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Matrix gla protein and alkaline phosphatase are differently modulated in human dermal fibroblasts from PXE patients and controls / Boraldi, Federica; Annovi, Giulia; Vermeer, C.; Schurgers, L. J.; Trenti, T.; Tiozzo, Roberta; Guerra, Deanna; Quaglino, Daniela. - In: JOURNAL OF INVESTIGATIVE DERMATOLOGY. - ISSN 0022-202X. - STAMPA. - 133:4(2013), pp. 946-954. [10.1038/jid.2012.460]

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09/01/2026 10:05

Matrix Gla Protein and alkaline phosphatase are differently modulated in human dermal fibroblasts from PXE patients and controls

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Short title: Ectopic calcifications in PXE fibroblasts

Abbreviations: ALP (alkaline phosphatase), CALU (calumenin), CM (calcifying medium), DMF (dimethylformamide), ER (endoplasmic reticulum), Gla (gamma carboxyglutamil residues), GRP-78 (glucose-regulated endoplasmic reticulum stress response protein -78), MGP (Matrix Gla Protein); dp-cMGP (dephospho-carboxylated-MGP); dp-ucMGP (dephospho-uncarboxylated-MGP), MK-4 (menaquinone), PDI (Protein disulfide isomerase), PXE (Pseudoxanthoma elasticum).

Abstract

Mineralization of elastic fibers in Pseudoxanthoma elasticum (PXE) has been associated with low levels of carboxylated Matrix Gla Protein, most likely as a consequence of reduced vitamin K availability. Unexpectedly, vitamin K supplementation does not exert beneficial effects on soft connective tissue mineralization in the PXE animal model. In order to understand the effects of vitamin K supplementation and in the attempt to interfere with pathways leading to the accumulation of calcium and phosphate within PXE mineralized soft connective tissues, we have performed *in vitro* studies on dermal fibroblasts isolated from control subjects and from PXE patients. Cells were cultured in standard conditions and in calcifying medium in the presence of vitamin K1 and K2 or of levamisole, an alkaline phosphatase inhibitor. Control and PXE fibroblasts were characterized by a similar dose-dependent uptake of both vitamin K1 and K2, thus promoting in all cell lines a significant increase of total protein carboxylation. Nevertheless, MGP carboxylation remained much less in PXE fibroblasts. Interestingly, PXE fibroblasts exhibited a significantly higher alkaline phosphatase activity. Consistently, the mineralization process induced *in vitro* by a long-term culture in a calcifying medium appeared unaffected by vitamin K, whereas it was abolished by levamisole.

Introduction

Pseudoxanthoma elasticum (PXE) is a genetic disorder associated to mutations in the ABCC6 gene (Le Saux et al., 2000) and characterized by progressive mineralization of elastic fibers affecting skin, eyes and the cardiovascular system (Quaglino et al., 2011). Compared to controls, fibroblasts from PXE patients exhibit *in vitro* and *in vivo* similar matrix gla protein (MGP) expression, but a significant reduction of carboxylated-MGP (cMGP) (Gheduzzi et al., 2007), a well known endogenous inhibitor of soft tissue calcification (Schurgers et al., 2008). Furthermore, PXE

fibroblasts are characterized by a down-regulation of protein disulfide isomerase (PDI) and an up-regulation of calumenin (CALU), two proteins of the endoplasmic reticulum that are involved in the vitamin K cycle (Boraldi et al, 2009). Vitamin K is a group of fat soluble vitamins that, in their reduced form, are needed for the posttranslational γ -carboxylation of glutamic acid (gla) residues in proteins involved in blood coagulation as well as in the mineralization process (Booth, 2009). Interestingly, the low vitamin K concentrations measured in the circulation of PXE patients (Vanakker et al, 2010) were not associated by an impairment of their blood coagulation system, indicating that the vitamin can reach the liver to be metabolized and used for the activation of coagulation factors, while it seems to be extruded or transported less efficiently from hepatocytes to the periphery (Borst et al., 2008; Brampton et al., 2011), thus affecting connective tissues. In this scenario, it has been suggested that vitamin K supplementation might be capable of restoring MGP carboxylation and to inhibit ectopic calcifications (Borst et al, 2008). However, recent studies in the PXE animal model (abcc6^{-/-} mice) demonstrated that vitamin K supplementation does not counteract the mineralization of soft connective tissues (Brampton et al., 2011; Gorgels et al, 2011; Jiang et al, 2011). Key question is whether PXE fibroblasts are able to utilize vitamin K and to restore an adequate MGP carboxylation.

Since both vitamin K1 and K2 can be used as diet supplements (Cranenburg et al, 2007), the aim of the present study was to investigate the effects of vitamin K1 (phylloquinone) and vitamin K2 (menaquinone-4, MK-4) supplementation on control and on PXE dermal fibroblasts, for a better understanding of the role of vitamin K-dependent carboxylation of MGP in PXE pathogenesis and in the negative outcome of treatments performed so far in the animal model.

Moreover, since ectopic calcifications are mainly due to calcium phosphate precipitation, we also investigated the possibility to interfere with the mineralization process, by modulating alkaline phosphatase (ALP) activity, that promotes hydroxyapatite formation by acting on phosphate-donor substrates (Orimo, 2010).

Results and Discussion

Assessment of blood parameters related to ectopic calcification

Calcification is subject to regulation on many different levels, both systemic and local. We have investigated in 79 PXE patients the levels of a number of circulating parameters known to be related to the development of ectopic calcifications and compared the values with reference values in control individuals (Table 1).

Calcemia and phosphatemia are always within normal range, thus confirming that in PXE: 1) deregulation of circulating levels of calcium and phosphate are only rarely observed, possibly as an additional unrelated clinical complication (Mallette and Mechanick, 1987); 2) ectopic calcification does not take place through the “calcification paradox” (Krueger et al., 2009), i.e. by increased circulating levels of calcium and phosphate due to decreased bone mineral density, as confirmed by the absence of altered bone metabolism in all patients investigated so far.

Similarly, activity of serum ALP activity never appeared up-regulated in patients (Table 1), which is consistent with the absence of a generalized calcification process. Since collagen fibrils never undergo mineralization in PXE and normal as well as calcified elastic fibers coexist within the same tissues, it is conceivable to hypothesize a local deregulation of pro- and anti-calcifying factors.

Finally, we have measured the levels of dp-ucMGP and dp-cMGP (Table 1); no significant difference were observed between control and PXE samples.

Although it cannot be excluded that altered, still unknown serum factor/s could be present in the circulation of patients, control subjects and PXE patients, or PXE patients with different extent/severity of clinical manifestations cannot be discriminated on the basis of these parameters.

Vitamin K uptake by dermal fibroblasts and cell viability

Few data are available in the literature concerning the ability of fibroblasts to respond to *in vitro* vitamin K treatments (Canfield et al., 1987; Ross et al., 1991) and no data are available on human

dermal fibroblasts in primary culture. In addition, the demonstration that vitamin K supplementation does not counteract soft connective tissue mineralization, in the PXE animal model (Brampton et al., 2011; Gorgels et al., 2011; Jiang et al., 2011;), poses the question whether PXE fibroblasts are unable to utilize the vitamin (Boraldi et al., 2009).

