Accelerated Bone Resorption, Due to Dietary Calcium Deficiency, Promotes Breast Cancer Tumor Growth in Bone


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Abstract
The skeleton is a major site of breast cancer metastases. High bone turnover increases risk of disease progression and death. However, there is no direct evidence that high bone turnover is causally associated with the establishment and progression of metastases. In this study, we investigate the effects of high bone turnover in a model of breast cancer growth in bone. Female nude mice commenced a diet containing normal (0.6%; ‘Normal-Ca’) or low (0.1%; ‘Low-Ca’) calcium content. Mice were concurrently treated with vehicle or osteoprotegerin (1 mg/kg/d s.c.; n = 16 per group). Three days later (day 0), 50,000 Tx-SA cells (variant of MDA-MB-231 cells) were implanted by intratibial injection. On day 0, mice receiving Low-Ca had increased serum parathyroid hormone (PTH) and tartrate-resistant acid phosphatase 5b levels, indicating secondary hyperparathyroidism and high bone turnover, which was maintained until day 17. Osteoprotegerin increased serum PTH but profoundly reduced bone resorption. On day 17, in mice receiving Low-Ca alone, lytic lesion area, tumor area, and cancer cell proliferation increased by 43%, 24%, and 24%, respectively, compared with mice receiving Normal Ca (P < 0.01). Osteoprotegerin treatment completely inhibited lytic lesions, reduced tumor area, decreased cancer cell proliferation, and increased cancer cell apoptosis. Increased bone turnover, due to dietary calcium deficiency, promotes tumor growth in bone, independent of the action of PTH. Breast cancer patients frequently have low dietary calcium intake and high bone turnover. Treatment to correct calcium insufficiency and/or treatment with antiresorptive agents, such as osteoprotegerin, may be of benefit in the adjuvant as well as palliative setting. [Cancer Res 2007;67(19):9542–8]

Introduction
Breast cancer is one of the most common malignancies in women and affects approximately one million women per year worldwide. Approximately 70% of patients who develop advanced breast cancer will have secondary tumors in the bone (1). These bone metastases frequently produce osteolytic bone lesions by activating local osteoclasts to break down existing bone and often result in significant morbidity, including pain, fracture, immobility, and spinal cord compression (2, 3).

In established bone metastases, a “vicious cycle” seems to exist between tumor and bone cells. Proresorptive factors, such as parathyroid hormone (PTH)–related protein, released from proliferating tumor cells, increase osteoclastic bone destruction by acting on osteoblasts to increase expression of receptor activator of nuclear factor-κB ligand. As bone breaks down, growth factors, such as transforming growth factor-β and insulin-like growth factors, are released from the bone matrix in active forms to stimulate tumor growth leading in turn to further bone destruction (4). In animal models of breast cancer metastasis, antiresorptive treatment with either bisphosphonates or osteoprotegerin has shown skeletal protection and reduced tumor growth (5–8). Therefore, bone resorption seems to provide the cellular microenvironment that enhances growth of breast cancer metastases in bone.

Several clinical studies showed that in patients with newly diagnosed cancer, high bone turnover is associated with a significantly higher risk of future skeletal-related events, disease progression, and death (9–15). However, thus far, there is no direct evidence indicating that high bone turnover is causally associated with the establishment and progression of metastatic bone disease. Of note, secondary hyperparathyroidism due to low dietary calcium intake and/or vitamin D deficiency is frequent in middle-aged and elderly women in whom breast cancer is also common (16, 17); elevated bone remodeling and progressive bone loss typically occur in these individuals (18). Taking these facts into consideration, we hypothesized that increased bone turnover may enhance the growth and destructive potential of breast cancer metastases in bone. In this study, we therefore evaluated whether induction of high bone turnover as a consequence of a calcium-deficient diet in mice promotes the establishment and growth of breast cancer cells in bone.

