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Collagen Cross-Linking Influences Osteoblastic Differentiation

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Abstract Osteoblasts synthesize collagen matrix, which itself regulates the differentiation of precursor cells into mature osteoblasts. They express lysyl oxidase (LOX), which is involved in the collagen cross-linking process. Lathyrogens, like ß-aminopropionitrile (ßAPN), inhibit the formation of a stable matrix. The aim of the present study was to investigate the influence of cross-linking on osteoblastic differentiation. MC3T3-E1 cells were seeded and treated with or without 400 μM ßAPN for 1 week. Thereafter, living cells were removed and, on this extracellular matrix, new MC3T3-E1 cells were seeded and cultured for 1 week without ßAPN. RNA was isolated, and expression of specific marker genes was determined by quantitative reverse transcription-polymerase chain reaction. Changes in specific cross-links after ßAPN treatment were measured with Fourier-transform infrared spectroscopy. The collagen matrix that formed showed a significant reduction of two major cross-links of bone collagen, deH-DHLNL and pyr, compared to control cultures. Gene expression studies showed an increase of collagen α1 (I) (COL1A1) to 150%. Expression of LOX and osteocalcin (OCN) mRNA was significantly downregulated to about 75%. When fresh MC3T3-E1 cells were seeded on this altered matrix without ßAPN, COL1A1 mRNA expression was upregulated (140%), OCN was downregulated (60%), and LOX mRNA expression remained unaffected. These results indicate that ßAPN treatment not only disrupts collagen cross-link formation but also affects osteoblastic activity and expression. In conclusion, the disrupted matrix produced in the presence of lathyrogen influences, even in its absence, the expression of osteoblastic genes.

Keywords Osteoblast · Cross-link · Lysyl oxidase · Osteocalcin · Collagen · Lathyrogen

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Collagen is the most abundant molecule in vertebrae, accounting for about 30% of total proteins. It is synthesized as procollagen and undergoes a variety of intra- as well as extracellular processing and modification steps [1].

Intracellular modifications of newly synthesized collagen polypeptides include hydroxylation of specific lysyl and prolyl residues and glycosylation of hydroxylysine residues [2]. Hydroxyproline is important for the formation and stabilization of the triple-helical domains. Hydroxylysyl directs certain cross-linking pathways, and it is the site of glycosylation. Glycosylation itself is a steric impairment and hinders staggering and lateral packing of collagen fibers within the cell [3]. When the triple-helical domains are formed, procollagen is packed into saccules and transported to the extracellular space via the Golgi complex [4].

Subsequent extracellular events include removal of C- and N-terminal procollagen peptides, which is performed by procollagen C-proteinases and procollagen N-proteinase, respectively [1]. The resulting tropocollagen molecules assemble into collagen fibrils and are stabilized by covalent cross-linking. Collagen cross-linking occurs as a result of oxidative deamination of ε-amino groups of lysine and hydroxylysine residues, which then form highly reactive aldehyde moieties that initiate a series of spontaneous condensation reactions leading to formation of covalent cross-links [2, 3].

The maturation process of collagen requires at least 10 different enzymes, many of which are unique to collagens or proteins with collagenous sequences [3]. The process of

cross-linking itself implies both enzymatic catalysis and a series of condensation reactions to form intra- and intermolecular cross-links. At present, six major naturally occurring collagen cross-links have been established. They are dehydrohistidinohydroxymerodesmosine (deH-HHMD), histidinohydroxylysinonorleucine (HHL), pyridinoline (pyr), deoxypyridinoline (d-pyr), pyrrole (prl), and deoxypyrrole (d-prl). The patterns of cross-linking and their molecular distributions vary from tissue to tissue. These patterns seem to be tissue- rather than collagen type-specific and probably are related to the physiological function of distinct tissues [2].

The importance of normal cross-link formation for healthy tissue is displayed by a number of genetically inherited diseases. In Ehlers-Danlos syndrome type VI, which is characterized by connective tissue dysfunctions such as kyphoskoliosis or ruptures of the eye and the aorta, the level of hydroxylation is reduced to 17% of normal bone due to a deficiency in lysyl oxidase (LOX) enzyme activity [5]. In homocystinuria, homocysteine is suggested to inhibit LOX, impairing subsequent cross-linking [6]. Long extremities, lens subluxation, and mental retardation go together with this disease. Conversely, overhydroxylation, as a consequence of retarded triple helix formation, can be observed in different forms of osteogenesis imperfecta [7]. Inhibition of LOX by lathyrogens such as ß-aminopropionitrile (ßAPN) and homocysteine causes reduced bone strength and fragility of connective tissue because of a disturbance in cross-linking.

