Thrombin, but not bradykinin, stimulates proliferation in isolated human osteoblasts, via a mechanism not dependent on endogenous prostaglandin formation

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Osteolysis or osteosclerosis often occurs in bone tissue adjacent to chronic inflammatory processes. Numerous cytokines and inflammatory mediators have been implicated as osteoclast-activating agents, explaining inflammation-induced bone resorption. In many cases, the cause of the sclerosis seen in these lesions is less thoroughly investigated. We have studied the effects of thrombin and bradykinin, 2 inflammatory mediators, on the rate of proliferation in isolated human osteoblasts (hOBs).

Thrombin, at and above 1 U/mL, stimulated the rate of thymidine incorporation into hOBs. The absolute cell number also increased, as measured by an assay based on the detection of cell metabolism. A synthetic peptide ligand for the thrombin receptor enhanced the rate of [^{3}H]thymidine incorporation in hOBs, indicating that thrombin-induced proliferation is mediated via the tetheric thrombin receptor. The thrombin-induced proliferation was not affected by indomethacin, excluding prostanoids as mediators of this effect. Bradykinin did not affect either the rate of thymidine incorporation, or number of cells in long-term cultures of hOBs.

In conclusion, the inflammatory mediator, thrombin, stimulates proliferation in isolated human osteoblasts probably via the recently described G-protein-coupled tetheric thrombin receptor. Thrombin may therefore be involved as a mediator of inflammation-induced sclerosis and bone formation.

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Inflammation in the vicinity of bone may induce resorption and/or stimulation of bone formation (Lerner 1994). Numerous cytokines and low molecular-weight mediators produced by the inflammatory process are known to influence bone turnover (Lerner 1994, Ralston and Grabowski 1996). The precise role of these mediators in inflammatory osteosclerosis is not known. Thrombin and bradykinin are two mediators of the inflammatory process that have been found to stimulate bone resorption via enhanced prostaglandin formation in osteoblasts (Weksler et al. 1978, Tatakis et al. 1989, Frohlander et al. 1991). However, their effects on bone formation are less well characterized. Thrombin has been shown to induce proliferation in the human osteosarcoma cell line SaOS-2 (Tatakis et al. 1991) and in the murine osteoblastic cell line MC3T3-E1 (Suzuki et al. 1996), which suggests a possible role of thrombin in the development of osteosclerosis. Thrombin may also have direct effect on osteoblast differentiation since it affects the expression of phenotypic markers of the osteoblast lineage, e.g., down regulation of the synthesis of alkaline phosphatase and up-regulation of collagen type I secretion (Lerner 1994, Suzuki et al. 1996). The effects of thrombin in cultures of human bone cells have not been studied and there are no reports regarding the effects of bradykinin on human osteoblastic proliferation. We have therefore studied the effects of thrombin and bradykinin on the rate of DNA synthesis and proliferation in primary cultures of isolated human osteoblasts (hOBs).

Material and methods

Material

Alpha modification of Eagle's medium (α-MEM) was purchased from Sigma Chemical Co., St. Louis, MO, USA. Penicillin, streptomycin, L-glutamine, trypsin-EDTA, phosphate-buffered saline (PBS) and fetal calf serum (FCS) were purchased from SVA, Uppsala, Sweden, and the Alamar Blue growth indicator from AccuMed, Westlake, OH, USA. Human recombinant insulin-like growth factor-I (IGF-I) was kindly provided by PharmaciaUpjohn, Stockholm, Sweden. Thrombin and bradykinin were purchased from Sigma-Aldrich, Stockholm, Sweden. The synthetic thrombin receptor activator peptide (TRAP) (with the amino-acid sequence: SFLLRN) was purchased from Ross-Petersen AS, Horsholm, Denmark. The fura-2 acetoxymethylester was supplied by Calbiochem, La Jolla, CA and the PGE2-RIA kit by Du Medical, Stockholm, Sweden.