Materials and Methods

Breast cancer cell line. The estrogen-independent bone-seeking human breast cancer cell line, Tx-SA, was derived from the cell line MDA-MB-231 and was obtained from Dr. Toshi Yoneda (M. D. Anderson Cancer Center, San Antonio, TX; ref. 19). All tissue culture media and supplements were supplied by Invitrogen unless otherwise stated. The cell line was cultured in DMEM supplemented with 10% FCS (JRH Biosciences) and 1% penicillin-streptomycin solution. To assess whether osteoprotegerin had direct effects on cell growth in vitro, 10,000 Tx-SA cells were plated in DMEM medium with 2% FCS per well in 12-well tissue culture plates and allowed to adhere overnight. Osteoprotegerin (10, 100, and, 1,000 μg/mL) or vehicle (PBS) was added to the media on day 0, with replacement every 48 h without washing. Cells were trypsinized and cell numbers were counted at days 1, 3, and 5 by trypan blue exclusion. Experiments were done two times.

Mouse model of breast cancer growth in bone. Four-week-old female BALB/c nu/nu mice (Animal Resources Center, Canning Vale, Western Australia, Australia) were used for the experiments. Mice were maintained under specific pathogen-free, temperature-controlled conditions throughout this study at the animal facilities of the ANZAC Research Institute in accordance with Institutional Animal Welfare Guidelines and an approved protocol. All mouse manipulations were done inside a laminar-flow hood under aseptic conditions while maintaining general anesthesia with i.p. injection of ketamine/xylazine (75/10 mg/kg), unless otherwise noted.

References

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At day 3, mice were placed on a diet with normal (0.6%; ‘Normal-Ca’) or low (0.1%; ‘Low-Ca’) calcium content (Specialty Feeds) and then treated with vehicle (0.9% sterile saline) or osteoprotegerin (1 mg/kg/d s.c.), using a recombinant construct of osteoprotegerin containing amino acids 22 to 194 of human osteoprotegerin fused to the Fc domain of human immunoglobulin G (kindly provided by Amgen, Inc.). The dose of osteoprotegerin was selected to produce rapid and profound inhibition of bone resorption represented in vivo. Patients received osteoprotegerin (1 mg/kg/d s.c.) or vehicle (0.9% sterile saline) or osteoprotegerin (1 mg/kg/d s.c.), using a recombinant construct of osteoprotegerin containing amino acids 22 to 194 of human osteoprotegerin fused to the Fc domain of human immunoglobulin G (kindly provided by Amgen, Inc.). The dose of osteoprotegerin was selected to produce rapid and profound inhibition of bone resorption. In total, 1,800 projections were collected at a resolution of 6.93 μm/pixel. Reconstruction of sections was done using a modified Feldkamp cone-beam algorithm with beam hardening correction set to 50%. VGStudio MAX 1.2 software (Volume Graphics GmbH) was used to obtain three-dimensional visualization of bone from reconstructed sections.

Measurement of lytic lesions. Lytic bone areas of injected and sham-injected tibiae were measured on digitally recorded radiographs using interactive image analysis software (ImageJ, NIH). At each time point (days 10, 14, and 17), changes in the size of osteolytic lesions were calculated and growth of lytic lesions was compared between Normal-Ca and Low-Ca group of mice from day 10 to day 17.

Histologic assessment. Harvested tibiae were fixed for 36 h in 4% paraformaldehyde buffered with 0.1 mol/L phosphate buffer (pH 7.4) and decalcified in 10% EDTA at 4°C for 2 weeks. The tissues were then processed and embedded in paraffin. Five-micron sections were cut from each specimen and stained with H&E for routine histologic examination. Histochemical examination for tartrate-resistant acid phosphatase (TRACP), as a marker for osteoclasts, was done by using naphthol AS-BI phosphate (Sigma Chemical) as a stain for the reaction product; incubation was done for 10 min at room temperature. The proportion of Ki67-positive cells and apoptotic TUNEL-stained cells was determined by counting positive and negative cells in five random fields of nonneoplastic areas of tumors in a representative section in each bone specimen (400 magnification).