Despite a certain heterogeneity observed between cells from different individuals, a similar dose-dependent vitamin K uptake is clearly detectable in both control and PXE fibroblasts (figure 1), although, the uptake of phylloquinone (figure 1a) is higher than that of menaquinone (figure 1b) at comparable doses in the culture medium. These data clearly demonstrate that fibroblasts can take up and accumulate vitamin K and that this trait is not affected by the pathologic phenotype (figure 1).

In the light of these results, further experiments were performed with the lowest (0.1 μ M) and the highest (100 μ M) vitamin concentrations. The highest concentration was chosen to guarantee the possibility to quantify the effects of vitamin supplementation, even in short-term cultures (48h); the lowest concentration was chosen to follow the intracellular uptake and to look for eventual cumulative effects in long-term cultures (≥ 20 days).

Evaluation of cell viability demonstrates that, in standard culture conditions (i.e. DMEM), both vitamins, even at very high concentrations and for long-term cultures (>20 days), never appeared to inhibit cell growth or to induce cell death (data not shown), as demonstrated for K2 in different tumor cell lines (Ogawa et al., 2007), where the pro-apoptotic effect of the vitamin could be the consequence of metabolic pathways associated to the neoplastic phenotype. Consistently, vitamin K has been safely used in many long-term *in vivo* treatments (Iwamoto et al., 2009), supporting the feasibility to use these dietary compounds for therapeutic strategies (McCann and Ames 2009).

As far as the effects of vitamin K on the cellular redox balance, in contrast to the menadione-induced generation of reactive oxygen species (ROS) (Loor et al., 2010), vitamin K1 and K2 supplementation never induced oxidative stress. In particular, vitamin K1 never modified the intracellular accumulation of $O_2^{\cdot -}$ and H_2O_2 (data not shown), whereas vitamin K2, at the highest dose (100 μ M) significantly ($p \leq 0.05$) reduced the level of $O_2^{\cdot -}$ in both control and PXE fibroblasts.

In particular, values, measured as fluorescence arbitrary units, were 0.70 ± 0.07 in controls; 0.55 ± 0.07 in controls+K2; 1.11 ± 0.06 in PXE and 0.66 ± 0.07 in PXE+K2, in agreement with previous observations that menaquinone can act as positive modulator of oxidative stress (Li et al., 2009).

Endoplasmic reticulum-proteins involved in vitamin K cycle

To exclude that vitamin K supplementation may be associated to ER-stress, having a negative effect on vitamin K-dependent carboxylation and favoring the calcification process (Liberman et al., 2001), we have investigated the expression of GRP-78, a well known marker of ER-stress (Zhang and Kaufman, 2006). Data indicate that GRP-78 expression (figure 2a and b) was very similar in control and in PXE fibroblasts and remained unmodified after treatments, clearly demonstrating that intracellular accumulation of vitamin K is not associated to ER-stress.

Moreover, since PXE fibroblasts are characterized by chronic oxidative stress (Pasquali Ronchetti et al., 2006) and the redox environment within the ER is known to affect the carboxylation process, we investigated if vitamin K supplementation can modify the expression of other molecules that, in the ER, are involved in vitamin K cycle, as PDI (figure 2a and c) and CALU (figure 2a and d), that are respectively down- and up-regulated in PXE (Boraldi et al., 2009). Both vitamins K1 and K2, independently from concentration, similarly down-regulate the expression of CALU in all cell strains (figure 2a and d), whereas changes were negligible in the case of PDI (figure 2a and c), indicating that the effect of vitamin K supplementation on the expression of ER-proteins involved in vitamin K cycle was not pathway-specific.

Protein carboxylation and MGP expression

Gla-residues were evaluated on total protein extracts, as markers of protein carboxylation. Data reveal that control and PXE fibroblasts have a comparable number of bands of similar intensity

(figure 3a and 3b), suggesting that the whole carboxylation process in PXE fibroblasts is equivalent to that of controls.

Possibly due to the low sensibility of this parameter, low doses of vitamin K1 and K2 are not associated to significant differences in total densitometry of bands. By contrast, high doses of vitamin K1 and K2 markedly up-regulated band's intensity in all cell strains, indicating that the carboxylation process is similarly modulated in both control and PXE fibroblasts (figure 3a and b).

Moreover, by comparing data on the intracellular vitamin K content (figure 1) and the amount of carboxylated proteins (figure 3a and b), it appears that vitamin K2, although present inside cells at lower levels compared to phylloquinone, has a similar effect on the carboxylation process, in agreement with previous findings that menaquinone is more efficient than phylloquinone in extra-hepatic tissues (Wallin et al., 2008).

On the basis of the positive effects of vitamin K on the carboxylation process, we have looked for changes in MGP expression and maturation. Unexpectedly, vitamin K supplementation never induced a significant MGP up-regulation. In particular, at high doses, vitamin K1 significantly reduced MGP protein expression in PXE fibroblasts (figure 3f), whereas both in control and PXE fibroblasts vitamin K2 down-regulated MGP, at gene and protein levels (figure 3 c-f). Moreover, treatment with vitamin K2, at all doses, did not increase cMGP (figure 3g and h), whereas vitamin K1 appeared to up-regulate cMGP, although only in control fibroblasts (figure 3g).

All these data are in agreement and help understand the observations by Brampton and coworkers (Brampton et al., 2011) that MGP remained undercarboxylated in the muzzle tissue of *abcc6* ^{-/-} mice despite the increase in circulating levels of vitamin-K. In particular, results from the present study demonstrate that in PXE, in a general setting of vitamin K availability and adequate carboxylation process, MGP is not sufficiently carboxylated. It could be suggested that the defect may specifically involve MGP and that the extent of gamma-carboxylation could be related not only on the bioavailability of cofactors, but also on the binding affinity between proteins and gamma-glutamylcarboxylase (Berkner, 2008).

Surprisingly, in the absence of any effect on cell counts and/or cell viability, K2 negatively modulated MGP expression, apparently in contradiction with the statement that *in vivo* vitamin K2 is specifically active in modulating the calcification process (Spronk et al, 2003; Beulens et al, 2009), although, in most cases, positive effects, observed either *in vitro* and/or *in vivo*, have to be related to supplementation with menaquinones with longer chains or by the simultaneous presence of other dietary components and/or to healthier life style behavior (Rees et al., 2010). Moreover, it cannot be excluded that these results could be due to a different behavior of fibroblasts compared to smooth muscle cells, or, as it has been described in other cell types (Goritz et al., 2007; Mertsch et al., 2009), to the association of low MGP expression with low proliferation/migration of cells, as an additional effect of vitamin K2 supplementation

Alkaline phosphatase activity

ALP is considered a marker of the calcification since it degrades phosphate donors, releasing inorganic phosphate and decreasing the concentration of inhibitors such as pyrophosphate (Orimo 2010). Evaluation of ALP activity in fibroblasts cultured in standard medium clearly shows that values are elevated in PXE fibroblasts and remain unmodified by vitamin K, whereas a significant inhibition can be measured after levamisole supplementation (Table 2).

