Response of normal and osteoporotic human bone cells to mechanical stress in vitro

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Sterck, Jozien G. H., Jenneke Klein-Nulend, Paul Lips, and Elisabeth H. Burger. Response of normal and osteoporotic human bone cells to mechanical stress in vitro. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E1113–E1120, 1998.—Bone adapts to mechanical stress, and bone cell cultures from animal origin have been shown to be highly sensitive to mechanical stress in vitro. In this study, we tested whether bone cell cultures from human bone biopsies respond to stress in a similar manner as animal bone cells and whether bone cells from osteoporotic patients respond similarly to nonosteoporotic donors. Bone cell cultures were obtained as outgrowth from collagenase-stripped trabecular bone fragments from 17 nonosteoporotic donors between 7 and 77 yr of age and from 6 osteoporotic donors between 42 and 72 yr of age. After passage, the cells were mechanically stressed by treatment with pulsating fluid flow (PFF; 0.7 ± 0.03 Pa at 5 Hz for 1 h) to mimic the stress-driven flow of interstitial fluid through the bone canaliculi, which is likely the stimulus for mechanosensation in bone in vivo. Similar to earlier studies in rodent and chicken bone cells, the bone cells from nonosteoporotic donors responded to PFF with enhanced release of prostaglandin E2 (PGE2) and nitric oxide as well as a reduced release of transforming growth factor-β (TGF-β). The upregulation of PGE2 but not the other responses continued for 24 h after 1 h of PFF treatment. The bone cells from osteoporotic donors responded in a similar manner as the nonosteoporotic donors except for the long-term PGE2 release. The PFF-mediated upregulation of PGE2 release during 24 h of postincubation after 1 h of PFF was significantly reduced in osteoporotic patients compared with six age-matched controls as well as with the whole nonosteoporotic group. These results indicate that enhanced release of PGE2 and nitric oxide, as well as reduced release of TGF-β, is a characteristic response of human bone cells to fluid shear stress, similar to animal bone cells. The results also suggest that bone cells from osteoporotic patients may be impaired in their long-term response to mechanical stress.

prostaglandin E2; nitric oxide; osteoporosis

The increasing number of osteoporotic fractures makes osteoporosis an important health risk of our time. The cause of bone loss in patients with osteoporosis is multifactorial, but in all cases an imbalance between bone resorption and bone formation is the underlying mechanism (7, 31). Bone mass and the mechanical performance of the skeleton are affected by a variety of factors, both local and systemic. Systemic control results from a number of calcium-regulating hormones such as parathyroid hormone, calcitonin, and vitamin D as well as growth hormone and sex hormones. Local control is exerted primarily by mechanical demands resulting from gravity and the stressing of bone by muscular contraction. Many studies have shown that bone as a tissue adapts to these mechanical demands to produce an optimized structure in terms of mass and geometry (10, 13, 16). Bone mass diminishes with increasing age as a result of changes in circulating levels of hormones, in particular decreased estrogen levels after menopause (30, 42, 47), but possibly also because of decreased anabolic effects of mechanical loading as a result of declining levels of physical activity. Osteocytes, osteoblasts, and lining cells are anatomically in an appropriate position to detect mechanical strain and are extremely sensitive to mechanical stress in vitro (23). Therefore, these bone cells are thought to play a role in the response to skeletal loading as mechanosensors that can transduce the physical stimuli into biochemical signals (23).

The pathogenesis of osteoporosis is complicated by the fact that it is a multifactorial disorder. Diseases, drugs, and environmental influences such as chronic liver or kidney disease, an excess of thyroid hormones, low calcium intake, alcoholism, and smoking appear to exaggerate the normal bone loss that occurs with aging (34, 37). Immobilization may also contribute to the loss of bone in osteoporosis (46). However, the relationship of osteoporosis with an abnormal bone cell mechanosensitivity has not been studied as far as we know. Impaired responsiveness of bone cells to mechanical stimuli might lead to less effective adaptation of the bone tissue and thereby to a reduced ability to carry the prevalent loads resulting from normal usage.