Bone histomorphometry. Histomorphometric analysis of the proximal tibia live bone at day 17 was used as an index of the tumor burden. Cortical bone area was measured in the same sections. Osteoclasts were identified as TRACP-positive multinucleated cells associated with bone surfaces. The number of osteoclasts at the bone surface was counted in each section (×200 magnification) and osteoclast number per millimeter of bone surface was calculated. Bone volume and osteoclast surface were also measured at the same time. Osteoblast surface was calculated on H&E-stained sections following morphologic identification of osteoblasts associated with bone surfaces (×400 magnification).

Biochemical measurements. Serum calcium, PTH, osteocalcin, and TRACP5b were measured at days 0 and 17. Serum calcium levels were measured by standard autoanalyzer techniques. TRACP5b, a marker of bone resorption, was analyzed by ELISA (sensitivity, 0.1 U/L; intra-assay variation, 2.2%; Suomen Bioanalytiikka). Serum levels of PTH and osteocalcin (a marker of bone formation) were analyzed by immunoradiometric assay [PTH: sensitivity, 3 pg/mL; intra-assay variation, 7.9%; osteocalcin: sensitivity, 0.1 ng/mL; intra-assay variation, 3.1%; Immutopics International].

Statistical analysis. All data were presented as the mean ± SE and statistics were done using one-way ANOVA, followed by Bonferroni’s adjustment where there were multiple comparisons by means of SPSS 15.0 for Windows (SPSS, Inc.). Significance was accepted where P < 0.05.

Results

Osteoprotegerin does not affect Tx-SA cell growth in vitro. At doses of 10 to 1,000 μg/mL, given over 5 days, osteoprotegerin had no detectable effect on the increase in cell numbers (Fig. 1) or the morphology of Tx-SA cells in vitro (data not shown).

Dietary calcium deficiency induces hyperparathyroidism. At the time of tumor inoculation (day 0), animals receiving Low-Ca for 3 days were found to have decreased serum calcium and increased serum PTH, osteocalcin, and TRACP5b levels, indicative of secondary hyperparathyroidism and accelerated bone turnover. Treatment with osteoprotegerin decreased serum calcium and increased serum PTH levels in mice on both diets; these effects were most pronounced in mice receiving the Low-Ca diet. In contrast with vehicle-treated mice, osteoprotegerin treatment profoundly reduced serum osteocalcin and TRACP5b levels in mice on either diet, signifying reduced bone remodeling in these animals (Table 1). At day 17, when mice were sacrificed, animals on Low-Ca
levels. Taken together, these data show that 3 days of a low-calcium diet induced hyperparathyroidism and both accelerated bone resorption and formation; in contrast, treatment with osteoprotegerin increased PTH levels but inhibited both bone resorption and formation.

Effects of low dietary calcium and/or antiresorptive treatment on intratibial tumor growth and bone destruction. Seventeen days after tumor cell implantation, lytic bone lesions were established in 100% of the vehicle-treated animals in both the Normal-Ca and Low-Ca groups, as determined by plain X-rays and micro-CT (Fig. 2A). In animals receiving Low-Ca alone, lytic lesion area was significantly increased by 42% on day 17 compared with animals on Normal-Ca diet (Fig. 2B). In contrast, no lytic lesions were detected in any of the mice treated with osteoprotegerin. No bony lesions were seen in any of the sham-injected legs.

Compared with mice on Normal-Ca, animals fed the Low-Ca diet showed significantly lower cortical bone area (9% decrease) and significantly higher tumor area (24% increase; Fig. 3A–C) 17 days after tumor implantation. For both Low-Ca and Normal-Ca groups, treatment with osteoprotegerin significantly increased cortical bone area (Fig. 3A–C). Osteoclast numbers at the tumor bone still had significantly decreased serum calcium and increased PTH and osteocalcin levels, but TRAcP5b levels were similar to animals on Normal-Ca. At day 17, mice receiving osteoprotegerin on either diet continued to show low serum calcium, osteocalcin, and osteoprotegerin. In contrast, treatment with osteoprotegerin increased PTH levels but inhibited both bone resorption and formation.