Calcification assay

Independent from their ability to induce mineralization *in vivo*, cells in normal culture conditions are not able to mineralize, due to the presence in the culture medium of bovine serum factors, as fetuin, that acts as a potent calcification inhibitor. Therefore, in order to induce the calcification process *in vitro*, a complex environment is necessary such as that provided by addition of β -glycerophosphate (a source of organic phosphate), dexamethasone (a glucocorticoid that up- and down-regulates osteogenic or inhibitor molecules, respectively) and ascorbic acid (an essential co-factor for collagen synthesis as a fundamental substrate for mineral deposition) (Buranasinsup et al.,

2006). Starting from 10 days of culture in this specific calcifying medium, areas of mineralization can be visualized in all fibroblast strains, progressively increasing with time (figure 4). The heterogeneous distribution of calcifications on the cell surface indicates that this phenomenon is not due to a passive diffuse precipitation, but it is the result of specific cell-dependent processes. Treatment with levamisole inhibited the mineralization process, whereas vitamin K supplementation was always ineffective in all cell strains (figure 4). The observation that menaquinone, at the lowest concentration, actually favored the accumulation of mineral deposits, could be due to the fact that, as observed in osteoblastic cell lines, vitamin K2, differently from vitamin K1, has a transcriptionally regulatory function on extracellular matrix genes (Koshihara et al., 2003; Ichikawa et al., 2006), consistent with the use of vitamin K2 as potential anti-osteoporotic agent (Iwamoto et al., 2009).

Evaluation of ALP activity (figure 4) in these long-term culture conditions indicates that enzyme activity: 1) is higher in PXE compared to control fibroblasts; 2) is not significantly modified by vitamin K supplementation, 3) is influenced by the extracellular environment, due to the progressive accumulation of matrix molecules, as in the case of cells cultured in CM. To be noted that CM favors extracellular matrix deposition (figure 1 Supplementary material) leading to a thick layer of matrix covering and partially embedding the cell monolayer and for this reason at 30 days the matrix is so dense that it negatively interferes with protein solubilization unabling appropriate ALP activity determination. Surprisingly, for long periods of culture in CM (≥ 20 days), the highest dose of vitamin K2 was associated to changes in cellular morphology leading to areas of cell detachment in both control and PXE fibroblasts (figure 4). However, it is worth mentioning that no toxic effects were noticed on cells grown in standard medium supplemented with vitamin K2 (at all doses and for long times of culture) (Figure 2 Supplementary material), nor in CM alone or in the presence of phylloquinone (data not shown). It could be therefore hypothesized that menaquinone may interact with some CM component(s), thus negatively interfering with cell viability.

Conclusions

Despite the well known limits provided by the simplifications usually associated with *in vitro* models, never the less studies on cultured human dermal fibroblasts isolated from healthy subjects as well as from PXE patients, maintaining *in vitro* several characteristics related to their pathologic phenotype (Quaglino et al., 2011), allow direct investigation of the response of human peripheral mesenchymal cells, avoiding interferences due to dietary absorption properties and/or transport capabilities in the circulation. From *in vivo* studies on the PXE animal model it was suggested that, due to still elusive mechanisms, availability of vitamin K is not a limiting factor in the pathology of PXE and/or that vitamin K-dependent inhibition of calcification may not be preponderant in the development of the disease (Brampton et al., 2011). Present results demonstrate that vitamin K1 and K2 can be taken up and accumulated at similar levels by control and PXE fibroblasts, that the carboxylation process can be consequently similarly up-regulated in both cell strains, although MGP cannot be adequately carboxylated, even at increased levels of vitamin K. It can be therefore excluded that PXE fibroblasts are not capable to utilize the vitamin, thus suggesting that altered MGP characteristics/properties could at least contribute to defective carboxylation.

Within this context and given its peculiar matrix localization on the elastic component (Gheduzzi et al., 2007), MGP may contribute to the selective mineral deposition on elastic fibers. Moreover, the observation that in an *in vitro* calcification model, both vitamin K1 and K2 are ineffective in inhibiting the mineralization process, also in control fibroblasts, i.e. in cells that do not exhibit reduced cMGP, may underline the importance and the complexity of the extracellular environment in mineral deposit formation and in regulating cell behavior. In the development of ectopic calcifications, not a single component is prevalent or unique, but the unbalance ratio between many different factors locally controlling calcium and phosphate homeostasis, as well as the characteristics of the extracellular matrix, may be decisive in the question whether a tissue will start to calcify. Therefore, beside vitamin-K dependent molecules, other factors, including the upregulation of ALP, must be taken into account for future therapeutic options

Materials and Methods

More detailed information on materials and methods is available at Supplementary Materials and Methods.

Patients

PXE patients were diagnosed on the basis of clinical manifestations, histopathological findings and identification of ABCC6 gene mutations (Gheduzzi et al., 2004).

Laboratory parameters (calcemia, phosphatemia, alkaline phosphatase activity) were measured in the plasma of 79 patients according to the Clinical Pathology Laboratory routine procedures.

Plasma levels of dp-ucMGP and of dp-cMGP were measured in the same patients by sandwich ELISAs techniques already developed at VitaK BV (Maastricht, The Netherlands) (Cranenburg et al, 2010). The different sensitivity of the two antibodies does not allow a direct comparison of the amount of two forms of MGP and evaluations have to be done between controls and PXE within the same parameter.

Cells and treatments

Human dermal fibroblast cultures were obtained in accordance with the Declaration of Helsinki protocol and the guidelines of the ethical committee of the Modena University Faculty of Medicine, after written informed consent from 5 clinically healthy females, (38 \pm 7 years) which did not exhibit any sign of genetic, metabolic or connective tissue disorders and from 6 PXE patients (40 \pm 10 years).

Confluent fibroblasts were treated with 0.1, 1, 10 and 100 μ M phylloquinone (vitamin-K1, Sigma, St Louis-MO) or menaquinone (MK-4) (vitamin-K2, Sigma) or with 100 μ M levamisole (Sigma). Cells grown in the presence of DMF (Sigma) alone were used as control of treatments.

Unless otherwise specified, cells from each individual were kept separate during all experiments.

Fibroblast's morphology was evaluated by phase-contrast microscopy and cell viability was assessed by cell count.

High-performance liquid chromatography

Confluent fibroblasts were treated with vitamins for 48h, washed in buffer, trypsinized and centrifuged. Cell pellets were stored at -80°C and sent to VitaK (Maastricht-The Netherlands) for measuring, on cell extracts, vitamin K1 and K2 intracellular concentrations by HPLC, using a reversed-phase column with online zinc reduction and fluorescence detection (Schurgers et al., 2007).