The intrinsic capacity of bone cells to respond to mechanical stress may be studied with the use of cultures of isolated bone cells in vitro. This approach also allows comparison of bone cells derived from osteoporotic patients with control donors. Bone cells from younger control and older osteoporotic donors were found to differ in their response to cyclic strain, measured as enhanced cell proliferation and release of transforming growth factor-β (TGF-β) (29). Although the exact mechanism whereby bone cells sense the effect of stress placed on a bone organ is not known, recent theoretical (5, 48) as well as in vivo (45) and in vitro experimental (20, 23) evidence agrees that flow of interstitial fluid is likely involved. Deformation (strain) of bone tissue as a result of load causes flow of interstitial fluid through the lacunar-canicular porosity of bone (32). It has been calculated that this flow of fluid over the osteocyte surface may trigger a cellular response (48). Indeed, several in vitro studies have shown that fluid flow causing a fluid shear stress of 0.5–1 Pa rapidly increases prostanoïd production in
animal bone cells (20, 23, 36). In addition, human (22), mouse (20), and chicken (20) bone cells all rapidly respond to fluid flow with an enhanced release of nitric oxide (NO). NO has been implicated in a number of conditions of orthopedic interest, including inflammation, arthritis, aseptic loosening of joint prostheses, sepsis, ligament healing, and osteoporosis (8). The modulation of NO production by cells within the bone microenvironment may therefore be a sensitive mechanism for local control of bone remodeling. Prostaglandins, particularly prostaglandin E2 (PGE2), are important local regulators of bone metabolism (35) and have been shown to play a central role in the ability of the skeleton to respond to mechanical stress (49). Other molecules likely involved in mediating local gain and loss of bone tissue are locally produced growth factors such as TGF-β. TGF-β is an abundant noncollagenous protein in bone (41), it is produced by bone cells (11), and it can modulate the growth and differentiation of bone cells in vitro. We have recently found that mouse bone cells react to mechanical stress with changes in TGF-β production (19).

The aim of this study was to test whether bone cells derived from osteoporotic patients respond differently to mechanical stress than cells derived from age-matched controls. To verify their bone cell phenotype, bone-derived cell cultures were monitored for production of osteocalcin, a bone-specific protein, and alkaline phosphatase (ALP) activity under basal and 1,25-dihydroxyvitamin D3 [1,25(OH)2D3]-stimulated conditions. We then tested the response of the bone cells to mechanical stress by examining changes in the release of PGE2, NO, and TGF-β1 in response to pulsating fluid flow (PFF). Both short-term and long-term responses were studied, i.e., immediately after 1 h of treatment and 24 h later.

**MATERIALS AND METHODS**

Donors. Transiliac bone biopsies were obtained from 17 control donors (10 males, 7 females) between 7 and 77 yr of age with a mean age of 29 ± 7 yr. They were all without metabolic bone disease and entered the hospital for maxillofacial surgery (cleft palate or mandibular reconstruction using iliac crest bone) or orthopedic surgery (elective joint replacement or humerus reconstruction after trauma). In addition, similar bone biopsies were obtained from six osteoporotic (OP) donors (1 male, 5 females) with a mean age of 61 yr ranging between 47 and 72 yr. They were diagnosed as OP by their low bone mineral density in the lumbar spine (t-score less than –2.5) in combination with at least one vertebral fracture (type I osteoporosis). Histology showed no mineralization defect in these patients. They were all treated with calcium (500 mg/day) and vitamin D (400 IU/day), and one of them had been treated with the bisphosphonate pamidronate for 1 yr. For statistical analysis, the data of the OP group were contrasted with the non-OP controls of the same age range consisting of six donors (4 males, 2 females) between 44 and 77 yr of age with a mean age of 67 yr (Table 1). The protocols were approved by the ethical review board of the Academic Hospital, Vrije Universiteit, and all donors gave informed consent.