Recent clinical/epidemiological data [8–11] show a definite correlation between homocysteine in the blood and fracture risk. Homocysteine is known to interfere with LOX action [6], thus altering collagen posttranslational modifications and collagen cross-link profiles. Moreover, if the clinical findings are true, then this interference and cross-link profile alteration would be expected to be more prominent in actively forming bone surfaces where LOX is active.

In the present study, we investigated the effects of the lathyrogen ßAPN, a collagen cross-linking inhibitor, on matrix deposition and osteoblastic gene expression as monitored by quantitative reverse transcription-polymerase chain reaction (QRT-PCR) in MC3T3-E1 osteoblastic cell cultures. The results indicate that the disrupted matrix produced in the presence of the well-known lathyrogen has, even in its absence, a lasting effect on the expression of osteoblastic genes in new generations of cells.

Materials and Methods

Cell Culture and Matrix Production

Each experiment consisted of a triplicate run, and three experiments per treatment and time point were performed. MC3T3-E1 cells, a clonal preosteoblastic cell line derived from newborn mouse calvaria, were cultured in humidified air under 5% CO_2 at 37°C. Culture medium was α-minimum essential medium (α-MEM; Biochrom, Berlin, Germany) supplemented with 5% fetal calf serum (Biochrom), 50 µg/mL ascorbic acid (Sigma, Vienna, Austria), and 10 µg/mL gentamycin (Sigma). For propagation, cells were subcultured twice a week using 0.001% pronase E (Roche, Mannheim, Germany) and 0.02% EDTA in Ca^{2+}- and Mg^{2+}- free phosphate-buffered saline (PBS) before reaching confluence. MC3T3-E1 cells were seeded in culture dishes at a density of 20,000/cm^2 and cultured overnight. On the next day, the medium was changed and the cells were treated with or without 400 µM ßAPN (Sigma) for 1 week to gain two to three cell layers [12]. This concentration was chosen based on previously published reports [13, 14]. Pictures of the cultures were taken by an Olympus (Hamburg, Germany) DP10 fixed on a Nikon (Dusseldorf, Germany) TMS microscope and shown without further processing.

Matrix was either used for reseeding experiments or investigated for mRNA expression and Fourier-transform infrared (FTIR) analysis.

Matrix Preparation for Reseeding Experiments

For reseeding experiments, living cells were removed by treatment with sodium deoxycholate (DOC) after being cultured for a total of 7 days. To optimize the concentration of the detergent, cells were plated in 24-well plates at a density of 50,000/cm^2 and grown in the medium as described above. After 1 week, different concentrations of DOC dissolved in PBS were added to the cell layers and incubated for 10 min at 4°C. To verify that all cells were removed with this treatment, a cell proliferation and cytotoxicity assay (EZ4U; Biomedica, Vienna, Austria) was used, according to the protocol of the supplier.

For reseeding experiments, fresh MC3T3-E1 cells were seeded onto the extracellular matrix (ECM) at a density of 20,000/cm^2 and cultured for 1 week. Pictures of the cultures were taken by an Olympus DP10 fixed on a Nikon TMS microscope and shown without further processing.

Cell Multiplication (DNA Amount) and Alkaline Phosphatase Activity

MC3T3-E1 cells were seeded in culture dishes at a density of 20,000/cm^2 and cultured overnight. On the next day, the medium was changed and the cells were treated with or without 400 µM ßAPN for 1 week. Before reseeding and after 1 week of reseeding, cell number and ALP activity were analyzed.

For determination of cell number (DNA amount), cell layers were washed with PBS and frozen with 1 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM EDTA. During thawing, Hoechst dye (Polysciences, Warrington, PA) was added (1 μg/mL) and, after an incubation of 15 min at room temperature, the fluorescence of the DNA was measured (excitation 360, emission 465 nm).

Thereafter, alkaline phosphatase (ALP) activity was measured with *p*-nitrophenylphosphate (2.5 mg/mL in 0.1 M diethanolamine buffer [pH 10.5], 150 mM NaCl, 2 mM $MgCl_2$) by incubation of the cell layers for 15 min at room temperature. Absorption was measured in a microplate reader at 405/490 nm. ALP activity (units per milligram DNA) was estimated using a standard curve prepared from calf intestinal ALP (Roche).

