Matrix vesicle-mediated mineralization depends on a balance between annexins and fetuin-A which may be modulated by TNAP and calcium channel inhibitors

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INTRODUCTION

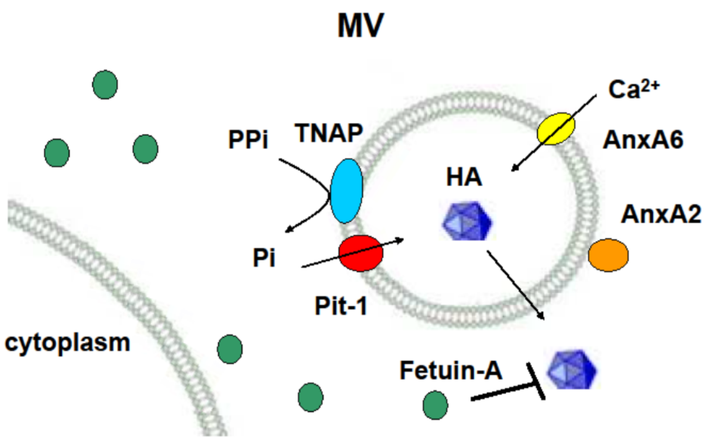
Bone mineralization is initiated by matrix vesicles (MVs), cell-derived structures released into the extracellular matrix which are nucleation sites for hydroxyapatite (HA) formation. It is suggested that annexins are mineralization-stimulating membrane proteins that exhibit ion channel activity and facilitate the influx of Ca^{2+} into MVs. The process is also regulated via enzymatic degradation of inhibitory pyrophosphate by tissue-nonspecific alkaline phosphatase (TNAP). Another layer of control is exerted by circulating, mineralization-inhibiting protein fetuin-A. The objective of our study was to examine the roles of annexins and fetuin-A in MVs function during physiological and pathological mineralization.

METHODS

We used two human cell lines: osteoblastic hFOB1.19 and osteosarcoma Saos-2. These cells were stimulated for mineralization for 7 days by osteogenic factors (0.28 mM ascorbic acid and 7.5 mM β-glycerophosphate) treatment. We compared cell morphology, intracellular distribution of proteins and formation of HA in control and levamisole (TNAP inhibitor)- or K-201 (a calcium channel inhibitor)-treated cells. We detected calcium nodules by Alizarin Red-S staining of cell cultures. We then isolated MVs from these cells by collagenase digestion and determined TNAP activity using pNPP as a substrate. Finally, we used Western blot method to identify differences in annexins and fetuin-A profile and expression in both cell lines.

RESULTS

Fig. 1. Schematic representation of proteins involved in MV-mediated mineralization