Isolation of human osteoblast-like cells

Trabecular bone was obtained from the iliac crest of patients undergoing bone-graft procedures. The specimens were cut into small fragments, 1–2 mm in diameter, thoroughly rinsed with PBS, and cultured in 78 cm^2 tissue culture flasks containing α-MEM supplemented with antibiotics (100 U/mL of penicillin, 100 μg/mL of streptomycin, 1.25 μg/mL of amphotericin-B), 2 mM L-glutamine and 10% FCS. After 3–4 weeks, the culture dishes were confluent with cells that had migrated from the trabecular bone. The cells were detached with trypsin-EDTA (0.05/0.02% w/v), and seeded in multi-well culture dishes in which the subsequent experiments were performed, as described below. Only first-passage cells were used in these experiments and for each experiment, cells from a new patient were used. We detected no difference between the various donors. The data are the results of at least three separate experiments. The project was approved by the ethics committee of the local hospital.

Measurement of $[Ca^{2+}]_i$

Human osteoblasts were seeded onto glass coverslips, in α-MEM with 10% FCS, and cultured for 24 hrs. Thereafter, the cells were incubated for 30 min at 37 °C with 2.0 μM fura-2 acetoxymethylester. After rinsing with HEPES buffered saline (125 mM NaCl, 5.9 mM KCl, 0.5 mM $MgCl_2$, 1.3 mM $CaCl_2$, 25 mM HEPES, adjusted to pH 7.4) containing 3 mM glucose and 0.1% BSA, the coverslips were used as the bottom of an open chamber. The chamber was inserted into an inverted microscope (Nikon Diaphot, Tokyo, Japan) in a climate box maintained at 37 °C. The microscope was equipped for dual wavelength epifluorescence microfluorometry. The excitation wavelength was altered by a computer-controlled filter changer and the Bimica Software (Bergström Instruments, Solna, Sweden), changing between 340 and 380 nm filters. Emitted light was collected through a 510 nm interference filter and measured with a Nikon DC photometer P101. Individual cells were centered in the 25 μm measuring field of the fluorometer. $[Ca^{2+}]_i$ was calculated from the 340/380 nm fluorescence ratio, using a Kd of 224 nM.

Thymidine incorporation assay

Human osteoblasts were seeded in 24-well culture plates at a density of 10,000 cells/well. They were left to adhere in α-MEM supplemented with 10% FCS and antibiotics for 24–48 hrs, after which the medium was changed to serum-free α-MEM. After 24 hrs of serum starvation, test substances were added to the medium with 0.5% FCS and, 24 hrs later, the cells were pulsed with 0.6 μCi [^{3}H]methyl thymidine for 24 hrs. Cells were harvested by trypsination and transferred to a 96-well Milipore multi-screen HV opaque Durapore filter plate. The filters were washed and the DNA was precipitated by ethanol, before counting in a microbeta liquid scintillation counter.

Alamar Blue proliferation assay

Osteoblastic cells were plated in 96-well culture plates at a density of 2,000 cells/well in α-MEM containing 10% FCS and antibiotics. They were allowed to adhere for 24 hrs. Thereafter, a medium containing the experimental agents and 5% FCS was added and the plates were incubated for different periods. Half of the medium was replenished every fourth day. At the end of the experiments, the medium was removed and the cells