Expression Analysis by QRT-PCR

mRNA was extracted using an mRNA Isolation Kit (Roche), and cDNA was synthesized from the mRNA using the 1st Strand cDNA Synthesis Kit (Roche). The obtained cDNA was subjected to PCR amplification with a real-time cycler using TaqMan Gene Expression Master Mix (Applied Biosystems) and TaqMan primers (Applied Biosystems) that amplify COL1A1 and LOX. Glyceraldehyde phosphatede hydrogenase (GAPDH) was used as a housekeeping gene for normalization, amplified in the same tube. Because no tested TaqMan probe for osteocalcin (OCN) was available, SYBR-Green (Roche) PCR was performed using 5'-CCAGTCCCACACAGCAGCTT-3' as forward and 5'-GATCTGGGCTGGGGACTGAG-3' as reverse primer. All PCRs were performed in triplicate.

After 10 min of initial denaturation at 95°C, PCR was performed with 60 cycles: 10-s denaturation at 95°C, 30-s annealing and extension at 60°C. Quantification was achieved using the comparative threshold cycle method according to the manufacturer's protocol. Expression was quantified using the 2(−Delta Delta C (T)) method [15].

FTIR Imaging

For collagen cross-link analysis, the cells were fixed in alcohol, scraped off the culture dishes, and transferred onto barium fluoride windows, where they were air dried. Following this, spectra were obtained in transmission with a Bruker (Vienna, Austria) Equinox 55 spectrometer coupled to a Bruker Hyperion 3000 FTIR microscope equipped with a motorized stage (±1 μm) and a x15 objective. The spectra were baseline-corrected in the amide I and II spectral area ($\sim$1,500–1,700 cm^{-1}); water vapor was subtracted and then subjected to second derivative spectroscopy and curve fitting routines as described previously [16]. The type and amount of collagen cross-links were determined as previously described [16]. Moreover, to investigate whether the noncollagenous organic matrix changed as a result of ßAPN treatment, the underlying bands at $\sim$1,646, 1,654, 1,674, and 1,683 cm^{-1} were also monitored and their percentage area recorded. These subbands have been reported in the literature to correspond to protein conformation of random coil, α helix, β structure, and β turn, respectively [17].

Statistical Analysis

Statistical analyses were performed by analysis of variance (Scheffe's post hoc test) or Student's *t*-test using Prism 4.03 (GraphPad Software, San Diego, CA). $P \leq 0.05$ was considered significant, and the data are presented as means ± standard deviation (SD).

Results

MC3T3-E1 Cell Cultures

During prolonged culture time, MC3T3-E1 cells produce numerous cell layers embedded in ECM that consists mainly of collagen [12, 18]. For our experiments, MC3T3-E1 cells were grown for 1 week, a period sufficient to produce about three cell layers [12]. To alter cross-link formation catalyzed by LOX, the cultures were treated with 400 μM ßAPN and compared to controls (untreated cultures). This treatment did not cause visible changes in the appearance of the cells (data not shown).

Effects of ßAPN on Cell Multiplication and ALP Activity

Firstly, the effects of ßAPN on cell multiplication of MC3T3-E1 cells were measured after 1 week of culture. Figure 1A demonstrates that the ßAPN treatment had no influence on cell multiplication. Next, we analyzed whether the matrix, modified by ßAPN has an influence on cell proliferation of reseeded cells. Figure 1B shows that after 1 week of culture there was no significant change ($P = 0.06$).

Secondly, the influence of ßAPN on ALP activity, an early marker of cell differentiation, was measured after 1 week of culture. During this culture time, ALP activity was not significantly changed ($P = 0.06$, Fig. 2A). After reseeding, the cultures showed no difference (Fig. 2B) as well.

Effect of ßAPN Treatment on Collagen Cross-Links

The results of the FTIR spectroscopic determination of two of the major bone collagen type I cross-links, pyr and