Flow cytometry

Intracellular levels of O_2^- and H_2O_2 were estimated by FACS after incubation of fibroblasts with 1 μ M DH_2 or with 2 μ M H_2DCF -DA (Molecular Probes, Eugene-OR), respectively (Boraldi et al., 2009). Experiments were performed three times in duplicate.

Protein extraction and Western blot

Cells were homogenized in RIPA buffer with protease inhibitors (Sigma), centrifuged and supernatants collected and stored at -80°C until analysis. After measurement of protein concentration (Bradford, 1976) proteins were separated by 1D or 2D-PAGE (Gheduzzi et al., 2007; Boraldi et al., 2009). After separation, proteins were transferred to nitrocellulose and incubated with anti-GRP78, anti-PDI (Abcam, Cambridge-UK); anti-CALU, anti-MGP (Santa Cruz Biotechnology, Santa Cruz-CA); anti- γ -Gla-residues (American Diagnostica, Stamford-CT); anti-cMGP (Vascular Products, Maastricht-The Netherlands) and thereafter with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam).

1D-PAGE experiments were performed at least two times with all different cell lines. In the case of cMGP, due to the low abundance of the protein, analyses by 2D-PAGE were carried out on proteins extracted from a pool of fibroblasts from 6 patients and from 5 controls and data are expressed as mean values \pm SD of replicates.

Quantitative real-time RT-PCR

Total RNA was isolated from cells using the RNeasy Protect cells Mini kit (Qiagen, Milano-Italy) and reversely transcribed using Superscript III (Invitrogen, Monza-Italy) and Oligo dT₁₈ primers (Invitrogen). cDNAs were amplified on a iCycler (BioRad, Hercules-CA) using SYBR[®] GreenER[™] qPCR SuperMix (Invitrogen). MGP gene expression in each sample was normalized against CLK2 and quantified (Pfaffl, 2001). Experiments were performed two times in triplicate.

Alkaline phosphatase activity

ALP activity was measured spectrophotometrically at 405 nm on control and PXE fibroblasts and values of optical density were related to cellular protein concentration (Bradford, 1976). Experiments were performed three times in triplicate.

***In vitro* calcification and Von Kossa staining**

Fibroblasts were routinely cultured in 6-well plates (BD-Falcon, Franklin Lakes-NJ) up to confluence, then the standard medium (DMEM + 10% FBS, Gibco, Monza-Italy) was replaced by the same medium or by the calcified medium (i.e. standard medium plus 10mM β -glycerophosphate, 50 μ g/ml ascorbic acid and 10 nM dexamethasone, Sigma) (Buranasinsup et al., 2006). Vitamin K1 and K2 (0.1 μ M and 100 μ M) and levamisole (100 μ M) were added to each type of culture media. After 10-20-30 days, cells were stained with the Von Kossa method and areas of mineralization quantified on digital images. Experiments were performed three times.

Data analysis

Data are expressed as mean values \pm SD of all measurements and compared by Mann Whitney or by Anova test using GraphPad software (San Diego-CA).

Conflict of interests

The Authors state no conflict of interest.

Acknowledgements

This work has been supported by grants from FCRM (EctoCal) and PXE International. Authors are especially indebted to Stefan Pantus for his technical help in HPLC determination, and gratefully acknowledge Prof. Ivonne Ronchetti for the critical reading of the manuscript.

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Table 1: Plasma levels of selected ectopic calcification-related parameters

	Controls*	PXE
Calcium mg/dL	8.6 -10.3	9.53 ± 0.4
Phosphate mg/dL	2.4 - 4.8	3.4 ± 0.6
ALP activity U/L	40 -129	66.5 ± 37.7
dp-ucMGP pmol/L	426 ± 235	300 ± 221
dp-cMGP pmol/L	1865 ± 597	1169 ± 603
dp-ucMGP/dp-cMGP	0.23 ± 0.09	0.24 ± 0.12

* Normal reference range/values

Table 2: Alkaline phosphatase activity in control and PXE fibroblasts treated for 48 hours after confluence.

	DMEM	DMEM + Vitamin K1		DMEM + Vitamin K2		DMEM + Levamisole
		0.1 μ M	100 μ M	0.1 μ M	100 μ M	100 μ M
Control §	1 \pm 0.2	0.9 \pm 0,2	1.2 \pm 0.05	1 \pm 0.1	1 \pm 0.09	0.2 \pm 0.09**
PXE §	1.5 \pm 0.3*	1.3 \pm 0,1	1.1 \pm 0.2	1.1 \pm 0.3	1.1 \pm 0.2	0.1 \pm 0.07**

§ Enzyme activity is measured in confluent fibroblasts. Values are normalized to those of control fibroblasts cultured in DMEM, set as one-fold, and expressed as mean values \pm SD.

* $p \leq 0.05$ PXE vs Control fibroblasts

** $p \leq 0.01$ Levamisole treatment vs DMEM

Figure Legends

Figure 1.

Amount of vitamin K1 and K2 taken up by control and PXE dermal fibroblasts.

Intracellular determinations of phyloquinone (vitamin K1, a) and menaquinone (vitamin K2, b) content in fibroblasts from control and PXE patients grown *in vitro* for 48h after confluence in the presence of the vitamin (0.1, 1, 10 and 100 μ M). As controls, cells were cultured in the presence of comparable amounts of DMF without vitamin K. A dose-dependent uptake can be measured in both control and PXE cells (a linear regression analysis is shown in small graphs in the upper-left side of each panel).

Figure 2.

Effect of vitamin K1 and K2 on the expression of ER-related proteins in control and in PXE fibroblasts.

Expression of GRP-78 (b), PDI (c) and CALU (d) evaluated by Western blot on control (left panels) and PXE (right panels) fibroblasts, cultured for 48 hours after confluence with vitamin K1 or K2 (0.1 and 100 μ M). Data in histograms are expressed as mean values \pm SD. Representative WBs of control (lines 1,3,5,7,9) and PXE (lines 2,4,6,8,10) fibroblasts in the presence of DMF (lines 1 and 2), 0.1 μ M K1 (lines 3 and 4), 100 μ M K1 (lines 5 and 6), 0.1 μ M K2 (lines 7 and 8), 100 μ M K2 (lines 9 and 10) are shown at the top of the figure (a).

* $p < 0.05$ treatment vs DMF (dotted line set at 1).

Figure 3.

Effect of vitamin K1 and K2 on carboxylated proteins in control and in PXE fibroblasts.

Determination of Gla-residues (a,b) and MGP expression (c-h) in control and PXE confluent fibroblasts grown for 48h in DMEM + 10% FBS (DM) supplemented with the solvent alone (DMF)

or with vitamin K1 and K2 (0.1 and 100 μ M). Representative WBs for Gla-residues are shown in panels a and b, whereas values on top of each line represent densitometric analyses of all bands obtained in duplicate using all cell strains. MGP expression was analyzed by RT-PCR (c,d) and WB (e,f), whereas cMGP was evaluated by 2D-WB (g,h). Data are expressed as mean values \pm SD.

* $p < 0.05$ treatment vs DMF (dotted line set at 1); § $p < 0.05$ PXE vs control fibroblasts within the same experimental condition.

