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Homocysteine Decreases Chondrocyte-Mediated Matrix Mineralization in Differentiating Chick Limb-bud Mesenchymal Cell Micro-mass Cultures

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The differentiating chick limb-bud mesenchymal cell micro-mass culture system has been used as a model for monitoring the effects of matrix modification on cell-mediated calcification. In this study, we show that treating these micro-mass cultures with homocysteine (Hcys) impairs cartilage calcification. Cultures were treated from day 2 to day 7 with two nonphysiological concentrations of Hcys equivalent to 100× and 1000× avian serum levels (0.36 and 3.6 mmol/L), and from days 9–13 with one tenth the concentration. Mineralization assays were done at days 16, 19, and 21, and matrix and cell properties were examined between days 5 and 21. Mineral accretion, based on differential ^{45}Ca uptake (mineralizing minus control cultures), was significantly reduced in the high-Hcys-concentration group, and slightly reduced in the low-Hcys-concentration group. Electron microscopy at culture day 21 showed that the collagen matrix was less abundant and its banding pattern less obvious in the Hcys-treated groups than in the untreated cultures. Pyridinoline (Pyr) and deoxypyridinoline (d-Pyr) contents were not detectable in day 21 cultures with either 0.36 or 3.6 mmol/L homocysteine, whereas values in mineralizing and nonmineralizing controls ranged from 0.06 to 0.08 and 0.03 to 0.06 (moles/mole collagen) for Pyr and d-Pyr, respectively. Fourier transform infrared (FTIR) imaging also indicated a decreased content of pyridinoline cross-links. Hcys caused other matrix changes as well. Whereas at culture day 5 there was no significant difference in the number of chondrocyte nodules formed, by day 11 the proteoglycan content (measured by Alcian blue dye binding at 595 nm) was significantly reduced in both mineralizing and control cultures in the high- and low-Hcys groups. In contrast, there were no detectable differences in type X collagen and alkaline phosphatase staining in the mineralizing cultures with or without Hcys supplements. Because vital dye stains and electron microscopy studies indicated that cells in the control and experimental groups did not differ in terms of viability, the observed differences cannot be attributed to toxicity. Thus, Hcys treatment, which causes matrix disorganization, decreases the ability of the matrix to support mineralization. (Bone 28:387–398; 2001)

Key Words: Mineralization; Homocysteine; Micro-mass cell culture; Collagen cross-links; Infrared imaging.

Introduction

Differentiating chick limb-bud mesenchymal cells plated in micro-mass culture recapitulates the events involved in matrix mineralization in the chick growth plate in ovo.[6] This system has been used extensively for the analysis of cell-matrix interactions in the regulation of cartilage calcification.[6–8,10–13] Type I, II, IX, and X collagens are expressed in these cultures,[12] and the mineralization that occurs is associated with both thin (type II) and thick (type I) collagen fibrils.[7,10,12] To test the hypothesis that alterations in collagen cross-links would alter matrix-mediated mineralization, homocysteine (Hcys), an agent known to block collagen cross-link formation,[27,62] was added to these cultures and time-dependent alterations in mineral and matrix properties were monitored.

Several studies have attempted to assess the role of collagen cross-links in the process of mineralization. For example, inhibition of collagen cross-links by β-aminopropionitrile (BAPN), which inhibits the enzyme responsible for the generation of the reactive aldehydes involved in collagen cross-linking (lysyl oxidase),[14,35,61] causes a decrease in bone mineralization both in vitro and in vivo.[55,60,65] Other cross-linking inhibitors have also been reported to decrease mineralization in animal models.[32,35,36] Several bone diseases are associated with abnormal collagen cross-linking. Osteoporotic avian[31] and primate[70] bones and osteopetrotic rat bones,[68] for example, have shown alterations in collagen cross-links relative to normal controls. Collagen cross-links are also reduced in aging and degenerating human intervertebral disks,[54] in which aberrant calcification is common.[20] It has also been observed that treatment of bioprosthetic aortic valves with glutaraldehyde prior to implantation, which leads to cross-linking of collagen (albeit biochemically distinct from physiological cross-linking), causes accelerated calcification when compared with untreated controls.[45] All these observations seem to suggest that collagen cross-linking may have important implications for the mineralization, as well as the strength and mechanical integrity, of collagenous tissues.

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There is evidence in the literature, however, that the association between collagen cross-links and mineralization may not be so apparent. For example, lysyl oxidase requires pyridoxal phosphate (PLP) for its activity,[36,43] and collagen cross-links are present in lesser amounts in PLP-deficient chicks.[21] Electron-microscopic (EM) examinations of articular cartilage collagen from these chicks have revealed abnormally large collagen fibers,[38] altered growth plates, and desiccated articular cartilage (Masse, personal communication). However, Fourier transform infrared (FTIR) and ash-weight analyses of the bones of these chicks showed no abnormalities in mineralization, even though the cross-link profile and collagen solubility was altered.[39] This finding should be examined with two facts in mind: First, PLP is important for amino acid metabolism in the rest of the body and its deficiency may bring on systemic changes, as reflected by altered plasma amino acid composition. Plasma homocysteine concentrations are also elevated in PLP-deficient animals.[41,60,62,63] Second, collagen cross-links are altered in the disease homocystinuria, a genetic connective tissue disease, characterized by long extremities and digits, pex cavus, lens subluxation, and mental retardation.[62] This connective tissue disorder is thought to be caused by the reaction of collagen aldehyde groups with homocysteine with the formation of a stable thiazine ring; the resulting paucity of available aldehyde groups does not allow normal cross-linking of collagen, thereby decreasing its stability.[27] Other experiments have shown that penicillamine, as well as homocysteine, impairs collagen cross-linking by reacting with the aldehydes generated by lysyl oxidase.[61] Although these compounds do not inhibit the synthesis of reactive aldehydes, they do interfere with cross-link formation by not allowing steps subsequent to aldehyde formation to take place. In contrast to the aldehyde-deficient lathyrism (e.g., BAPN treatment), penicillamine-induced lathyrism is characterized by increased aldehyde content.[15] This alteration in aldehyde distribution might also affect mineral deposition.

Materials and Methods

The objective of this study was to test the hypothesis that preventing collagen cross-linking by exogenous addition of D,L-homocysteine (Hcys) would decrease collagen-based mineralization in the differentiating chick limb-bud mesenchymal cell micro-mass culture system. For this study, day 5 (chondrocyte nodule production), day 11 (maximum matrix synthesis), and day 21 (maximum mineral accretion)[12] were selected as key endpoints.