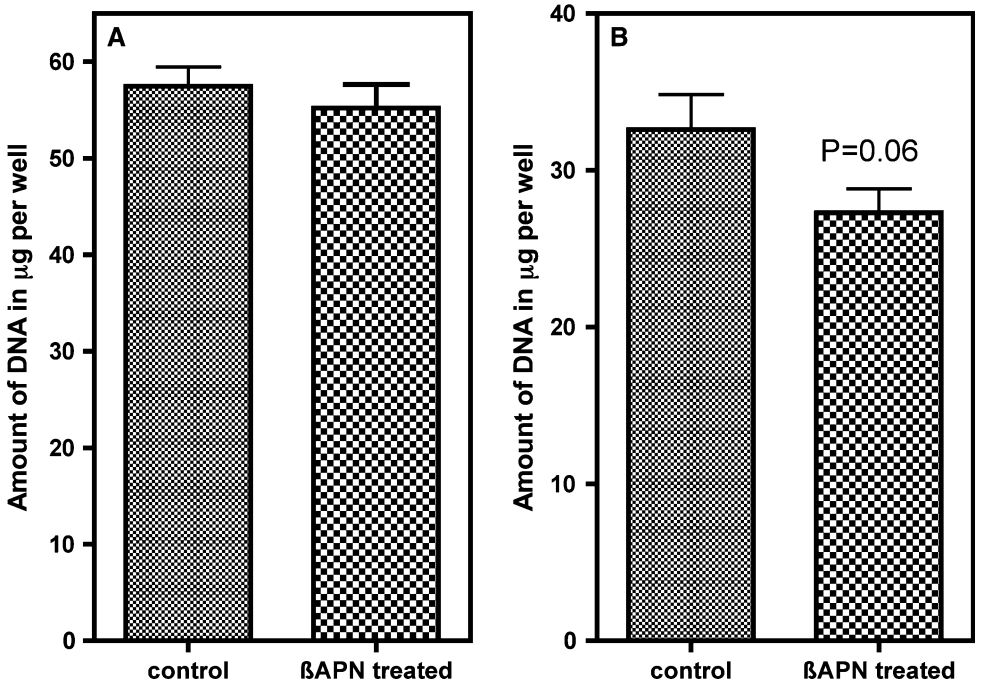

Fig. 1 Effects of ßAPN on cell multiplication of MC3T3-E1 cultures before reseeding (**A**) and after 1 week of culture of reseeded MC3T3-E1 cells (**B**). Amount of DNA, as a surrogate of cell multiplication, was estimated after 1 week of culture with and without ßAPN treatment (**A**). Cells were reseeded to matrix either untreated or treated with ßAPN and cultured for 1 week without ßAPN (**B**). Again, the DNA amounts of the cultures were estimated by measuring the fluorescence after staining the nuclei with Hoechst dye. No difference was found between untreated and treated cultures before reseeding (**A**). The reseeded cells tended to proliferate slower, but this did not reach significance (**B**). Bars represent the mean ± SD, $n = 4$

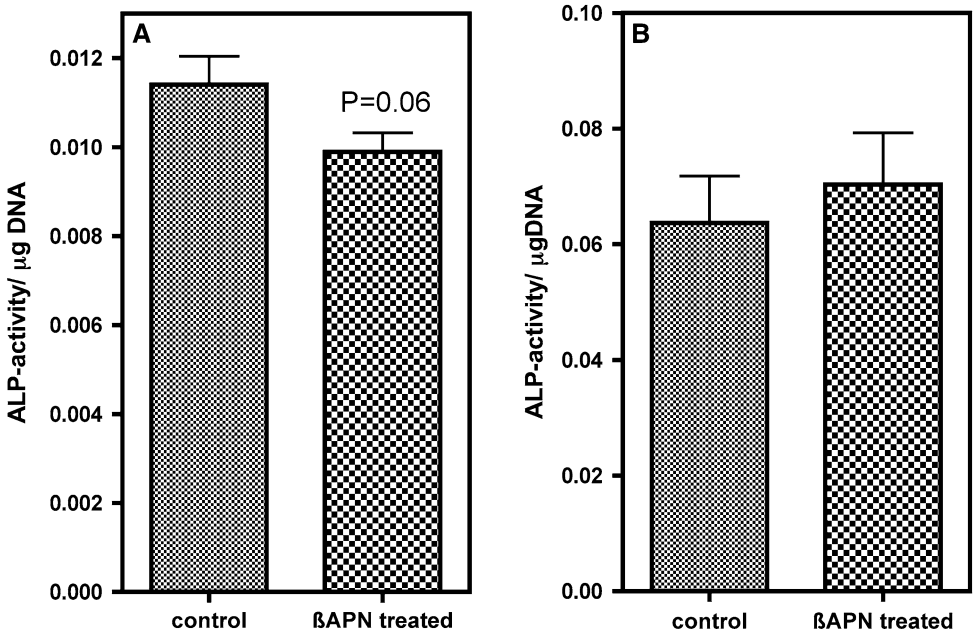

Fig. 2 Effects of ßAPN on ALP activity of MC3T3-E1 cultures before reseeding (**A**) and after 1 week of culture of reseeded MC3T3-E1 cells (**B**). ALP activity was analyzed with *p*-nitrophenylphosphate (2.5 mg/mL). Absorption was measured at 405/492 nm and compared to a standard curve. ßAPN-treated cultures showed slightly reduced ALP activity, but this did not reach significance (**A**). No difference was found between untreated and treated cultures after reseeding (**B**). Bars represent means ± SD, $n = 4$