**Table 1. Sex, age, and treatment data of 6 osteoporotic donors and 6 non-osteoporotic donors that served as age-matched control group**

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age (yr)</th>
<th>Treatment</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Treatment</th>
</tr>
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<tr>
<td>Female</td>
<td>47</td>
<td>Ca/vitD</td>
<td>Male</td>
<td>44</td>
<td>Max</td>
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<tr>
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<td>57</td>
<td>Ca/vitD/pam</td>
<td>Male</td>
<td>55</td>
<td>Max</td>
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<tr>
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<td>63</td>
<td>Ca/vitD</td>
<td>Female</td>
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<td>Max</td>
</tr>
<tr>
<td>Female</td>
<td>66</td>
<td>Ca/vitD</td>
<td>Male</td>
<td>73</td>
<td>Or</td>
</tr>
<tr>
<td>Male</td>
<td>67</td>
<td>Ca/vitD</td>
<td>Female</td>
<td>75</td>
<td>Or</td>
</tr>
<tr>
<td>Female</td>
<td>68</td>
<td>Ca/vitD</td>
<td>Male</td>
<td>77</td>
<td>Or</td>
</tr>
</tbody>
</table>

Osteoporotic (OP) donors were diagnosed as such based on low bone mineral density (dual X-ray absorptiometry measurement) and at least 1 vertebral fracture. Nonosteoporotic (non-OP) control donors entered hospital for reconstructive maxillofacial surgery (Max) or elective orthopedic surgery (Or). Ca/vitD, treated with calcium and 1,25-dihydroxyvitamin D3 [1,25(OH)2D3]; Ca/vitD/pam, Ca/vitD plus treatment with 150 mg/day pamidronate.

Bone specimens were placed in cold sterile PBS and dissected within 1 h after removal. Bone specimens were minced into small fragments, washed extensively and repeatedly with PBS, and incubated with 2 mg/ml of collagenase (type II, Worthington) for 2 h at 37°C in a shaking water bath. The collagenase-treated bone fragments were washed once with medium containing 10% fetal bovine serum (FBS, Gibco, Paisley, UK) to inhibit collagenase activity and were transferred to 25- or 75-cm2 flasks (Nunc, Roskilde, Denmark) depending on the amount of bone tissue obtained. Roughly 5–10 mg of bone fragments were added per square centimeter of flask surface. The bone fragments were cultured in DMEM (Gibco) supplemented with 100 U/ml of penicillin (Sigma, St. Louis, MO), 50 µg/ml of streptomycin sulfate (Sigma), 50 µg/ml of gentamycin (Gibco), 1.25 µg/ml of Fungizone (Gibco), 100 µg/ml of ascorbate (Merck, Darmstadt, Germany), and 10% FBS. Culture medium was replaced 3 times/wk. Bone cells started to migrate from the bone chips after 4–14 days. When the cell monolayer growing from the bone fragments reached confluence, after 2–5 wk, cells were trypsinized using 0.05% trypsin (Difco Laboratories, Detroit, MI) and 0.02% EDTA (Sigma) in PBS for 5–10 min and plated at 25 × 10³ cells/well in six-well culture dishes (Costar, Cambridge, MA) containing 3 ml of DMEM with 10% FBS. Cells were grown until subconfluence, when they were challenged with 1,25(OH)2D3 or PFF.

The characterization of bone cell cultures: response to 1,25(OH)2D3 expression of von Willebrand factor. To test their osteoblastic phenotype, cell cultures were incubated for 3 days in the presence or absence of 10⁻⁸ M 1,25(OH)2D3 as follows. The medium of the subconfluent six-well plate cultures (see above) was replaced by fresh medium containing 2% FBS and 10⁻⁸ M vitamin K₁ (Hoffmann-La Roche, Basel, Switzerland) (27, 33, 44) with or without 10⁻⁸ M 1,25(OH)₂D₃. Subsequently, osteocalcin was measured in the conditioned medium by radioimmunoassay (Incstar, Stillwater, MN) with the use of an antibody raised against bovine osteocalcin. The detection limit amount was 0.2 ng/ml. All osteocalcin values were corrected for the amount of osteocalcin in medium with 2% FBS. In addition, ALP activity was determined in the cell lysate by using p-nitrophenyl phosphate (Merck) as a substrate at pH 10.3, according to the method as described by Lowry (25). The assay was performed in 96-well microtiter plates, and the absorbance was read at 410 nm using a Dynatech MR7000 microplate reader (Dynatech, Billinghurst, UK).