<table>
<thead>
<tr>
<th>Table 1. Group-specific and longitudinal changes in serum calcium, PTH, and bone markers</th>
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<tr>
<td><strong>Serum assay</strong></td>
</tr>
<tr>
<td>Calcium (mmol/L)</td>
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<tr>
<td>PTH (pg/mL)</td>
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<tr>
<td>mTRAcP5b (units/L)</td>
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<td>Osteocalcin (ng/mL)</td>
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<tr>
<td>Day 17 (n = 8)</td>
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<td>Calcium (mmol/L)</td>
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<td>PTH (pg/mL)</td>
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<tr>
<td>mTRAcP5b (units/L)</td>
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<td>Osteocalcin (ng/mL)</td>
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NOTE: Data are expressed as mean ± SE. Abbreviations: mTRAcP5b, serum mouse TRAcP5b; OPG, osteoprotegerin.
* P < 0.05.
† P < 0.01 versus Normal-Ca.
‡ P < 0.05 versus Normal-Ca osteoprotegerin.

Table 2. Day 0 histomorphometric data

<table>
<thead>
<tr>
<th></th>
<th>Normal-Ca</th>
<th>Low-Ca</th>
<th>Normal-Ca OPG</th>
<th>Low-Ca OPG</th>
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</thead>
<tbody>
<tr>
<td>BV/TV (%)</td>
<td>11.67 ± 0.47</td>
<td>9.03 ± 0.62*</td>
<td>21.22 ± 1.20 †</td>
<td>20.56 ± 0.70 †</td>
</tr>
<tr>
<td>NOc/BS</td>
<td>7.19 ± 0.23</td>
<td>9.33 ± 0.35*</td>
<td>3.06 ± 0.24 †</td>
<td>2.96 ± 0.38 †</td>
</tr>
<tr>
<td>Oc.S/BS (%)</td>
<td>5.41 ± 1.46</td>
<td>5.92 ± 1.93*</td>
<td>17.92 ± 1.36 †</td>
<td>19.29 ± 0.71 †</td>
</tr>
<tr>
<td>Ob.S/BS (%)</td>
<td>24.60 ± 1.57</td>
<td>38.03 ± 2.31*</td>
<td>11.96 ± 1.21 †</td>
<td>12.59 ± 0.50 †</td>
</tr>
</tbody>
</table>

NOTE: Data are expressed as mean ± SE. n = 5 per group. Abbreviations: BV/TV, bone volume % tissue volume; NOc/BS, osteoclast number per millimeter bone surface; Oc.S/BS, osteoclast surface % bone surface; Ob.S/BS, osteoblast surface % bone surface.
* P < 0.05.
† P < 0.01 versus Normal-Ca.
interface were similar in Normal Ca and Low-Ca groups (14.83 ± 2.34/mm versus 15.84 ± 2.30/mm, respectively). Osteoclast differentiation and activity were almost completely inhibited by osteoprotegerin treatment, as no TRAcP-positive osteoclasts were detected in sections derived from these groups.

Tissues harvested from animals receiving a Low-Ca diet showed a 26% increase in the numbers of Ki67-positive, proliferating cancer cells, whereas both groups showed similar numbers of TUNEL-positive apoptotic cells. For both animals on Low-Ca and on Normal-Ca, treatment with osteoprotegerin decreased the number of Ki67-positive, proliferating cells by 45% and 47% and increased numbers of TUNEL-positive cells by 170% and 179%, respectively (Fig. 4A–D).

**Discussion**

Several clinical studies in cancer patients suggest that the level of bone resorption at the time of diagnosis is a strong predictor of skeletal-related events. In particular, high bone resorption rates seem to be associated with rapid disease progression (e.g., bone metastases), poor survival, and even death (10–14, 20–22). For example, Coleman et al. (14) found that cancer patients with high urinary N-telopeptide (NTX-I) levels (i.e., high bone resorption rates) had a 2-fold increased risk of skeletal complications and disease progression and a 4-to-6-fold increased risk of death on study compared with patients with low NTX-I levels. Although these data are intriguing, most if not all studies on the association between bone turnover and tumor outcome have been observational and provide no direct evidence that high bone turnover is causally associated with the establishment and progression of metastatic bone disease. Hence, whether bone turnover markers are useful for the prediction of skeletal-related events in cancer patients remains a controversial question.