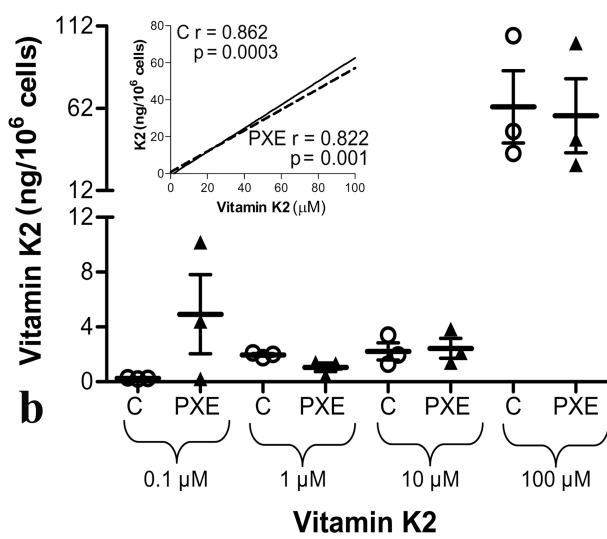
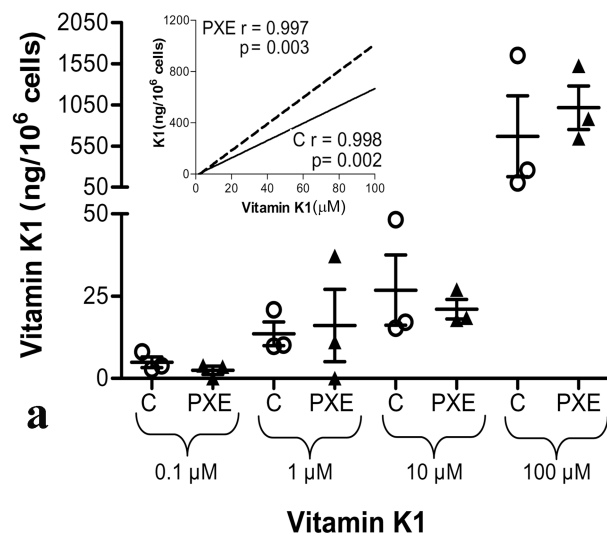
Figure 4.

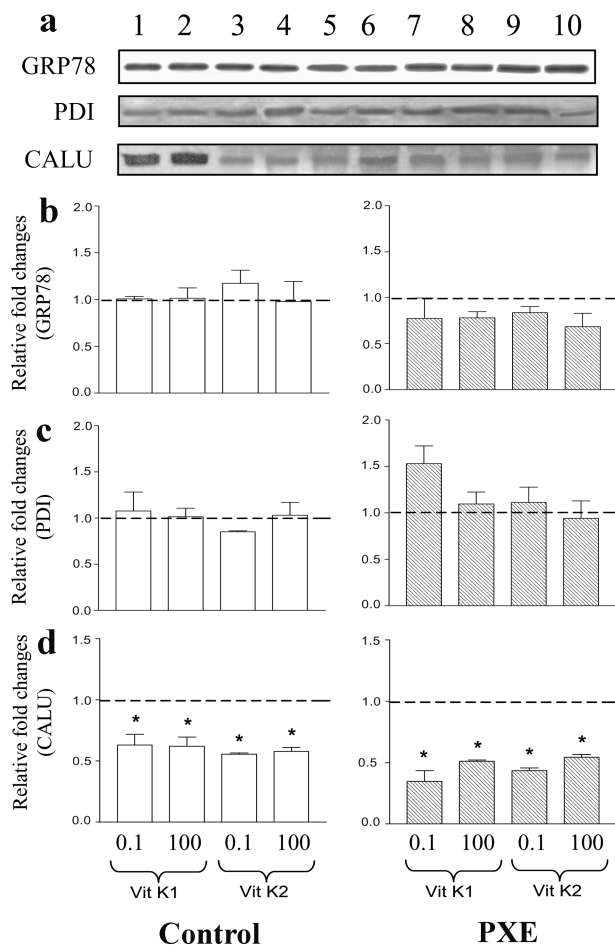
In vitro calcification assay.

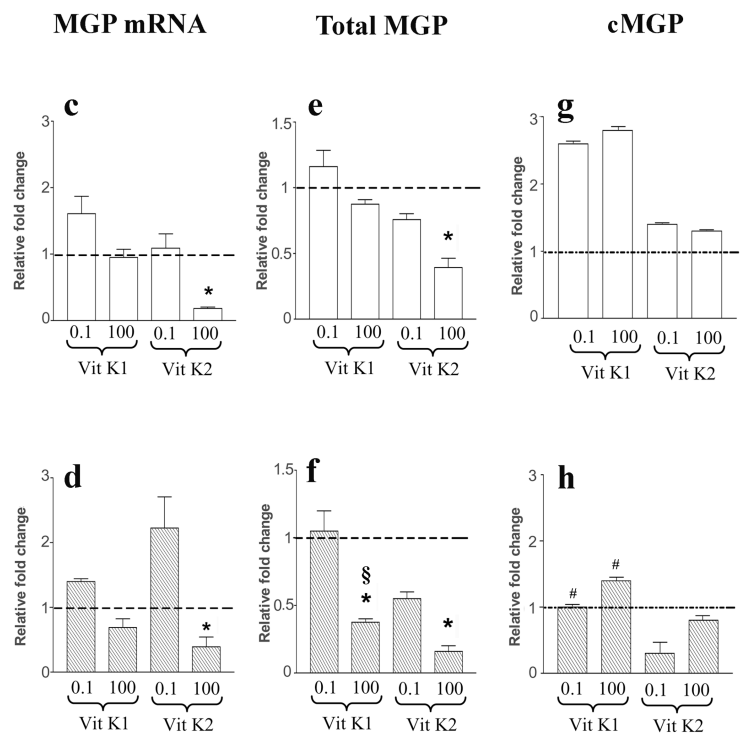
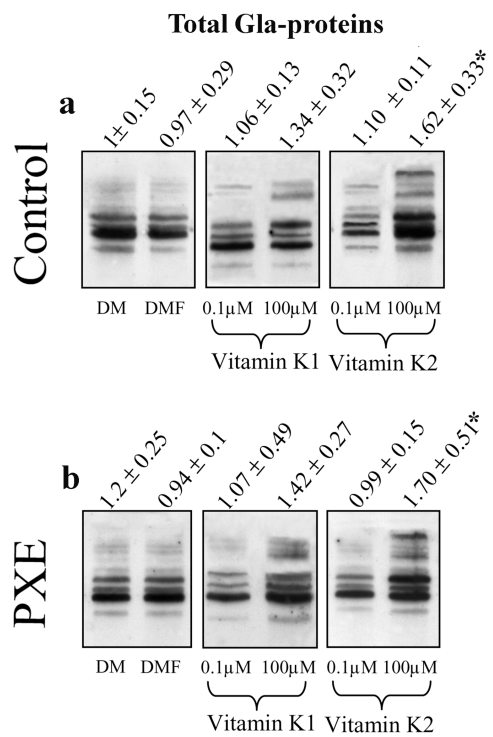
Control and PXE fibroblasts were cultured in standard medium (DMEM) or in calcified medium (CM) supplemented with vitamin K1, K2 or levamisole. Calcification is visualized at 30 days by light microscopy as dark precipitates (upper panels). In the lower part of the figure, the extent of mineralization (number of virtual meshes containing von Kossa positive precipitates in each culture plate) is reported at different time points, together with ALP activity (fold changes normalized to control fibroblasts cultured for 10 days in CM plus DMF). Data are expressed as mean values \pm SD.