Culture Conditions

The micro-mass cell culture system has been described elsewhere.[12] In brief, fertilized White Leghorn eggs (Truslow Farms, Chestertown, MD) were maintained in a humidified incubator at 37°C. At 4.5 days, when the embryos corresponded to stages 21–24 according to the classification of Hamburger and Hamilton,[24] the embryos were sterilely withdrawn from the eggs and their limb buds removed and placed into 0.9% USP-grade saline (Abbott Laboratories, North Chicago, IL). Cells were released from the limb buds by digestion for 7 min in 5 mL 0.25% (wt) trypsin/0.53 mmol/L ethylene-diamine tetraacetic acid (EDTA; Gibco, Grand Island, NY). The reaction was quenched by the addition of 5 mL of low-calcium (0.3 mmol/L) Dulbecco's modified Eagle medium (DMEM; Formula 80-0303 AJ, Gibco). Following vortexing, the cells were separated from debris by passage through two layers of 20 μm Nitex membrane (Tetko, Inc., Ardsley, NY), counted with a hemocytometer, checked for viability by trypan blue dye exclusion, and pelleted in the cold at 2300 rpm. For all experiments, viability at plating (day 0) exceeded 98%. Cells were resuspended in DMEM supplemented with 50 U/mL penicillin, 25 μg/mL streptomycin, and 10% fetal calf serum (Gibco), containing 1.3 mmol/L Ca, and plated, using the micromass technique,[1] at a density of 0.75 million cells per 20 μL drop in 35 × 10 mm Falcon dishes. After 2 h in a humidified atmosphere of 5% CO_2 at 37°C, 2 mL of medium was added. Medium was changed every 48 h, with 25 μg/mL ascorbic acid and 0.3 mg/mL glutamine added with each change of medium from day 2. For mineralizing cultures, the inorganic phosphate content was adjusted by adding 3 mmol/L KH_2PO_4 from day 2 onward, making the total inorganic phosphate content of the media 4 mmol/L. Nonmineralizing control cultures had a basal inorganic phosphate content of 1 mmol/L, and were not further supplemented with phosphate. Three different concentrations of Hcys (0, 0.36, and 3.6 mmol/L) were added. The supplement concentrations represent 100× and 1000× the avian serum concentration.[59] The cultures were supplemented with Hcys on days 2, 4, and 7. On days 9, 11, and 13 (after the matrix formation stage was known to have reached a plateau[12]) the Hcys dosage to each group was reduced to one tenth of the original. No Hcys was added after day 15 because of the matrix degeneration observed when addition continued beyond day 16.

Nodule Count and Proteoglycan Content

To determine the effect of the presence of Hcys on cartilage nodule formation, the number of Alcian blue-stained cartilage nodules per micro-mass culture at day 5 were counted at ×25 magnification. Cultures fixed with 90% ethanol were stained for 6 h in the cold with 0.5% Alcian blue GX (Sigma Chemical Co., St. Louis, MO) in 0.1N HCl. The number and size of the nodules were recorded using the METAMORPH image analysis system (Universal Imaging Corp., West Chester, PA).

The proteoglycan content of the culture was estimated based on Alcian blue dye binding as modified from Farndale et al.[18] In brief, on day 11, the cultures were fixed with 90% ethanol and stained for 6 h in the cold with 0.5% Alcian blue GX (Sigma) in 0.1N HCl. The proteoglycan/dye mixture was extracted into 4 mol/L guanidine hydrochloride for 24 h at 4°C, and the absorbance of the extract measured at 595 nm.

Mineralization Assays

Mineralization was monitored based on ^{45}Ca uptake, histology, and FTIR imaging. To monitor the total calcium uptake, 1 μCi/mL ^{45}Ca (Amersham, Arlington Heights, IL) was added to mineralizing and similarly treated nonmineralizing cultures on alternate days from culture day 7 onward. Based on previous studies,[11,12] the experiments were terminated at day 21, the time when mineral accumulation had reached a plateau value. At days 16, 19, and 21, the medium was removed from labeled cultures, cultures were washed with fresh cold media, and the mineralized or control matrix was transferred into scintillation vials and hydrolyzed (1 h, 60°C) in 0.2 mL 4N HCl. The ^{45}Ca incorporation, determined by scintillation counting, was expressed per micro-mass culture. Differential ^{45}Ca uptake was calculated as the difference between similarly treated mineralizing and nonmineralizing cultures at the same timepoint. For presentation purposes, data were normalized to the differential uptake at 21 days in the absence of Hcys. Unlabeled cultures were examined at day 21 using EM and FTIR.

Electron Microscopy, Histology, and Vital Stains

The appearance of cells and mineral in the Hcys-supplemented and Hcys-free cultures was examined by electron microscopy. Cultures collected at days 16 and 21 were washed with 0.05 mol/L cacodylate buffer and maintained in EM fixative (0.5 gluteraldehyde, 2% paraformaldehyde, 0.05 mol/L cacodylate buffer [pH 7.2]) in the culture dish for 4 h at 4°C. They were then stored at 4°C in 0.05 mol/L cacodylate buffer containing 7% sucrose. After removal from the dish, cultures were postfixed with 2% aqueous osmium, dehydrated in a graded series of alcohols, and embedded in Spurr's resin. Thin sections were collected on ammoniated water containing bromothymol blue as an indicator for pH above 8.0. Lead citrate and alcoholic uranylacetate-stained sections were examined on a Phillips CM12 electron microscope.

Cartilage cells in culture were evaluated by in situ vital staining (Kit L-3224, Molecular Probes, Eugene, OR) to determine the ratio of live:dead cells. The method utilizes fluorescent calcein, which enters live cells and is captured in the cytoplasm of metabolically active cells. Propidium dimer stains the nuclear DNA red in dead or membrane-leaky cells. The stains were added to the culture media and the cultures incubated at 37°C for 10 min. PBS was used to rinse off the stain and media, and the spots were visualized by fluorescent microscopy, resulting in live cells with green cytoplasm and dead or dying cells indicated by red staining nuclei.

Localization of phenotypic markers of hypertrophic chondrocytes (type X collagen and alkaline phosphatase) was performed in cultures with and without Hcys supplementation. For collagen localization, diaminobenzidine-streptavadin (Dako) secondary antibodies were used to localize type I, II, and X collagens (type I primary antibodies from Southern Biotechnology, Birmingham, AL; type II and X primary antibodies from the Hybridoma Bank, University of Iowa). The azo-dye method was used to monitor alkaline phosphatase activity (Kit 86R, Sigma). The presence of mineral in these sections was localized using the standard von Kossa technique (5% aqueous silver nitrate on slides exposed to sunlight).

FTIR Imaging

FTIR imaging was performed using a Stingray infrared system (Model FTS 6000, BioRad, Cambridge, MA), which consists of a mercury-cadmium-telluride (MCT) focal plane array detector coupled to an infrared microscope and a step-scan interferometer with a spectral resolution of $\backsim 8$ cm^{-1}. Detailed methodology is described elsewhere.[41] In brief, the instrument consists of an infrared microscope with a 64 × 64 element array detector that allows 4096 spectra to be recorded simultaneously from areas of 400 μm × 400 μm. Both ethanol fixed and air-dried mineralizing and nonmineralizing cultures with zero, low-, and high-Hcys supplements were analyzed. Following acquisition, the spectra were transferred off-line and zero-corrected for the baseline in the spectral areas of amide I ($\backsim 1590$–1700 cm^{-1}) and ν_1,ν_3 PO_4 ($\backsim 900$–1200 cm^{-1}) using GRAMS/32 (Galactic Software, Manchester, NH). The areas of these two peaks were calculated and exported as ASCII files into ORIGIN (Microcal Software, Inc., Cambridge, MA), and mineral:matrix ratios calculated as an indication of mineral content.[53] Using the amide I peak, which is attributed to the peptide bond C=O stretch, the 1660–1690 peak intensity ratio was calculated, providing an indication of collagen maturity.[50,51]

The collagen maturity calculation was based on previously described analyses of model compounds,[50,51] and the infrared spectral characteristics of collagen in solution.[34] Of the various underlying bands in the amide I region of the collagen spectrum, two are of particular interest: one at $\backsim 1660$–1663 cm^{-1}, and one at $\backsim 1690$ cm^{-1}. The former is a feature of the triple helical structure of the collagen molecule. The 1690 cm^{-1} peak contribution is negligible in pyridinoline-rich samples, and increases in value in pyridinoline-free samples. Thus, maximal 1660/1690 ratios are found in mature collagen, whereas lower values are found in collagen with immature (reducible) cross-links.

For each of the infrared parameters, three-dimensional surface area graphs were generated. For statistical analyses, histograms were created using ORIGIN (Microcal Software) to plot the number of pixels with each range of calculated values. Results are presented as integrated area or intensity distribution, with each pixel in the image corresponding to an area of 7 μm × 7 μm.