The present study is the first to show in a murine model of breast cancer growth in bone that accelerated bone turnover at the time of tumor implantation strongly promotes cancer cell proliferation and tumor growth in bone. Our results further suggest that increased bone turnover, rather than the low-calcium diet, hypocalcemia or secondary hyperparathyroidism per se, produced the observed effect as osteoprotegerin treatment reduced tumor growth in mice on a low-calcium diet while enhancing hypocalcemia and secondary hyperparathyroidism. These results support the concept that high bone turnover enhances breast cancer tumor growth in bone.

A diet low in calcium has been shown to increase bone remodeling in different animal models (23–26). In the present study, a low-calcium diet fed for 3 days resulted in mild hypocalcemia, secondary hyperparathyroidism, and increased bone remodeling as indicated by both increased numbers of osteoclasts and osteoblasts and higher circulating levels of TRAcP5b and osteocalcin. Hypocalcemia, secondary hyperparathyroidism, and elevated osteocalcin levels persisted throughout the study period until day 17. By then, however, osteoclast number and bone resorption rates (assessed by serum TRAcP5b) in the low-calcium diet group were similar to those in the group receiving a normal calcium diet. This discrepancy could be due to the adaptation of mice to a low-calcium diet or, alternatively, to proresorptive tumor effects dominating the effects of elevated systemic PTH levels. In contrast, osteoclast number was significantly reduced after 3 days of treatment with osteoprotegerin, and at day 17, almost no osteoclasts could be seen histologically, consistent with the known ability of osteoprotegerin to block osteoclast differentiation (27). The reduced TRAcP5b and

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Effects of low-calcium diet and/or osteoprotegerin treatment on the size of osteolytic lesions in Tx-SA–injected tibiae of nude mice. A, representative X-ray (a–d) and micro-CT (e–g) images of the tibia of mice inoculated by intratibial injection with Tx-SA cells. Images were taken 17 days after tumor cell inoculation [normal calcium diet (Normal-Ca) with vehicle (a and e), low-calcium diet (Low-Ca) with vehicle (b and f), normal diet plus osteoprotegerin (Normal-Ca OPG; c and g), and low-calcium diet plus osteoprotegerin (Low-Ca OPG; d and h)]. Osteolytic lesions on X-rays are darkened areas within the bone (arrows). B, mean area of osteolytic lesions from day 10 to day 17. Osteolytic areas were measured from X-ray images as described in Materials and Methods. Mice on Low-Ca had a 42% larger lesion area (P < 0.01) compared with Normal-Ca by day 17. Treatment with osteoprotegerin inhibited the formation of osteolytic bone lesions completely.

Points, mean (n = 16 in each group); bars, SE; *, P < 0.01 versus Normal-Ca. OPG, osteoprotegerin.
osteocalcin levels and reduced osteoclast and osteoblast surfaces at day 0 indicate that, in mice treated with osteoprotegerin, tumor cells were injected into an environment of profoundly reduced bone remodeling.

When breast cancer cells were injected intratibially in mice with high bone turnover, cancer cell growth was enhanced, as indicated by increased numbers of proliferating cells. These mice also had larger lytic lesions evident on radiographs and decreased bone volume compared with mice fed a normal diet. In contrast, osteoprotegerin-treated mice on either low calcium or normal diet exhibited significantly reduced tumor growth with increased cancer cell apoptosis and reduced cancer cell proliferation. Bone in tumor-bearing tibiae was well preserved in osteoprotegerin-treated mice on either diet. Interestingly, treatment with osteoprotegerin had similar inhibitory effects on tumor growth on both diets despite the presence of more severe hyperparathyroidism in mice on a low-calcium diet. Taken together, these findings provide support for the hypothesis that preexisting high levels of bone remodeling advance tumor growth by creating a more fertile soil that supports and promotes tumor proliferation.