dehydrodihydroxylysinonorleucine (deH-DHLNL), are shown in Fig. 3A and B, respectively. The results are presented as percent area of the two subbands at ~1,660 and 1,690 cm^{-1}, which have been shown to correspond with the amount of the two previously mentioned cross-links [16]. As can be seen, there was a marked reduction in both cross-links as a result of ßAPN ($P < 0.001$). There was no statistical difference in the percent area of the subbands representative of random coil, α helix, β structure, and β turn (data not shown).

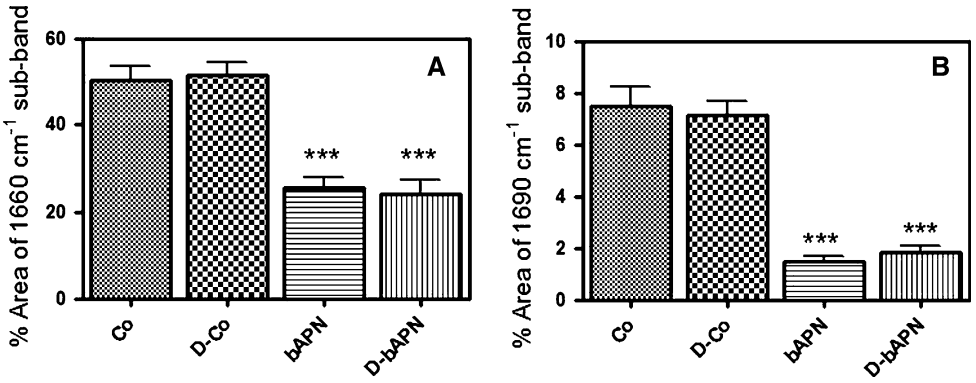

Fig. 3 Spectroscopic determination of relative amounts of pyr (**A**) and deH-DHLNL (**B**) collagen cross-links in control (Co), control after treatment with DOC (D-Co), ßAPN-treated (bAPN), and ßAPN-treated after DOC treatment (D-bAPN) cell cultures. There was no difference between Co and D-Co or between bAPN and D-bAPN cultures. On the contrary, there were significant differences between Co and bAPN and between D-Co and D-bAPN cultures. ***$P \leq 0.001$

Effect of ßAPN Treatment on Gene Expression at the mRNA Level

Next, a possible effect of ßAPN treatment on gene expression (COL1A1, LOX, and OCN) was investigated. The results are shown in Fig. 4A–C and, as can be seen, there was a significant increase in COL1A1 mRNA levels and a significant decrease in LOX and OCN mRNA levels as a result of ßAPN treatment.

Preparation of Matrix for Reseeding Experiments

To prepare disrupted matrix, cells were seeded as previously described in the presence of 400 µM ßAPN for 1 week. To remove living cells from their matrix, increasing concentrations of DOC in PBS were used. Thereafter, cellular activity was measured using the formazan-based EZ4U assay. As Fig. 5 demonstrates, at and above a concentration of 0.1% DOC no further cellular activity was evident; for safety reasons, we used 0.5% DOC in PBS to remove living cells from the ECM. Figure 6B shows that this treatment resulted in the removal of living cells from the ECM, leaving little holes. Moreover, DOC treatment for removal of living cells had no statistical effect on either collagen cross-link compared to its appropriate control (Fig. 3A, B; $P > 0.05$).

Reseeded Cell Cultures on Previously Synthesized Intact and Disrupted Matrix

After removal of the cells from the ECM by DOC, fresh MC3T3-E1 cells were seeded onto the ECM resulting from cultures that were treated previously with or without ßAPN for 1 week. Again, no visible differences were observed between cells adhering to ECM resulting from ßAPN-treated vs. untreated cultures (Fig. 6C, D).