Collagen Cross-link Analysis

Mineralizing and nonmineralizing cultures developed in the presence of 0 and 3.6 mmol/L Hcys were collected at day 21 and used for analysis of collagen cross-links. In brief, the cultures were rinsed in 0.9% NaCl, and lyophilized. Twenty-five to 30 individual micro-mass cultures were pooled for these analyses. Because preliminary analyses indicated that there were no detectable differences between mineralizing and nonmineralizing cultures, the analyses focused on the mineralizing cultures. After lyophilization, the dried samples were reduced with standardized NaB_3H_4 and hydrolyzed with 6N HCl, and aliquots of the hydrolysates were subjected to amino acid and cross-link analyses.[67,69] Following determination of hydroxyproline by amino acid analysis, an aliquot of each hydrolysate of known hydroxyproline content was subjected to cross-link analysis on a Waters 600E high-performance liquid chromatography system fitted with an ion-exchange column (Model AA911, Transgenomics, Inc., Omaha, NE) linked to an on-line fluorescence flow monitor (Model 821-FP, Jasco Spectroscopic Co., Tokyo, Japan) and a liquid scintillation flow monitor (Flo-one Beta, Packard Instruments, Meriden, CT). The reducible cross-links (deH-DHLNL, dehydrodihydroxylysinonorleucine; deH-HLNL, dehydrohydroxylysinonorleucine; deH-HHMD, dehydrohistidinohydroxymerodesmosine) were identified and quantified in their reduced forms (DHLNL, HLNL, and HHMD, respectively). The reducible cross-links, as well as the nonreducible, fluorescent cross-links (Pyr, pyridinoline; d-Pyr, deoxypyridinoline) were quantified as moles/mole of collagen as described previously.[69]

Data Analysis

For all data, comparisons were made to nontreated mineralizing or nonmineralizing cultures as indicated based on analysis of variance (ANOVA). Statistical significance was assumed for $p < 0.05$ (Student's *t*-test).

Results

Nodules

To determine the effect of Hcys on mesenchymal cell differentiation, the cultures stained with Alcian blue were examined at day 5. As shown in **Figure 1**, at day 5 (3 days after the addition of Hcys), there were no significant differences in the number of nodules among the groups. However, nodules formed in the presence of 3.6 mmol/L Hcys appeared slightly smaller when compared with the nontreated group (data not shown).

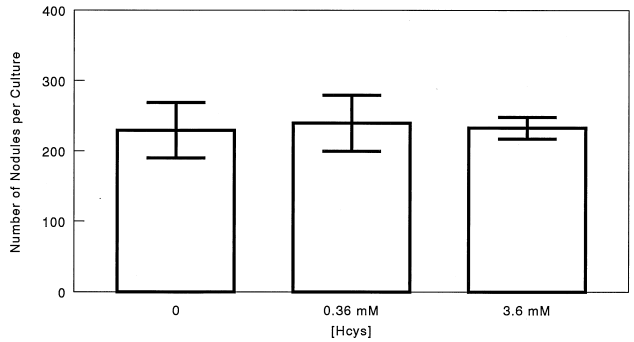

Figure 1. Number of cartilage nodules present on day 5 is not significantly altered by supplementation with 0, 0.36, and 3.6 mmol/L Hcys. Values are mean ± SD for three to five independent cultures.

Proteoglycan Content

Between days 7 and 11, the chondrocytes produced a proteoglycan-rich matrix that was then modified prior to calcification; the effect of Hcys on total proteoglycan content was examined at day 11. Hcys treatment caused a dose-dependent, statistically significant reduction ($p < 0.05$) in the proteoglycan content of both mineralizing and nonmineralizing cultures (**Figure 2**). The decreases were significant for both high and low doses for the mineralizing cultures, but in the nonmineralizing cultures significance was only reached when the initial Hcys concentration was 3.6 mmol/L.

^{45}Ca Uptake

The effect of Hcys on cell-mediated calcification was monitored based on uptake of ^{45}Ca. This uptake, corrected for binding to the matrix, parallels the accumulation of mineral in the culture.[11] Treatment with Hcys caused a significant reduction in ^{45}Ca uptake as a function of time (**Figure 3**). In mineralizing culture administered 3.6 mmol/L Hcys, ^{45}Ca uptake was reduced to the basal uptake of cultures that did not have any additional phosphate supplementation (nonmineralizing controls). Mineralizing cultures treated with 0.36 mmol/L Hcys had intermediate values. Nonmineralizing controls showed equivalent low ^{45}Ca uptake. Data are presented as the differential calcium uptake (mineralizing − control) for each condition, normalized relative to the maximum differential uptake in untreated cultures at 21 days.

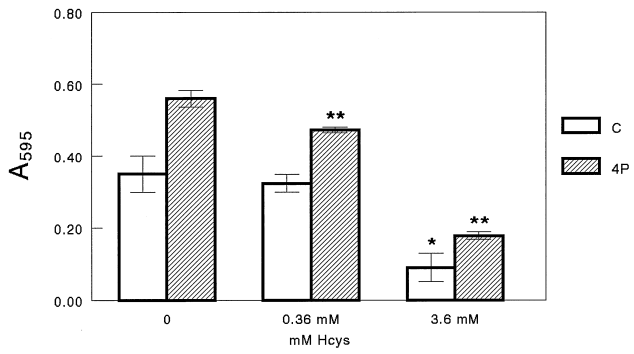

Figure 2. Proteoglycan content in mineralizing and control cultures is decreased on day 11 by the addition of Hcys. Mean ± SD of absorbance of the guanidine-HCl extractable Alcian blue/PG complex. Significance relative to Hcys-free cultures, $p < 0.05$ is shown for nonmineralizing (*) and (**) mineralizing cultures. Open bars, nonmineralizing cultures (C); hatched bars, mineralizing cultures (4P).

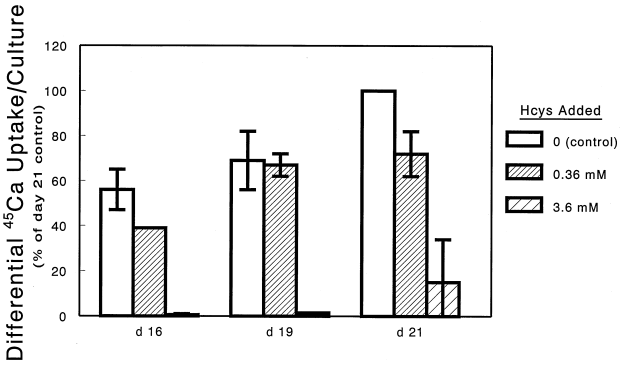

Figure 3. ^{45}Ca uptake is decreased on days 16, 19, and 21 in the presence of Hcys supplementatiobn. Data, which show differential uptake (difference between mineralizing and nonmineralizing controls subjected to the same experimental protocol), are presented as percent of untreated mineralizing cultures at day 21. Values are mean ± SD for three to five independent experiments.

Histology

To verify that the effect of Hcys addition was at the level of matrix synthesis and mineralization, and not due to cell degeneration, a series of light- and electron-microscopic studies were performed. For light microscopy, cartilage cells in culture were stained for type X collagen, for alkaline phosphatase activity, and for mineral distribution using von Kossa stain. In addition, cultures with and without Hcys treatment were stained by vital dye techniques and also for type II collagen to indicate whether significant numbers of cells were dead within the cell culture mass, and to verify the synthetic activity of the cells. Electron microscopy was used to determine the cellular morphology, the distribution of mineral, and the condition of the collagen matrix.