Consistent with the findings of this study, enhanced cancer cell growth in bone has occasionally been reported in association with treatments that increase bone resorption rates. Price et al. (28) conducted a study in which MDA-MB-231 cells were injected into nude mice by the intracardiac route, after which mice were treated with the heat shock protein 90 inhibitor, 17-allylamino-17-demethoxygeldanamycin (17-AAG). 17-AAG treatment enhanced tumor formation, is the dominant effector on tumor growth as bone remodeling rates can provide a bone microenvironment conducive to tumor growth. Low-calcium diet increased indices of both bone resorption and bone formation and simultaneously promoted tumor growth. Conversely, osteoprotegerin treatment suppressed indices of bone resorption and bone formation while also suppressing tumor growth. The parallel changes in bone resorption and bone formation reflect the close coupling of these processes (33). However, it is likely that bone resorption, rather than bone formation, is the dominant effector on tumor growth as bone resorption can potentially support tumor growth through the release of growth factors from bone matrix and remove the physical constraints on tumor growth (31, 32).

Calcium deficiency is common and its prevalence increases with age (34, 35). Vitamin D deficiency is also widespread and promotes calcium deficiency as it results in impaired intestinal calcium absorption (16, 17, 36–38). In both conditions, PTH secretion may be increased to compensate for inadequate serum calcium concentrations, leading to secondary hyperparathyroidism, which in turn induces an acceleration in bone turnover (16, 17, 36–38). Furthermore, the menopausal decrease in endogenous estrogen levels is associated not only with a significant increase in bone turnover but also with a substantial decrease in intestinal calcium absorption (39), which

![Figure 3. Effects of low-calcium diet and/or osteoprotegerin on tumor and bone area in Tx-SA–injected tibia of nude mice. A, representative H&E-stained sections of tibiae from mice at day 17 following intratibial inoculation with Tx-SA cells. The cortical bone in Normal-Ca (a) and Low-Ca mice (b) can be seen to be perforated (arrow) adjacent to the tumor mass (7), which fills the medullary cavity. Note the preservation of cortical bone and the accumulation of primary and secondary spongiosa (5) in ‘Normal-Ca OPG’ (c) or ‘Low-Ca OPG’ (d). Magnification, ×40. B, when compared with Normal-Ca, Low-Ca increased tumor area by 24% and osteoprotegerin treatment significantly decreased tumor area by 46% and 45% in mice on Normal-Ca or Low-Ca, respectively. C, when compared with Normal-Ca, Low-Ca decreased cortical bone area and osteoprotegerin increased cortical bone area in combination with normal or low-calcium diet. Columns, mean (n = 16 per group); bars, SE. *, P < 0.05; **, P < 0.01 versus Normal-Ca. OPG, osteoprotegerin.](cancerres.aacrjournals.org)
further aggravates dietary calcium deficiency in older women. Of note, the incidence of breast cancer increases after the menopause and continues to increase with advancing age (40, 41), thus coinciding with a peak in calcium deficiency and bone turnover. In this sense, our experimental findings may have clinical implications in that high bone turnover in postmenopausal women with breast cancer may increase their risk of disease progression to bone. Our study also confirms that reversing accelerated bone turnover through anti-resorptive treatment can reduce tumor growth. Although human studies are required to directly assess the associations between the incidence of metastatic bone disease and indices of calcium and bone homeostasis, our results do point to a causal relationship between accelerated bone turnover and skeletal tumor growth.

In conclusion, in mice, a diet low in calcium strongly promotes breast cancer cell growth in bone. Because treatment with osteoprotegerin reduced tumor growth while at the same time enhancing hypocalcemia and secondary hyperparathyroidism, it is likely that the rapid tumor growth observed under these conditions is due to increased bone resorption rather than to hypocalcemia or secondary hyperparathyroidism. Our findings may have clinical implications because many older cancer patients, much like the older population in general, are deficient in calcium and vitamin D and may present with accelerated bone turnover. Treatment of breast cancer patients to decrease bone turnover through correction of calcium or vitamin D deficiency may improve patient outcomes in the adjuvant as well as palliative settings. Our study also supports the concept that the use of antiresorptives, such as bisphosphonates or osteoprotegerin, to suppress bone turnover below normal levels, may slow disease progression in addition to reducing the risk of skeletal morbidity.

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References

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