Effect of Disrupted Matrix on Gene Expression in Reseeded Cells in the Absence of ßAPN at the mRNA Level

After 1 week of culture on untreated and ßAPN-treated matrix, mRNA was isolated from the reseeded cells and

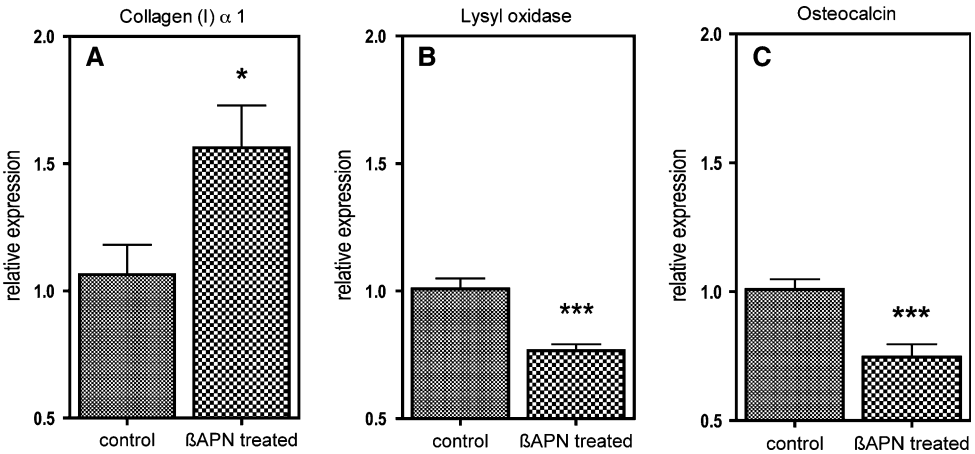

Fig. 4 Effects of ßAPN on the mRNA expression of collagen α1 (I) (**A**), lysyl oxidase (**B**), and osteocalcin (**C**) in MC3T3-E1 cells. MC3T3-E1 cells were cultured with and without ßAPN for 1 week. Thereafter, mRNA was isolated and gene expression was analyzed by QRT-PCR. Gene expression was normalized to GAPDH. Bars represent the mean ± SD, *$P \leq 0.05$, ***$P \leq 0.001$, $n = 3$

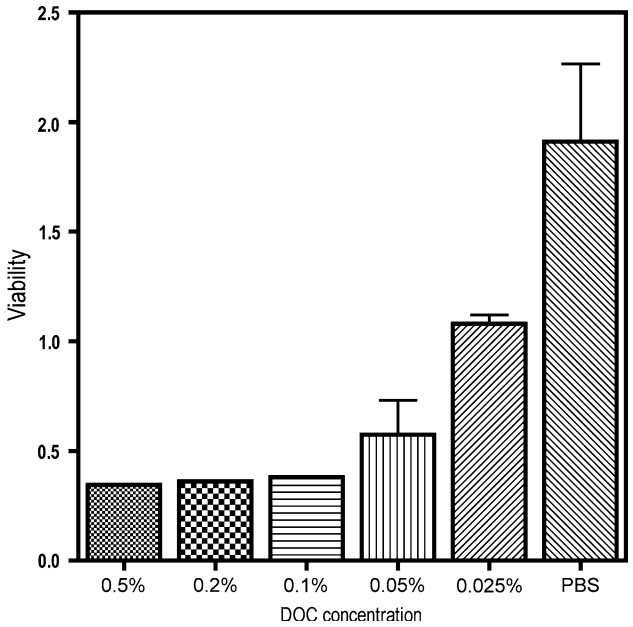

Fig. 5 Cell viability after treatment with DOC. Living cells were removed after 1 week of culture with different concentrations of DOC dissolved in PBS. At and above a concentration of 0.1% DOC, no viable cells could be found

analyzed for gene expression by QRT-PCR. As demonstrated in Fig. 7, in MC3T3-E1 cells seeded onto ECM modified by ßAPN, COL1A1 mRNA levels were significantly increased compared to controls (ECM from untreated cultures, Fig. 7A). OCN was downregulated significantly (Fig. 7C). In contrast, the influence on LOX mRNA regulation did not reach significance ($P = 0.06$, ns; Fig. 7B).