Light microscopy. Vital dye staining (**Figure 4**A) indicated that a few dead cells were randomly found in both Hcys-free and Hcys-treated cultures, but in low numbers. In all the cultures, independent of Hcys treatment, within the calcifying nodules (Figure 4B) many of the chondrocytes were hypertrophied or degenerate. Staining for type II collagen (not shown) also demonstrated a normal distribution throughout all the cultures independent of Hcys treatment, indicating that the great majority of cells were normally functioning chondrocytes. The hypertrophic cells were identified based on staining for type X collagen and, in the day 19 cultures, stained cells were distributed throughout the entire culture dish. The intensity of type X staining was much greater in the cultures that did not receive exogenous Hcys (Figure 4C), as compared with Hcys-treated cultures (Figure 4D), suggesting that cells were differentiating more slowly after Hcys treatment. The mineral deposition as illustrated by the von Kossa reaction was confined to mature nodules in the Hcys-free cultures (**Figure 5**A) with only light reactivity in the nodules of the Hcys-treated cultures (Figure 5B). At day 21 of culture, both the Hcys-free and the Hcys-treated cultures contained deposits of mineral (see EM results). The extent of mineral deposition and numbers of individual nodules containing mineral and/or hypertrophic chondrocytes were greater in those cultures that did not receive exogenous Hys as compared with the Hcys-treated cultures.

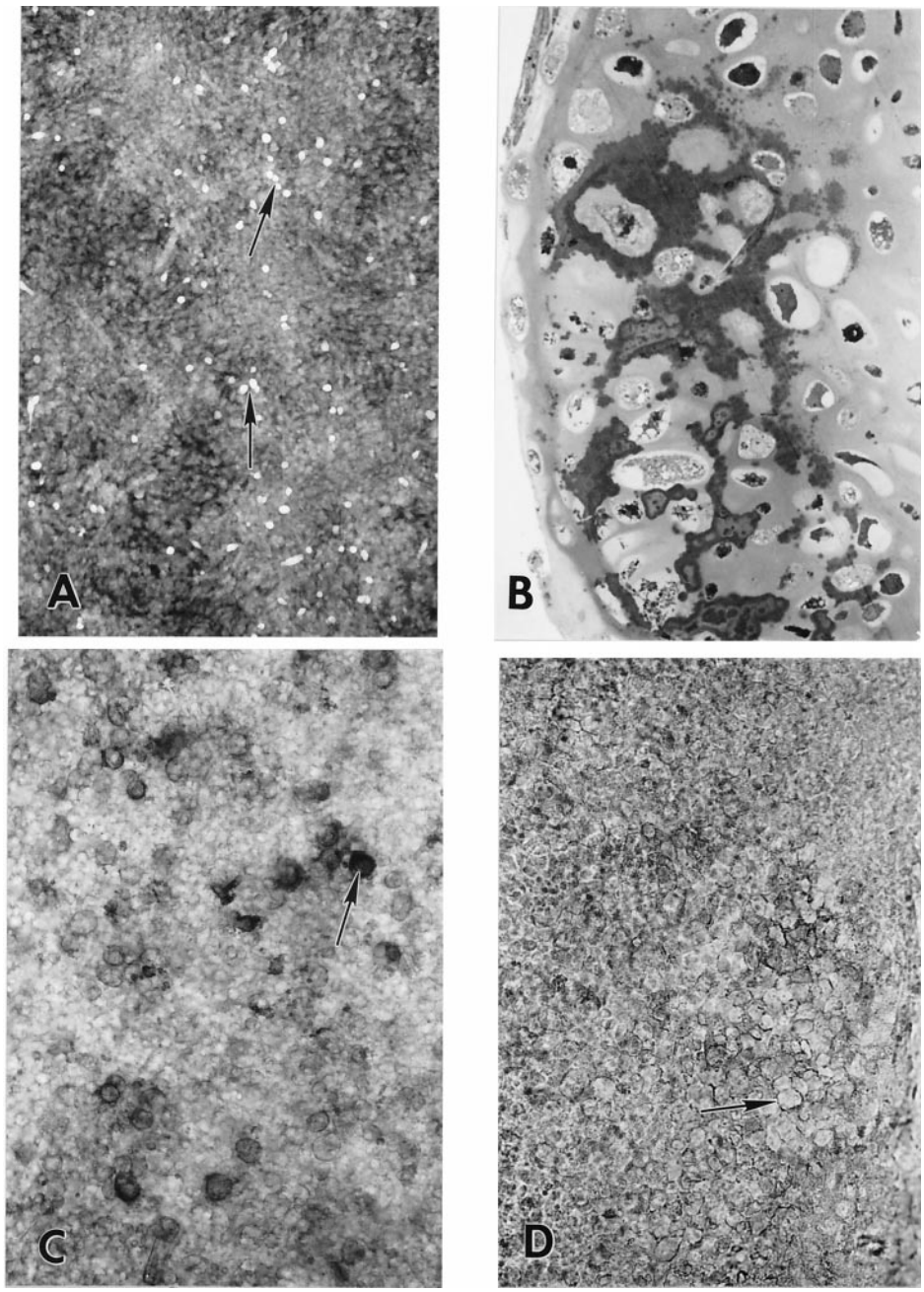

Figure 4. Effect of Hcys supplementation on cell viability. (A) Typical vital staining of chondrocytes in Hcys-treated mineralizing culture at day 19. The white objects (arrows) are cells containing red fluorescence due to cell death. This distribution of dead cells was equally spread among high-dose, Hcys-treated (shown here) and low-dose, untreated cultures (not shown). Original magnification ×45. (B) Calcifying nodule within a day 21 culture that was not supplemented with Hcys. The black deposits of mineral are spread between cells, most of which are hypertrophic or degenerate. This cell death is a normal response during mineralization of cartilage and occurred more often in the non-Hcys treated cultures that contained more mineral. von Kossa stain; original magnification ×180. (C) Chondrocytes in day 19 mineralizing cultures stained for type X collagen to demonstrate hypertrophic cartilage cells. In this non-Hcy-treated culture, there are prominent diaminobenzidine deposits around large cells (arrows), indicating the presence of type X collagen. Original magnification ×180. (D) Chondrocytes stained for type X collagen in a comparable culture supplemented with high-dose Hcys. There is weak staining (arrows) at the periphery of many cells, but the hypertrophic condition is not as well developed as in the nontreated control in (C). Original magnification ×180.

Electron microscopy. The fine structure of chondrocytes and matrix from cultures not treated with Hcys (**Figure 6**A and **Figure 7**A) was compared with that of Hcys-treated cultures (Figures 6B and 7B). The chondrocytes in cultures not supplemented with Hcys showed intact nuclei and typical cytoplasmic components of mitochondria, Golgi saccules, and vacuoles. The surrounding matrix was only partially mineralized. Figure 7A illustrates that the collagen fibrils formed a uniform matrix between the cells with more densely packed fibrils adjacent to the cell. The Hcys-treated chondrocytes (Figure 6B) showed a normal nucleus with mitochondria in the cytoplasm. The photograph presented was chosen to show that, in the Hcys-treated cultures, although mineral was difficult to locate, there were a few areas of extensive mineralization. The Hcys treatment resulted in collagen fibrils that were smaller in diameter and with decreased amounts of collagen found between adjacent cells (Figure 7B), in contrast to cultures that were not supplemented with Hcys (Figure 7A).