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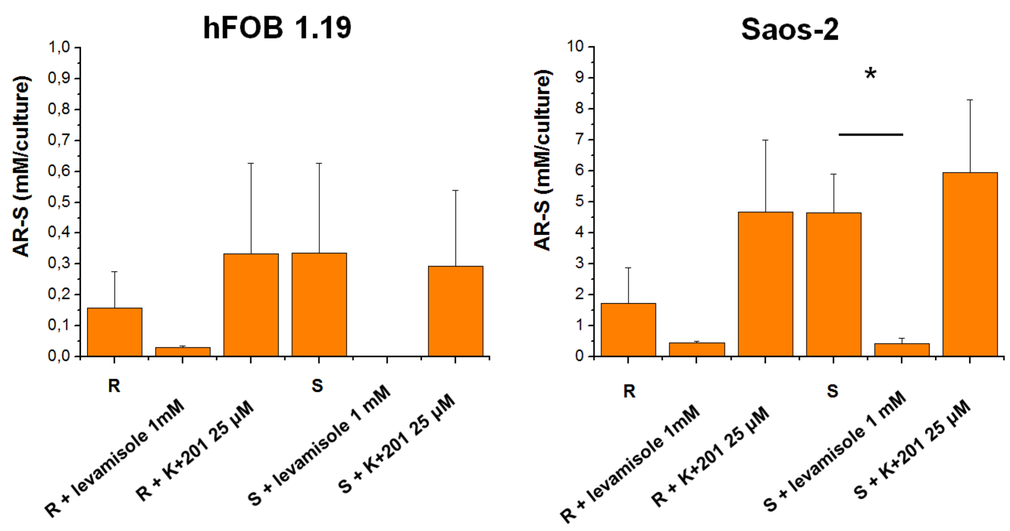
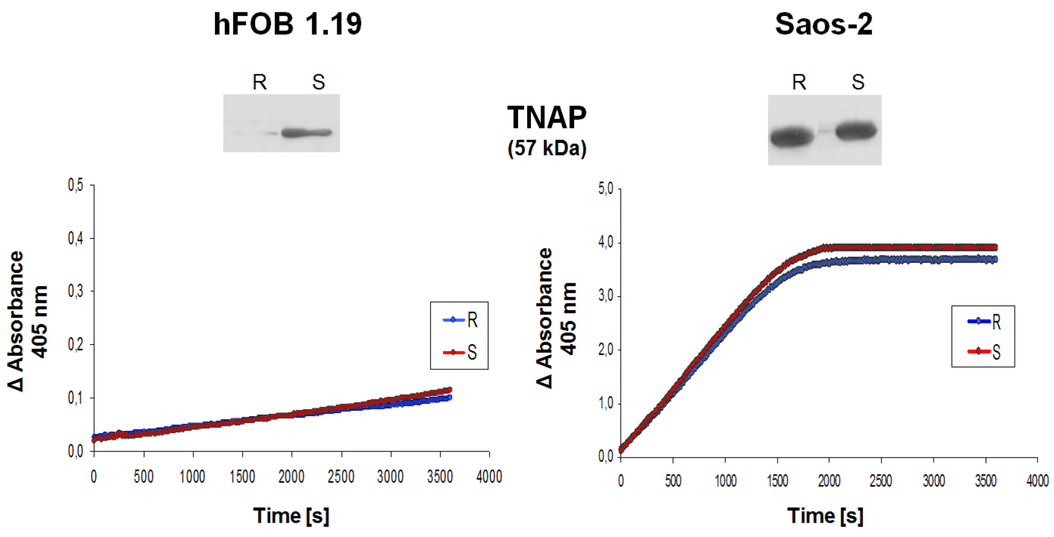
Abbreviations:
TNAP – tissue nonspecific alkaline phosphatase,
Pit-1 – phosphate transporter 1,
PPi – inorganic pyrophosphate,
Pi – inorganic phosphate,
AnxA6 – annexin A6,
AnxA2 – annexin A2
HA – hydroxyapatite.

Fig. 2. The effect of levamisole and K-201 inhibitors on mineralization of hFOB and Saos-2 cells



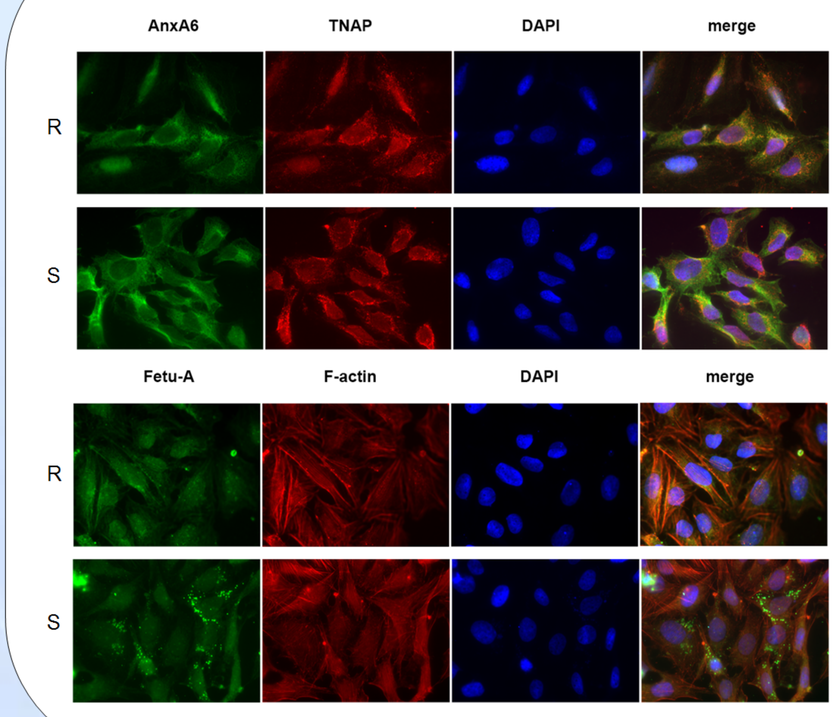
Cells were incubated for 7 days in the absence (R - resting) or presence (S - stimulated) of osteogenic factors, and additionally treated with levamisole, the TNAP inhibitor or K-201, a calcium channel inhibitor. Then, calcium nodules were stained with Alizarin Red S (AR-S) for 30 min. AR-S was solubilized in cetylpyridinium chloride for quantitative analysis. Absorbance was measured at 562 nm. Data were calculated from four independent experiments as means ± sē (*p<0.01) using ANOVA one-way software.

