules, including...
VEGF from tissues *in situ* (15–18, 26–28). In this study, we performed microdialysis 1 week after viral infection and measured the extracellular VEGF. In line with our earlier data, the addition of tamoxifen decreased soluble VEGF in all treatment groups, E+Addl-70-3 versus E+TAM+Addl-70-3 (17.8 ± 1.6 versus 11.1 ± 0.95 pg/mL; \( P = 0.0072; n = 5 \) in each group; Fig. 4) and E+AdMMP-9 versus E+TAM+AdMMP-9 (24.5 ± 4.9 versus 11.3 ± 1.45 pg/mL; \( n = 5 \) in each group, \( P = 0.02 \); Fig. 4). In the AdTIMP-1 group, there was a slight but nonsignificant decrease after tamoxifen addition.

In the estradiol-treated animals, the injection of AdMMP-9 caused a slight but nonsignificant increase of VEGF, E+Addl70-3 versus E+AdMMP-9 (17.8 ± 1.6 versus 24.5 ± 4.9 pg/mL; \( n = 5 \) in each group, \( P = 0.19 \); Fig. 4).

**AdMMP-9 treatment increased endostatin levels *in vivo***. Endostatin is a 20-kDa COOH-terminal cleaved fragment of collagen XVIII, which inhibits angiogenesis by decreasing endothelial cell proliferation and migration and by increasing endothelial cell apoptosis (29–31). As endostatin is generated in the...
extracellular space exclusively, it is imperative to quantify this protein in this compartment. Microdialysis allows for such measurements and this technique was used for sampling of endostatin 1 week after adenovirus tumor injections.

The addition of tamoxifen resulted in a significant increase of endostatin in all treatment groups, E+Addl70-3 versus E+TAM+Addl70-3 (288 ± 15 versus 415 ± 22 pg/mL; n = 5–7 in each group, P = 0.0029; Fig. 5), E+AdMMP9 versus E+TAM+AdMMP9 (489 ± 64 versus 721 ± 53 pg/mL; n = 5–7 in each group, P = 0.019; Fig. 5), and E+AdTIMP-1 versus E+TAM+AdTIMP-1 (263 ± 32 versus 433 ± 48 pg/mL; n = 5–7 in each group, P = 0.019; Fig. 5).

AdMMP-9 increased the levels of endostatin in a similar fashion as tamoxifen treatment, E+Addl70-3 versus E+AdMMP-9 (288 ± 15 versus 489 ± 64 pg/mL; n = 5–7 in each group, P = 0.04; Fig. 5). The increase of endostatin was more pronounced in the tamoxifen-treated animals, E+TAM+Addl70-3 versus E+TAM+AdMMP-9 (415 ± 22 versus 721 ± 53 pg/mL; n = 5–7 in each group, P = 0.0008; Fig. 5).

AdMMP-9–treated tumors exhibited decreased microvessel area. To investigate the functional effects of the MMP-9–induced increase of endostatin levels, microvessel area was scored in tumor sections from all treatment groups. Tumors were collected at the end of treatment at day 24. Tumors, which exhibited high levels of endostatin 1 week after adenovirus injections, had a decreased microvessel area at the end of therapy. Treatments with tamoxifen or AdMMP-9 caused a similar decrease in microvessel area compared with control tumors, E+Addl70-3 versus E+TAM+Addl70-3 (1.72 ± 0.2 versus 0.3 ± 0.033%; n = 9 in each group, P < 0.0001; Fig. 6) and E+Addl70 versus E+AdMMP-9 (1.72 ± 0.2 versus 0.2 ± 0.03%; n = 9 in each group, P < 0.0001; Fig. 6).

E+AdTIMP-1–treated tumors showed a nonsignificant decrease in microvessel area compared with E+Addl70-3 (0.34 ± 0.06 versus 1.72 ± 0.2%; n = 9 in each group, P = 0.09; Fig. 6).

Discussion

MMP activity is generally discussed in terms of the ability of MMPs to induce tissue destruction that promotes tumor invasion and angiogenesis. However, here we show that MMP-9 can induce quite the opposite effect. In our model of established breast cancer in nude mice, overexpression of MMP-9 by adenoviral gene transfer caused tumor regression and decreased angiogenesis. A possible mechanism behind this finding may be increased release of antiangiogenic fragments such as endostatin, which was found in the present study. Contrary to AdMMP-9 gene transfer, we show that injection of AdTIMP-1 into the breast cancer tumors had no effect on tumor growth compared with estradiol-treated animals injected with the control vector, Addl70-3. Cotreatment with AdMMMP-9 and tamoxifen had a synergistic effect on tumor regression compared with either treatment alone, whereas AdTIMP-1 counteracted the beneficial effects of tamoxifen on tumor regression and caused tumor progression compared with the control animals treatment with Addl70-3-TAM.