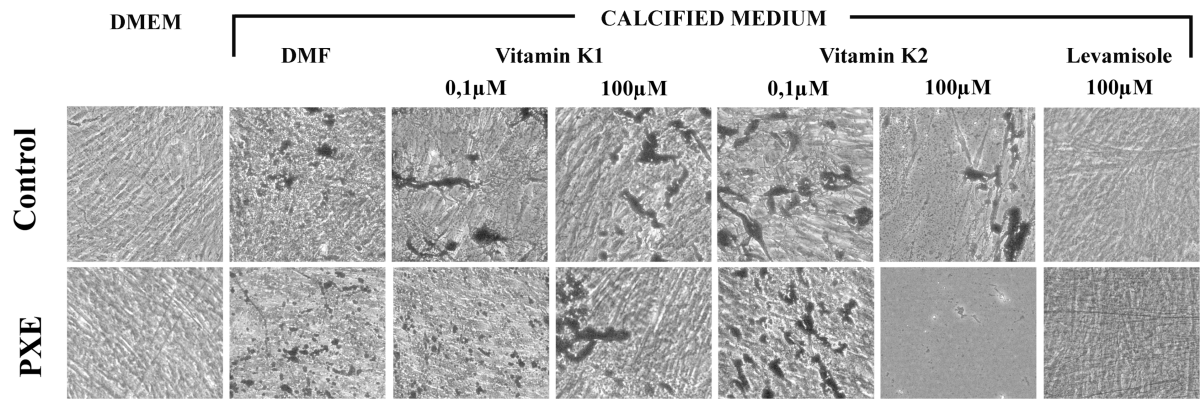
#occurrence of cellular morphological alterations; * $p < 0.05$ PXE vs Control

n.d. (not determined) due to areas of cell death or to thickness and partial insolubility of the deposited extracellular matrix, in the case of mineralization and ALP activity, respectively.









	DMEM	CALCIFIED MEDIUM					
		+ DMF	+ Vitamin K1		+ Vitamin K2		+ Levamisole
			0.1μM	100μM	0.1μM	100μM	
Mineralization							
10 days							
Control	0.0±0.0	0.2±0.4	0.4±0.5	0.6±0.9	0.7±1.2	0.8±1.0	0.0±0.0
PXE	0.0±0.0	0.2±0.4	0.4±0.9	0.8±1.3	0.3±0.8	0.4±0.5	0.0±0.0
20 days							
Control	0.0±0.0	0.3±0.5	1.4±1.9	2.6±4.3	0.7±1.2	2.0±1.6 [#]	0.0±0.0
PXE	0.0±0.0	1.2±1.4	1.8±2.5	4.6±5.9	3.4±6.8	1.7±2.9 [#]	0.0±0.0
30 days							
Control	0.0±0.0	9.9±9.1	9.4±10	9.0±13	26.0±19.8	n.d.	0.0±0.0
PXE	0.0±0.0	14.4±14.4	12.0±8.5	11.9±8.1	22.2±14.1	n.d.	0.0±0.0
ALP activity							
10 days							
Control	0.4±0.04	1.0±0.3	0.7±0.1	0.6±0.06	0.72±0.3	0.4±0.1	0.5±0.07
PXE	1.2±0.6 [*]	2.2±0.7 [*]	2.3±0.6 [*]	1.8±0.6 [*]	2.9±1.3 [*]	1.1±0.1 [*]	1.2±0.2 [*]
20 days							
Control	1.5±0.5	2.0±0.1	1.7±0.3	1.9±0.4	2.3±0.8	0.6±0.4 [#]	1.4±0.5
PXE	2.6±0.2 [*]	3.2±1.3	2.4±0.5	2.3±0.5	4.4±1.5	0.9±0.4 [#]	1.6±0.2
30 days							
Control	1.5±0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PXE	2.1±0.1 [*]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

SUPPLEMENTARY MATERIAL

Materials and Methods

Patients

PXE patients were diagnosed on the basis of skin and eye clinical alterations further confirmed by light and electron microscopy on skin samples and by identification of ABCC6 gene mutations according to procedures already described (Gheduzzi et al, 2004).

Blood was taken from 79 PXE patients by venipuncture, collected in appropriate tubes and stored for 1 hr at room temperature. Serum was collected after centrifugation at 3000xg for 10 min and aliquots (0.5 mL) frozen and stored at -80°C until use. For plasma, blood collected into Na-citrate containing tubes was centrifuged at $3500 \times g$ for 10 min at 4°C. Aliquots of plasma-citrate were stored at -85°C until use.

Laboratory parameters (calcemia, phosphatemia, alkaline phosphatase activity) were measured according to the routine procedures standardized in the Clinical Pathology Laboratory accredited by Regione Emilia Romagna for analytical activities and certified to UNI EN ISO 9001-2008. As controls, normal laboratory reference values were used.

Age and gender of patients were recorded in order to properly analyze and compare data.

Circulating MGP determinations

Plasma levels of desphospho-carboxylated MGP (dp-cMGP) or desphospho uncarboxylated MGP (dp-ucMGP) were measured by sandwich ELISAs techniques already developed at VitaK-BV (Maastricht, The Netherlands) (Cranenburg et al, 2010). In brief, microtiter plates were coated with monoclonal antibodies against the non-phosphorylated MGP 3-15 aa sequence. After blocking, either patient plasma or standards were transferred to the plates. The standards were synthetic peptides based on the non-phosphorylated 3–15 aa sequence and either the non-carboxylated 35–54

aa. sequence or carboxylated 35–54 aa. sequence, linked with a hydrophilic spacer (Pepscan, Lelystad, The Netherlands). After incubation and washing, the standards or the samples were detected using biotinylated monoclonal antibodies against the uncarboxylated or carboxylated 35-54 aa. sequence in human MGP, incubated with streptavidin-peroxide with 3,3',5,5'-tetramethylbenzidine.

Cells and treatments

Human dermal fibroblast cultures were obtained in accordance with the Declaration of Helsinki protocol and the guidelines of the ethical committee of the Modena University Faculty of Medicine, after written informed consent from 5 clinically healthy females, (38 ±7 years) which did not exhibit any sign of genetic, metabolic or connective tissue disorders and from 6 PXE patients (40 ±10 years).