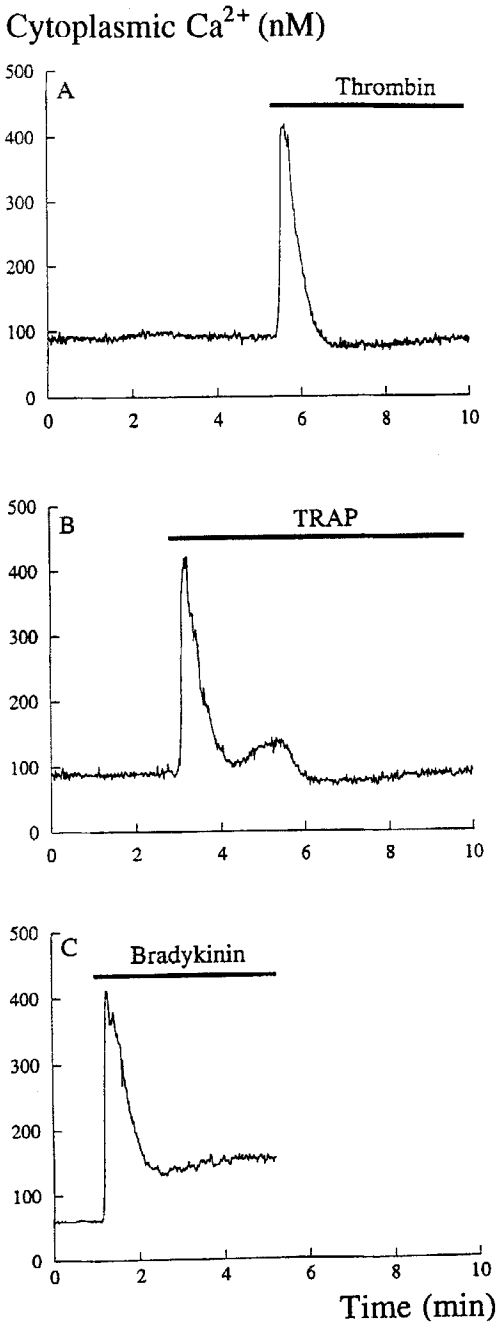

Figure 1. Effects of thrombin, TRAP and bradykinin on $[Ca^{2+}]_i$ in isolated human osteoblast-like cells. Human osteoblast-like cells were seeded onto glass coverslips, in α-MEM with 10% FCS, and cultured for 24 hrs. Thereafter, the cells were loaded with the Ca^{2+}-sensitive probe fura-2. After rinsing, cells were stimulated with thrombin (top), TRAP (middle) and bradykinin (bottom). Cytoplasmic calcium was measured in individual cells and the figure shows typical responses to the 3 agonists.

were rinsed in PBS before adding D-MEM, without phenol red or FCS, containing 10% (v/v) Alamar Blue. The wells were incubated with Alamar Blue solution for 5 hrs before measurements. The plates were brought to a fluorometer equipped with a xenon lamp and a broad band interference filter exciting fluorescence at 544 nm. The light emitted from a vertical light path on each well was read at 590 nm. The fluorescence thus obtained has been shown to be directly proportional to the number of cells in each well (Jonsson et al. 1997).

Prostaglandin E_2 measurements

Osteoblastic cells were plated in 24-well culture plates at a density of 2,000 cells/well in α-MEM containing 10% FCS and antibiotics. They were allowed to adhere for 24 hrs. Thereafter, fresh medium without FCS but containing the experimental agents was added and the plates were incubated for different periods. At the end of the experiments, the medium was removed and the amount of PGE2 analyzed, using a commercially available RIA.

Results

To obtain functional evidence of receptors for thrombin and bradykinin on human osteoblasts, hOB cultures were exposed to thrombin (10 U/mL), bradykinin (1 μM) or the synthetic thrombin receptor activator peptide (TRAP). Cytoplasmic calcium was measured in individual cells. The hOBs responded to all 3 agonists with an increase in cytoplasmic calcium (Figure 1). Subsequent experiments demonstrated that hOB cells also respond to thrombin and bradykinin rapidly increasing the formation rate of PGE2 (data not shown). From these data we conclude that the primary cultures of isolated human osteoblasts contain functional receptors for both thrombin and bradykinin.