Cross-link Analysis

Hcys treatment reduced Pyr and d-Pyr cross-links in both nonmineralizing and mineralizing cultures. In cultures that were not supplemented with Hcys, the Pyr content was 0.07–0.1 mol/mol collagen (nonmineralizing) and 0.08 mol/mol collagen (mineralizing). The d-Pyr content was 0.03–0.06 in the untreated groups. Pyr was not detectable in the cultures supplemented with 3.6 mmol/L Hcys, nor was d-Pyr. The reducible cross-links did not vary significantly in the supplemented and nonsupplemented

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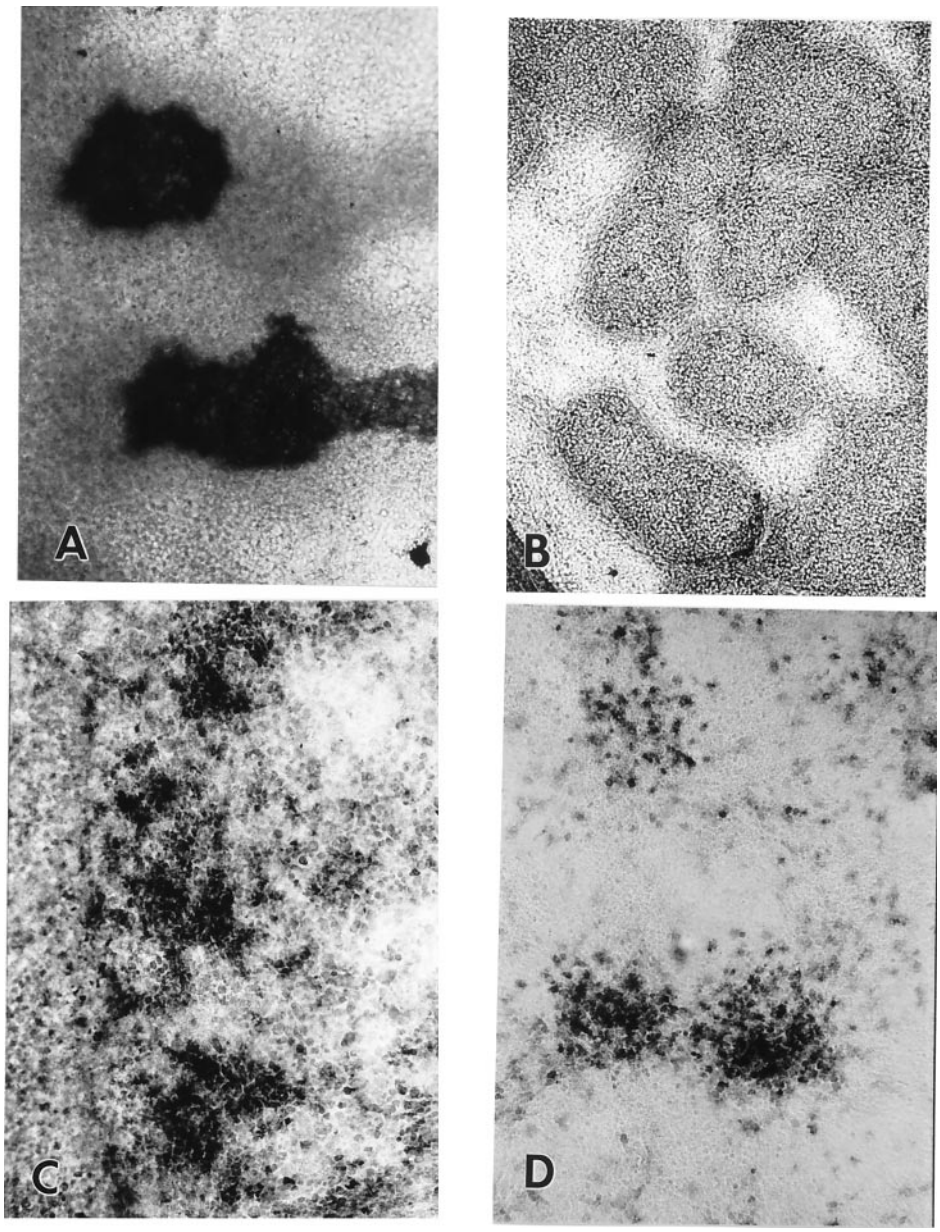

Figure 5. Light-microscopic evaluation of the effects of Hcys on mineralization in the differentiating mesenchymal cell micro-mass cultures. (A) von Kossa stain in the non-Hcys-treated day 21 culture shows mineral deposited as a black deposit among two distinct nodules. Original magnification ×90. (B) von Kossa stain of Hcys-treated day 21 culture demonstrates reduced mineralization represented by the light reaction among several nodules. However, none of the nodules stain to the same intensity as in the nontreated control (A). Original magnification ×90. (C) Alkaline phosphatase activity is shown as dark deposit of azo-dye products among chondrocytes of this day 19 mineralizing culture. The enzyme appears to be associated with individual nodules forming in the culture. Original magnification ×90. (D) Alkaline phosphatase activity as found in Hcys-treated day 19 cultures. The reaction is similar to the control cultures (C); however, there are fewer cells involved and fewer nodules containing enzyme activity within the culture. Original magnification ×90.

cultures, although values tended to be higher in the supplemented cultures (data not shown).

FTIR Imaging

FTIR spectra (**Figure 8**) of cultures in the presence and absence of Hcys were distinctly different. Spectral images of the matrix with and without Hcys are shown in Figure 8a,e,f. In the presence of Hcys, the peak of the amide I band found in control cultures and calcifying tissues at 1660 cm^{-1}, and attributable to the collagen triple helical structure,[34] had shifted to 1651 and 1647 cm^{-1}, with the greatest shift at the highest Hcys concentration, indicative of an increase in the contribution of pyridinoline-free molecules. FTIR images demonstrated a decrease in the 1660/1690 peak intensity ratio, with increasing Hcys concentration, indicative of the decrease in mature collagen cross-links.[50,51] The spectral characteristics of the mineral (Figure 8a–d) in the cultures were not altered, although the decreased mineral content in Hcys-treated cultures was apparent. Histograms, detailing the pixel distributions (y axis) for the 1660/1690 ratios (x axis) in the images from these cultures are presented in **Figure 9**. The shift to lower ratios with increasing Hcys content was seen in both the control and mineralizing cultures. In addition, in the mineralizing cultures with 3.6 mmol/L Hcys, the total number of pixels (7 μm × 7 μm sites) having spectra typical of collagen was markedly decreased.

Discussion

This study has demonstrated that exogenous homocysteine addition in concentrations known to be teratogenic in the developing chick[59] reduces mineralization in the differentiating chick limb-bud mesenchymal cell micro-mass culture system. Hcys has multiple effects in this culture system. It alters the matrix organization, limits formation of pyridinoline and deoxypyridinoline cross-links, and decreases proteoglycan content. Each