Fig. 1. Diagram of apparatus to apply pulsating fluid flow (PFF).

To test the presence of endothelial cells in the cell cultures, monolayers were stained for expression of the endothelial cell-specific factor VIII, von Willebrand factor (vWF), by immunofluorescence using a monoclonal vWF antibody.

PFF. PFF was generated with the use of a flow apparatus containing a parallel-plate flow chamber as described earlier (20, 23) (Fig. 1). The apparatus contained 13 ml of medium that was pumped over the cells in a pulsatile manner by a revolving pump. This resulted in a pulsating (5 Hz) fluid shear stress of 0.7 ± 0.03 Pa and an estimated peak stress rate of 12.2 Pa/s. During an experiment, the apparatus was placed in a 37°C incubator and connected to a gassing system that maintained a pH of 7.4 in the medium using 5% CO2 in air. For PFF experiments, cells were trypsinized and plated onto polylysine-coated (50 µg/ml; poly-L-lysine hydrobromide, mol wt 15-30 × 104; Sigma) glass slides, which served as the bottom of the flow chamber. Cells were plated at 5 × 10⁵ cells/glass slide (size 2.5 × 6.5 cm) and preincubated overnight in DMEM with 10% FBS, resulting in a subconfluent monolayer. Then the medium was changed to DMEM supplemented with 2% FBS, antibiotics, and 100 µg/ml of ascorbate, and the cells were incubated for 1 h in the absence (static control) or presence of PFF. Subsequently, the glass slide with the cells was removed from the flow apparatus, a culture well was created around the cells by securing a rectangle silicone rubber ring (Dow Corning, Midland, MI) on the glass slide, 1 ml of fresh culture medium was added, and the cells were postincubated for 24 h in the absence of PFF (post-PFF). Static control cultures were cultured in 13 ml of static medium during the first “PFF” hour and in 1 ml of medium thereafter.

NO. NO was measured as nitrite (NO₂⁻) accumulation in the conditioned media. NO₂⁻ is the stable end product of NO, and its concentration has been shown to be a good reflection of NO production. The amount of NO₂⁻ release was determined using Griess reagent (12) consisting of 1% sulfanilamide, 0.1% naphthylethenediamine dihydrochloride, and 2.5 M H₃PO₄. Serial dilutions of NaNO₂ were used as standard curve. Briefly, 75 µl of conditioned medium were mixed with 75 µl of Griess reagent and incubated for 15 min at room temperature under continuous shaking. The assay was performed in 96-well microtiter plates, and the absorbance at 540 nm was determined using a Dynatech MR 7000 microplate reader.

PGE₂. PGE₂ concentrations in the conditioned medium were measured by an enzyme immunoassay system (Amersham, Buckinghamshire, UK) with the use of an antibody raised against mouse PGE₂. The detection limit was 16 pg/ml. The absorbance was read at 450 nm using a Dynatech MR 7000 microplate reader.

TGF-β. TGF-β1 concentrations in the conditioned medium were determined by an ELISA system using an antibody raised against porcine TGF-β1 (Promega, Madison, WI). Total (latent and biologically active) TGF-β1 was measured after thermal activation of the samples for 5 min at 75°C (3). Biologically active TGF-β1 was below the detection limit of the assay (25 pg/ml). The absorbance was read at 450 nm using a Dynatech MR 7000 microplate reader.

Protein. After 24 h post-PFF, the protein content of the cell layer was measured using a BCA protein assay reagent kit (Pierce, Rockford, IL) (43). The assay was performed in 96-well microtiter plates, and the absorbance at 570 nm was determined using a Dynatech MR 7000 microplate reader.