We then investigated the effects of thrombin and bradykinin on the rate of hOB cell proliferation. Thrombin, at and above 1 U/mL, stimulated thymidine incorporation into hOB cells (Figure 2A). The cell number also increased, as measured by the Alamar Blue proliferation assay (Figure 3A). Bradykinin (1–1000 nM) did not affect the

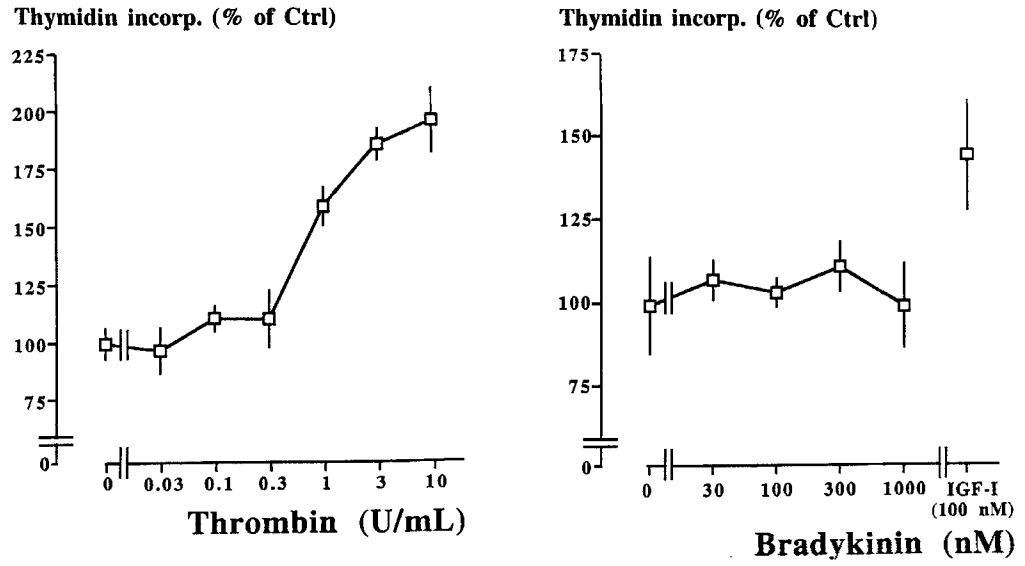

Figure 2. Effects of thrombin (left) and bradykinin (right) on thymidine incorporation in isolated human osteoblast-like cells. Human osteoblast-like cells were seeded in 24-well culture plates at a density of 10,000 cells/well. They were left to adhere in α-MEM supplemented with 10% FCS and antibiotics for 24–48 hrs after which the medium was changed to serum-free α-MEM. After 24 hrs of serum starvation test substances were added to the medium with 0.5% FCS. 24 hrs later, the cells were pulsed with 0.6 μCi [^{3}H]methyl thymidine for 24 hrs. The incorporation of [^{3}H]methyl thymidine was determined, as described in Methods. The figures show typical dose-response experiments and the results are expressed in relation to the incorporation rate of untreated controls. The data are mean ± SEM in 6 wells in each group. IGF-I was used as a positive control.