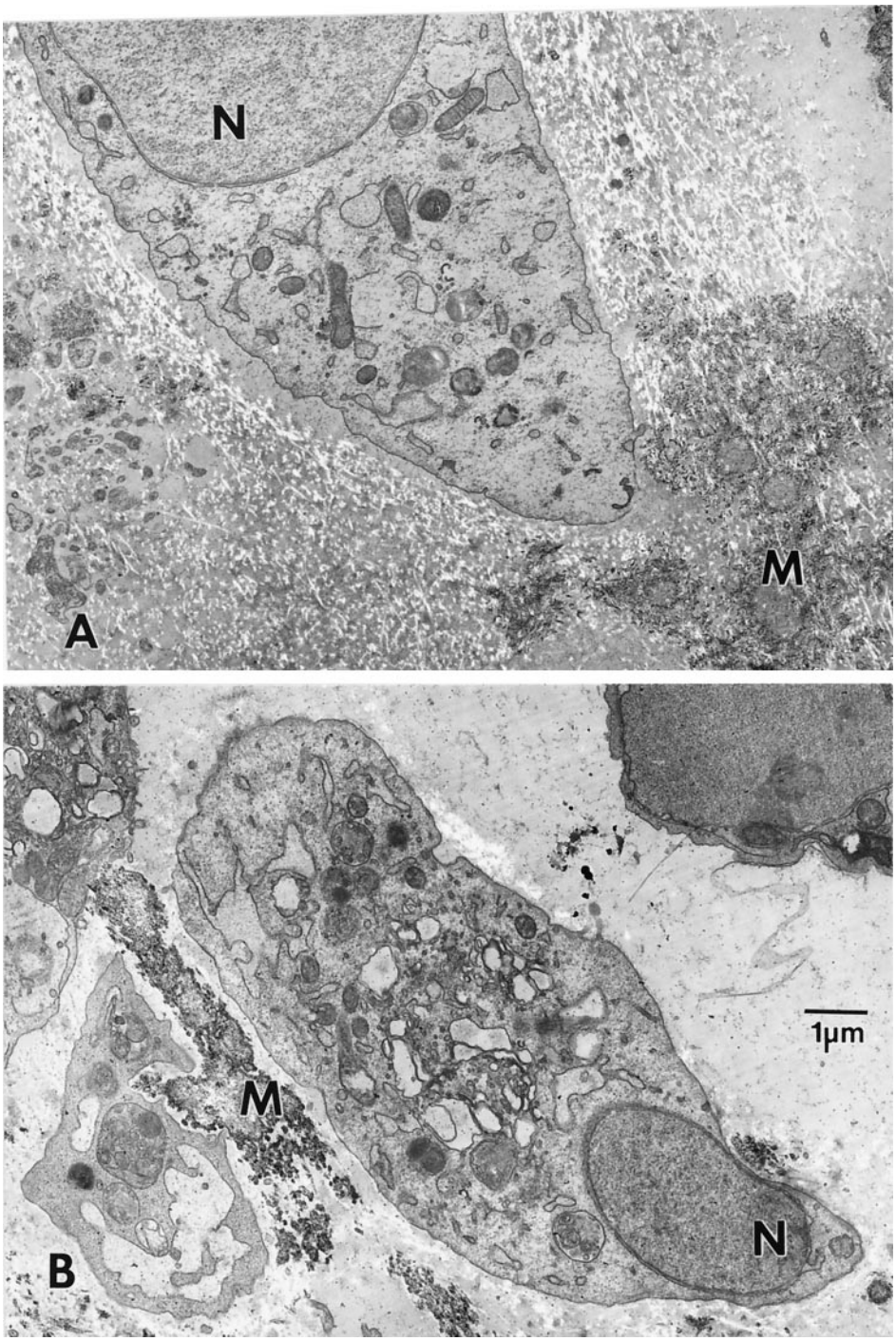

Figure 6. Effect of Hcys on cellular morphology in day 21 cultures not supplemented with Hcys (A) and those treated with high-dose Hcys (B). (A) Chondrocyte in a mineralizing nodule. The nucleus (N) is evident, and the adjacent cytoplasm contains mitochondria, endoplasmic reticulum, and some vacuoles. The matrix contains a very light deposit of mineral (M), indicating that the underlying collagen is just beginning to calcify. Original magnification ×9500. (B) Chondrocyte in a mineralizing nodule. The nucleus (N) shows normal euchromatic appearance, with cytoplasmic organelles of mitochondria, Golgi vacuoles, and endoplasmic reticulum. This photograph was chosen because it shows heavy deposits of mineral (M) in those regions where mineralization was occurring; however, few such regions could be located in the Hcys-treated cultures. Original magnification ×9500.

of these can directly or indirectly affect the ability of the matrix to support mineralization.

Both viable cells and a mature cartilage matrix are required for matrix mineralization in this culture system.[7,8,11,12] It has previously been shown that chondrocyte differentiation is accelerated when mesenchymal cell cultures are plated on type I collagen matrices,[7] thus the development of an abnormal matrix may affect the development of cells. An abnormal matrix was the most striking feature of the Hcys-treated cultures. The EM results showed that Hcys treatment resulted in irregular deposition of collagen in the extracellular compartment, and the fibrils were smaller in cross section than those in control cultures. This is not surprising, because disruption of collagen cross-links with other agents has been shown to prevent an orderly arrangement of the extracellular matrix.[4] The abnormal deposition of collagen in the extracellular matrix may lead to abnormal cell-matrix interactions, and therefore the chondrocytes may not be receiving the appropriate differentiation signals from the environment.[4] Indeed, collagen structure and alignment is known to be important for deposition of physiological mineral.[33] Furthermore, there is evidence that type I collagen is essential for mineralization in the micro-mass cell culture system.[10] However, it is not clear

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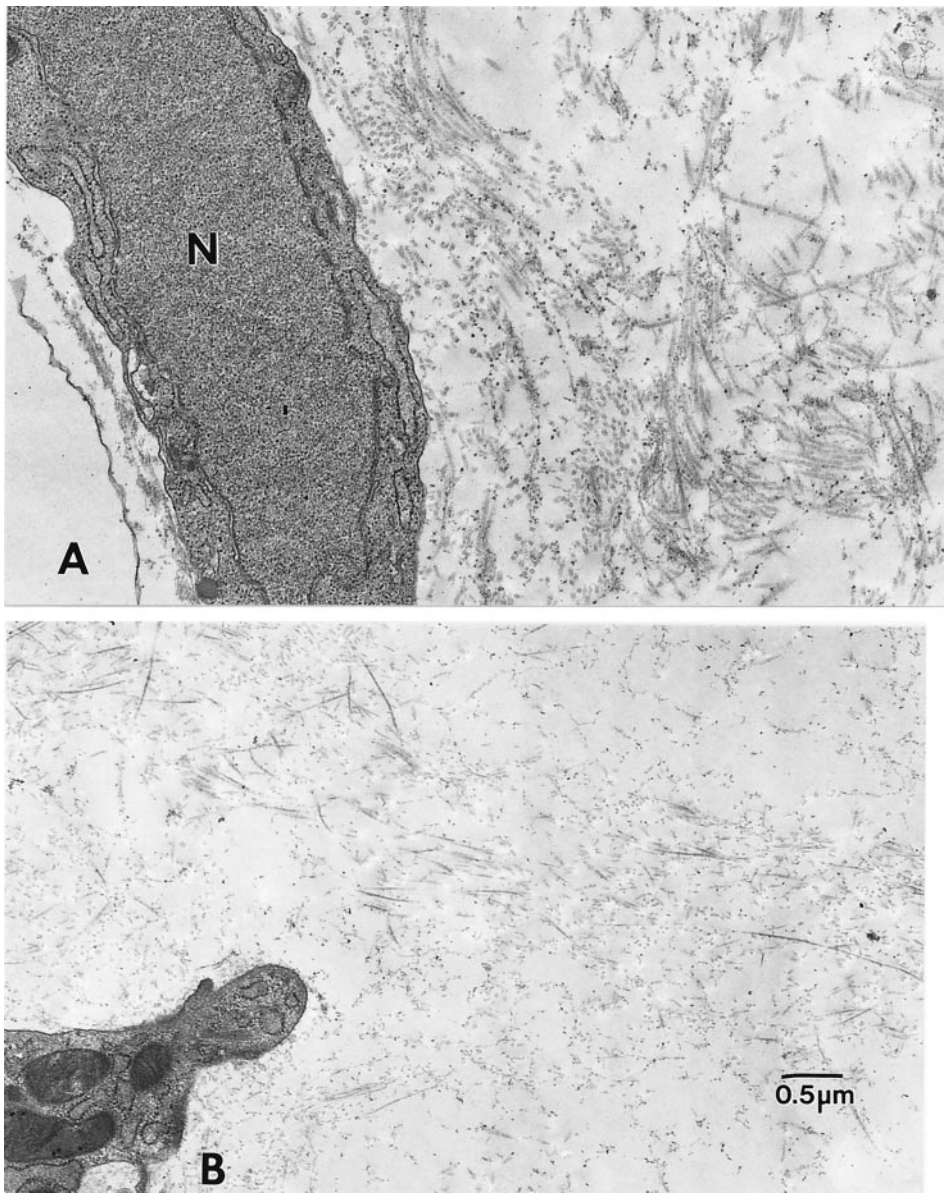

Figure 7. Electron micrographs demonstrating the effect of Hcys on collagen fibril appearance. The collagen fibrils are more densely arranged within the matrix of the nontreated cultures (A), as compared with the high-dose Hcys-treated cultures (B). In addition, the fibrils in the nontreated cultures often seem to accumulate near the cell and not in the intercellular spaces, which is a distribution effect not often seen in the treated cultures. Original magnification ×19,000.

whether collagen functions as a template for other proteins that promote mineralization, or if collagen itself acts as a nucleator of mineral crystals, or both. To this end, the results of the Hcys treatment indicate that, in addition to other proteins and molecules associated with collagen, the matrix organization and fiber size may also play an important role in the mineralization process.