Confluent fibroblasts were treated for 48h with phylloquinone (vitamin-K1, C₃₁H₄₆O₂, 450.7Da, Sigma, St. Louis, MO) or with menaquinone (MK-4) (vitamin-K2, C₃₁H₄₀O₂, 444.65Da, Sigma) at the concentration of 0.1, 1, 10 and 100µM. Due to their water insolubility, vitamins were dissolved in dimethylformamide (DMF, Sigma). Cells grown in the presence of DMF alone were used as control of treatments. Unless otherwise specified, cells from each individual were kept separate during all experiments.

Treatment with ALP inhibitors was performed with 100µM levamisole (Sigma) on cells at confluence.

Fibroblast's morphology was evaluated by phase-contrast microscopy and cell viability was assessed by cell count.

High-performance liquid chromatography (HPLC)

Vitamin K1 and K2 intracellular concentrations were measured by HPLC of cell extracts. Briefly, confluent fibroblasts were treated with vitamins for 48h, washed in buffer, trypsinized and

centrifuged. Cell pellets were stored at -80°C and sent to VitaK (Maastricht, The Netherlands) for analysis. After thawing, cell pellets were taken up in 500µL of distilled water, sonicated for 15sec at 12µm and supplemented with internal standard, followed by heating at 70°C for 30min and shaking vigorously for 5min. After cooling, 2.5mL of chloroform and 2.5mL of methanol were added followed by vigorous shaking and centrifugation for 10min at 1500g. The lower layer was transferred to a glass tube and organic solvents were evaporated until dryness under a stream of nitrogen at 50°C. The residue was taken up in 4 mL of n-hexane and vitamin K was eluted with 6 mL of 3% (v/v) diethyl ether in n-hexane, collected and organic solvents were removed by evaporation under a stream of nitrogen at 50°C. The residue was dissolved in 80 µL of isopropanol and analyzed by HPLC using a reversed-phase column with online zinc reduction and fluorescence detection. Vitamin K1-25 (a synthetic form of vitamin K1 containing 5 isoprenoid residues) was used as internal standard (Schurgers et al., 2007).

Flow cytometry

For each measurement, cells were incubated with appropriate stains, and analyzed on an EPICS XL flow cytometer (Coulter, Milan, Italy) (Boraldi et al, 2009). Ten thousand events were collected and evaluated for each cell type using a WINMDI 2.8 program. Experiments were performed three times in duplicate.

Superoxide anions (O_2^-) detection

Intracellular levels of O_2^- were estimated by incubating fibroblasts with 1 µM dihydroethidium probe (DH_2) (Molecular Probes, Eugene, OR) for 60 minutes at 37 °C . Fluorescence was measured at the emission wavelength of 575 nm.

Hydrogen peroxide (H_2O_2) detection

Trypsinized cells, resuspended in PBS, were stained with H_2DCF -DA (Molecular Probes) at the final concentration of 2µM for 30 min at 37°C. Fluorescence was measured at the emission wavelength of 520 nm.

Protein extracts and Western blot

Cells were homogenized in RIPA buffer with protease inhibitors (Sigma), centrifuged and supernatants collected and stored at -80°C until analysis. After measurement of protein concentration (Bradford, 1976), $30\mu\text{g}$ of proteins/lane were loaded on 10-lane 1-DE 10% polyacrylamide gel, under reducing conditions (Boraldi et al, 2009). In the case of cMGP, proteins were extracted in 8 M urea, 2% CHAPS, 65mM dithioerythritol, 2% pH 3–10 ampholyte (GE-Healthcare, Chalfont St. Giles, UK) with trace of bromophenol blue. After measuring protein concentration (Bradford, 1976), 2D gel electrophoresis was performed (Boraldi et al, 2009).

After separation in 1D or 2D-PAGE, proteins were transferred to nitrocellulose (Gheduzzi et al, 2007; Boraldi et al, 2009) and incubated with the following primary antibodies: anti-glucose-regulated endoplasmic reticulum stress response protein-78 (GRP78) (Abcam, Cambridge, UK, diluted 1:1000); anti-calumenin (CALU) (Santa Cruz Biotechnology, Santa Cruz, CA, diluted 1:100); anti-protein disulfide isomerase (PDI) (Abcam, diluted 1:2000); anti- γ -carboxyglutamyl (Gla) residues (American Diagnostica, Stamford, CT, diluted 1:200); anti-matrix Gla protein (MGP) (Santa Cruz Biotechnology, 1:200 diluted) and anti- γ -carboxylated-MGP (cMGP) (Vascular Products, Maastricht, The Netherlands, diluted 1:5000). After incubation with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam, diluted 1:5000), Western blots (WB) were visualized using Super Signal West Pico (Pierce, Rockford, IL). Densitometry of protein bands was performed using the ImageQuant TL v2005 software (GE-Healthcare).

1D-PAGE experiments were performed at least two times with all different cell lines, whereas in the case of cMGP, due to the low abundance of the protein, analyses by 2D-PAGE were carried out on proteins extracted from a pool of fibroblasts from 6 patients and from 5 controls.

Quantitative real-time RT-PCR

Total RNA was isolated from cells using the RNeasy Protect cells Mini kit (Qiagen, Milano, Italy), 3µg of total RNA were reverse transcribed using Superscript III (Invitrogen Life Technologies, Monza, Italy) and Oligo dT₁₈ primers (Invitrogen Life Technologies) and cDNAs were amplified on a iCycler (BioRad, Hercules, CA) using SYBR® GreenER™ qPCR SuperMix (Invitrogen Life Technologies), according to manufacturer's instructions. Primers were designed using the Universal ProbeLibrary (www.universalprobelibrary.com) and synthesized by Invitrogen: MGP (5'-CCCTCAGCAGAGATGGAGAG-3' and 3'-GCTTCCCTATTGAGCTCGTG-5') and CLK2 (5'-GCACCATAGCACCATTGTC-3' and 3'-GCACCATAGCACCATTGTC-5'). Efficiency of primers was 96.5% and 98.75%, respectively. Thermal cycling parameters were set to 50°C for 2min, 95°C for 3min, 45 cycles of 95°C for 30s, an annealing temperature of 60°C for 30s and 72°C for 30s, followed by melting curve analysis with a temperature ranging from 95 to 55°C. Gene expression in each sample was normalized against the housekeeping gene (CDC-like kinase, CLK2) and quantified using the Pfaffl method (Pfaffl, 2001). Analyses were carried out two times in triplicate.

Alkaline phosphatase activity

Fibroblasts were detached by enzymatic treatment and washed three times with PBS. Cells were lysed by appropriate buffer containing 0.1% Triton X-100. Cell lysate (20µL) was mixed with 100µL Tris-glycine buffer pH 10.3 (50mM Tris-HCl, 100mM glycine and 2mM MgCl₂) and 100 µL of p-nitrophenyl phosphate (Sigma). The reaction mix was incubated at 37°C for 30 min and the reaction stopped by adding 50µl of 3M NaOH. Absorbance was read at 405 nm in a microplate reader. Enzymatic activity was normalized to total protein concentration using bovine serum albumin as a standard for the Bradford protein detection method (Bradford, 1976).