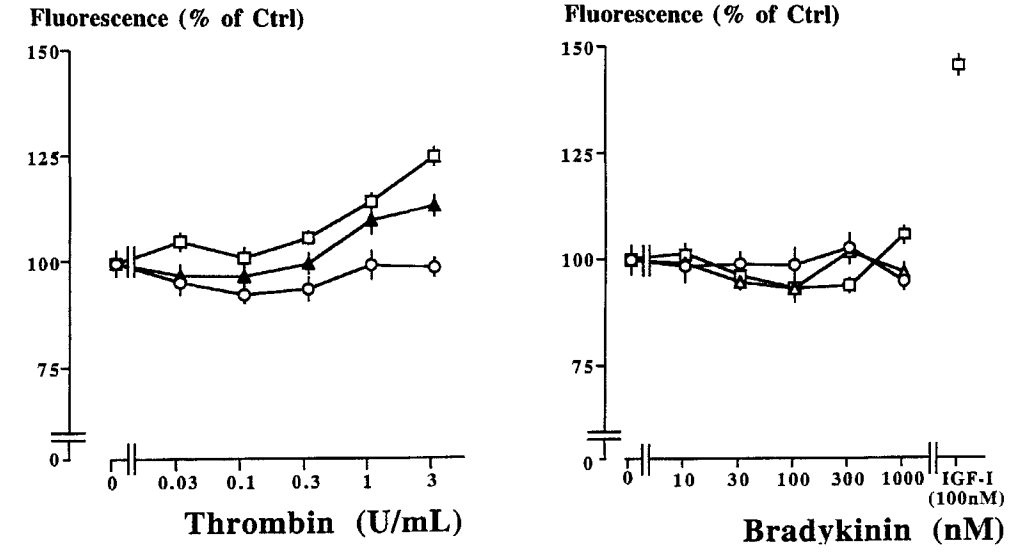

Figure 3. Effects of thrombin (left) and bradykinin (right) on cell proliferation, as determined by the Alamar Blue assay. Human osteoblast-like cells were plated in 96-well culture plates at a density of 2,000 cells/well in α-MEM containing 10% FCS. They were allowed to adhere for 24 hrs. Thereafter, a medium containing the experimental agents and 5% FCS was added and the plates were incubated for different periods. At the end of the experiments, proliferation was determined using the Alamar Blue assay, as described in Methods. The data are mean ± SEM of 16 wells in each group and presented as percent of control, with control values arbitrarily set to 100%. IGF-I was used as a positive control. ○ 4 days in culture, ▲ 8 days in culture, □ 12 days in culture.

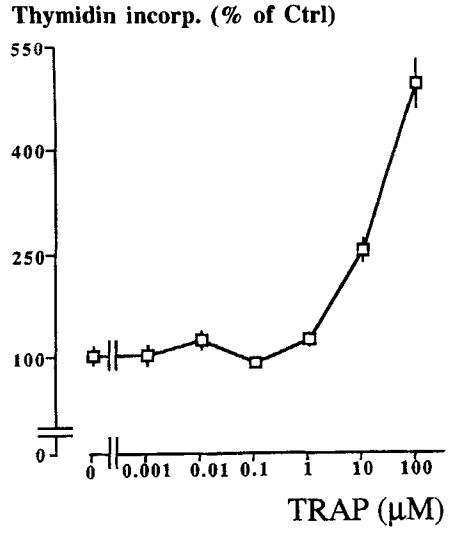

Figure 4. Effects of TRAP on thymidine incorporation in human osteoblasts. For method, see Figure 2. The figure shows a typical dose-response experiment and the results are expressed in relation to the incorporation rate in untreated controls. The data are mean ± SEM of 6 wells in each group.

rate of thymidine incorporation (Figure 2B), nor did bradykinin affect the number of cells in long-term cultures of hOBs (Figure 3B). TRAP also enhanced the rate of thymidine incorporation in hOBs, indicating that thrombin-induced proliferation is mediated via the G-protein coupled thrombin receptor (Figure 4). Since both thrombin and bradykinin could induce PGE_2 formation in hOBs and, since prostaglandins are known to be involved in the regulation of cell proliferation in osteoblasts, we investigated possible involvement of prostaglandin formation in the mitogenic response. However, thrombin-induced proliferation was not inhibited by indomethacin 1 μM (Table), clearly demonstrating that thrombin-induced proliferation in hOBs is not mediated via endogenous prostaglandin formation.