Type II, IX, X, and I collagens are produced in differentiating mesenchymal cell cultures.[12] These cultures mineralize in the presence of 4 mmol/L inorganic phosphate or a source of phosphate ions.[6,12] Recent studies using immunoblocking have demonstrated that some of the mineral deposition occurs on a type I collagen matrix.[12] Because similar processes form type I, type II, and other collagen cross-links,[16,17,52] although these cross-links differ in composition, it is likely that several types of collagen in the culture are affected by Hcys.

Collagen cross-links play an important role in the biochemical and mechanical properties of both bone and cartilage.[23,29] Their structure, localization, and quantity appears to be tightly regulated and highly tissue-specific.[16,17,56–58,70] During the course of maturation of these tissues, complex changes occur in their cross-link profiles.[2,30,70] In brief, two pathways for collagen cross-link synthesis have been proposed, one based on lysine aldehyde and the other on hydroxylysine aldehyde. First, prior to triple-helix formation, the specific lysine residues of nascent collagen peptides are hydroxylated intracellularly by lysyl hydroxylase. The extent of hydroxylation can vary substantially, depending on the tissue, possibly due to the differential expression pattern of various isoforms of the enzyme in the respective tissues.[69] The next step is the oxidative deamination of specific telopeptide lysine or hydroxylysine by the copper-dependent enzyme lysyl oxidase.[61] Subsequent nonenzymatic condensation reactions lead first to the formation of reducible imminium cross-links, such as dehydrodihydroxylysinonorleucine and dehydrohydroxylysinonorleucine, which play an important role in the organization and immobilization of collagen in the extracellular environment.[52] The amount of such reducible cross-links declines with age.[54,56] With time, these iminium cross-links mature into more complex tri-/tetravalent cross-links such as the nonreducible pyridinium cross-links Pyr and d-Pyr, and histi-

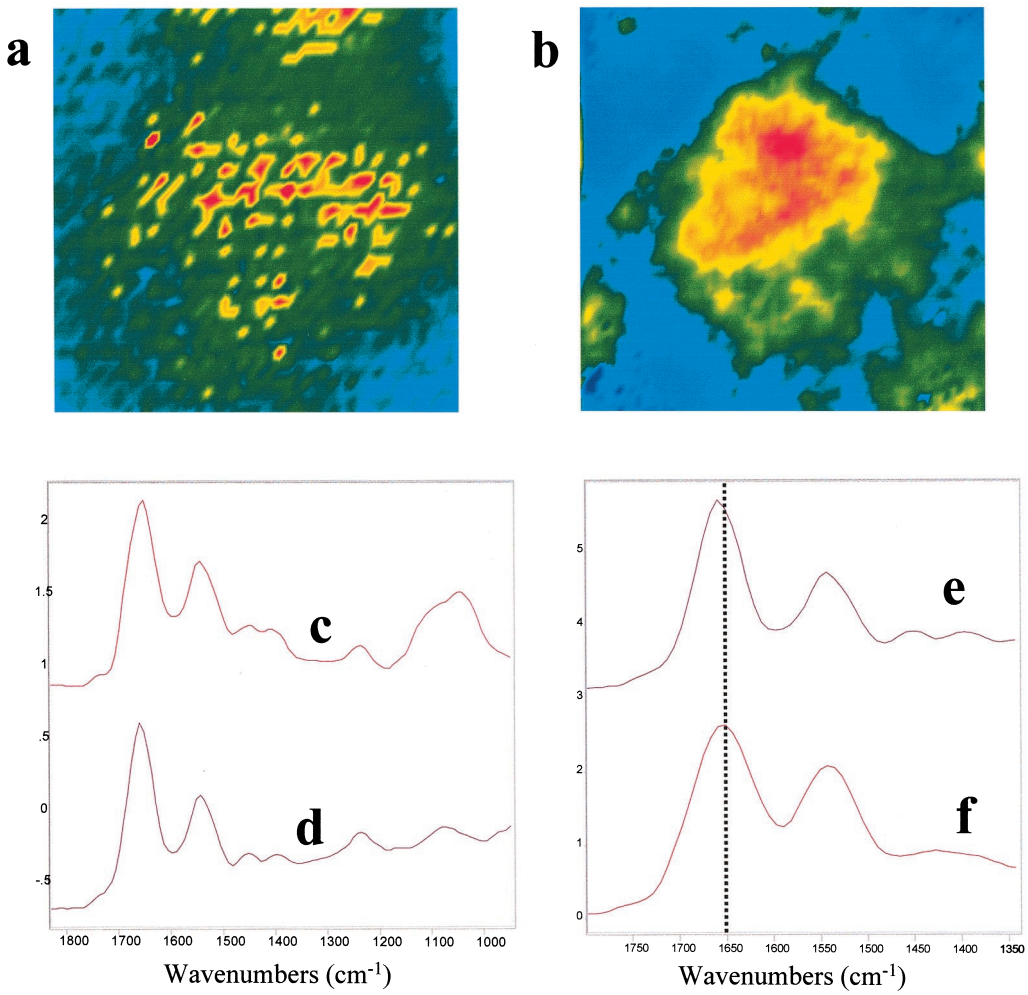

Figure 8. Fourier transform infrared imaging demonstrates the effect of Hcys on collagen and mineral maturity. Figure (a) (day 21, mineralizing, no Hcys addition) is an image of the collagen matrix in a typical chondrocyte nodule, whereas (b) illustrates the mineral distribution in the same nodule. The colors indicate relative intensity, with red the highest, yellow intermediate, and blue/black the lowest. Trace (c) is a spectrum extracted from the image in (b) showing a typical mineral peak at 900–1200 cm^{-1}; this peak is markedly diminished in (d), which represents the spectrum in a mineralizing culture treated with high-dose Hcys. Traces (e) (untreated) and (f) (high Hcys) show the protein amide I and amide II bands, with the shift from the 1660 cm^{-1} subband, which occurs with Hcys addition (f), indicated by a dotted line.

dine-involved cross-links.[69] The mechanism of formation of the pyridinium cross-links is a subject of controversy. Pyr is abundant in highly hydroxylated collagens such as those in cartilage, whereas d-Pyr is found primarily in calcified tissues such as bone and dentin.[22] Interestingly, one of the few noncalcified tissues where lysylpyridinoline is found in appreciable quantity is hypertrophic cartilage,[48] which suggests that it may have some role in the mineralization process.