Statistical analysis. Results are expressed as means ± SE. The effects of treatment with 1,25(OH)₂D₃ were analyzed using a paired two-tailed Student’s t-test. The effects of treatment with PFF were analyzed using Wilcoxon’s signed rank test because the data did not meet the requirements for normal distribution. The response of the OP group was contrasted with the non-OP control group using the Mann-Whitney U test. Differences were considered significant at P < 0.05.

RESULTS

Cells became visible as outgrowth of the collagenase-stripped bone chips after 1 or 2 wk in culture. They formed a subconfluent layer within 2–3 wk, when they were passaged. The speed of cell outgrowth (measured as time of first visible cell outgrowth, time of monolayer subconfluence) was somewhat faster in the younger donors below 44 yr of age than in the older donors. The cell growth characteristics of the OP group were not different from the non-OP control group (data not shown).

Three days of treatment with 10⁻⁸ M 1,25(OH)₂D₃ increased ALP activity and osteocalcin release in all non-OP control donors (Table 2) as well as in the OP group (Fig. 2). Basal as well as 1,25(OH)₂D₃-stimulated ALP activity was similar in the non-OP control group [basal, 6 ± 3 nmol p-nitrophenol (PNP)·h⁻¹·µg protein⁻¹; 1,25(OH)₂D₃-treated cultures, 14 ± 6 nmol PNP·h⁻¹·µg protein⁻¹] and in the OP group [basal, 7 ± 3 nmol PNP·h⁻¹·µg protein⁻¹; 1,25(OH)₂D₃-stimulated conditions, 15 ± 6 nmol PNP·h⁻¹·µg protein⁻¹]. The same applied to osteocalcin release [non-OP control group: basal, 93 ± 41 ng/mg protein; 1,25(OH)₂D₃-treated cultures, 123 ± 31 ng/mg protein].
Table 3. Effect of PFF on PGE2, NO, and TGF-β1 release in bone cell cultures from 17 nonosteoporotic donors

<table>
<thead>
<tr>
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<th>−PFF</th>
<th>+PFF</th>
<th>+PFF/−PFF</th>
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<tr>
<td>PGE2, ng/mg protein</td>
<td>64±21</td>
<td>110±32*</td>
<td>1.8</td>
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<tr>
<td>NO, nmol/mg protein</td>
<td>262±82</td>
<td>603±133†</td>
<td>2.3</td>
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<tr>
<td>TGF-β1, ng/mg protein</td>
<td>18±5</td>
<td>12±4*</td>
<td>0.7</td>
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<tr>
<td>PGE2, ng/mg protein</td>
<td>49±20</td>
<td>91±28†</td>
<td>1.9</td>
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<tr>
<td>NO, nmol/mg protein</td>
<td>372±130</td>
<td>379±116</td>
<td>0.4348 NS</td>
</tr>
<tr>
<td>TGF-β1, ng/mg protein</td>
<td>4±1</td>
<td>6±1</td>
<td>0.5754 NS</td>
</tr>
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</table>

Values are means ± SE of duplicate cell cultures of 17 donors. Results were analyzed using Wilcoxon’s signed rank test. PFF, pulsating fluid flow; PGE2, prostaglandin E2; TGF-β1, transforming growth factor-β1; +PFF/−PFF, ratio of treatment with PFF to that without PFF; NS, not significant. *P < 0.05 and †P < 0.01.
been treated with PFF was significantly lower in OP than in non-OP control cells, in both an absolute sense and as the ratio of +PFF to −PFF (Δ+PFF, −PFF: non-OP control, 81 ± 22 PGE2 ng/mg protein; OP, 6 ± 2 PGE2 ng/mg protein, P < 0.005, Mann-Whitney U test; ratio of +PFF to −PFF: non-OP control, 5.1 ± 1.5; OP, 1.5 ± 0.2, P < 0.02, Mann-Whitney U test).