Effect of indomethacin on thrombin-induced cell proliferation. Thymidine incorporation (cpm *SEM*)

	– Indomethacin		+ Indomethacin	
Ctrl	864	*123*	1028	*188*
FCS 5%	12238	*422*	13248	*513*
Thrombin 1U/mL	2026	*96*	1906	*136*
Thrombin 10U/mL	2846	*224*	3241	*313*

Discussion

The mechanisms by which an inflammatory process affects bone are complex and involve a large number of signaling molecules that act on different types of cells. Thrombin, a serin protease that plays an important role in the blood coagulation cascade through its cleavage of fibrinogen to fibrin, has been reported to have hormone-like receptor-mediated effects in various types of cells, including bone cells (Fenton 1988). On activation, these receptors signal via intracellular calcium and prostaglandin formation and the thrombin receptor responds to the activating peptide in a manner previously described (Santulli et al. 1995). Most of these effects seem to be mediated via a unique receptor mechanism, in which the biological effect of thrombin is elicited by proteolytic cleavage of the thrombin receptor in its N-terminal extension. The proteolysis results in exposure of a new amino terminus, acting as a tetheric ligand, which induces receptor activation and subsequent interaction with G-proteins (Vu et al. 1991).

The thrombin receptor belongs to the 7 transmembrane spanning receptor family (Sugama et al. 1992, Tiruppathi et al. 1992) and has been shown to mediate well-known inflammatory phenomena, such as vasodilatation (Pinheiro et al. 1993), increased vascular permeability (Garcia et al. 1993) and neutrophil-endothelial cell adhesion (Sugama et al. 1992, Tiruppathi et al. 1992). Recently, it has been shown that the thrombin receptor is expressed on the cell surface of the osteosarcoma cell line SAOS-2 and that its activation results in intracellular Ca^{2+} release (Jenkins et al. 1994). These cells also respond to thrombin with increased proliferation, suggesting that thrombin could mediate an increase in bone formation (Tatakis et al. 1989). As it is not clear whether one can extrapolate results from rat or murine cell lines to non-transformed human bone cells, we have used primary cultures of normal human osteoblast-like cells in this report. This is currently believed to be the most reliable cell culture system since it reflects processes occurring in human

bone and we have recently optimized an isolation technique providing cells with a mature osteoblastic phenotype (Jonsson et al. 1999). Using these cells, we here demonstrate that thrombin stimulates proliferation in non-transformed human osteoblasts and that the mechanism appears to involve the tetheric G-protein coupled thrombin receptor. Endogenous prostaglandin formation is apparently not involved as a part of the mitogenic mechanism, since the effect of thrombin on thymidine incorporation was not affected by indomethacin. This is in contrast to the effect of thrombin on bone resorption, since it has been shown that thrombin stimulates mineral mobilization and bone matrix breakdown in neonatal rat calvariae by means of a pathway that is at least partially prostaglandin-dependent (Lerner and Gustafson 1988).

The nonapeptide bradykinin is generated by the inflammatory process via proteolytic cleavage of the circulating substrate kininogen by various forms of kallikreins. Bradykinin affects vascular permeability and mediates the sensation of pain (Regoli and Barabe 1980). Bradykinin has also been suggested as a mediator of inflammation-induced bone resorption (Lerner 1994). There is evidence that osteoblasts possess both type 1 and type 2 bradykinin receptors (Ljunggren and Lerner 1990, Ljunggren et al. 1991). The latter signals an increase in cytoplasmic Ca^{2+} (Ljunggren et al. 1991). A subsequent enhanced formation rate of prostaglandins is believed to cause the bone-resorbing effect that has been demonstrated for bradykinin (Gustafson and Lerner 1984). Despite the very similar effects of thrombin and bradykinin in bone cultures, e.g., a rise in cytoplasmic calcium, stimulation of prostaglandin formation, and an induction of bone resorption, we failed to detect any effect of bradykinin on the growth rate of isolated human osteoblasts. We conclude that bradykinin is probably not involved as a mediator of the sclerotic process seen in inflammation. Furthermore, the discrepancy suggests that thrombin has a specific effect on osteoblast proliferation, mediated via the known thrombin receptor.

In conclusion, inflammatory processes adjacent to bone may cause osteosclerosis, an effect that probably involves proliferation of osteoblasts. We show that the inflammatory mediator thrombin, via its known cell surface receptor, stimulates proliferation in osteoblasts. Thrombin may therefore be a mediator of inflammation-induced sclerosis.

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