The impaired mineralization in the Hcys-treated cultures may be explained in terms of the distribution of collagen cross-links noted when mineralized and nonmineralized tissues are compared. For example, in the chick growth plate, while there is a decrease in the content of pyridinoline cross-links going from the proliferating zone to the mature hypertrophic cell zone where mineralization commences, d-Pyr first appears in the prehypertrophic zone.[20,46,47] In addition, it has been demonstrated that in mineralizing turkey leg tendon, d-Pyr is associated with mineralizing collagen matrix, but not with the collagen that never mineralizes.[69] Similarly, a decrease in Pyr content of bone has been correlated with reduced strength and mineral density of both rat and rabbit bones.[35,49]

In addition to effects on collagen, Hcys treatment also decreased the proteoglycan content of both the mineralizing and the nonmineralizing cultures. Hcys has been shown to affect proteoglycan synthesis in the aorta,[40] and recently the mechanism of action has been explained by the Hcys activation of the latent matrix metalloproteinase (MMP-2) at low concentrations of Hcys, and the inhibition of MMP-2 activity at higher concentrations.[5] Several metalloproteinases, including MMP-2,[28,44] are present in epiphyseal cartilage. Thus, it is possible that, even with the concentrations of Hcys used in the micro-mass culture system, Hcys may be activating matrix degradation as it does in arterial walls. The effects of Hcys on MMP-2 are quite complex, but the observations in this system are consistent with such a mechanism. Increased proteoglycan degradation, in addition to affecting mineralization, would contribute to matrix disorganization. In earlier studies of this culture system, decreases in proteoglycan content were associated with increased mineralization,[13] rather than the decreased mineralization found in the present study. The decreased mineralization in the face of decreased proteoglycan content suggests that the principal effect of Hcys in this system is on collagen-mediated mineralization.

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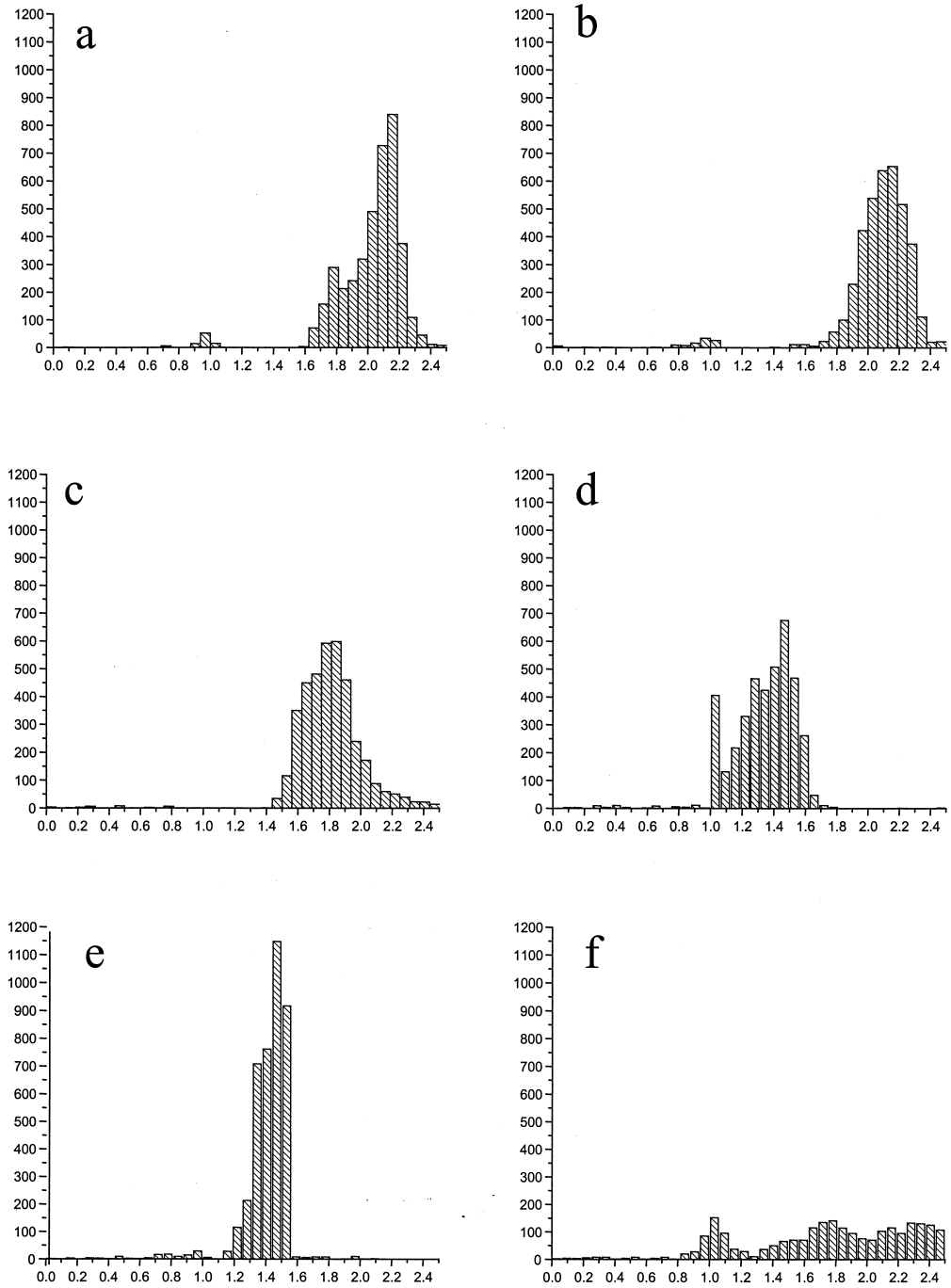

Figure 9. Histograms illustrating the effect of Hcys supplementation on collagen maturity. The number of pixels (y axis) with the indicated 1660/1690 intensity ratio (x axis) in infrared images from nonmineralizing (a,c,e) and mineralizing (b,d,f) cultures treated with 0 (a,b), 0.36 mmol/L (c,d), and 3.6 mmol/L (e,f) Hcys. Note the shift to the left as Hcys concentration increases.

However, it must be noted that, in other systems, the matter of whether cartilage-specific proteoglycans (aggrecan) inhibit or promote the calcification process is a disputed issue.[9,25,26]

The results of this study, which associate an agent that is known to inhibit collagen cross-link formation with impaired mineralization in chick limb-bud mesenchymal cell cultures, are in agreement with clinical and laboratory observations. For example, reduction of collagen cross-links in Bruck syndrome, due to a mutation in a bone-specific telopeptide (lysyl hydroxylase), also results in decreased mineralization.[3] Similarly, in Ehlers–Danlos syndrome type VI (another disease of defective lysyl hydroxylation), there is abnormal tissue formation manifested by kyphoscoliosis, weak arterial walls, and osteoporosis.[66] It is not possible to extrapolate the results in the cultures directly to human diseases, because the Hcys levels that significantly blocked mineralization were 100× normal (chick) levels. Such Hcys concentrations are teratogenic in developing chick embryos, but not cytotoxic.[59] It is known, however, that homocys-

teinuria is associated both with abnormalities in collagen cross-link formation and with osteopenia.[37] The results of the chick limb-bud culture study suggest a mechanism for the impaired mineralization in such patients.

There are three reasons why we believe that the decreased mineral formation in the presence of Hcys cannot be attributed to toxicity. First, vital dye staining of cultures at days 16 and 21 did not reveal any differences in viability among the different groups. Second, the cells in the Hcys-supplemented cultures, although fewer in number, produced type II and X collagens and alkaline phosphatase, although at lower levels. Finally, EM studies failed to demonstrate any significant evidence of cellular damage among the Hcys-treated groups when compared with controls. Therefore, we conclude that cell toxicity was not a major factor in these outcomes, and that Hcys impairs mineralization by disrupting the matrix constituents.

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