Control samples were set as 1. Experiments were performed at least three times in triplicate.

***In vitro* calcification and Von Kossa staining**

Fibroblasts were routinely cultured in 6-well plates (BD-Falcon, Franklin Lakes, NJ) up to confluence, then the standard medium (DMEM + 10% FBS, Gibco, Monza, Italy) was replaced by the same medium with or without vitamin K1 and K2 (0.1 μ M and 100 μ M) or levamisole (100 μ M), or by the calcified medium (i.e. standard medium plus 10mM β -glycerophosphate, 50 μ g/mL ascorbic acid and 10 nM dexamethasone, Sigma) (Buranasinsup et al, 2006) with or without vitamin K1 and K2 or levamisole. Media were changed three times a week. After 10-20-30 days, cells were stained with the Von Kossa method (fixation in 3.5% formaldehyde, washes and staining with 5% silver nitrate for 30 minutes under a UV lamp). Plates were photographed and digital images overlapped with a 100 mesh virtual grid. Areas of calcification were quantified as the number of meshes containing von Kossa positive precipitates in each fibroblast cell culture plate and data expressed as mean values \pm SD. Experiments were performed three times.

Data analysis

Data are expressed as mean values \pm SD of all measurements and compared by Mann Whitney or by Anova test with significance at $p < 0.05$. Statistical data were obtained using GraphPad software (San Diego, CA).

SUPPLEMENTARY MATERIAL

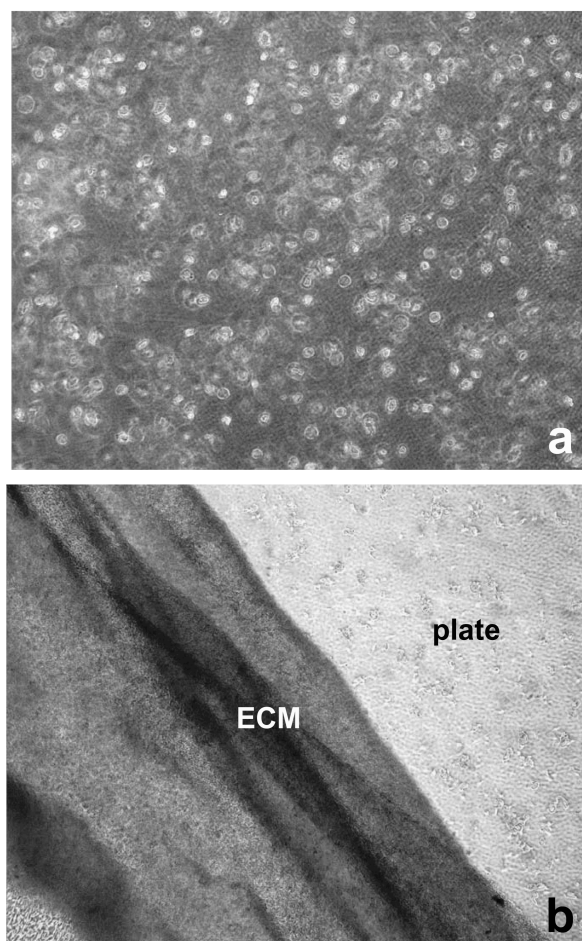


Figure 1S: Human dermal fibroblasts cultured for 30 days in DMEM (a) or in CM (b). Upon trypsinization cells cultured in DMEM are easily detached from the plastic surface, appearing as round translucent single floating cells. By contrast, cells cultured in CM (b), independently from mineral deposition, are covered and partially embedded in a dense layer of matrix molecules (ECM), whose synthesis is favored by the dexamethasone and the ascorbate present in CM. After treatment with trypsin, this layer is detached from the plate as a continuous sheet that cannot be homogenously solubilized by usual protein extraction procedures.

SUPPLEMENTARY MATERIAL

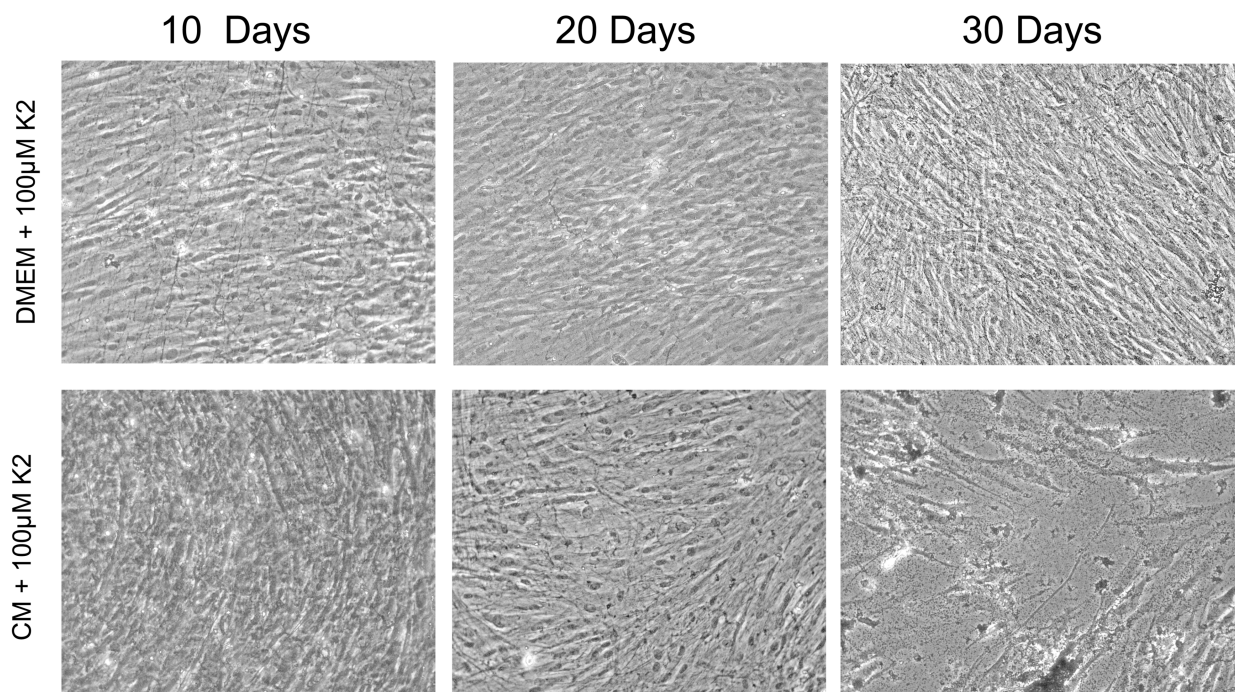


Figure 2S: Control fibroblasts observed at the inverted microscope. Vitamin K2 100µM-supplementation does not affect cell morphology and viability when cells are cultured in standard medium (DMEM), whereas it leads to changes in cell morphology (20 days) and to cell death (30days) when the vitamin is added to the calcifying medium (CM). Similar results can be visualized also for PXE fibroblasts.