Discussion

In the present study, the hypothesis tested was that, in addition to interfering with collagen cross-link formation, lathyrogens such as ßAPN have an affect on osteoblastic gene expression. The present study shows that blocking LOX by ßAPN treatment resulted in a marked decrease of the two main cross-links pyr and deH-DHLNL, which is in agreement with previously published reports where inhibition of LOX by ßAPN in MC3T3-E1 cell cultures resulted in increased accumulation of abnormal collagen fibrils, as determined by solubility studies and electron microscopy [19]. Moreover, the present results confirmed the hypothesis in that osteoblastic differentiation is also altered by the impaired structure of the ECM. Indeed, when cells were grown in the presence of ßAPN, three markers (COL1A1, LOX, and OCN) had altered expression at the mRNA level due to the presence of ßAPN. This should not be attributed to a toxic effect of ßAPN because no influence on cell multiplication could be found, indicating no toxic influence of this reagent on osteoblast-like cells. The lack of significance for downregulation of ALP activity could be attributed to the short duration of the experiments

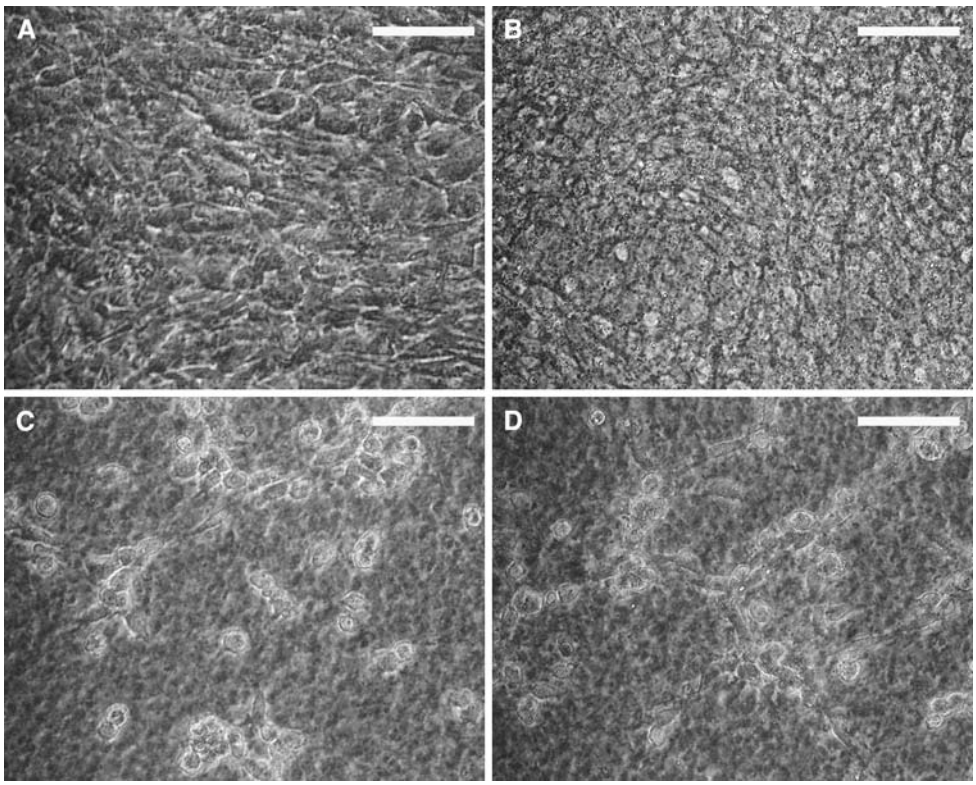

Fig. 6 Phase-contrast micrographs of MC3T3-E1 cells treated with ßAPN (**A**) and ßAPN-treated cells after DOC treatment (**B**). Small "holes" are noticeable in **B**, most probably due to the absence of the cells. Phase-contrast micrographs of reseeded MC3T3-E1 cells. MC3T3-E1 cells were cultured without (**C**) and with (**D**) ßAPN, and after removal of living cells by DOC, fresh MC3T3-E1 cells were seeded onto the matrix. There is no noticeable morphological difference between the two cell cultures. Bars = 100 µm