Fig. 3. Tissue nonspecific alkaline phosphatase activity of hFOB and Saos-2 cells



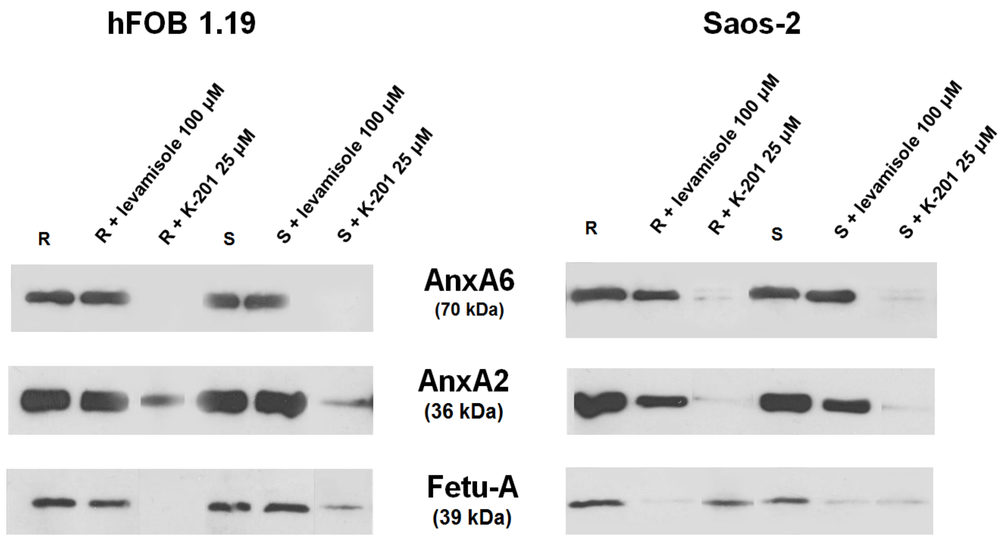
Cells were incubated for 7 days in the absence (R - resting) or presence (S - stimulated) of osteogenic factors. After 7 days of treatment, cells were digested by 2 U/ml collagenase at 37ºC for 3 h, collected in lysis buffer (0,5 % Triton X-100, 50 mM Tris-HCl, pH 7,4, 80 mM NaCl, 10 µg/ml protease inhibitor cocktail) and sonicated. TNAP activity was measured by ALP ELISA Assay (pNPP substrate) in samples with equal protein content.

Fig. 4. The localization of mineralization-related proteins



Saos-2 cells were incubated for 7 days in the absence (R - resting) or presence (S - stimulated) of osteogenic factors, then immunostainings for AnxA6-FITC or fetuin-A-FITC and TNAP-TRITC or F-actin-Rhodamine together with nucleus-DAPI were performed. A typical photos from three independent experiments, taken using Axio Observer.Z1 Transmission Light Microscopy (Zeiss) with PlasDIC and fluorescent filters, are shown. Yellow color indicates proteins colocalization.

Fig. 5. The expression of mineralization-related proteins after levamisole and K-201 treatment of the cells



Protein bands were analysed by SDS-PAGE in samples with equal protein content. Cells were incubated for 7 days in the absence (R - resting) or presence (S - stimulated) of osteogenic factors, and additionally treated with levamisole or K-201 inhibitor. After 7 days of treatment, the cells were digested by 2 U/ml collagenase at 37ºC for 3 h, collected in lysis buffer and sonicated.

CONCLUSIONS

- Levamisole blocked mineralization of hFOB and Saos-2 cells. On the other hand, K-201 did not have a significant effect on hFOB cells whereas stimulated mineral deposition in Saos-2 cells.
- TNAP activity in osteosarcoma cells was at least 30 times higher than in hFOB cells and reached the maximum after 25 min.
- We observed differences in annexins and fetuin-A profile in MVs from resting versus stimulated cells, but the expression of annexins and fetuin-A was similar in both cell lines.
- Levamisole decreased the level of fetuin-A in Saos-2 cells, but did not influence the annexin level, whereas K-201 decreased the content of fetuin-A and annexins in both cell lines.

SUMMARY

Understanding of the role of annexins and fetuin-A as biomarkers in TNAP-regulated function of MVs may provide novel insights into the mechanisms of physiological and pathological mineralization and may help to develop therapeutic strategies on the basis of the use of TNAP and calcium channel inhibitors to prevent pathological mineralization.

ACKNOWLEDGEMENTS

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Conflict of Interest Declaration

We have no conflict of interest to declare.