When using viral gene transfer, one obstacle may be the difficulty in measuring the efficacy of the infection by detection of bioactive protein expression within the tissue. In the present study, we do not only detect increased levels of the proteins after gene delivery but we can also in a unique manner directly quantify the activity of the adenovirally delivered MMP-9. By perfusing microdialysis catheters with the fluorescent substrate for MMP-9, we show that the injection AdMMP-9 directly into the tumor induced increased
MMP activity. The in vivo MMP activity after AdMMP-9 gene delivery was enhanced by tamoxifen, and animals treated with the combination of AdMMP-9 and tamoxifen exhibited a synergistic effect on tumor regression. This may be explained by the ability of tamoxifen to induce cell cycle arrest and thus slow cell proliferation in breast cancer cells. Genes delivered via adenoviruses remain extrachromosomal and a cell cycle arrest will thereby prolong gene expression. A prolonged gene expression by tamoxifen may also explain that tumors treated with AdTIMP-1 counteracted the therapeutic tamoxifen effects on breast cancer growth and instead caused tumor progression despite adequate antiestrogen doses of tamoxifen.

MMP activity has generally been positively associated with increased invasiveness of cancers and the angiogenic switch by releasing bioactive molecules such as VEGF from the ECM and thereby enhancing tumor angiogenesis and progression (4, 5, 25, 32). However, no previous study has directly measured VEGF released in tumor tissue in vivo after MMP-9 treatment. We have previously shown that microdialysis is a valid technique for sampling of extracellular soluble VEGF in vivo in both normal and cancerous breast tissue in animals as well as in humans (15–18, 26, 28). In the present study, we performed microdialysis and sampled VEGF 1 week after AdMMP-9 and AdTIMP-1 injections. As previously shown, the addition of tamoxifen in all treatment groups, Addl70-3, AdMMP-9, and AdTIMP-1, caused a decrease of extracellular soluble VEGF in situ. Interestingly, we found, as previously suggested (25), a slight but nonsignificant increase of extracellular VEGF after AdMMP-9 intratumoral injections in animals without tamoxifen treatment. This increase, however, was not of enough biological importance to enhance angiogenesis in these tumors because all of the AdMMP-9–injected tumors exhibited tumor regression and decreased angiogenesis.

An increasing number of recent studies have suggested that MMPs may negatively regulate tumor progression and angiogenesis through the release of angiogenic inhibitors. MMP-9–deficient mice lacking the antiangiogenic fragment tumstatin exhibit accelerated tumor growth and increased angiogenesis when inoculated with Lewis lung carcinomas (9). Moreover, decreased tumor growth and angiogenesis have been shown in MMP-9–overexpressing mice, effects that were attributed to increased production of angostatin (33, 34). We have previously found that tamoxifen induces increased MMP-9 activity, which resulted in increased endostatin generation in breast cancer in vivo and thereby decreased angiogenesis and tumor regression (10, 19). Previous studies have investigated knockout MMP-9 mice or MMP-9–overexpressing mice and cell lines and modulations of endogenous MMP-9. To our knowledge, no previous study has investigated direct treatment of established solid tumors with MMP-9. Our results in the present study confirm previous reports of a beneficial effect of endogenous MMP-9; therefore, it is plausible that this effect may also be found in established tumors treated with MMP-9. Our results suggest that one of the mechanisms involved in tumor regression may be the release of endostatin after MMP-9 treatment. In our model, intratumoral AdMMP-9 injection resulted in a significant increase of released interstitial endostatin. The levels of endostatin were almost doubled after AdMMP-9 injections and although some VEGF was simultaneously released, the biological consequence in the AdMMP-9–injected tumors was decreased angiogenesis and tumor regression. Moreover, our study shows that elevating MMP-9 levels in established late-stage tumors may be an effective tumor treatment and not only beneficial in very early stage cancer disease as previously suggested (35).

Tumor progression in animals treated with AdTIMP-1 strengthens the notion of a beneficial therapeutic effect of AdMMP-9 in our breast cancer model. TIMP-1 is an endogenous natural inhibitor of MMP-9, and in tumors injected with AdTIMP-1, the levels of MMP-9 protein analyzed by ELISA were decreased compared with Addl70-3–treated controls. The role of TIMP-1 in cancer biology is complex and contradictory. Similar to MMPs, both tumor-protective actions and tumor-enhancing action have been reported (20, 36–38). Our results in the present study are in line with recent
studies emphasizing TIMP-1 as a tumor-enhancing factor in cancer progression (20, 36, 37).

Taken together, our data illustrate the complex role of the MMP/TIMP system in tumor biology. MMPs may facilitate ECM degradation, thereby promoting tumor invasion and angiogenesis; however, they may also block angiogenesis by producing antiangiogenic fragments such as endostatin. These latter effects may explain the negative results in clinical trials using MMP inhibitors (35, 39). For successful targeting of the MMP/TIMP system in future cancer therapy, a better understanding of the function of specific MMPs and TIMPs in specific tumors is necessary. Exploring MMP/TIMP effects at different tumor stages as well as the possible dose-response relationship of tumor promotion/inhibition is also needed. Hitherto, the main focus on MMP function has been on their modulation of tumor invasiveness and angiogenesis and to a much lesser extent on their protective roles in cancer progression. It is clear that these effects are potent and need much more attention in future research. Elevating levels of MMP-9 and the thereby generation of angiogenesis inhibitors either directly by gene therapy, as shown in this article, or protein delivery or indirectly via small molecular inducers of MMP-9, may be one promising future therapeutic approach against breast cancer that should be explored.

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