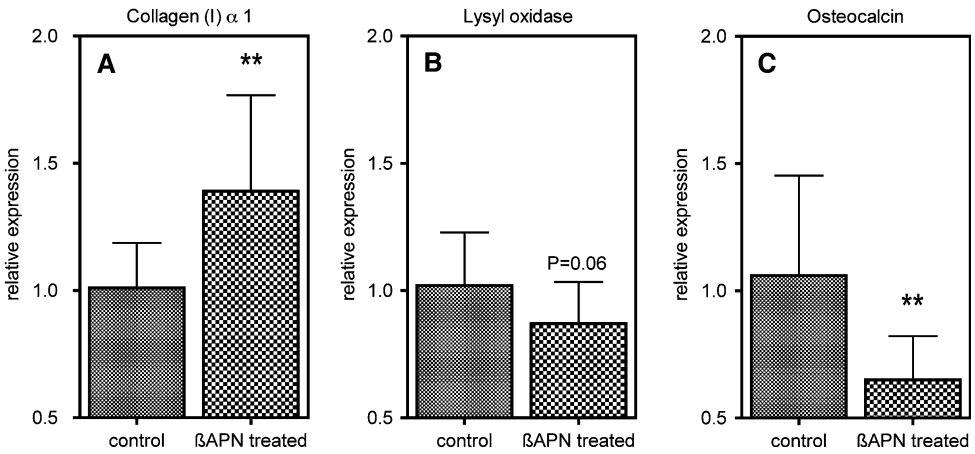

Fig. 7 Effects of disrupted matrix on the mRNA expression of collagen α1 (I) (**A**), lysyl oxidase (**B**), and osteocalcin (**C**) in MC3T3-E1 compared to controls. MC3T3-E1 cells were seeded on the previously disrupted matrix and cultured for 1 further week. Thereafter, mRNA was isolated and gene expression was analyzed by QRT-PCR. Gene expression was normalized to GAPDH. Bars represent means ± SD, $^{**}P \leq 0.01$, $n = 3$

because no continuous differentiation process was possible and it is well known that mRNA processing, translation, and transport of ALP to the cell membrane take a long time. However, the downregulation of mRNA expression of late marker proteins is not a contradiction because regulation of mRNA is usually a quick process. MC3T3-E1 cells are a heterogeneous population of osteoblasts with different differentiation status [18, 20], and their phenotype changes during prolonged culture time. This suggests that in the beginning of the culture time most osteoblasts are in a preosteoblastic situation, expressing no OCN. However, some cells exist in a differentiation status expressing low levels of OCN mRNA. Using QRT-PCR, these very low levels could be detected easily and any change of expression determined. This change can be induced by variation of the hormonal condition, changes in growth factor environment, or as suggested by our experiments, deficiency of collagen cross-links.

Based on these results, a second hypothesis was tested: a disrupted matrix will have an effect on osteoblastic expression even in the absence of lathyrogens. Again, the results partially confirmed this hypothesis in that COL1A1 and OCN expression at the mRNA level was altered, while there was no effect on LOX expression compared to control cultures. Again, no statistically significant influence was found on cell multiplication and ALP activity. These results, when considered together, indicate that, in addition to the well-known effects of lathyrogens on collagen cross-link formation, they influence osteoblastic gene expression as well and this effect lingers due to the disrupted matrix even in the absence of the substances themselves. Furthermore, indirect analysis based on protein conformation suggests that there were no detectable changes in the noncollagenous organic matrix due to ßAPN treatment. These results emphasize the importance of a well-formed ECM for proper osteoblastic differentiation.

In recent years, an association between homocysteine levels (a lathyrogen with the same course of action as ßAPN) and fracture risk has emerged in the clinical literature [8–11, 21–40]. Although attempts to explain this association focused on bone turnover and bone mineral density (BMD) changes, one should not forget that one of the effects of high homocysteine levels is disruption of collagen cross-link formation [41–44].

The results of the present study show that lathyrogens such as ßAPN and most likely homocysteine, as it has the same course of action as ßAPN, potentially have a dual effect on osteoblasts and bone strength. First, they interfere with the collagen cross-link formation process. The importance of collagen intermolecular cross-links to the mechanical performance of bone is very apparent in lathyrism [45] as well as in the pyridoxine-deficient chick model [42]. In this B_6-deficient chick, the mineral in the cortical bone remains unaltered (both quantity and composition) compared to controls, whereas higher amounts of extractable collagen and altered cross-links are exhibited. The B_6-deficient animals exhibit a decreased fracture load and offset yield load. These data suggest that although proper cortical bone mineralization occurred, the alterations of the collagen and collagen cross-links resulted in changes to bone mechanical performance. In addition to this effect, they may alter the gene expression of osteoblasts as the results of the present study indicate (changes at least in COL1A1, LOX, and OCN at the mRNA level), with possible consequences on the differentiation of osteoblasts and their precursor cells.

Taken together, the present results, that collagen I is upregulated and OCN downregulated on ßAPN-treated matrices compared to controls, could indicate that cross-linking accelerates differentiation of osteoblasts. Moreover, eradicating the presence of the interfering lathyrogen may not relieve the potential harmful effects due to the presence of the altered matrix which, through some unknown feedback mechanism, still alters osteoblastic expression. Although caution should be exercised not to overinterpret the results of the present study as results obtained in a cell

culture system may not be directly extrapolated to animal and human tissue, further investigation is warranted, especially in view of the recent correlation between homocysteine levels and fracture in humans.

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