

Purinergic upregulation of osteocyte RANKL expression in response to local microinjury *in vitro*

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INTRODUCTION: Osteocyte apoptosis triggers bone remodeling *in vivo*, but it is the viable neighboring osteocytes ('bystanders') rather than the apoptotic cells themselves which produce the osteoclastogenic cytokine RANKL⁽¹⁾. Purinergic signals, particularly ATP, released from apoptotic cells, have been implicated as a critical signal between dying cells and their surviving neighbors⁽²⁾. However, it is not clear whether such ATP release from dying osteocytes plays a direct role in triggering RANKL expression in bystander cells. Investigations into early osteocyte signaling have been limited by lack of *in vitro* systems that reproduce the unique spatial and communication constraints of osteocytes *in vivo*, as these cells communicate with each other only by direct contact of their processes via gap junctions or by extracellular molecules moving through the tiny lacunar-canalicular fluid spaces surrounding osteocytes. We developed a novel micro-nanofluidic device with two chambers separated by a barrier permeated by channels whose diameter (< 1 μ m) approximates that of canaliculi in bone⁽³⁾. Cells cultured in one or both chambers can send or receive signals only through those channels, either by direct contact (dendrites can grow into the channels) or via solute around the processes. Using this approach, we tested the *hypothesis* that "bolus" purinergic signals like those released from apoptotic osteocytes upregulate RANKL expression in surviving bystander osteocytes.

METHODS: Device Design: The Macro-micro-nano (M μ n) device was designed in AutoCAD and printed on two chrome on quartz masks (Advance Reproductions) with submicron resolution. The design consisted of two 1mm wide x 10mm long x 50 μ m high cell seeding chambers (each with inlet and outlet ports) separated by a 100 μ m wide barrier containing 1 μ m wide x 5 μ m high channels, 10 μ m apart (Fig 1). Details of the device fabrication are reported elsewhere⁽⁴⁾. Final devices were cast in PDMS and then bonded to coverslips for cell culture. The M μ n was also designed with a specialized heating element to stress only one cell chamber in order to selectively induce apoptosis in that compartment⁽⁵⁾. **RANKL Reporter Osteocytes** were created to assess response in bystander cells by transduction of MLO-Y4 cells with lentiviral particles (Cyagen) bearing an mCherry reporter driven by the RANKL promoter (Genecopoeia). Stable transductants were selected with puromycin for 14 days. **Cell Seeding and Purinergic Stimulation:** RANKL reporter MLO-Y4 cells were seeded in one side of the M μ n at 10⁶ cells/mL once a day for 3 days, then cultured an additional 4 days with daily medium replacement. On Day 7, medium with 20nM ('physiological' concentration) and 1 μ M (high concentration) of ATP, UTP or adenosine ($n \geq 3$ devices for each molecule and concentration) were flowed at 10 μ L/min (low shear stress) into the non-seeded side of the M μ n for 10 min while medium alone was flowed into the seeded region. Simultaneous medium replacement sequestered the purinergic molecules to one side. Flow was then stopped, permitting molecules to passively diffuse through the channels. RANKL reporter cells were imaged by brightfield and fluorescence microscopy at 1, 6, and 12 hr post-exposure and RANKL expression was determined as number of osteocytes with $\geq 50\%$ mean fluorescence intensity of the maximal value for unstimulated control cells. Change in reporter expression was examined both temporally and spatially relative to distance from the channel array. RANKL and OPG gene expression were also measured by qPCR, with a GAPDH reference. Statistical differences were determined at $p < 0.05$ by ANOVA with post-hoc Tukey tests.

RESULTS: ATP exposure resulted in approximately 2-fold increase in the number of RANKL expressing osteocytes and a 3 to 4-fold increase in RANKL gene expression (data not shown) for both the physiological and high concentrations of ATP at 6 and 12 hours post-treatment. In contrast, UTP exposure at the low concentration did not affect osteocyte RANKL expression. Adenosine had no effect on RANKL or OPG expression. Data are summarized in Fig 2 & 3. Numbers of RANKL-expressing osteocytes decreased as a function of distance from the "canalicular" ATP source (Fig 3).

DISCUSSION: We recently reported that the M μ n device allows selective induction of osteocyte apoptosis in one cell compartment, triggering osteocytes in the other compartment to upregulate RANKL expression⁽⁴⁾. This system replicates critical features of osteocyte behavior observed at bone microdamage sites *in vivo* (i.e., osteocyte apoptosis activates RANKL expression in nearby bystander osteocytes). The current studies revealed that controlled continuous exposure of osteocytic cells to extracellular ATP delivered through the canaliculi-like channels of the M μ n device is sufficient to upregulate RANKL. Bolus ATP release is employed by apoptotic cells to signal bystander cells⁽⁶⁾. The current studies suggest that such ATP when released from dying osteocytes might serve as the trigger for RANKL expression in bystander osteocytes. Moreover, the specificity of the effect for ATP over UTP also supports proposed roles for the Panx1 channel and purinergic receptors (e.g. P2X7) in this pathway^(1,7). We also found that RANKL-expressing osteocytes decreased with distance from the canalicular ATP source in the M μ n device. Moreover, this decay pattern for the number of RANKL-expressing osteocytes in M μ n device was similar to that reported for RANKL-expressing osteocytes in bone surrounding microdamage sites in living bone⁽⁸⁾, suggesting that this investigative approach models both key temporal and spatial components of osteocyte response to challenge.

SIGNIFICANCE: Studies of osteocyte behavior *in vitro* have largely relied on bulk delivery of experimental stimuli in culture medium. Use of a micro-nanofluidics device that models key spatial constraints of the *in vivo* osteocyte environment revealed that extracellular ATP delivered through canaliculi-like channels is sufficient to upregulate osteocyte RANKL expression. This finding supports the concept that bolus ATP release from apoptotic osteocytes is key to activation of neighboring bystander osteocytes to initiate bone remodeling.

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ACKNOWLEDGEMENTS: NIH Grants AR041210 and EY026752. NSF Grant CBET0939511. CUNY Advanced Science Research Center.

Fig 1: M μ n Schematic

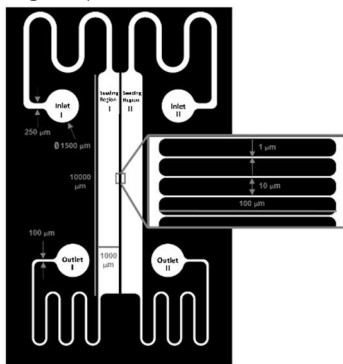
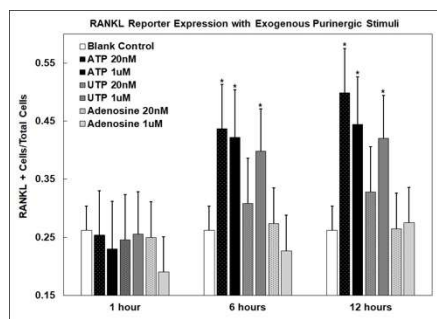


Fig 2: RANKL Expressing osteocytes with treatment



* - Indicates statistical difference from unstimulated control, $p < 0.05$.

Fig 3: RANKL Expressing osteocytes distribution