The pattern of NO release was similar in the OP group and the non-OP control group, i.e., a twofold increase after 1 h of PFF treatment that was not continued during post-PFF incubation (Fig. 4, A and B). Also, the pattern of TGF-β release was similar. In both the non-OP control group and the OP group, TGF-β release was diminished by 1 h of PFF treatment (non-OP control: −PFF, 14 ± 7 ng TGF-β/ng protein; +PFF, 11 ± 7 ng TGF-β/ng protein, P < 0.05; OP: −PFF, 14 ± 5 ng TGF-β/ng protein; +PFF, 9 ± 3 ng TGF-β/ng protein, P < 0.05) but not during the 24 h postincubation after PFF treatment (Fig. 5, A and B).

DISCUSSION

The present study shows that cell cultures derived from human trabecular bone biopsies respond to fluid shear stress in a manner that is largely similar to the response of embryonic chicken or neonatal mouse calvarial bone cells (20, 22, 23). Primary cultures of untransformed cells derived from human bone explants have been shown to express an osteoblast-like phenotype (1, 2, 44). In the present study, all cultures tested responded to 1,25(OH)2D3 treatment with enhanced release of osteocalcin and enhanced ALP activity, both markers of the osteoblastic phenotype (2, 38). In addition, staining for vFF, a characteristic of endothelial cells, was negative.

One-hour treatment with PFF increased PGE2 release by the bone cell cultures, an effect that was also found in chicken osteocytes and in mouse bone cells (20, 22, 23). This response continued after stopping PFF application, leading to a twofold increase of PGE2 concentration in the culture medium after 24 h postincubation. Such a sustained upregulation of PGE2 re-
TGF-β1 release has also been observed in chicken and mouse bone cells (22, 23). In mouse bone cell cultures, Northern blot analysis showed that PFF treatment induced the expression of prostaglandin G/H synthase (PGHS)-2 [or cyclooxygenase (COX)-2] (17). PGHS, or COX, is a key enzyme regulating prostaglandin synthesis and is present as two isoforms, COX-1, the constitutive isoform, and COX-2, the inducible enzyme (24, 39, 50). Induction of COX-2 provides a cellular mechanism for continued upregulation of prostaglandin release, even when the cell stress itself is removed. Because expression of COX-2 but not COX-1 seems to be required for inducing an adaptive response to mechanical stress in bone in vivo (9), the in vitro data by Klein-Nulend et al. (17) suggest that continued upregulation of PGE2 release after a stress experience is part of the signal transduction pathway involved in mechanical adaptation of bone. In this respect, the reduced long-term response of the OP group is remarkable. In none of the five OP bone cell cultures tested did PFF treatment induce a continu-
effects on bone (3, 6, 26). In addition, the kinetics of the TGF-β response to stress seems to be time variable because long-term (3 days) treatment with cyclic stress has been reported to increase TGF-β production by bone cells (19, 29). This increase was reduced in bone cells from OP patients (29). The short-term inhibitory effect of stress as studied in the present paper was not altered in OP patients. Although it is clear that TGF-β release by bone cells may be modulated by mechanical stress, the details of this effect and its relation with osteoporosis need further study.

In sum, the present study shows that bone cells cultured from human trabecular bone fragments respond to fluid shear stress with a transient modulation of NO and TGF-β release and a more sustained upregulation of PGE_2 release. The sustained response was reduced in cells from OP patients, an observation that warrants further study.

We express gratitude to Drs. L. Smeeele, J. C. Netelenbos, J. Baart, and J. van Boven for assistance in obtaining the bone biopsies. We gratefully acknowledge the staff of the Endocrine Laboratory (Dr. C. Pop-Snijders, H. H. M. van der Schouw) for performing the osteocalcin assays.

The research of J. Klein-Nulend was made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences, and that of J. G. H. Sterck was made possible by Vrije Universiteit-Universitair Stimulering Fonds Grant 91/23.

This work was presented in part at the 1996 World Congress on Osteoporosis, Amsterdam, The Netherlands, May 1996. Address for reprint requests: E. H. Burger, ACTA-Vrije Universiteit, Dept. of Oral Cell Biology, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands.

Received 25 September 1997; accepted in final form 26 February 1998.

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